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Small Molecule DNA and RNA Binders

From Synthesis to Nucleic Acid Complexes

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Preface

The ultimate goal of most organic-medicinal chemists is to see the small molecule that they have synthesized become a useful drug for the treatment of human diseases. Unfortunately, even with modern technology this is an extremely rare event. In most cases, the compounds designed and synthesized (generally with pain and passion) have a brief existence that does not exceed the first biological activity assay. The valley between chemistry and therapeutics is deep and difficult to cross but nevertheless the two disciplines are intimately associated. It is our goal to help construct a bridge between the makers of the small molecules and the users. Over the past two decades, a relatively large number of useful anticancer and anti-parasitic drugs have been discovered or rationally designed based on the principle of nucleic acids recognition. A better understanding of the molecular rules that govern interactions between small molecules and the many sequences and structures of DNA and RNA is pivotal to the development of novel drug candidates. How does the drug adapt to the nucleic acid target (and *vice versa*)? How do nucleic acid structures affect ligand binding? How do small molecules read the genetic information? These types of questions continue to excite our scientific curiosity and the quest for better DNA/RNA binders drives modern researchers much as the search for the Holy Grail did the ancients.

Design and development of nucleic acid targeted drugs is a challenging enterprise but real breakthroughs have been made in recent years and many are reported here. This volume is intended to give the reader an up-to-date view of the current status and expected developments in research involving ligand-nucleic acid recognition. This book was built on a discussion among the three of us on how chemistry, biophysical chemistry and pharmacology serve our field to help design new drugs. Our different but complementary view angles on the subject prompted us to edit this volume focussed on DNA/RNA recognition by a variety of small molecules: peptides, intercalators, groove binders, metal complexes. The various DNA structures that can be targeted by drugs are also considered and the field of natural products is partially covered. Altogether, the 25 chapters of this volume survey most of the drug categories that bind, bond or cleave nucleic acids. The reader will notice the diversity of small molecules mentioned here, from marine products to platinum complexes, from G4-binders to RNA cleaving agents, from abasic site selective agents to aptamers, as well as the panel of biophysical and

biochemical approaches routinely used to investigate the structures and dynamics of drug-nucleic acids complexes. The portraits of specific drug families (anthracyclines, indolocarbazoles, bleomycins, ...) are also thoroughly presented. The amalgam was deliberately chosen to cross ideas of organic chemists and biophysicists and those more interested in the therapeutic end point of the research.

The volume starts with a general introduction (magisterially presented in a British style) and then it flies over the world, from several countries in Europe (Spain, Italy, France, Czech Republic, UK) to the USA, via Japan and New Zealand, illustrating the essential international character of the research (and the friendly atmosphere of the edition). Inevitably we have neglected (mostly for consideration of space) a number of interesting areas that should have been cited here, such as clinical applications. But the gallery of molecules presented in this volume must be considered as a live exhibit to explore and to use for further drug design. Come on in, and like us, become fascinated by the “small molecules” that bind or bite the genetic material in its many forms. We hope you will also find examining this volume an enriching experience.

The enterprise was very exciting and proceeded smoothly (with no delay!) thanks to the enthusiastic contribution of all the authors. We are grateful to everyone for delivering their manuscript on time (and in some cases even well before the deadline!) and for making our task as editors such a memorable one. We also thank our “artist” Christian Coulombeau who kindly drew the front cover, sort of a railway to the future. Finally, we shall dedicate this volume to our colleagues who left the world too quickly to contribute (Marc Leng, David S. Sigman, and Peter A. Kollman in particular).

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1

Forty Years On

Michael J. Waring and L. P. G. Wakelin

1.1**Early Experiments Prior to Molecular Modeling**

The quest to understand specific interactions between drugs and nucleic acids dates back a long time – more than 40 years. Even though the concept of gene targeting could not be explicitly formulated until much later, there were early realizations that DNA could provide a fine receptor for drugs. A major turning point in the history of drug binding to DNA, the publication in 1961 of the intercalation hypothesis by Leonard Lerman [1], in many people's estimation represents the true birth of the subject, but it would be wrong to neglect mention of the contributions of earlier workers. These workers knew they were dealing with drug–nucleic acid interactions and must have had some inkling of the future importance of the topic. Among them were the histologists who employed dyes such as aminoacridines to stain cells and tissue sections, particularly the fluorescent dye acridine orange, whose capacity to cause nuclei to fluoresce bright green while the cytoplasm fluoresced red was a valuable tool in histology and cell biology. Indeed in the researches of these pioneers can be found the first evidence that particular dyes can react differently with different kinds of nucleic acid-containing structures and therefore that the small molecules must be capable of some form of discrimination based upon what we would today call molecular recognition. From the variable and sometimes capricious performance of substances such as acridines employed as stains it could also be surmised that depending upon the solvent conditions a given dye might react in more than one way with its 'receptor,' foreshadowing the concept of heterogeneity in binding that was later to occupy the attention of biophysicists.

At the same time, thanks to the seminal work of Paul Ehrlich half a century earlier, the usefulness of dyes – particularly aminoacridines – as antiseptics and antimalarials was widely recognized so that the connection between cell staining and useful biological activity was more than implicit. Thus it happened at a critical moment that the potency of proflavine as a mutagen was recognized. This led to the brilliant experimental work of Crick, Brenner and colleagues [2] showing that exposure of bacteriophage-infected bacteria to proflavine produced frameshift mutations – a phenomenon that enabled them to deduce the triplet nature of the

genetic code. Meanwhile, the careful experiments of Peacocke and Skerrett [3] on the interaction of proflavine with purified DNA were under way and the first truly quantitative measurements of a reversible drug–DNA binding reaction became available, complete with a proper description of the metachromatic shift in the absorption spectrum, application of spectrophotometry and equilibrium dialysis to determine genuine binding constants, and clear evidence of the occurrence of secondary binding after saturation of the strong primary binding sites had been accomplished.

Now all the elements were in place for Lerman, at that time working in the Cambridge MRC Laboratory of Molecular Biology with Crick and Brenner, to get to work on the intercalation hypothesis. Stone and Bradley [4] disposed of the secondary interaction of acridine orange with nucleic acids by attributing it to the formation of stacked aggregates of dye bound externally to the polyanion.

Two other pre-intercalation areas of endeavor must be mentioned, the first of which is the action of the antibiotic actinomycin D. Actinomycin had been discovered in the 1940s and was the first antibiotic found to be highly active against certain tumors – indeed, through the 1950s and early 1960s it was reckoned to be the most potent anticancer agent available in the arsenal of chemotherapy. The antibiotic was known to be capable of inhibiting nucleic acid synthesis in susceptible cells, a process that was consequently identified as a prime target for anti-cancer chemotherapy. The discovery of mRNA and the process of gene transcription owes much to the earnest work of early cell biologists who showed that actinomycin was an exquisitely selective inhibitor of transcription by virtue of its specific inhibitory action on the newly discovered enzyme RNA polymerase; that in turn was attributable to tight but reversible binding of actinomycin to the double-helical DNA template [5]. These discoveries firmly established the business of ligand–DNA interaction as a matter of concern to biologists, clinicians, and a breed of pharmacologists who later emerged as key players in founding what was to become the illustrious discipline of molecular interactions.

The second area of endeavor, though it had little influence on the development of ideas about reversible ligand–nucleic acid interactions, is the remarkable work of people like Kohn, Brooks, and Lawley on nitrogen mustards and comparable alkylating agents used for cancer chemotherapy [6, 7]. We should recall that nitrogen mustards were the very first chemicals used to treat cancer, prompted by unhappy events that occurred during the Second World War; it is indeed salutary that so evidently worthy a purpose as the alleviation of suffering from one of humanity's most dreaded diseases should have come about in such an inauspicious manner. The determined attentions of a few medically minded individuals capable of grappling with rather complicated and messy chemistry did a lot to clarify the mechanisms of action of these highly reactive substances, and again the critical target turned out to be DNA. Painsstaking analysis of the products formed *in vitro* and *in vivo* when cells were exposed to mustards eventually identified the N7 position of the guanine ring as the most reactive (i.e. nucleophilic) site for alkylation of DNA, and the perceived correlation of anticancer activity with the possession of two alkylating centers spaced some five atoms apart led to the concept that bi-functional reactivity must be crucial for therapeutic effect.

1.2

Formulation of Molecular Models and Mechanisms of Binding to DNA

Before we return to the historic turning point at which the intercalation hypothesis was born, it is logical to finish consideration of the early alkylating agents by referring to their identification as crosslinking agents capable of covalently linking the complementary strands of the DNA double helix. At first it was thought that this action would adequately explain their cytotoxic activity through inhibiting the progress of the replication fork, but more recently the possible contribution of *intra*-strand crosslinks and DNA–protein crosslinks has brought this assumption into question [8, 9]. Meanwhile other types of powerful alkylating agents have been discovered that do not necessarily form interstrand crosslinks but are endowed with excellent biological activity. Moreover, an early twist to the tale of covalent reaction with DNA came with the finding that the antibiotic mitomycin C must be activated by reduction prior to forming inter-strand DNA crosslinks: this discovery added impetus to the idea of bioreductive activation of pro-drugs, particularly for cancer treatment, which has become an important focus for the efforts of several groups of drug designers (see Chapter 9). There is also a complex relation between bond-forming and bond-breaking interactions with nucleic acids that can be seen with several DNA-binding compounds described elsewhere in this volume (see Chapters 3 and 23).

A unifying theme that runs through these lines of work, and indeed throughout the volumes of this publication, is the extraordinarily sophisticated chemistry that attends the reaction of many compounds with nucleic acids, not to mention the amazing biosynthetic capabilities of the organisms that produce those substances that are of natural origin. Neither should we belittle the remarkable inventiveness and achievements of the organic chemists who increasingly are succeeding in their efforts to design strategies to come up with novel DNA-reactive compounds for chemotherapy as well as other purposes.

Returning to the historical thread, we go back to the year 1961, which was when the first reasonably explicit model for binding of a drug to the double helix – the intercalation hypothesis – was proposed for the interaction of aminoacridines like proflavine with DNA [1]. It is no secret, though not often appreciated outside laboratories of molecular biology, that the notion of frameshift mutation furnished a degree of inspiration for the model. However, the idea of intercalation was not universally acclaimed: indeed it was greeted with profound skepticism in certain quarters. Lerman's original evidence, drawn from observations of changes in viscosity, sedimentation coefficient, and X-ray diffraction from oriented fibers of DNA, was perfectly reasonable so far as it went. But that was not far enough to satisfy many of the “real” structure solvers, who made it clear that they were not going to believe the postulate unless and until it had been verified by their own favorite “direct” technique as opposed to the admittedly rather indirect evidence adduced by Lerman. One of the present authors remembers conversations including such phrases as “do you believe in intercalation?,” as if it were an article of faith akin to religion. In due course, experiments were devised to verify or disprove the hypothesis, eventually to the satisfaction (or conversion) of the most hardened skeptics.

One early experiment was the circular DNA unwinding test, based upon the generally (but not quite universally) agreed expectation that intercalation must locally unwind the double helix [10]. It worked, and confirmed ethidium together with aminoacridines and several other interesting ligands, including actinomycin, as intercalators [11]. By the same token, antibiotics like netropsin and distamycin were identified as something else: minor groove binders as we now know [11]. It also became clear that different drugs unwind the helix by different angles when they intercalate, and the test even unearthed certain ligands that could unwind the double helix somewhat without apparently intercalating in the usual sense: steroidal diamines and triphenylmethane dyes [12, 13]. Questions still remain to be answered about these ligands. Of course a legacy of this early work is the detailed understanding of higher order structure, especially circularity, of DNA which studies on drug interactions have helped to elucidate. Thirty-five years after it was first shown to unwind circular DNA, ethidium is still routinely used to isolate plasmids.

Perhaps because of the seminal contributions of physical (bio)chemists during the early years of probing mechanisms of drug–nucleic acid interaction, the study of reaction kinetics soon emerged as a powerful tool for throwing light on the forces involved [14]. Don Crothers, an influential advocate of the kinetic approach, used to remark that the study of kinetics was uniquely valuable, if only because it added a new dimension – time – to the analysis of the phenomena. He was absolutely right. A highlight was the discovery that some ligands which bound well but not outrageously tightly to DNA could be characterized by on-rates and off-rates many orders of magnitude slower than ostensibly comparable substances. The anthracycline antibiotic nogalamycin is a good case in point; its slow association and dissociation kinetics are attributable to the disruption of base pairing needed to “thread” its bulky sugar substituents through the double helix [15]. Slow dissociation kinetics have been correlated with improved biological activity, and underlie the success of Phillips’ relatively recent assay for transcription termination at particular drug-binding sites on DNA [16]. Direct ligand transfer between binding sites on DNA without involving complete dissociation from the polymer was evidenced many years ago and has given rise to the “shuffling” concept whereby ligands are supposed to migrate one-dimensionally along a DNA molecule in search of better (tighter) binding sites [17, 18].

1.3

Specificity of Nucleotide Sequence Recognition

Although the value of DNA and, to a lesser extent, RNA as a target for selective drug action had been evident from the outset, it also quickly became apparent that few known drugs showed much, if any, selectivity for binding to particular nucleotide sequences. Yet the holy grail of selectively suppressing gene expression was conceived early on, together with the realization that to attain this end it would be necessary to recognize moderately long stretches of base pairs. Eventually it was

calculated that one might need to recognize a sequence composed of a number of base pairs in the high teens in order to identify a single targeted site in the human genome. The first experiments aimed at examining drug-binding preferences were crude and laborious to say the least, consisting of little more than attempts to detect different levels of binding to nucleic acids from different sources. Scatchard plots were employed to determine affinity constants, together with the frequency of binding sites, initially by simple and inappropriate means that were eventually much improved by better theoretical treatments like those of McGhee and von Hippel [19]. Sometimes the available methods (spectroscopy, equilibrium dialysis, etc.) were simply inapplicable because of the poor aqueous solubility of the ligands under investigation and alternative techniques had to be devised, such as solvent partition analysis used for the quinoxaline antibiotics [20]. Much effort was required just to establish a preference for, say, GC-rich DNA. Then the steady development of chemical methods for polynucleotide synthesis began to extend the range of synthetic, defined sequences available to the investigator and furnished substrates that could be used to examine whether or not a particular sequence would support interaction with a drug of interest.

A quantum leap occurred in the early 1980s with the invention of footprinting methodology in several laboratories at much the same time, using enzymes or Dervan's cleverly designed synthetic reagent MPE-Fe(II) to cut a cloned radio-labeled DNA fragment [21–23]. At a stroke it became possible to identify exactly where the preferred binding sites for a ligand were on a substrate that amounted to a real gene or a chosen fragment of a known gene. Although only semiquantitative at first, methods were quickly developed to adapt the technology to provide passable binding constants so that a true thermodynamic comparison of ligand affinity and capacity to discriminate between different sites could be gained in a single experiment or series of experiments. The power of the footprinting technique can be gauged from the reports of sequence-selectivity to be found in several chapters of this book. With its application, a substantial database of binding affinities for different sequences has been amassed, so that it is now becoming possible to enquire about general mechanisms that underlie the recognition of particular base-pair sequences, such as whether binding occurs predominantly in the major groove, the minor groove (much the most common with small molecules), or occasionally both.

Some workers have focused attention on the distinction between “digital” and “analog” readouts of sequence information, based on the notion that microstructural variation in the exact parameters of the double helix (groove width, for example) can sensitively reflect nucleotide sequence heterogeneity and therefore afford a means of sequence recognition that is independent of direct, specific contacts with the base pairs themselves. One of the techniques that can throw light on such questions involves looking at the behavior of DNA molecules containing unnatural nucleotide substitutions, which have the effect of shifting, removing, or adding specific base substituents. Such experiments have amply confirmed the dominant role of the 2-amino group of guanine in directing many ligands to their preferred binding sites, and have also thrown light upon related questions like the

role of base pair substituents in modulating groove width, reactivity towards alkylating agents, helix curvature or flexibility, and the sequence-dependent winding of DNA around the histone octamer in nucleosome core particles [24].

While footprinting and related gel methodology continues to play a major role in studies of this sort it has recently been joined by the elegant but simple method of competition dialysis, whereby the relative binding of a test ligand to many different types of nucleic acids can be assessed at the same time [25]. This method is of particular interest for investigating structure-specific binding of drugs to nucleic acids or indeed other polymers, whether natural or synthetic.

1.4

Details at the Atomic and Molecular Levels

Insight into the structure and dynamics of intercalation complexes has progressed by a close synergy between theoretical and experimental approaches, which today has developed to the point at which it is now possible to give a complete molecular description of what a drug–DNA complex looks like at the atomic level, and how its constituent atoms move in solution. The techniques that have proved invaluable in this quest are X-ray crystallography, NMR spectroscopy, quantum chemistry, molecular mechanics, and molecular dynamics. Lerman himself used X-ray fiber diffraction data from proflavine–DNA complexes as part of the initial evidence he marshaled for the intercalation hypothesis [1], and Fuller and Waring adopted the technique to produce the first molecular model of the ethidium–DNA complex [26].

These early attempts at model building took an important step forward at the beginning of the 1970s when Sobell and colleagues solved the crystal structure of a 2:1 actinomycin–deoxyguanosine complex, which enabled them to construct a fairly precise intercalation model based upon purely geometrical constraints [27]. Later in the 1970s the commercial availability of DNA and RNA dinucleoside monophosphates made possible crystallographic and NMR studies of intercalated mini-duplexes of ethidium and aminoacridines such as 9-aminoacridine, proflavine, and acridine orange. The crystallographic studies by Sobell, Neidle, Rich, and their colleagues provided the first truly atomic description of intercalation complexes, and unequivocally proved that the DNA duplex could indeed stretch so as to sandwich acridine and phenanthridine chromophores between two base pairs [28–30].

These were seminal studies that not only provided insight into the fine details of individual drug–DNA complexes, but also revealed modifications to the geometry of the sugar–phosphate backbone generally required to open the intercalation cavity. Armed with the latter information, Neidle and others were able to construct molecular models of suitably modified B- and A-DNA duplexes containing stereochemically sound intercalation cavities [31, 32]. This provided the means for many investigators, Neidle, Pullman, and Hopfinger prominent amongst them, to use

molecular mechanics and related methods to propose plausible structures for intercalation complexes of individual drugs [33–35].

Meanwhile, developments by Pullman and others in the application of quantum mechanical methods to determine the molecular electrostatic potential surrounding DNA, led to the important realization that the electrostatic field is strongly dependent on helical geometry, and that for B-type duplexes the field strength is strongest along the floor of the major and minor grooves [36, 37]. Moreover, it transpired that the intensity of the field in the grooves is sequence-dependent, so that for some sequences the potential is greatest in the minor groove, whereas for others, the global maximum is to be found in the major groove [36, 37]. Naturally, these sites of maximum potential provide “hotspots” for binding of cations, and positively charged, or highly dipolar, ligands. Interestingly, these studies also made clear that there are strong end effects for short oligonucleotides, so that the field strength is greatest in the middle of a short DNA fragment [36, 37]: a phenomenon that provides a potential motive force for driving the movement of DNA-bound ligands along the helix. These studies, in common with molecular mechanics-based modeling, emphasized the theoretical importance of electrostatics in determining the mode and strength of DNA–ligand interactions, and provided general insights into how intercalating agents and minor groove binders might interact with DNA.

In parallel work, Manning, Record, and their colleagues applied polyelectrolyte theory to DNA in order to establish a theoretical framework for interpreting the experimental observation that the DNA affinity of positively charged ligands is strongly ionic strength-dependent [38, 39]. Polyelectrolyte theory provides the fundamental insight that the electrostatic field of DNA causes the condensation of mobile cations in the region of its surface, so that for a B-type duplex the local concentration is about 1 M. It is the displacement of these mobile cations into the bulk solvent of lower ionic strength by fixed positively charged ligands that provides the entropic source that drives the observed dependency of affinity on salt concentration. In this way, polyelectrolyte theory links DNA molecular electrostatic potential and thermodynamics to enable a comprehensive description of the major forces that hold DNA–ligand complexes together: a union that Wilson, Chaires, Graves, and many others have used to great effect [40–42].

Structural studies made another quantum leap in the 1980s, courtesy of organic chemists, who provided the means to synthesize longer DNA fragments, initially in solution, and later by solid phase methods. The availability of self-complementary hexa-, octa-, and dodecanucleotides led to a truly wonderful explosion of crystallographic and NMR studies of DNA complexes containing intercalators and minor groove binders. At this time we were treated to crystal structures of the complexes of the quinoxaline bisintercalators echinomycin and triostin A, complete with a switch from Watson–Crick to Hoogsteen pairing in the base pairs immediately flanking the intercalated chromophores [43, 44], a multitude of complexes of anthracyclines related to adriamycin and daunomycin [e.g. 45], and to the complexes of the threading anthracycline nogalamycin [46]. A little later came the actinomycin–DNA structures [47]. It was at this time that NMR spectroscopy be-

gan to reveal its true power in providing near-atomic resolution structures in solution, a result of the union of NOESY data with molecular dynamics simulations, and we saw consonance with the crystallographic structures for the quinoxaline antibiotics nogalamycin, actinomycin, and the anthracyclines [48–52].

NMR even began to steal a march on crystallography by yielding solution structures of the luzopeptin, chromomycin, and pluramycin DNA complexes [53–55]. We still await the crystal structures. Simultaneously, much progress was made in the crystal and solution structures of DNA complexes of a multitude of minor groove-binding ligands encompassing oligopyrrole ligands such as netropsin and distamycin, bisbenzimidazoles related to Hoechst 33258, bisquaternary ligands and the bisamidine ligands related to pentamidine [56–58]. The NMR studies of these systems also provided information concerning the internal dynamics of the complexes, such as base pair breathing rates, and on occasions could define binding kinetics as well [59–61].

As the 1980s moved into the 1990s, developments in quantum mechanics and molecular dynamics began to lift traditional molecular modeling, based primarily on molecular mechanics calculations or rudimentary molecular dynamics performed *in vacuo*, out of the realm of the purely theoretical into simulations that can provide realistic descriptions of the complete drug:DNA:solvent:cation system. These calculations have progressed in the hands of the NMR spectroscopists and enlightened molecular modelers to the point where we can now determine atomic charge distributions by *ab initio* quantum mechanical methods, and formulate molecular dynamics simulations using more reliable force-fields that include the DNA, the drug, water, and cations [62–66]. In addition to the obvious advantages that these new developments bring by including solvent and counterion to deal properly with electrostatics and solvation, most significantly they intrinsically include hydrophobic bonding and entropy effects. We often forget the importance of these latter phenomena, because most DNA-binding ligands are charged, but should remember that they frequently dominate the energetics of drug–receptor complexes in the wider world of pharmacology, and are particularly significant in determining the DNA affinity of neutral ligands such as actinomycin, echinomycin, and luzopeptin.

Much of the fundamental work in this area was pioneered by Peter Kollman, who provided the community with AMBER, one of the most important computation tools [67]. His recent sudden death in his prime is a sad loss for us all, and he will be sorely missed by colleagues grateful for his contribution.

So, today, we stand in the happy position of being able to recruit crystallography, NMR spectroscopy, and molecular dynamics to provide a true atomic description for practically any DNA–ligand system. The limitations on the experiments are that the complex must crystallize and diffract, or, for NMR, the lifetime of the complex must be sufficiently long to allow build-up of NOE signals before the ensemble dissociates. The limitations on molecular dynamics are now reduced to concerns about computational power: a problem that is rapidly vanishing for most laboratories. Recent achievements of note are the crystal structures of a bisanthracycline complex [68], a DNA–PNA triplex [69], a PNA duplex [70], complexes

with the sequence-selective hairpin polyamide ligands of Dervan [71, 72], and complexes of the acridinecarboxamide topoisomerase poisons [73, 74]. These works afford eloquent testimony to the current sophistication of our field, and have provided profound insights into the molecular determinants of drug–DNA complexes that confer upon the ligands their important biological properties.

One of the greatest surprises of recent times is the curious structure formed by the acridinecarboxamides, and similar ligands, with $d(\text{CGTACG})_2$ in which four duplexes come together to exchange, by strand invasion, their terminal cytosine bases and to provide an intercalation cavity that accommodates two drug molecules [75, 76]. This structure is surely beyond our wildest imaginings and one can only wonder at the marvels of the solid state. However, it serves to remind us that we should be cognisant of the possibility of unexpected structures in circumstances where concentrations are high, water activity low, and mobility restricted: the cellular nucleus, for example.

A newcomer to the scene is atomic (scanning) force microscopy which promises to revolutionize the study of ligand–receptor interactions in general, including drug–nucleic acid complexes. Although barely out of its infancy, AFM has already produced excellent images of single DNA molecules with or without bound drugs. These studies have convincingly illustrated the changes in contour length and altered supercoiling of circular DNA caused by intercalative binding [77]. The singular advantages of AFM lie in its ability to acquire images in vacuum, under liquid, or in air at ambient, elevated, or cryogenic temperatures – not to mention the extreme economy in its requirements for materials when single molecules are the objects of examination. As the technology evolves to produce better images at higher resolution we can expect to admire genuine pictures of drugs attached to nucleic acid molecules under “wet” conditions in an aqueous environment. Perhaps what readers of this book have been seeing in their mind’s eye for many years.

1.5

Identification of Motifs for Drug Design

As we noted at the beginning of this chapter, medicinal chemists have constantly sought pointers to mechanisms that might serve as a springboard for drug design, and latterly have been keeping a watchful eye on motifs arising from structural work that might provide a rational basis for designing new nucleic acid-binding ligands. Since the middle of the twentieth century numerous pointers or “leads” have emerged from the study of nucleic acids and their interactions with small molecules. At first the intercalation hypothesis itself attracted attention to the peculiar susceptibility of helical structures to deformation by binding of planar aromatic molecules, and the manifest existence of several useful drugs acting in this way encouraged the design and synthesis of many more. The endeavor received something of a boost with the discovery 13 or 14 years later of bis-intercalation, by echinomycin [78] and diacridines [79, 80] in particular, that

clearly indicated the potential advantages of tighter binding and sequence-selectivity to be gained by synthesizing novel bis-intercalators. This was a task comfortably within the capabilities of organic chemists, and also afforded opportunities for early computer modelers to flex their muscles. The need to respect the limitations of the neighbor exclusion hypothesis [81] was recognized along with a whole new area of structure–activity relations. Sadly, despite considerable effort few synthetic bis-intercalators ever reached the clinic.

In parallel, attempts were made to modify classical minor groove binders so as to endow them with the capacity to recognize GC as well as AT base pairs, termed lexitropsins, but still no new drugs emerged [82]. Next came combilexins, in which intercalators were combined with other DNA-interactive moieties such as minor groove binders, alkylators and free radical generators or other mediators of polynucleotide strand cleavage [83–85]. Notwithstanding the failure of these programs to deliver quickly a new generation of wonder drugs (a notion which appears unrealistically optimistic in retrospect), they helped to build up a strong corpus of knowledge and experience which will have a lasting influence on the business of drug design.

As the concept of specific gene targeting gained momentum in the last years of the 1990s, four fresh approaches to the recognition of biologically meaningful sequences of base pairs made their appearance. The first was triple helix formation, dating back to a very early observation from Rich's laboratory in 1957, in which a third polynucleotide strand sequence specifically occupies the major groove of the double helix. Its most common manifestation relies upon Hoogsteen pairing with the intact Watson–Crick base pairs and is restricted to polypurine tracts in the DNA target, but other motifs are possible and drug stabilization of such structures is important (see Chapter 14). The second approach, invented by Ole Buchardt in Copenhagen and now championed by Peter Nielsen [86] uses synthetic polyamide (or peptide) mimics of polynucleotides, termed PNA, to form double-stranded, triple-stranded, or strand-displacement complexes with a DNA target. The third approach, with its origins in the zinc-finger motif identified by Aaron Klug and his colleagues as underlying the sequence-specific recognition of DNA-response elements by many proteins including hormone receptors and transcription factors, relies upon exploiting the natural motif(s) employed by peptides and proteins to recognize DNA [87]. More can be read about this topic in Chapter 16.

The fourth approach, arising from a chance observation that the minor groove binder distamycin could form a 2:1 complex with double-helical DNA by squeezing two antibiotic molecules side-by-side into the minor groove [88] was immediately recognized by Peter Dervan as a new motif endowed with tremendous potential for generating sequence readout by application of a new twist to the old lexitrop-sin concept. Dervan synthesized dimeric derivatives of *N*-methylpyrrole and *N*-methylimidazole ring-containing polyamides linked like a hairpin, showed that they could recognize and bind to targeted DNA sequences with subnanomolar affinity, and formulated a set of pairing rules governing the recognition of all four Watson–Crick base pairs in the minor groove [89]. Recently it has been found that

a similar motif is applicable to synthetic diamidine minor groove binders as recounted in Chapter 1.

1.6

Actions on Nucleoproteins, Chromatin, and Enzymes

Much of the most recent research on small molecule DNA and RNA binders has, quite rightly, been devoted to studies on more biologically relevant systems – building upon the detailed chemistry and physico-chemical principles established in studies on interactions with purified nucleic acids occupying the last four decades of the twentieth century. During the latter part of that period advances in biochemistry and cell biology rendered it possible to begin to make serious efforts aimed at understanding how drugs might affect nucleic acids in their native state – particularly the nucleoproteins of the cell nucleus. Early experiments on chromatin–drug interactions had generally been inconclusive to say the least, but with the rapid advances that followed the isolation of nucleosome core particles, together with better understanding of chromatin structure, it became feasible to design experiments with nucleic acid-binding drugs that might yield useful, concrete information.

Of course, binding of many ligands to the DNA in chromatin or core particles often does little more than disrupt the DNA–protein association so that the integrity of the structure is gradually impaired and eventually lost, releasing the DNA (presumably with lots of drug bound) from the histones and any other chromosomal proteins. However, some drugs do seem to have a peculiar effect on the winding of DNA around the histone octamer at moderate concentrations, provoking a change in the rotational orientation as judged by hydroxyl radical or enzyme attack. The altered rotational orientation may be as much as 180 degrees, and a similar effect can be produced by incorporating unnatural nucleotides into the DNA which alter its flexibility [90, 91]. Needless to say, the local unwinding of the double helix associated with intercalation could play a significant part in this phenomenon, though some minor groove binders can produce it too.

More recently the involvement of specific enzymes in the correct functioning, packaging, and maintenance of chromosome structure has been clarified to the extent that novel drug actions of great therapeutic importance and promise can be envisaged. We have already referred to the transcription stop assay that can be used to determine drug-binding sites where RNA polymerase activity is terminated most effectively [16]. Another enzyme of crucial importance for the functioning of chromosomes in replication and transcription is topoisomerase, whose two principal forms (topo I and topo II α and β) are both targets for drug attack via inhibition or poisoning. Small molecules that act on these enzymes, several of them high on the list of newly developed anticancer agents, are described in detail in Chapters 19, 20, and 21. But perhaps the most interesting and remarkable enzyme to have emerged as a novel drug target is telomerase, the enzyme responsible for the

maintenance of chromosome ends in eukaryotes, which is a reverse transcriptase that operates to extend the telomeric structures at the ends of chromosomes. Those structures are characterized by multiple repeats of a short DNA sequence rich in guanine nucleotides which predisposes them to form peculiar quadruplex structures that represent a potential target for attachment of drugs, thereby inhibiting telomerase activity and the preservation of chromosome ends. Since telomere maintenance appears to be correlated with malignancy, the G-quadruplex structures could offer a marvelous target for selective suppression of cancer cells as described in Chapters 12 and 13. where drug design strategies are described using molecular modeling based upon the accumulated experience recounted throughout this book.

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2

Targeting HIV RNA with Small Molecules

Nathan W. Luedtke and Yitzhak Tor

2.1

Introduction

The central dogma of biology states that RNA is selectively transcribed from DNA and serves as a messenger to be translated into proteins [1, 2]. Recent discoveries detailing the molecular workings of the cell have inspired new generations of chemists to search for a deeper understanding of the biophysical world. The majority of this book presents important advances in the study of DNA; this chapter is one of the few to explore RNA. We have, therefore, taken the liberty of presenting a general background on why RNA is such a fascinating biomolecule and why RNA small molecule recognition holds great promise for the future.

Far from being a passive carrier of genetic code, RNA is intimately involved in a wide range of biological processes including chemical catalysis and information storage. The vast majority of cellular RNAs form higher order structures with other cellular components to facilitate the exchange of biochemical information. Successful molecular recognition of RNA often precedes catalytic events that are essential to a wide range of cellular activities, including: initiation of DNA replication [3], extension of the telomeric regions of chromosomes [4], splicing of pre-mRNA [5], and iron chelation [6]. In addition, RNA serves as the primary genome of most pathogenic viruses [7].

2.1.1

Translation

Unique patterns of gene expression rely upon an interplay between recognition events and catalytic activities of RNA–protein complexes. Ribosomal RNA accounts for the vast majority of total cellular RNA (80%) and provides both the molecular scaffold and enzymatic activities needed for protein translation [8]. The key step of translation occurs in the ribosome's A-site, where codon–anticodon recognition decodes the mRNA. Upon a correct codon–anticodon match between mRNA and the anticodon loop of tRNA, the ribosome's peptidyltransferase activity catalyzes the formation of a new peptide bond between the amino acid-charged tRNA in the A-site and the growing protein chain on the tRNA in the P-site. Studies have

shown that the prokaryotic ribosomes stripped of protein are still capable of limited peptidyltransferase activity [9]. In accordance with this result, recent crystal structures show that the peptidyltransferase active site is composed entirely of rRNA [10]. A single, unusually basic adenosine may be the key player in the mechanism of peptidyl transfer [11, 12].

Transfer RNAs, at 15% of total cellular RNA, are the most common type of “soluble” RNA (i.e. lacking associated protein). The binding of tRNA to the ribosomal A-site is mediated by extensive RNA–RNA interactions (including rRNA–tRNA, and mRNA–tRNA binding). Through these and other important interactions, the ribosome can distinguish the relatively small energetic differences between cognate and non-cognate codon–anticodon pairing to achieve an astounding fidelity of translation (over 99.9% accuracy) [13].

The transport, translation efficiency, and stability of individual messenger RNAs are controlled by numerous protein–RNA, ribonucleoprotein–RNA, and RNA–RNA interactions [14–16]. Upon transcription from DNA, ribonucleoprotein complexes called spliceosomes excise the introns from pre-mRNA and from other heterogeneous RNAs. Some organisms are capable of intron excision (splicing) without protein assistance, and provided the first examples of RNA enzymes (or ribozymes) [17]. The translation efficiency of individual mRNAs is regulated at many levels, including the binding of the 5′ and 3′ untranslated regions (UTRs) of the mRNA by proteins [18], microRNAs [19], and even small molecules [20].

2.1.2

RNA Viruses

Viral epidemics have accounted for more human deaths than all known wars and famine combined. Over 65% of the known families of viruses use RNA for a primary genome and cause many of the modern-day plagues, including: AIDS, cancer, hepatitis, smallpox, ebola, and influenza [7]. Most viruses are, however, benign. Approximately 42% of the human genome is composed of transposable elements that multiply by reverse transcription, using an RNA intermediate similar to that of a retrovirus [21]. In general, reverse transcription is a highly error-prone process allowing viral elements to evolve rapidly under selective pressures (such as antiviral drugs). About 8% of the human genome is made up of repetitive genomic elements known as “retrovirus-like elements” [21]. Their structures closely resemble those of retroviruses, carrying the open reading frames common to all retroviruses (Gag, Pol, Env) flanked by 5′ and 3′ long terminal repeats. Overall, the human genome is composed of approximately 50% self-repeating, parasitic sequences. Compare this with the unique (non-repeated) genes, encoding for only ~5% of the human genome!

2.2

Small Molecules that Modulate RNA Activity

The ability of RNA to facilitate the essential biochemical activities needed for information storage, signal transduction, replication, and enzymatic catalysis has

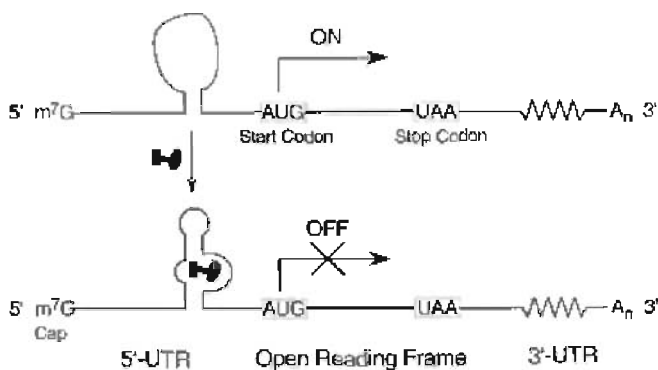


Fig. 2.1. The mature mRNA of an artificial gene construct is actively translated in the absence of small-molecule binding. Upon binding of the aptamer by its cognate small molecule, the translation of a reporter gene is deactivated.

distinguished it as a good candidate for being the central biomolecule in a prebiotic world [22]. If such an “RNA world” did exist, then small molecule–RNA interactions most likely played a key role in the regulation of RNA replication and in modulating RNA enzymatic activity [23].

An impressive conceptual proof demonstrating the ability of small organic molecules to regulate gene expression *in vivo* has recently been illustrated in the context of an artificial gene construct [20]. An RNA aptamer located in the 5' UTR region of an mRNA, has been shown to inactivate translation of a downstream open reading frame upon the binding of its cognate small molecule (Fig. 2.1). (Note: Aptamers are RNA sequences that are selected from large libraries of RNA sequences for affinity to a predetermined small molecule. Through multiple rounds of mutation, selection, and amplification, the evolution of an RNA sequence to fit onto a predetermined small molecule can be effected. Selected sequences often demonstrate truly astonishing affinity and specificity to their cognate small molecule.) The mechanism proposed for translation inactivation involves the structural rearrangement of the aptamer into a rigid RNA–small molecule complex that cannot be correctly scanned by the ribosomal pre-initiation machinery. Natural systems have also provided examples of how small molecule–RNA binding (often accompanied by RNA structural rearrangements) can modulate RNA activity.

2.2.1

Magnesium (II)

Much like proteins, the primary sequence of an RNA directs its folding into a unique three-dimensional structure [24]. Correct RNA folding, however, typically relies upon the binding of divalent metal ions (especially Mg²⁺). Mg²⁺ exhibits a low to moderate affinity to many unrelated RNAs. Mg²⁺ binding affinities (*K_d*) range from 0.01 mM through 10 mM in the presence of 0.1–0.2 M of monovalent ions [25, 26]. Given the three-dimensional structure of an RNA, an electrostatic

contour map can be calculated, allowing for the theoretical prediction of the higher affinity Mg^{2+} -binding sites [27, 28].

The Mg^{2+} -induced folding of the *Tetrahymena thermophila* group 1 intron has become an important paradigm for RNA folding [29–31]. In the absence of Mg^{2+} , it adopts flexible structures dominated by duplex regions that are interrupted by internal bulges and stem loops. This secondary structure can largely be predicted from its nucleotide sequence using base-pairing and nearest neighbor rules [32–34]. Upon Mg^{2+} binding, it collapses into a more rigid, enzymatically active, tertiary structure with fewer available conformations. In at least one region of the group 1 intron, Mg^{2+} binding induces a rearrangement of the RNA secondary structure itself [35]. Our limited understanding of cation-binding interactions remains a major obstacle in the accurate prediction of a three-dimensional RNA structure given only its sequence.

There are some cases where small molecules other than metal cations can be used to facilitate the folding and enzymatic activity of RNA. Linear polyamines (like spermine) and aminoglycosides, displace Mg^{2+} from RNA, and have been shown to directly facilitate enzymatic activity of the hairpin and hammerhead ribozymes in the absence of divalent metal ions [36, 37].

2.2.2

Aminoglycosides

Aminoglycoside antibiotics are a diverse family of natural products that interfere with prokaryotic protein biosynthesis (Fig. 2.2). Their ability to non-specifically bind to RNA through electrostatic interactions was described over 20 years ago [39]. The aminoglycosides are also capable, however, of site-specific recognition of prokaryotic rRNA. Footprinting experiments indicated that the aminoglycosides bind to discrete locations within the ribosome [40]. Later experiments showed that aminoglycosides increase the affinity of tRNA to the ribosomal A-site [41], thus providing an attractive mechanism to explain their ability to decrease the fidelity of prokaryotic translation [42]. A recent crystal structure of three aminoglycosides (streptomycin, paromomycin, and spectinomycin) bound to the *Thermus thermophilus* 30S ribosomal subunit confirms the location of these distinct binding sites and provides a high-resolution picture of how RNA–aminoglycoside recognition occurs within a ribonucleoprotein complex [43]. This type of structural information will likely prove indispensable for structure-based design of aminoglycoside derivatives that will have an improved “fit” within their ribosomal binding pockets. Structural information cannot, however, answer basic questions related to the energetics involved in the binding of small molecules to their RNA “receptor.” Equilibrium binding constants must be measured in order to establish the actual energetic values associated with RNA–small molecule interactions.

Despite the structural details provided by aminoglycoside–RNA complexes, the energetic contributions made by the pendant hydroxyl groups of the aminoglycosides remain unclear. Hydrogen bonding between these groups and RNA are apparent in some structures [44], but the energetic contributions of hydrogen bond-

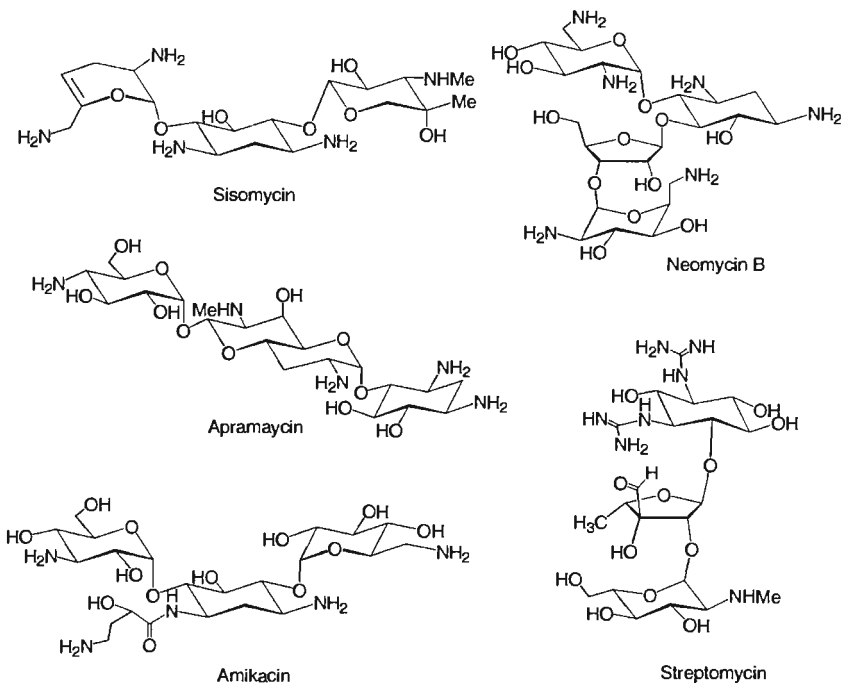


Fig. 2.2. Representative aminoglycosides. Five out of the six amino groups of neomycin B have pK_a values over 7, giving it a highly positive charge under physiological conditions [38].

ing in aqueous media is still debated [45]. In an attempt to determine their role in RNA affinity, the hydroxyl groups of tobramycin were systematically removed (Fig. 2.3). RNA binding was tested by measuring the HH16 ribozyme inhibitory activity of each aminoglycoside derivative [46]. Interestingly, the removal of

The chemical structure of tobramycin is shown with labels R¹, R², R³, and R⁴ indicating the positions of the hydroxyl groups on the 2-deoxy-4-amino-2,3,6-trideoxyribose moiety. R¹ is at the 2' position, R² is at the 3' position, R³ is at the 4' position, and R⁴ is at the 6' position.

Aminoglycoside	R ¹	R ²	R ³	R ⁴	Relative Ribozyme Cleavage Rate
Tobramycin	OH	OH	OH	OH	1.0
6"-Deoxytobramycin	H	OH	OH	OH	1.4
4"-Deoxytobramycin	OH	H	OH	OH	0.33
2"-Deoxytobramycin	OH	OH	H	OH	0.17
4'-Deoxytobramycin	OH	OH	OH	H	0.33

Fig. 2.3. Summary of hammerhead inhibition by deoxyto-
bramycin derivatives. A lower relative rate suggests better
RNA binding.

hydroxyls at positions R², R³, and R⁴ (Fig. 2.3) lead to aminoglycosides with *better* RNA binding. The current explanation of this “unexpected” result is that the hydroxyl groups decrease the basicity of neighboring amines. Removing certain hydroxyls, therefore, increases the overall positive charge, and hence RNA affinity of the resulting molecules. These dehydroxylated aminoglycosides have not yet been tested for the binding of other RNAs, so the roles of the hydroxyls in RNA specificity remain unknown.

2.2.3

Ligand Specificity

For the purpose of this discussion, specificity will be defined as the binding affinity (K_{eq}) of a small molecule to a particular RNA site divided by its average affinity to “all” other RNAs.

$$\text{Specificity} = \frac{K_{eq}(\text{interaction of interest})}{\text{average } K_{eq}(\text{other sites of interaction})} \quad (2.1)$$

For practical reasons, specificity is a relative term, where the affinity of the interaction of interest is weighted by the affinity of that same small molecule to “other” nucleic acids. The reported specificity is, therefore, always dependent on the selection of the “other” nucleic acids used for the comparison. In general, larger RNA ligands have a greater potential to exhibit higher RNA specificity. This explains why metal ions typically show a very low specificity for any single RNA.

High specificity is an essential factor for the effective modulation of *in vivo* RNA activity. Specificity is directly related to both bioavailability and receptor occupancy. Aminoglycosides, for example, are not ideal antibiotics. Their promiscuous binding to non-ribosomal RNAs and/or membrane components may be related to the multiple therapeutic side effects exhibited by these compounds [47–49]. Aminoglycosides bind to and inhibit the function of a wide range of unrelated RNAs with moderate activities ($IC_{50} = 0.1\text{--}100 \mu\text{M}$) [23, 50]. Aminoglycosides do, however, show excellent specificity for RNA over DNA (Section 2.5.3). Aminoglycosides have, therefore, served as suitable scaffolds for the synthesis of new small molecules targeted to RNA. These derivatives are shown to exhibit dramatically different RNA specificities and altered biological activities as compared to their parent aminoglycosides.

2.2.4

Goals

Notwithstanding the efforts of many groups towards the understanding of how small molecules bind to RNA, there are still no “rules” for the structure-based design of small molecules for a specific RNA tertiary fold. One obstacle is that there are still very few examples of small molecules that bind to natural RNA structures

with high specificity. There are other potential reasons as well. For example, RNA is a highly dynamic molecule known to occupy multiple conformations. The structural details of an RNA do not typically entail the potential structural changes it can adopt upon ligand binding. Issues related to induced fit add additional complexity to the problem.

The following fundamental questions remain largely unanswered:

- How do electrostatic interactions affect the RNA specificity of a small ligand?
- How does one design small RNA ligands that exhibit high specificity for a pre-determined RNA target?

To help answer these questions, we have addressed a number of goals:

1. To design and synthesize new small molecules that are targeted to a pre-determined RNA site, in this case the HIV-1 RRE (Rev Response Element).
2. To use novel fluorescence-based methodologies to rapidly characterize the affinity and specificity of the new RRE ligands.
3. To conduct experiments in a systematic fashion so that general questions about RNA–small molecule recognition can be rigorously addressed.

To evaluate the “higher order” biological impacts of RNA binding, we have chosen the HIV-1 Rev–RRE interaction as our model system. This way, new RNA ligands that show promising activities, may eventually prove themselves as future antiviral agents. Our work, along with effort by many other groups, contributes to the growing body of knowledge that aids in the future rational design and use of small molecules directed to RNA [51–53].

2.3

The RRE and HIV Replication

The Rev Response Element (RRE) is an HIV-1 RNA structure essential for viral replication [54–56]. The Rev protein binds to the RRE and facilitates the export of HIV RNA out of the host nucleus, while protecting it from the cell's splicing machinery (Fig. 2.4). The importance of the “underspliced” HIV RNA is two-fold. First, this RNA serves as an open reading frame for proteins that are essential to the construction of new viral particles (including Gag, Pol, and Env). Second, the unspliced HIV RNA is packaged into the outgoing viroids, serving as the primary genome. Successful inhibition of Rev–RRE binding, therefore, prevents the production of new viral particles in two ways: the proteins essential for viroid construction are never translated, and the future HIV genomic RNA becomes highly spliced.

The RRE serves both as the high-affinity Rev-binding site and as part of a highly conserved open reading frame for the fusion domain of the gp41 Env protein (essential for the CD4-dependent viral invasion of the host cell) [57]. This dual function is likely responsible for the RRE's low mutation rate [58]. The RRE is, therefore, a highly attractive target for small molecule therapeutics since mutations that

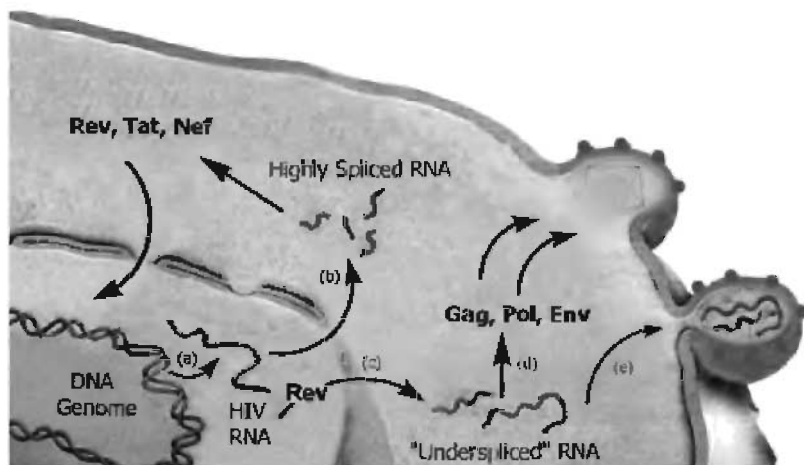


Fig. 2.4. Production of viral particles in an HIV-infected cell. (a) The Tat–TAR interaction facilitates the transcription of viral RNA. (b) In the absence of Rev activity, the HIV RNA becomes highly spliced and the proteins Rev, Tat, and Nef are translated. (c) HIV’s “late”

replication phase is initiated by the Rev–RRE interaction. (d) From the unspliced and singly spliced HIV RNA, the proteins Gag, Pol, and Env are translated. (e) Unspliced RNA is packaged into outgoing viral particles.

can lead to drug resistance should be impeded. Through the efforts of a number of research groups, many details regarding the sequences, structures, and dynamics of the Rev–RRE interaction have been revealed [54–56]. The 116-amino-acid Rev protein has at least three functional domains, including an arginine-rich motif spanning amino acids 34–50 (Fig. 2.5a). This positively charged domain binds to a single high-affinity site on the RRE (bold in Fig. 2.5b) with high affinity ($K_d = \sim 1$ nM) and high specificity. Additional Rev molecules then polymerize along the length of the RRE by utilizing both protein–protein and protein–RNA interactions. The oligomerization of Rev along the RRE is essential for Rev’s ability to transport the HIV genome out of the host’s nucleus while protecting it from the host’s splicing machinery.

If the Rev protein cannot bind to its high-affinity site on the RRE, subsequent Rev molecules do not bind along the length of RRE and the entire HIV genome becomes highly spliced, thereby breaking the viral replication cycle. For this reason, we have chosen the high-affinity Rev binding site on the RRE (bold in Fig. 2.5b and henceforth referred to as “the” RRE) as our target for small molecules.

2.4

Determination of RRE–Ligand Affinity and Specificity

Numerous methods are available for evaluating a small molecule’s ability to inhibit the binding of two macromolecules. Traditional techniques, such as native gel-shift electrophoresis, analytical ultracentrifugation, and equilibrium dialysis are limited

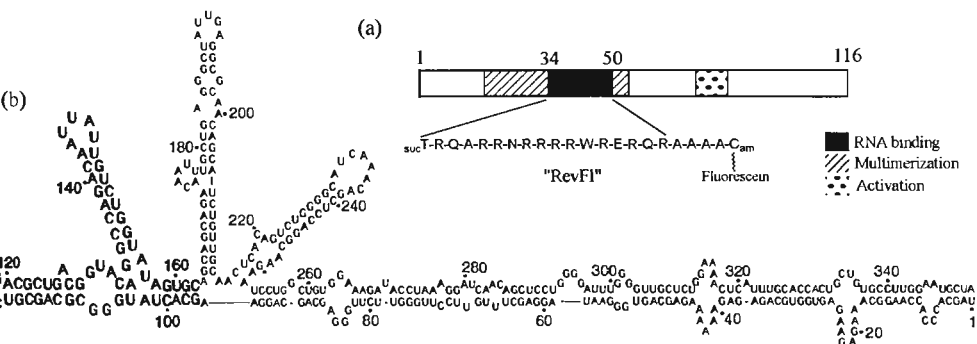


Fig. 2.5. (a) A domain map of Rev protein is shown with the sequence of its RRE-binding domain (amino acids 34–50). The peptide RevFl is used for fluorescence-based assays and is succinylated at its N-terminus, amidated at its C-terminus, and contains a four-alanine spacer to fluorescein. These modifications have

been shown to increase the helicity and RRE specificity of this peptide [59]. (b) The RRE secondary structure as defined by Mann [60] spans a total of 351 bases and contains a single high-affinity Rev-binding site (the bold G-rich bulge contained between bases 100 and 160).

in their inability to rapidly screen large numbers of potential inhibitors. Newer methodologies such as mass spectrometry and surface plasmon resonance are powerful tools, but hardware costs can be problematic. Fluorescence-based techniques have proven to be highly sensitive, versatile, and relatively inexpensive for the examination of RNA–ligand interactions [61–63]. Three fluorescence-based techniques are described for evaluation of the RRE affinity and specificity of natural and synthetic Rev–RRE inhibitors.

2.4.1

Fluorescence Anisotropy

Fluorescence anisotropy can be used to measure a small molecule's ability to inhibit the Rev–RRE interaction. The anisotropy value is directly proportional to the tumbling rate of a fluorescein-labeled Rev_{34–50} peptide "RevFl" (Fig. 2.5a). As RRE is added to the fluorescent Rev peptide, their association is observed by an increased anisotropy value (Fig. 2.6a). The increase is due to the slower tumbling rate of the RevFl–RRE complex compared to that of the free RevFl.

Once the RevFl–RRE complex is formed (indicated by high anisotropy values), an inhibitor "X" can be added. Competitive inhibitors will bind to the free RRE, and shift the three-way binding equilibrium towards release of the RevFl peptide from the RevFl–RRE complex (causing a decreased anisotropy of RevFl) (Fig. 2.6b). If the number of inhibitor-binding sites on the RRE is established by an independent method, the absolute affinity of the RRE–"X" binding interaction can be calculated (K_i is equivalent to K_d). In most cases, however, the binding stoichiometry of the inhibitor is not known, so its affinity to the RRE is evaluated by measuring the concentration of inhibitor needed to displace half of the Rev peptide from the RRE (defined as the ligand's IC_{50} value).

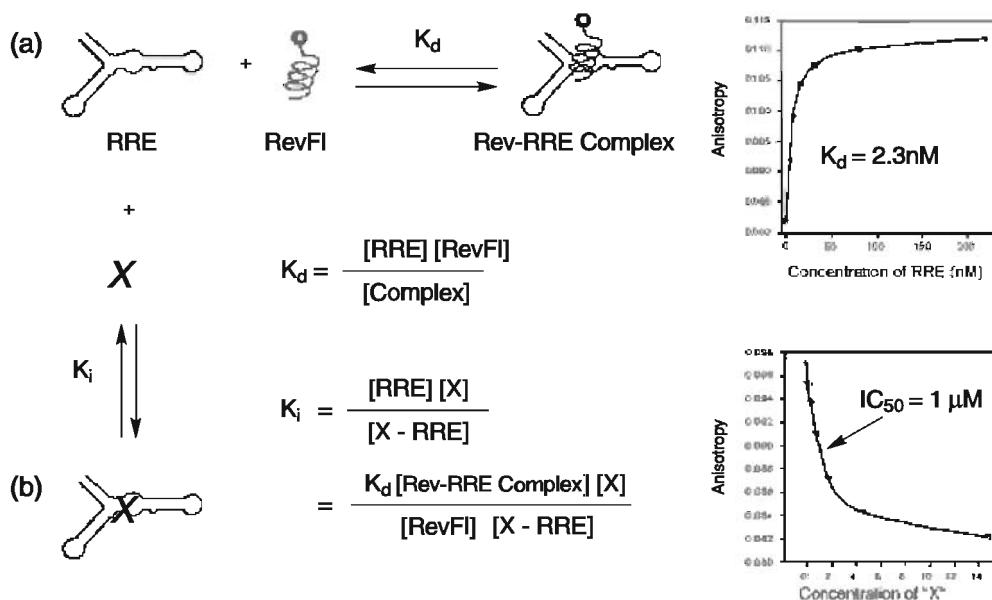


Fig. 2.6. (a) Association of RevFI with the RRE as evident by changes in the fluorescence anisotropy of RevFI. Since this is a simple two-state system, the change in fluorescence anisotropy is directly proportional to the fraction of RevFI bound by the RRE. By non-linear regression, analysis of the association data yields a binding constant for Rev–RRE

that is similar to the Rev protein (K_d approx. 2 nM). (b) Upon partial formation of the RevFI–RRE complex (using 10 nM RRE), an inhibitor “X” can be titrated. Binding of the RRE by “X” (in this case neomycin B) is apparent by the concentration-dependent decrease of RevFI’s anisotropy signal back to its value when free in solution.

2.4.2

Solid-Phase (Affinity-Displacement) Assay

A novel biophysical approach has been developed to rapidly characterize the relative affinity *and* specificity of a small molecule for the RRE [64]. Following immobilization of a biotinylated RRE transcript onto an insoluble solid support, one equivalent of the fluorescent Rev protein fragment “RevFI” is added. RevFI binds to the immobilized RRE with an affinity the same as measured by fluorescence anisotropy (K_d 2 nM). (Note: Calculating the binding constant of Rev–RRE from solid-phase data is different from in solution, because the volume occupied by the unbound RRE is the same volume occupied by the Rev–RRE complex, so that the actual surface volume does not matter. The $K_d(\text{solid phase}) = (\text{moles of free RRE}) \times (\text{concentration of RevFI in solution}) / (\text{moles of RevFI–RRE complex})$.) The fraction of RevFI bound to the immobilized RRE is easily determined by measuring the fluorescence intensity of the RevFI bound to the solid support versus that free in solution (Fig. 2.7a). As in solution, the immobilized Rev–RRE forms a highly specific complex that is not disrupted by addition of a vast excess of other nucleic acids (including DNA, tRNA, and polyA–polyU duplex RNA). (Note: We

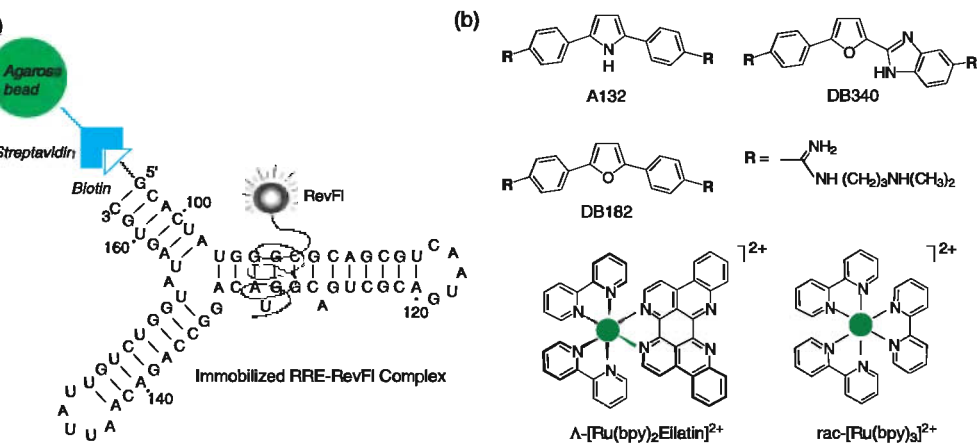


Fig. 2.7. (a) Immobilized Rev-RRE Complex. (b) Some of the compounds used to evaluate the solid-phase assay.

feel that immobilization of the RNA to the solid support by site-specific linkage through biotin-streptavidin provides a high level of environmental homogeneity for each RNA transcript, and that interactions between neighboring RNA transcripts do not occur. The bulky, hydrated matrix components (provided by agarose and streptavidin) are ideal conditions for the study of biologically relevant interactions (as proven by the countless macromolecular binding studies by ELISA and other related techniques.)

Following formation of the immobilized Rev-RRE complex, small molecule inhibitors are titrated in, and the fraction of RevFl free in solution is monitored to determine an IC_{50} value. Most biophysical methods, including fluorescence anisotropy, are not amenable to the analysis of complex mixtures of biomolecules or fluorescent ligands. Fluorescence anisotropy is found to be susceptible to artifacts when used to evaluate ligands that are fluorescent and/or quench the fluorescence of RevFl. The solid-phase assay has been shown to overcome these challenges and to produce displacement data that are consistent with native gel-shift electrophoresis experiments (Tab. 2.1). Another advantage of the solid-phase assay is that the specificity of an RRE ligand can be evaluated by comparing its activity in the

Tab. 2.1. IC_{50} values (μM) for Rev displacement by three methods.

Compound	Fluorescence anisotropy	Solid-phase assay	Gel-shift electrophoresis
Neomycin B	7	7	~7
A132	10	1.2	~1.2
DB182	1.5	0.4	~0.4
DB340	0.3	0.1	<0.1
$\text{rac-Ru}(\text{bpy})_3$	170	>10,000	>10,000
$\Lambda\text{-}[\text{Ru}(\text{bpy})_2\text{Ellatin}]$	1	2	~1

presence and absence of an excess of competing nucleic acids (examples in later sections).

2.4.3

Ethidium Bromide Displacement

The emission intensity of ethidium bromide typically increases upon binding of nucleic acids [65]. Subsequent displacement of ethidium is apparent by a decrease in total fluorescence upon addition of a competitive inhibitor. Displacement experiments can be conducted using a wide range of nucleic acids [62]. Ethidium, however, is known to have variable binding stoichiometries and variable affinities to different nucleic acids [66]. The IC_{50} values for a given inhibitor cannot, therefore, be directly compared between different nucleic acids.

2.5

New RRE Ligands

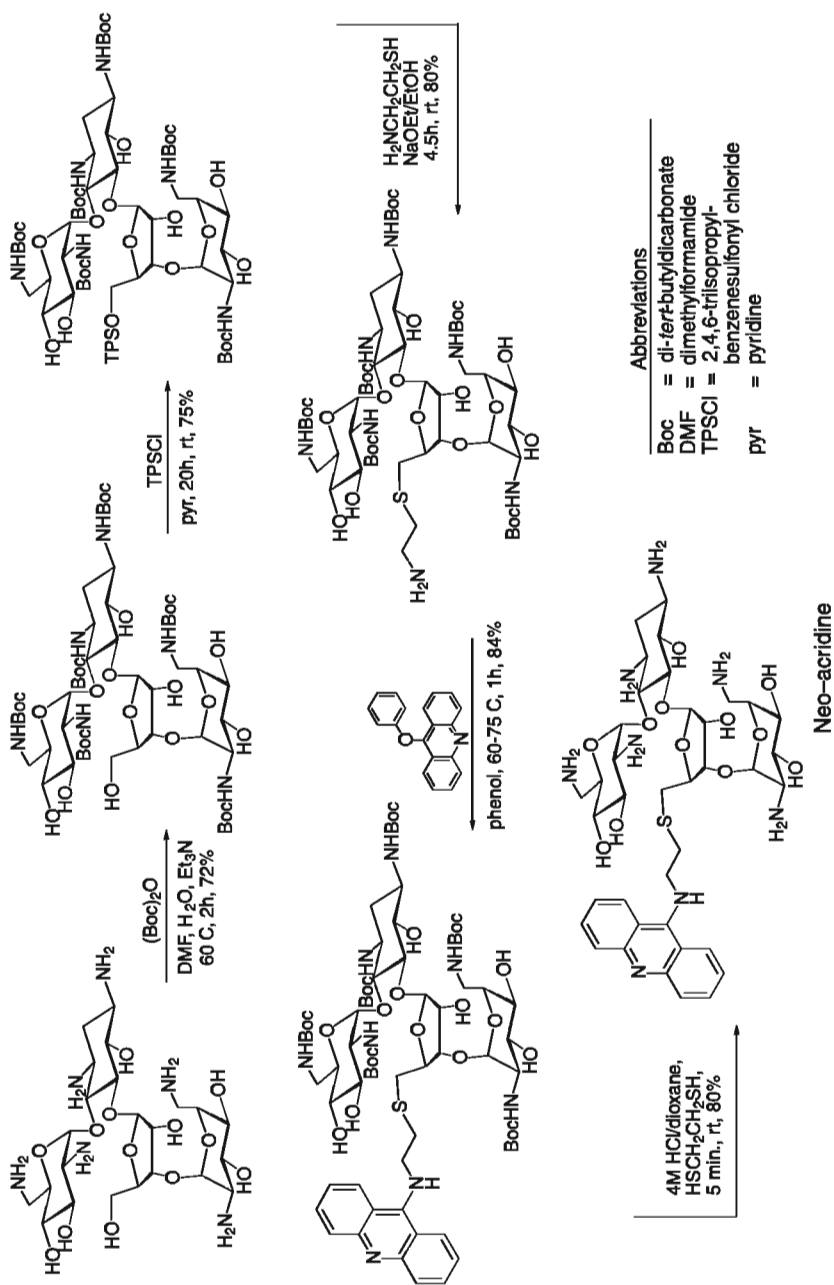
An important demonstration of small molecules binding to viral RNA was established for the aminoglycoside family of antibiotics. These compounds were reported as having moderate affinities to the RRE ($1 \mu M < IC_{50} < 100 \mu M$) [67]. Adverse side effects and poor anti-HIV activities have prevented the use of aminoglycosides as antiviral agents. In attempts to improve these properties, modifications to aminoglycosides have been made. The resulting compounds show dramatically different RRE affinity, specificity, and anti-HIV activities.

2.5.1

Neomycin–Acridine Conjugates

Both neomycin B and the Rev protein are reported to bind near the purine-rich bulge of the RRE [67]. Flanking this bulge is a single adenosine bulge. Intercalating agents are known to preferentially bind to duplex regions containing a bulged base [68]. To utilize both of these structural features, acridine, a well-studied intercalating agent, was conjugated to the 5' position of neomycin B (Scheme 2.1). (Note: A “single step” conjugation of acridine to an amine can potentially provide a shorter synthetic route, but the amines are essential for RNA binding). The resulting conjugate “neo–acridine” shows extremely high affinity for the RRE [69]. Both fluorescence anisotropy and the solid-phase assay indicate that neo–acridine binds to the RRE with an affinity comparable to that of the unlabeled Rev peptide itself (Tab. 2.2). At the time of publication, the binding between neo–acridine and the RRE was the highest affinity interaction between a natural RNA and small synthetic ligand reported to date. Enzymatic footprinting experiments showed that neo–acridine binds to the same portion of the RRE as does the Rev peptide (Fig. 2.8).

Neo–acridine was evaluated for nucleic acid specificity using the solid-phase assay (Tab. 2.2). By comparing the IC_{50} values measured in the absence of nucleic



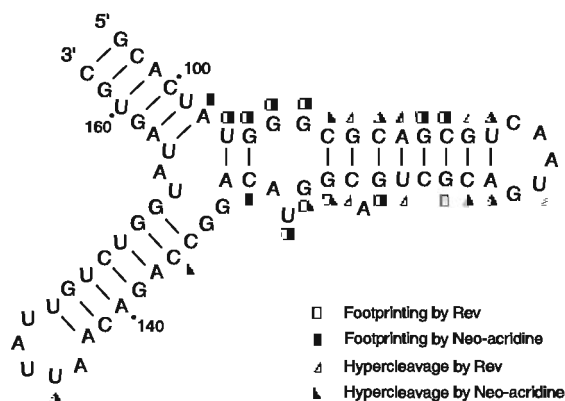
Scheme 2.1. Synthesis of neo-acridine.

Tab. 2.2 Approximate IC_{50} values (μM) for RevFl displacement by solid phase.

Compound	IC_{50}	IC_{50} with DNA	IC_{50} with tRNA
Rev (unlabeled)	0.035	0.080	0.085
Neo-acridine	0.040	0.45	1.2
Neo- <i>N</i> -acridine	0.045	0.15	0.50
Neomycin B	7	8	18
Neo-neo	0.055	0.11	3.5

acid with those measured in an excess of other nucleic acids, the RRE specificity of each compound can be evaluated. Even though both neo-acridine and the Rev peptide bind to the same location of the RRE with similar affinities, neo-acridine has a much lower specificity for the RRE compared with the Rev peptide. Neo-acridine, in fact, binds with high affinity to nearly every RNA and DNA evaluated thus far. The low RRE specificity of neo-acridine can be improved by decreasing the linker length between the two moieties by three atoms (Fig. 2.9). Neo-*N*-acridine has about the same affinity but a significantly improved RRE specificity compared with neo-acridine (Tab. 2.2). The shorter linker length provided by neo-*N*-acridine is believed to provide fewer degrees of freedom to the conjugate and hence fewer nucleic acids can bind to it with high affinity.

Ethidium displacement experiments confirm that neo-acridine has a much higher affinity to “non-specific” competitors (like calf thymus DNA) than does neo-*N*-acridine (Tab. 2.3). Other groups have also synthesized intercalator-glycoside conjugates [70]. Their approach, however, involves incorporation of a variable-length methylene spacer linking paromomycin to either pyrene or thiazole orange. Subsequent evaluation as A-site antagonists reveals that as the number of atoms in the linker increases, the A-site affinity decreases. This is a different trend as observed with our conjugates. It is possible that highly flexible, hydrophobic linkers (including polymethylene) introduce a penalty for RNA binding.

**Fig. 2.8.** Summary of enzymatic footprinting and hypercleavage observed for Rev₃₄₋₅₀ and neo-acridine.

Tab. 2.3. Approximate IC₅₀ values (μM) for ethidium displacement.

Compound	RRE	Calf thymus DNA
Neo-acridine	0.03	0.2
Neo-N-acridine	0.04	1.3
Neo-neo	0.5	0.45
Neo-N-neo	0.2	0.45

2.5.2
Dimeric Aminoglycosides

The dimerization of aminoglycosides had previously been reported to dramatically increase their ability to inhibit ribozyme activity [71]. Dimerization doubles the total charge of each compound, leading to a dramatically enhanced affinity to many RNAs, including the RRE. The Rev-RRE inhibitory activity of neomycin is enhanced by over 100-fold upon dimerization (Tab. 2.2). The solid-phase assay indicates, however, that the RRE specificity, relative to other RNAs, of neo-neo is even worse than that of neo-acridine (Tab. 2.2). As observed for the neomycin-acridine conjugates, RRE specificity is enhanced by decreasing the linker length between moieties (Fig. 2.9). Ethidium displacement experiments indicate that the dimer neo-N-neo has a higher RRE affinity than neo-neo, and that both compounds have the same affinity to a non-specific nucleic acid (calf thymus DNA) (Tab. 2.3).

2.5.3
Guanidinoglycosides

Effective recognition between Rev and the RRE is mediated, largely, by guanidinium groups present in the Rev’s arginine-rich domain [72]. Substitution of arginine at positions 35, 38, 39, or 44 with lysine leads to over a 20-fold loss of *in vivo* RRE binding [73]. We speculated, therefore, that the transformation of the amino groups of the aminoglycosides into guanidinium groups would increase their

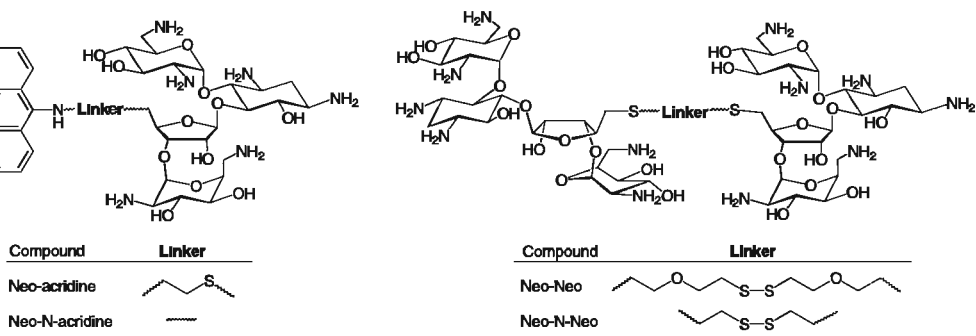
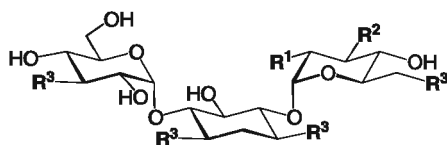
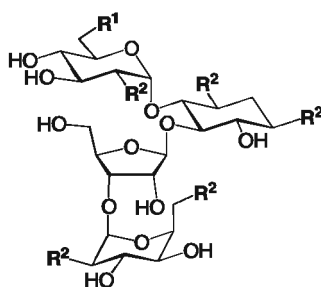


Fig. 2.9. Structures of the neo-acridine and dimeric neomycin conjugates.



Glycoside	R ¹	R ²	R ³
Kanamycin A	OH	OH	NH ₂
Guanidino-kanamycin A	OH	OH	NH(C=NH)NH ₂
Kanamycin B	NH ₂	OH	NH ₂
Guanidino-kanamycin B	NH(C=NH)NH ₂	OH	NH(C=NH)NH ₂
Tobramycin	NH ₂	H	NH ₂
Guanidino-tobramycin	NH(C=NH)NH ₂	H	NH(C=NH)NH ₂



Glycoside	R ¹	R ²
Paromomycin	OH	NH ₂
Guanidino-paromomycin	OH	NH(C=NH)NH ₂
Neomycin B	NH ₂	NH ₂
Guanidino-neomycin B	NH(C=NH)NH ₂	NH(C=NH)NH ₂

Fig. 2.10. Aminoglycosides and guanidinoglycosides used in these studies.

affinity and specificity for the RRE. We guanidinylated five aminoglycosides and used fluorescence anisotropy to evaluate the RRE affinity of the resulting “guanidinoglycosides” (Fig. 2.10 and Tab. 2.4) [74]. IC₅₀ values were measured at both 10 nM and 100 nM RRE (consistently using 10 nM of RevFl). By taking the ratio of these IC₅₀ values (Tab. 2.4), some information regarding the small molecule–RRE binding stoichiometries can be gleaned. A lower ratio suggests that more binding sites for the small molecule are capable of displacing the peptide. Surprisingly, the binding stoichiometries (for peptide displacement) are significantly different for kanamycin A versus kanamycin B, and for paromomycin versus neomycin B. This ratio, however, does *not* change for each compound upon guanidinylation, indicating that the RRE-binding stoichiometries of the guanidinoglycosides are the same as their aminoglycoside precursors. Differences in Rev–RRE IC₅₀ values are, therefore, proportional to the differences in RRE affinity. Upon guanidinylation of neomycin B and paromomycin, a five-fold increase in RRE affinity is obtained.

Tab. 2.4. IC₅₀ values (μM) for RevFI displacement by anisotropy.

Glycoside	10 nM RRE	100 nM RRE	Ratio (100 nM/10 nM)
Kanamycin A	100	750	7.5
Guanidino-kanamycin A	8	65	8.1
Kanamycin B	15	80	5.3
Guanidino-kanamycin B	0.7	3.5	5.0
Tobramycin	10	45	4.5
Guanidino-tobramycin	0.8	3.8	4.7
Paromomycin	12	65	5.4
Guanidino-paromomycin	3.4	18	5.3
Neomycin B	1	7	7
Guanidino-neomycin B	0.2	1.3	6.5

Upon guanidinylation of the kanamycin family of glycosides, over a 10-fold increase in affinity is gained (Tab. 2.4).

The solid-phase assay indicates that guanidinylation also significantly affects RNA specificity (Tab. 2.5). By dividing the IC₅₀ values obtained in the presence of competitor RNAs (in this case duplex poly(A)–poly(U)) with the IC₅₀ values obtained in the absence of other nucleic acids, a specificity ratio can be calculated (Tab. 2.5). A lower ratio indicates higher RRE specificity (relative to simple duplex RNA). Upon guanidinylation, this ratio decreases for all the glycosides (except for neomycin B), showing that, in general, guanidinylation increases RRE specificity of the aminoglycosides. Importantly, guanidinylation does *not* affect the RNA over DNA specificity of these glycosides (Tab. 2.5). This is, to our knowledge, the first indication that the identity of the basic group is not an important factor for the ability of aminoglycosides to preferentially bind RNA over DNA.

Analysis of the activity trends apparent for each family of glycosides (Tab. 2.5) suggests some fundamental relationships relating the total charge of a ligand to its

Tab. 2.5. IC₅₀ values (μM) for RevFI displacement by solid-phase assay.

Glycoside	IC ₅₀	IC ₅₀ with DNA	IC ₅₀ with RNA	RNA specificity ratio
Kanamycin A	700	750	1300	1.9
Guanidino-kanamycin A	55	55	60	1.1
Kanamycin B	90	90	170	1.9
Guanidino-kanamycin B	3	3	4.7	1.6
Tobramycin	45	50	130	2.9
Guanidino-tobramycin	3	3	4.7	1.6
Paromomycin	55	60	110	2.0
Guanidino-paromomycin	15	18	18	1.2
Neomycin B	7	8	16	2.5
Guanidino-neomycin B	1	1	4.5	4.5

RNA affinity *and* specificity. The total number of basic groups on each compound is roughly proportional to its RRE affinity, and the higher the ligand's affinity, the less likely it is to show specificity for the RRE. These same principles are also reflected in the dimeric aminoglycosides and may, therefore, reflect a general rule.

Other informative activity trends can also be observed (Tab. 2.5). By comparing compounds with the same number of basic groups (like kanamycin B, tobramycin, and paromomycin), additional structure–activity relationships can be inferred. For example, tobramycin is found to have a higher RRE affinity than kanamycin B, even though these two compounds differ only by a single hydroxyl (Fig. 2.10). This finding is consistent with the ability of hydroxyls to decrease the basicity of neighboring amines (giving tobramycin a higher overall charge *and* a lower RRE specificity than kanamycin B) [46]. Upon guanidinylation, the differences between kanamycin B and tobramycin are lost, because the pK_a of guanidinium groups are significantly less variable than those of amines.

Guanidinium groups are highly basic, capable of defined multidirectional hydrogen bonding, and their planar surface may facilitate stacking interactions. The higher basicity of the guanidinoglycosides, as compared to the aminoglycosides, should impart a higher total charge, and therefore higher RNA affinity. Their higher overall positive charge cannot, however, explain their improved RRE specificity (this would oppose the general relationships stated above). To understand why the guanidinoglycosides show improved RRE affinity *and* specificity (relative to the aminoglycosides), a number of urea-containing glycosides were synthesized [75]. Urea is an uncharged structural analog of guanidine. Both urea and guanidinium are capable of denaturing nucleic acids; therefore, the urea glycosides may have bound to RNAs that possess single-stranded character (like the purine-rich bulge of the RRE). The urea glycosides are, however, unable to inhibit Rev–RRE binding through 1 mM. This indicates that electrostatic interactions are critical for the guanidinoglycoside's affinity for RRE. Guanidinoglycoside–RNA binding has been shown (in a different system) to be much less dependent on the total ionic strength of the media compared to the aminoglycosides [76]. This suggests that stacking and/or other hydrophobic interaction(s) also mediate guanidinoglycoside–RNA binding. This may be the origin of the increased RRE specificity of the guanidinoglycosides.

The guanidinoglycosides are found to possess significantly improved RRE affinity, specificity, and anti-HIV activities [77] compared to the aminoglycosides (Fig. 2.11). Other groups have conjugated amino acids to aminoglycosides in order to mimic the Tat protein arginine-rich motif. Conjugation of arginine to kanamycin A is reported to increase its affinity and specificity for the TAR RNA [78]. This synthetic modification effectively doubles the number of basic groups present. Their findings suggest, however, that the guanidinium-containing side chains do not play the dominant role in RNA binding. The α -amines of the amino acids, in close proximity to the “core” glycoside, are reported to be essential for TAR binding. We have made similar observations with tobramycin–arginine conjugates in the Rev–RRE system. A tobramycin–arginine conjugate has the same number of basic groups and a similar RRE affinity as a tobramycin–aminoglycoside dimer (not shown). If

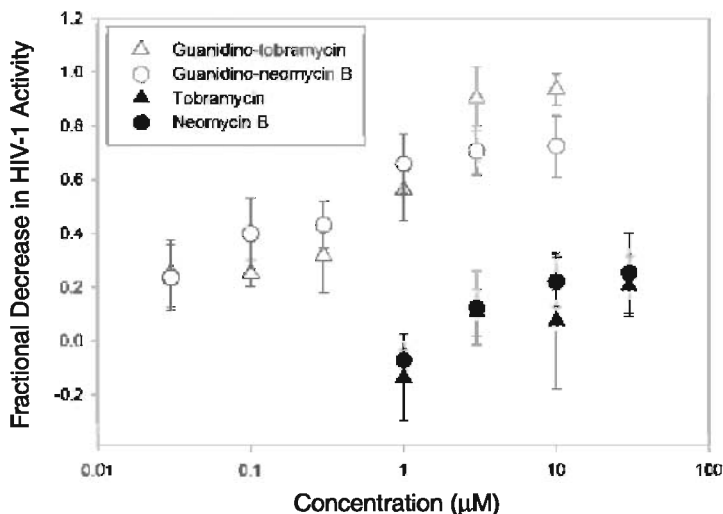


Fig. 2.11. Anti-HIV activities of aminoglycosides and guanidinoglycosides.

the α -amines of the arginine–tobramycin conjugate are blocked by acetylation, the resulting compound has the same number of basic groups as guanidino-tobramycin. Both guanidino-tobramycin and the acetylated tobramycin-arginine conjugate possess five guanidinium groups, and their comparison allows us to establish the contributions made by the flexible, hydrophobic, methylene linkages present in the arginine conjugates. We find that the acetylated tobramycin arginine conjugate has over a 20-fold lower RRE-binding activity than guanidino-tobramycin, indicating that a significant energetic penalty is introduced by the methylene linkages of the amino acids.

Neither neo-acridine, nor neo-*N*-acridine possess anti-HIV activity. The guanidino forms of these compounds, however, are active in HIV inhibition. Fluorescence microscopy indicates that mammalian cells show significant cellular uptake of the guanidino forms of these compounds but not of their respective amino forms. As reported for the poly-arginine peptides (including Tat and Rev) the guanidinoglycosides may be actively transported into eukaryotic cells.

2.6

Conclusions

Systematic studies of the binding interactions between small molecules and RNA are essential for deciphering the parameters that govern RNA recognition. As illustrated above, the RRE affinity, specificity, and antiviral activities of aminoglycosides can be dramatically altered through synthetic modifications. The amine groups present on aminoglycosides are essential for RNA binding, but can be substituted by other basic groups including guanidine. The resulting “guanidinoglycosides” have improved affinity and specificity for the RRE, and maintain RNA

over DNA specificity. Structure–activity studies in both families of glycosides have revealed trends that suggest general relationships between total charge of small ligands and their RNA affinity and specificity. Synthetic modifications that introduce extremely greasy and flexible functionalities onto aminoglycosides are likely to be detrimental to RNA binding. Polycyclic aromatic heterocycles, however, can greatly enhance the RNA-binding affinity, but can decrease the specificity of glycoside conjugates. The nucleic acid specificity of aminoglycoside dimers and of neomycin–acridine conjugates can be tuned by adjusting the length of the linkers that separate each moiety.

RNA plays a pivotal role in the replication of all organisms, including viral and bacterial pathogens. The development of small molecules that can selectively interfere with undesired RNA activity is a promising new direction for drug development. Continuing progress in the understanding of small molecule–RNA recognition may provide future scientists a means for selective targeting of a predetermined RNA regulatory element.

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3

RNA Targeting by Bleomycin

Sidney M. Hecht

Although the bleomycin (BLM) group antitumor antibiotics are best known for their properties as sequence-selective DNA-cleaving agents [1–8], several possible biochemical targets have actually been reported for these compounds. These include DNA and RNA nucleases, DNA ligase, both DNA and RNA polymerases [9–12], as well as lipid peroxidation [13–16]. Of particular note was the report by Magliozzo *et al.* in 1989 that bleomycin could release free bases from transfer RNAs [17]. The study of RNA cleavage by bleomycin has been studied by a few different laboratories; this chapter summarizes the status of those studies.

3.1

Activation of Bleomycin for Polynucleotide Degradation

Bleomycin-mediated DNA degradation is an oxidative process that requires oxygen and a redox-active metal ion [1–8]. The structural domain believed to be involved in metal binding and oxygen activation is indicated in Fig. 3.1 for bleomycin A₂. While any of several metal ions can support DNA cleavage by bleomycin in an experimental setting, under physiological conditions the relevant metal ion is likely to be Fe [18] or possibly Cu [19]. Bleomycin cleaves double-stranded DNA predominantly at 5'-GT-3' and 5'-GC-3' sequences; a subset of these sites is cleaved with high efficiency. Two sets of products are formed as a consequence of DNA degradation by activated metalbleomycins; both are believed to result from the abstraction of an H atom from C4' H of deoxyribose [1–8]. It may be noted that C4' H resides in the minor groove of DNA, the dimensions of which may be smaller than the size of the metal-binding domain of metalbleomycins. In fact, it has been suggested [20] that the 5'-GC-3' and 5'-GT-3' selectivity of cleavage by metalbleomycins reflects the fact that these sequences correspond to the widest, shallowest part of the minor groove in B-form DNA [21] (i.e. that structural element best able to accommodate the metal-binding domain). Consistent with this suggestion, it has been found that structurally altered DNAs predicted to have widened minor grooves exhibited enhanced cleavage at those sites [22–24].

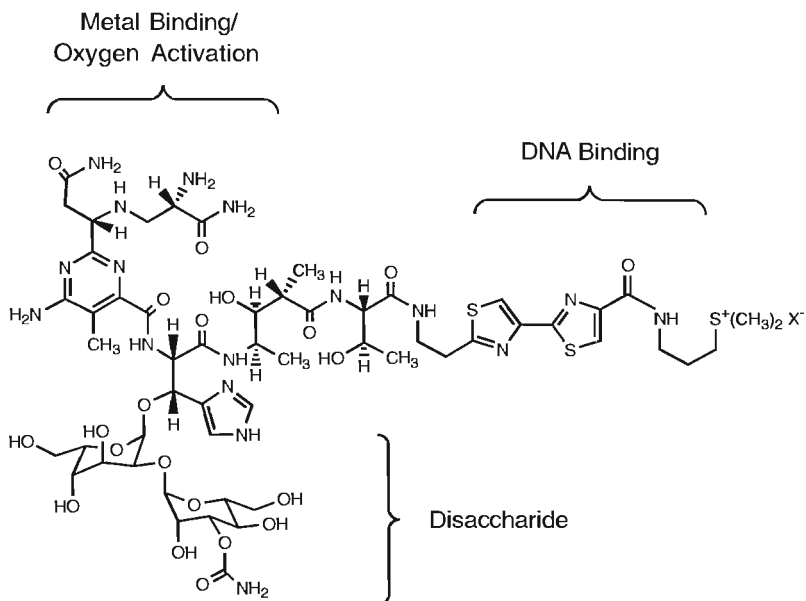


Fig. 3.1. Structure of bleomycin A₂. The individual domains are labeled.

3.2

Bleomycin-mediated Cleavage of Transfer RNAs and tRNA Precursor Transcripts

As noted above, Magliozzo *et al.* reported the first study of the cleavage of tRNAs by activated bleomycin [17]. The experiments involved relatively high concentrations of (non-radiolabeled) tRNAs and bleomycin, the clinically used mixture consisting primarily of BLM A₂ and BLM B₂. Treatment of unfractionated yeast tRNAs, or *Escherichia coli* tRNA^{Tyr} and tRNA^{Phe}, with activated Fe-BLM afforded tRNA breakage in a dose-dependent fashion. Significant cleavage was observed when 3 mM tRNA^{Phe} was treated with 0.3 mM activated Fe-BLM; also formed were products that co-migrated with adenine and uracil.

A survey of the susceptibility of a number of individual tRNAs and tRNA precursor transcripts was carried out by the Hecht laboratory using ³²P-end labeled substrates. Most of the substrates studied were not substrates for cleavage by Fe-BLM at any reasonable concentration. However, *Bacillus subtilis* tRNA^{His} precursor transcript was found to be cleaved with reasonable efficiency by 3 μM Fe(II)-BLM A₂ (Fig. 3.2) [25]. An ostensibly similar tRNA precursor, *E. coli* tRNA^{Tyr} precursor, was not a substrate for cleavage by Fe-BLM even when much higher concentrations were employed. It is also interesting that the tRNA^{His} precursor was cleaved at a single major site, unlike the cleavage of any DNA restriction fragment studied [1–8], all of which are cleaved at numerous sites.

The survey of tRNA structures revealed Fe-bleomycin-mediated cleavage of a *Schizosaccharomyces pombe* amber suppressor tRNA^{Ser} construct at two major sites,

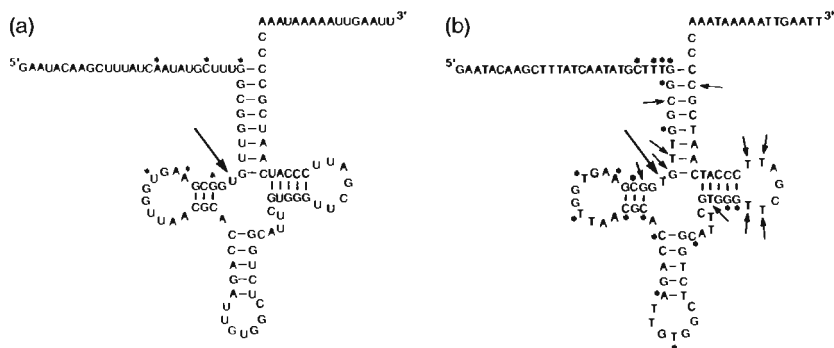


Fig. 3.2. (a) Structure of *Bacillus subtilis* tRNA^{His} precursor, indicating the major (arrow) and minor (asterisks) sites of cleavage by Fe(II)-bleomycin. (b) Cleavage of a DNA transcript having the same primary sequence as tRNA^{His} precursor.

one of which was in the D-loop while the other was within the 5'-leader sequence [26]. In comparison, no cleavage was noted for *E. coli* tRNA^{Cys} or yeast mitochondrial tRNA^{Asp} or tRNA^{Met} precursor constructs [26]. Of particular interest were the results of treatment of *E. coli* tRNA₁^{His} with Fe-BLM. Cleavage was observed at three major sites, but none of these was the same as the major or minor cleavage sites noted for *B. subtilis* tRNA^{His} precursor transcript [25, 26].

A more detailed study of the effect of tRNA conformation on susceptibility to cleavage by Fe-BLM was carried out using the crystallographically defined yeast cytoplasmic tRNA^{Phe} and tRNA^{Asp}, as well as *in vitro* tRNA transcripts related to these two mature tRNAs [27]. The two sites of cleavage of mature tRNA^{Asp} and four sites of cleavage of mature tRNA^{Phe} are shown in Fig. 3.3. In Fig. 3.4, four tRNA transcripts structurally related to tRNA^{Asp} and tRNA^{Phe} are shown. Transcript A has the same sequence as tRNA^{Asp} with the exception of a G₁-C₇₂ base pair introduced to facilitate *in vitro* transcription. This transcript has been shown to be activated efficiently by yeast aspartyl-tRNA synthetase [29]. As noted in Fig. 3.4, this transcript was cleaved at four sites, none of which was the same as the two sites cleaved in mature tRNA^{Asp}. A second tRNA^{Asp} substrate utilized was transcript F, which has an altered D-loop and a fifth nucleotide in the variable loop; both of these are characteristic of yeast tRNA^{Phe} [30].

As indicated in Fig. 3.4, this transcript was cleaved at five sites, namely U₁₇, G₁₉, A₂₁, U₂₅, and G₅₀. The cleavage at G₁₉ was at the same site as one of the major cleavage sites of yeast tRNA^{Phe} and the cleavage of the transcript at G₅₀ was close to the U₅₂ cleavage site in mature tRNA^{Phe}. However, the remaining cleavage sites in transcript F resembled neither the cleavage pattern of tRNA^{Phe} nor tRNA^{Asp}.

Also studied were transcripts B and D, each of which contains only one of the two alterations by which transcripts A and F differ. Transcript B, containing an additional uridine between A₄₆ and U₄₇, was cleaved at eight sites, indicating that the addition of a single uridine rendered the substrate significantly more susceptible to cleavage by Fe-BLM. The eight cleavage sites included U₁₆, C₂₉, and C₃₈,

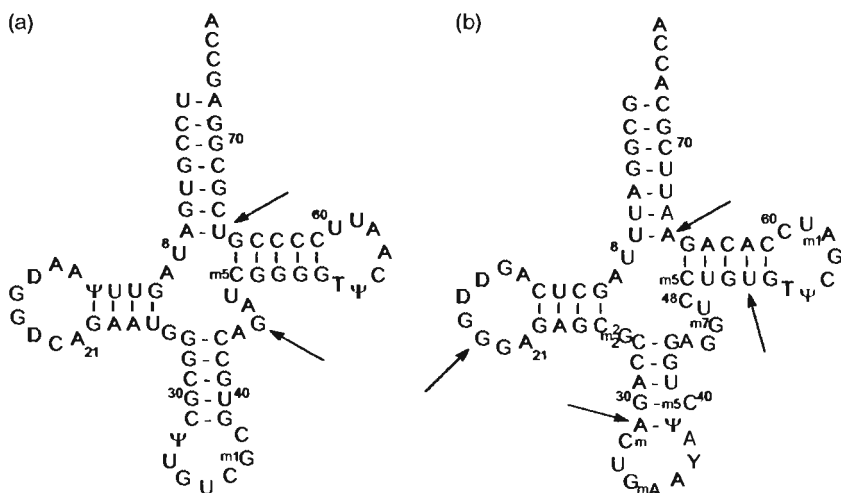


Fig. 3.3. Structures of (a) yeast tRNA^{Asp} and (b) yeast tRNA^{Phe} (right). The sites of Fe(II)-BLM A₂-mediated cleavage are indicated by arrows.

all of which were also cleaved in transcript A. In comparison, transcript D, having a D-loop identical with transcript F, was cleaved only at a single position (A₄₆) which was the single cleavage site in transcript A not cleaved in transcript B. In spite of the similarity of transcript D to transcript F, they showed no cleavage site in common.

Thus the overall picture that emerges from this survey is one of great sensitivity to changes in tRNA tertiary structure. While 5'-G-pyr-3' sequences were probably the most common sequence cleaved by Fe-BLM, many other sequences were also cleaved. Further, at least at the level of secondary structure analysis, cleavages occurred in nominally single- and double-stranded regions. More careful analysis at the level of tertiary structure suggests that many of the sites susceptible to cleavage reside at the junction between single- and double-stranded structures (i.e. adjacent to minor groove-like structures exhibiting local widening).

3.3

Other RNA Targets for Bleomycin

Early studies of RNA cleavage by bleomycin failed to detect any effect [31–35]. The earliest report of attempted cleavage of an RNA–DNA heteroduplex by Fe-BLM was described by Haidle and Bearden in 1975; they found that Fe-BLM degraded only the poly(dT) strand of a poly(rA)-poly(dT) heteroduplex [34]. This finding was confirmed and extended by Krishnamoorthy *et al.* using both poly(rA)-poly(dT) and poly(dA)-poly(rU) as substrates [36, 37]. Nonetheless, a subsequent study by Morgan and Hecht, using an RNA–DNA heteroduplex substrate formed by reverse



Fig. 3.4. Structure of four tRNA transcripts illustrating (arrows) the sites of cleavage by Fe(II)-BLM A_2 . Nomenclature for the transcripts was based on Giegé *et al.* [28]. Transcript A has the same sequence as mature yeast tRNA^{Asp}, but contained a C₁–C₇₂ base pair to facilitate transcription in lieu of the

normal U₁-A₇₂ base pair. Relative to transcript A, transcript B contained an additional nucleotide (U₄₇) in the variable loop, while transcript D has an altered dihydrouridine loop. Transcript F contained both the additional nucleotide in the variable loop and an altered dihydrouridine loop.

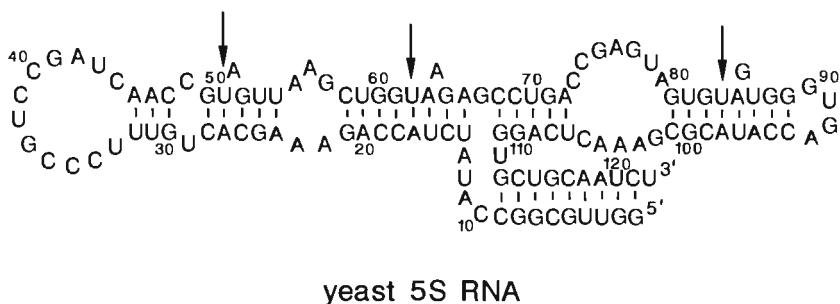


Fig. 3.5. Three sites of cleavage (arrows) of yeast 5S ribosomal RNA by Fe(II)-bleomycin.

transcription of *E. coli* 5S ribosomal RNA, clearly demonstrated cleavage at a limited number of sites on both the DNA and RNA strands [38]. It is interesting that the sites of cleavage on the DNA strand of the heteroduplex were found to be a subset of those cleaved on a DNA duplex having the same sequence.

The BLM-mediated cleavage of a ribosomal RNA, namely yeast 5S ribosomal RNA, has also been studied [26]. The three sites of cleavage are summarized in Fig. 3.5. All of these involved the uridine residue in a 5'-GUA-3' sequence. Interestingly each of the cleaved sequences was flanked by a one-base bulge one or two nucleotides to the 3'-side of the cleavage site, a feature that is reminiscent of DNA cleavage in proximity to bulges [22].

As regards cleavage of messenger RNAs, two reports document that these can be substrates for cleavage by Fe-BLM. The first involved the treatment of a 347-nucleotide substrate corresponding to the 5'-end of HIV-1 reverse transcriptase mRNA. This mRNA was found to be cleaved at no less than four sites by Fe-BLM [25].

Subsequently, Dix *et al.* [39] studied the cleavage of the iron regulatory element of ferritin mRNA using Fe-BLM as a probe. The wild-type sequence was cleaved solely at 5'-GU₁₇-3', which is believed to be at the junction of a single- and double-stranded region within the stem-loop structure. A functional mutant of the iron regulatory element in which the flanking region was altered by disruption of phylogenetically conserved base pairs, was cleaved at A₁₀ and A₁₁, i.e. within the double-stranded region on the opposite side of the stem-loop structure.

3.4

Characteristics of RNA Cleavage by Fe-BLM

In comparison with DNA cleavage, Fe-bleomycin-mediated RNA cleavage has a number of unique characteristics. While the cleavage of both substrates requires Fe²⁺ and oxygen for bleomycin activation, RNA cleavage is much more selective than DNA cleavage [20]. Further, while the RNA cleavage sites involve a disproportionate number of 5'-G-pyr-3' sequences, tertiary structure appears to be quite important in determining the sites of cleavage of RNA by Fe-BLM.

It should also be noted that the various RNA substrates described above are not cleaved with equal facility by Fe(II)-BLM. The best two substrates are the *B. subtilis* tRNA^{His} precursor [25] and the yeast 5S ribosomal RNA [26]. In addition to being susceptible to Fe-BLM-mediated cleavage at low micromolar concentrations, cleavage of these species was not eliminated in the presence of physiological concentrations of Mg²⁺ [26]. This contrasts with the cleavage of species such as yeast tRNA^{Phe}, which require greater concentrations of Fe-BLM and are not substrates in the presence of even 0.5 mM Mg²⁺ [40].

It seemed possible that some of the differences in effects of Fe-BLM on RNA as compared with DNA might be explained by differences in efficiency of binding of activated Fe-BLM. Accordingly, an experiment was designed in which the cleavage of *B. subtilis* tRNA^{His} precursor at 5'-GU₃₅-3' was monitored in the presence of increasing concentrations of the self-complementary dodecanucleotide d(GCGT₃A₃GCG), which also has a single preferred cleavage site (at 5'-GC₁₁-3') and binds BLM quite efficiently [41]. As shown in Fig. 3.6, when the tRNA^{His} precursor was present at a concentration of 2.3 μM, cleavage at 5'-GU₃₅-3' was readily



Fig. 3.6. Cleavage of tRNA^{His} precursor by Fe(II)-BLM in the presence of a DNA oligonucleotide. *B. subtilis* tRNA^{His} precursor was treated with Fe(II)-BLM A₂ in the presence of 5'-d(CGCT₃A₃GCG)-3'. Lane 1, tRNA^{His}

precursor alone (270 μM final nucleotide concentration); lane 2, 10 μM Fe²⁺; lane 3, 10 μM BLM A₂; lanes 4–13, 10 μM Fe(II)-BLM A₂ + 400, 300, 200, 150, 100, 50, 10, 5, 0.5, and 0 μM dodecanucleotide, respectively.

apparent in the presence of 10 μM d(CGCT₃A₃GCG). Thus activated Fe·BLM clearly binds to this good RNA substrate no less efficiently than it binds to a good DNA duplex substrate [26].

While the most obvious difference between DNA and RNA structures is at the level of the mononucleotide constituents, it is also true that the secondary and tertiary structures assumed by RNAs generally have no DNA counterparts. Therefore, we prepared “tDNA^{His}” (i.e. a DNA molecule having the same primary sequence as *B. subtilis* tRNA^{His} precursor). While there was no assurance that the tDNA would actually assume secondary and tertiary structures analogous to those of a tRNA, earlier studies with a tDNA analog of *E. coli* tRNA_f^{Met} having a riboadenosine at the 3'-end revealed that this species was activated by methionyl-tRNA synthetase [42]. Further, the tDNA analogs of *E. coli* tRNA^{Phe} and tRNA^{Lys} were also bound and activated by the cognate aminoacyl-tRNA synthetases [43].

As shown in Fig. 3.7, treatment of tDNA^{His} with 0.5 μM Fe(II)·BLM A₂ afforded a major cleavage band at 5'-GT₃₅-3' (i.e. exactly analogous to the major site of cleavage of tRNA^{His} precursor) [44]. Interestingly, higher concentrations of Fe(II)·BLM A₂ produced additional cleavage products and complete consumption of the intact tDNA. In comparison, no significant cleavage of tRNA^{His} precursor was apparent at Fe(II)·BLM A₂ concentrations below 2.5 μM , and concentrations as high as 250 μM produced no greater degradation of the tRNA^{His} precursor. The actual sites of tDNA^{His} and tRNA^{His} cleavage are summarized in Fig. 3.2. In addition to sharing the same major site of cleavage at U(T)₃₅, it is interesting that cleavage of the tDNA at less efficient cleavage sites involved a number of sequences other than the 5'-G-pyr-3' sequence that dominates the cleavage of duplex DNA by bleomycin. These observations argue that both DNA and RNA undergo cleavage by activated Fe·BLM that is based primarily on the overall shape of the oligonucleotide target.

Competition experiments analogous to that shown in Fig. 3.6 were also carried out. Treatment of radiolabeled tRNA^{His} (7–8 μM nucleotide concentration) with 25 μM Fe(II)·BLM A₂ in the presence of unlabeled tDNA^{His} at concentrations up to 80 μM had no significant effect on cleavage of the radiolabeled tRNA. When the experiment was repeated with unlabeled tRNA^{His}, no diminution of cleavage was observed in the presence of 16 μM unlabeled tRNA^{His}, but cleavage was virtually absent in the presence of 80 μM unlabeled tRNA^{His}. In contrast, while cleavage of 7–8 μM tDNA^{His} at T₃₅ by 1.25 μM Fe(II)·BLM A₂ was diminished only by 80 μM unlabeled tDNA^{His}, it was significantly diminished by the presence of even 8 μM tRNA^{His} and virtually absent at higher tRNA^{His} concentrations. The clear conclusion is that Fe(II)·BLM A₂ binds more tightly to tRNA^{His} than to tDNA^{His} [44].

Another interesting facet of tDNA^{His} cleavage by Fe(II)·BLM A₂ is shown in Tab. 3.1, which records the effect of Mg²⁺ on tRNA^{His} and tDNA^{His} cleavage. The diminution of cleavage of the two polynucleotides caused by Mg²⁺ is not dramatically different for the two substrates. Thus the effects of Mg²⁺ on Fe·BLM-mediated RNA cleavage are related to tertiary structure rather than any fundamental property of RNA (versus DNA) structure.

In view of the finding that the affinity of Fe(II)·BLM A₂ for tRNA^{His} was actually

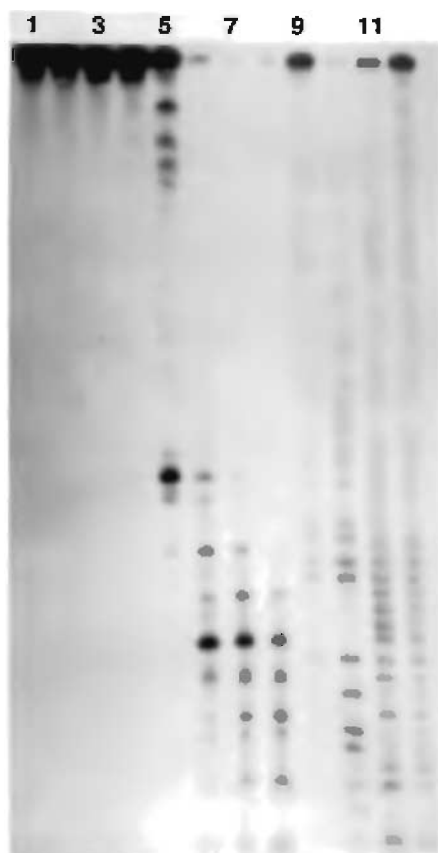


Fig. 3.7. Fe(II)-bleomycin-mediated cleavage of tDNA^{His} substrate. Lane 1, DNA alone (approx. 2.1 μ M nucleotide concentration); lane 2, 2.5 μ M BLM; lane 3, 2.5 μ M Fe²⁺;

lanes 4–8, 0.25, 0.5, 1.25, 2.5, and 5.0 μ M Fe(II)-BLM, respectively; lane 9, G lane; lane 10, G + A lane; lane 11, C + T lane, lane 12, C lane.

Tab. 3.1. Effect of Mg²⁺ on Fe-BLM-mediated cleavage of tRNA^{His} and tDNA^{His} precursors.

[Mg ²⁺] (mM)	Percentage cleavage	
	tDNA ^{His}	tRNA ^{His}
0	10.4	13.5
0.25	8.4	7.2
0.5	4.4	4.3
2.0	3.5	1.8
5.0	1.9	1.8

greater than that for tDNA^{His}, but the DNA substrate nonetheless underwent cleavage at lower concentrations of added Fe-BLM and to a greater extent, it seems reasonable to consider the factors that limit RNA cleavage. Since RNA cleavage is limited neither by binding affinity nor by the effects of divalent cation, two explanations seem possible. One of these is that Fe-BLM binds to RNA in a fashion that is relatively unproductive from the perspective of RNA cleavage. The other possibility is that reactive intermediates are formed from the RNA substrate but do not result in strand scission. Crich and Mo have suggested that the intermediate radicals produced from RNA sugars may have greater stability than those derived from DNA [45]; conceivably they could be “repaired” by H atom abstraction from some other species. Alternatively, it is possible that the degradation of RNA affords lesions analogous to the alkali-labile lesions in DNA [1–8, 46–48] that do not lead directly to strand scission. The latter possibility has been explored in the context of the actual chemistry of RNA degradation.

3.5

Chemistry of Bleomycin-mediated RNA Cleavage

The chemistry of BLM-mediated RNA cleavage is significantly more complex than that of DNA. Perhaps the most surprising finding was that of Keck and Hecht [49] which demonstrated a metal ion- and oxygen-independent pathway for RNA strand scission. The transformations occurred at all phosphodiester bonds within 5'-pyr-pur-3' sequences not involving modified nucleobases. The products of the transformation were shown to be 5'-oligonucleotides terminating in nucleoside 2,3'-cyclic phosphates and 3'-oligonucleotides having free 5'-OH groups (i.e. the products expected to result from phosphoryl transfer initiated by the 2'-OH group of the pyrimidine nucleotide). The facility of this transformation was comparable to that of the oxidative transformation of RNAs by activated Fe-BLM. In common with oxidative cleavage of RNA by metalloBLMs, the “nuclease-like” cleavage was also found to be diminished in the presence of Mg²⁺; for yeast tRNA^{Phe} no strand scission was observed when [Mg²⁺] was greater than 200 μM [49].

This transformation required the presence of a free primary amino group within the β-aminoalanineamide side chain. It was quite similar to the pattern of strand scission obtained with imidazole–intercalator conjugates prepared as RNase mimics [50], as well as a number of other reagents such as polyvinylpyrrolidone [51, 52]. The mechanism is not entirely clear, but the transformation occurs at sites susceptible to spontaneous hydrolysis [53], and presumably reflects the ability of the 2'-OH group on the pyrimidine nucleoside to assume a conformation conducive to phosphoryl transfer.

As regards the oxidative chemistry of RNA, the earliest evidence was provided by Magliozzo *et al.*, who reported the isolation of adenine and uracil nucleobases, identified by co-migration with authentic samples on TLC [17]. Also reported were base propenals, identified on the basis of their colorimetric response upon treatment with thiobarbituric acid. Given the presence of 2'-OH groups in the ribose

moieties of the substrate nucleotides, the actual structures of the base propenals would presumably have to be hydroxylated analogs of the base propenals formed from DNA [1–8].

The release of uracil concomitant with cleavage of U₃₅ in the *B. subtilis* tRNA^{His} precursor was established by the use of a tRNA^{His} transcript incorporating both [³²P]CMP and [5.6-³H]uridine radiolabels. Cleavage of the tRNA precursor substrate was monitored on a polyacrylamide gel, while release of [³H]uracil was determined in parallel by HPLC analysis [54]. [³H]Uracil equivalent to 83% of the number of tRNA strand breaks was detected. By the use of both 5'- and 3'-³²P end-labeled yeast 5S ribosomal RNAs, it was determined that the 5'-oligonucleotide cleavage products migrated on polyacrylamide gels as though they had 3'-phosphoroglycolate moieties [1–8, 41], while the 3'-oligonucleotide products co-migrated with oligonucleotides having 5'-phosphate termini [26]. These products, of course, are the same as those formed from DNA by the action of activated metalloBLMs and would be fully consistent with oxidation initiated by abstraction of an H atom from the C4' position of ribose.

A more detailed analysis was carried out by the use of self-complementary deoxyoligonucleotide containing a single *ribo*-cytidine or *ara*-cytidine (i.e. CGCTAGCG) (Fig. 3.8). As shown in the figure, treatment with Fe(II)-BLM under aerobic conditions afforded a product having the same chromatographic properties as CpGpCH₂COOH [25, 26] (i.e. the product anticipated from initial abstraction of C4' H).

From a chemical perspective, the radical formed by abstraction of C1' H of (deoxy)ribose would be expected to be of reasonable stability and it was suggested a number of years ago that this pathway might also be utilized by activated BLM in effecting DNA degradation [55]. Although no products of DNA degradation resulting from C1' H abstraction have yet been reported, it has been noted that it might be logical to anticipate the formation of such products from an A-form duplex in which C1' H resides prominently within the minor groove [20]. In fact, Absalon *et al.* [37] attempted to identify such products from the DNA strand of a homopolymeric DNA–RNA heteroduplex, but concluded that they were not formed. In comparison, activated Fe-BLM treatment of either the chimeric octanucleotide shown in Fig. 3.9, or else the isomeric C₃-*ara* octanucleotide, afforded products in addition to those formed by abstraction of C4' H [55]. As shown in Fig. 3.9, one of the formed products could be trapped as a dinucleotide quinoxaline derivative following treatment with 1,2-diaminobenzene. The isolation of this product constitutes strong supporting evidence for the abstraction of C1' H by activated Fe-BLM. The CpG-quinoxaline derivative constituted about 10% of the products derived from degradation of C₃-*ribo* CGCTAGCG, and 58% of those formed from C₃-*ara* CGCTAGCG.

As is clear from Fig. 3.9, the intermediate resulting from H atom abstraction from the C1' position would afford free nucleobase, but not lead directly to strand scission. An analogous product, the alkali-labile lesion [46–48] is a well-characterized product of Fe-BLM-mediated DNA degradation following C4' H abstraction, and could conceivably be formed from RNA as well. Unfortunately, due to

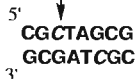


Fig. 3.8. Oxidative degradation of chimeric octanucleotides initiated by abstraction of C4' H.

the ability of all RNA phosphodiester linkages to alkali and other nucleophilic reagents, the selective cleavage of BLM-induced RNA lesions is not straightforward. However, evidence for the formation of a lesion of this type has been obtained by successive treatments of the RNA substrate with Fe(II)-BLM and then with hydrazine [54].

3.6

Significance of RNA as a Target for Bleomycin

While DNA has long been thought to constitute the critical therapeutic locus of action for bleomycin, there are observations which argue that this may not be the sole locus of action for the drug. Unresolved issues include the relatively poor cor-

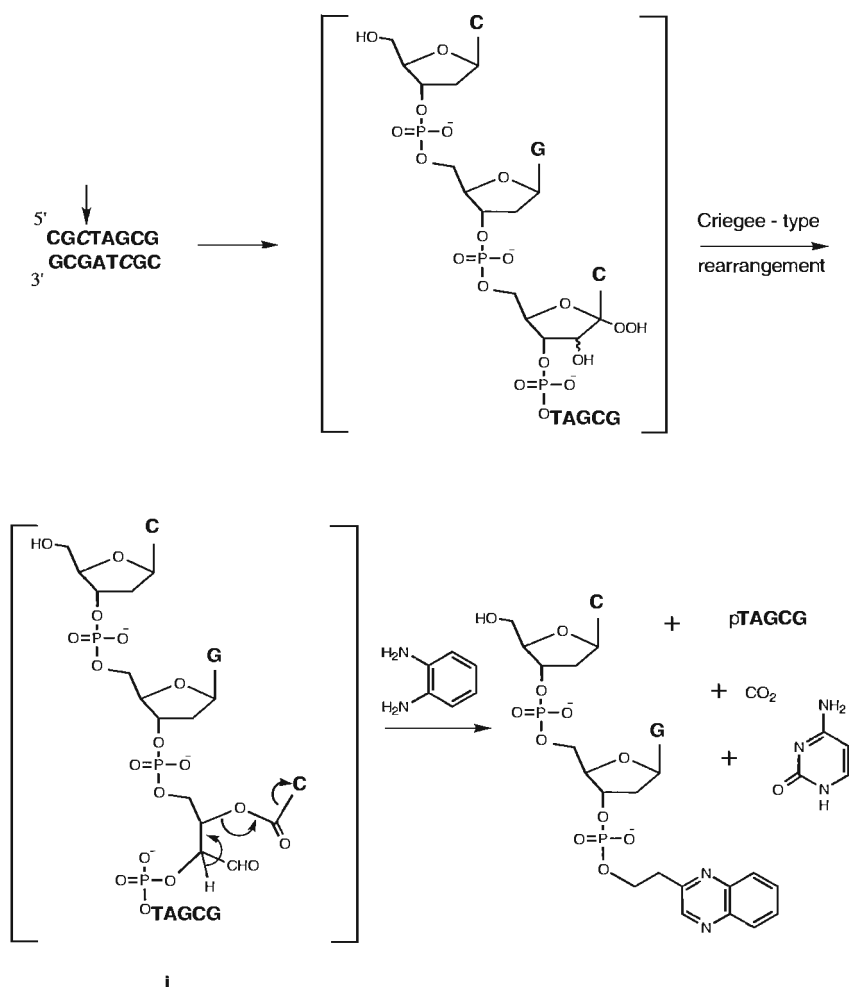


Fig. 3.9. Oxidative degradation of chimeric octanucleotides initiated by abstraction of C1' H.

relation between the ability of individual BLM congeners to mediate DNA strand scission and their ability to inhibit the growth of cultured KB cells, as well as the remarkably facile repair of BLM-mediated damage to chromatin [57].

That the lipid membrane may constitute an additional locus of action for BLM is suggested by the ability of the drug to effect lipid peroxidation [13–16] and by the finding that dibucaine, a local anesthetic known to increase membrane flexibility, rendered cultured KB cells susceptible to inhibition by a BLM analog that was incapable of DNA cleavage [58]. To the extent that the uptake of BLM by a cell is limiting to the expression of BLM cytotoxicity [59], lipid peroxidation could obviously alter uptake and thereby contribute importantly to potency of action.

There are reasons to consider RNA as a therapeutic target for BLM as well. If

drug uptake is limiting for the expression of cytotoxicity. The presence of RNA targets in the cytoplasm may facilitate the action of the drug since the nuclear membrane is likely to constitute a hindrance to nuclear entry as well. Additional advantages to an RNA target might be thought to include the apparent lack of intensive packaging of cytoplasmic RNAs, and the limited mechanisms for RNA repair. At least for a bacterial cell, it has been established that BLM is actually capable of intracellular RNA strand scission [60].

A key issue is the ability to kill cancer cells by destruction of one or more cellular RNAs. In fact, nature has provided good examples of cell killing by means of RNA targeting. These include the cytotoxic protein ricin, which functions by depurination of 28S ribosomal RNA [61]. Another good example is onconase, a cytotoxic member of the RNase A superfamily that appears to function at the level of tRNA degradation [62, 63].

One interesting strategy to resolve the issue of the actual locus of action of bleomycin would be to identify BLM analogs capable of functioning only at a single locus (i.e. either RNA or DNA degradation). The behavior of such species as anti-tumor agents could potentially result in the identification of the primary locus at which BLM expresses its antitumor effects. In this context it is worth noting that regardless of the actual locus of action of BLM itself, the elaboration of analogs of BLM capable of targeting critical RNAs with good selectivity could afford antitumor agents that function at novel cellular loci.

Acknowledgments

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4

Inhibitors of the Tat–TAR Interactions*Chimmanamada U. Dinesh and Tariq M. Rana*

4.1

Introduction

Acquired immune deficiency syndrome (AIDS) is one of the major causes of death in the recent history of humanity and the AIDS pandemic is not going away. As of the end of 2001, an estimated 40 million people worldwide – 37.2 million adults and 2.7 million children younger than 15 years – were living with AIDS. In spite of remarkable medical advances, HIV-1 infections are still increasing. Antiretroviral therapies have brought down the mortality rate significantly in developed countries. Unfortunately, the highest burden of AIDS is in nations that have the most limited medical resources. Worldwide, 95% of HIV-1 infections are in the developing world.

Enormous progress has been made in the development of antiretroviral agents. An understanding of the HIV replication mechanism first led to the development of “reverse transcriptase” (RT) inhibitors and later to the “protease” inhibitors. The nucleoside analog zidovudine (AZT) was one of the first RT inhibitors to be used for the treatment of AIDS. Several nucleoside, non-nucleoside, nucleotide RT inhibitors and protease inhibitors are currently available for AIDS therapy. However, no chemotherapeutic regimen is curative as yet.

Current AIDS therapies face three major problems: (1) first-line drugs are not effective in some patients, (2) newer medications have major side effects, (3) new drug-resistant strains of HIV are emerging. Therefore, there is a great need to find new drugs and treatment strategies. Given the pathogenesis of HIV mutants capable of resisting triple drug therapies, the identification of drugs that target HIV proteins other than reverse transcriptase and protease is a high priority for the development of new drugs.

HIV-1 is a complex retrovirus that encodes six regulatory proteins, including Tat and Rev, essential for viral replication. Inhibition of Tat and Rev function is an attractive target for new antiviral therapies. Both Tat and Rev are RNA-binding proteins and require specific interactions with RNA structures called TAR and RRE, respectively, for their function. In line with the focus of this chapter, Tat and its target RNA (TAR RNA) will be discussed in the following section.

4.2

Mechanism of Transcriptional Activation by Tat

The HIV replication cycle can be divided into two distinct phases (Fig. 4.1, see p. 60). During an early pre-integration stage, virus infects the cell and reverse transcriptase converts viral RNA into double-stranded DNA and the proviral genome is transported to the nucleus and integrated into the host genome. In the second post-integration stage, viral gene expression takes place from integrated proviral genome which is followed by viral assembly and maturation.

After the integration step of the HIV life cycle, the HIV proviral genome is transcribed by the human cellular machinery, including RNA polymerase II and other transcription accessory factors. Transcription from the viral long terminal repeat (LTR) is a complex process and is elegantly regulated at the elongation stage of transcription. The HIV-1 encodes a transcriptional activator protein, Tat, which is expressed early in the viral life cycle and is essential for viral gene expression, replication, and pathogenesis (for reviews, see Refs. [1–6]). Tat enhances the processivity of RNA Pol II elongation complexes that initiate in the HIV long terminal repeat (LTR) region. Mutational analysis of HIV-1 Tat protein has identified two important functional domains: an arginine-rich region that is required for binding to TAR RNA, and an activation domain that mediates the interactions with cellular machinery [7, 8]. Recent studies showed that Tat *trans*-activation function is mediated by a nuclear Tat-associated kinase, TAK [1, 2, 6]. The *trans*-activation domain of Tat interacts with TAK [9, 10], which was recently shown to be identical to the kinase subunit of P-TEFb [11, 12]. Tat interacts with the cyclinT1 (CycT1) subunit of P-TEFb and recruits the kinase complex to TAR RNA. Recruitment of P-TEFb to TAR has been proposed to be both necessary and sufficient for activation of transcription elongation from the HIV-1 long terminal repeat promoter [13]. The mechanism of Tat activation is summarized in Fig. 4.2 (see p. 60).

Neither CycT1 nor the P-TEFb complex bind TAR RNA in the absence of Tat, and thus the binding is highly cooperative for both Tat and P-TEFb [14, 15]. Tat appears to contact residues in the C-terminal boundary of the CycT1 cyclin domain that are not critical for binding of cyclinT1 to CDK9 [13, 16–23]. Mutagenesis studies showed that the CycT1 sequence containing amino acids 1–303 was sufficient to form complexes with Tat–TAR and CDK9 [13, 16–23]. Thus, the assembly of this complex appears to involve a series of adaptive interactions between the *trans*-activation and arginine-rich motif (RNA-binding) domains of Tat and their respective protein (CycT1) and nucleic acid (TAR) partners during transcription. Recent fluorescence resonance energy transfer studies using fluorescein-labeled TAR RNA and a rhodamine-labeled Tat protein showed that CycT1 remodels the structure of Tat to enhance its affinity for TAR RNA, and that TAR RNA further enhances interaction between Tat and CycT1 [24].

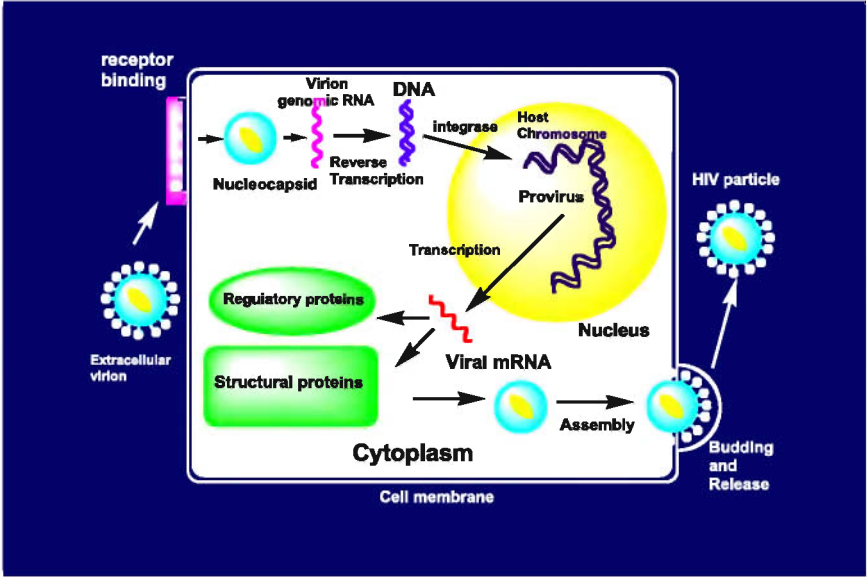


Fig. 4.1. Replication cycle of HIV. Virus initiates contact with receptors that is followed by entry and uncoating. Viral RNA is converted into DNA by the viral reverse transcriptase and the proviral genome is transported into the nucleus and integrated into the host genome. RNA polymerase II transcribes viral mRNA and the transcription of the proviral genome

requires the HIV regulatory protein Tat, which is expressed at low levels during early life cycle and is imported to the nucleus to activate transcription of viral genes. At later stages of the life cycle, structural proteins are expressed and progeny virus particles are assembled and released by a process of budding and subsequent maturation into infectious virus.

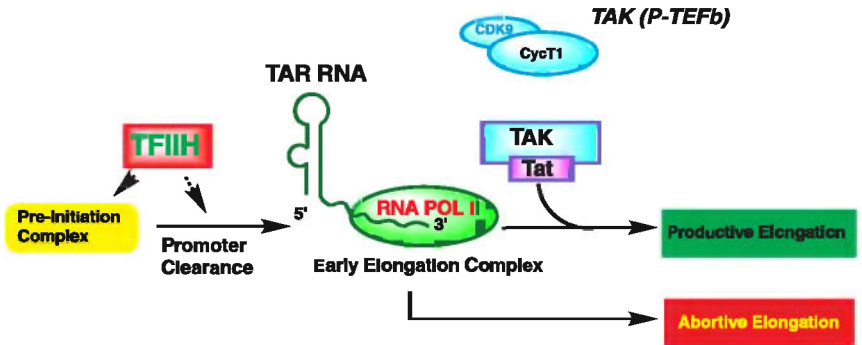
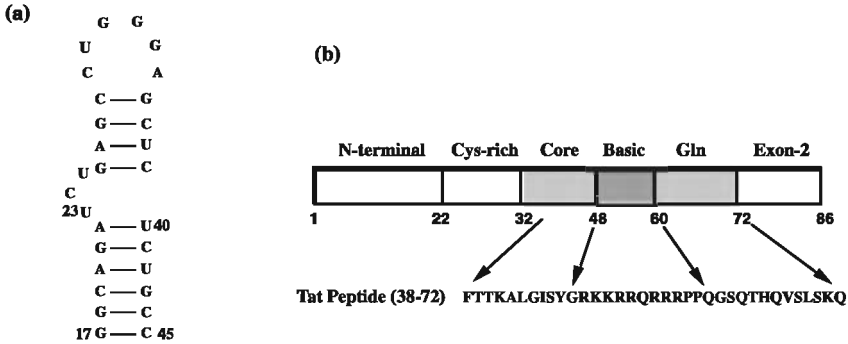


Fig. 4.2. Mechanism of transcriptional activation by Tat. Human RNA polymerase II initiates transcription from HIV promoter and TFIIH kinase assists in promoter clearance steps. TFIIH leaves the elongation complex when 30–36 nucleotides mRNA are transcribed [128]. Elongation is inefficient in the absence of

Tat protein. Tat recruits P-TEFb kinase complex (TAK – Tat-associated kinase) to TAR RNA via contacting the RNA and cyclinT1 component of the complex. Then, CDK9 kinase phosphorylates RNA polymerase II and other substrates in the elongation complex, which leads to processive transcription elongation [129].



TAR RNA

trans-activation response element

Fig. 4.3. (a) Sequence and secondary structure of TAR RNA used in structural studies. TAR RNA spans the minimal sequences that are required for Tat responsive-ness *in vivo* [29] and for *in vitro* binding of Tat-derived peptides [31]. Wild-type TAR contains two non-wild-type

base-pairs to increase transcription by T7 RNA polymerase [130]. Nucleotides critical for Tat binding are outlined. (b) Regions of the HIV-1 Tat protein and sequence of the Tat (38–72) peptide that recognizes Tat with high affinity and specificity.

4.3

Tat–TAR Interactions

As described above, the Tat protein is a potent transcriptional activator of the HIV-1 long terminal repeat promoter element. A regulatory element between +1 and +60 in the HIV-1 long terminal repeat which is capable of forming a stable stem-loop structure designated TAR is critical for Tat function.

Tat proteins are small arginine-rich RNA-binding proteins. HIV-1 Tat is encoded by two exons containing 86–101 amino acids in different HIV-1 isolates. Amino acids encoded by the first exon are both necessary and functional for TAR RNA binding and *trans*-activation *in vivo*. Tat protein is composed of several functional regions (Fig. 4.3). A cysteine-rich region (amino acids 22–37) contains seven cysteine residues; a “core” sequence (37–48) contains hydrophobic amino acids; a basic RNA-binding region (48–59) contains six arginines and two lysines and is a characteristic of a family of sequence-specific RNA-binding proteins; a glutamine-rich region at the C-terminus of the first exon contains several regularly spaced glutamines. In lentiviral proteins, only the basic and core regions are conserved. Although the integrity of the Cys-rich region is essential for *trans*-activation, this region does not appear to be directly involved in TAR RNA recognition. Based on mutational analysis, Tat can be divided into two functional domains. The first domain is the activation domain (amino acids 1–47) or cofactor-binding domain,

which is functionally autonomous and is active when recruited to the HIV-1 LTR via a heterologous RNA-binding protein [25]. The second functional domain contains the basic region and is required for both RNA binding and nuclear localization activities of Tat [26].

HIV-1 Tat protein acts by binding to the TAR (*trans*-activation responsive) RNA element, a 59-base stem-loop structure located at the 5' ends of all nascent HIV-1 transcripts [27]. TAR RNA was originally localized to nucleotides +1 to +80 within the viral LTR [28]. Subsequent deletion studies have established that the region from +19 to +42 incorporates the minimal domain that is both necessary and sufficient for Tat responsiveness *in vivo* (Fig. 4.3) [29]. TAR RNA contains a six-nucleotide loop and a three-nucleotide pyrimidine bulge which separates two helical stem regions [30].

Tat protein recognizes the trinucleotide bulge in TAR RNA. Key elements required for TAR recognition by Tat have been defined by extensive mutagenesis, chemical probing, and peptide-binding studies [31–35]. Tat interacts with U23 and two other bulge residues, C24 and U25, which act as spacers because they can be replaced by other nucleotides or linkers [34, 36]. In addition to the trinucleotide bulge region, two base pairs above and below the bulge also contribute significantly to Tat binding [32, 34]. Phosphate contacts below the bulge at positions 22, 23, and 40 are critical for Tat interactions [33, 34, 37]. Chemical crosslinking studies showed that a TAR duplex containing a trisubstituted pyrophosphate replacing the phosphate at 38–39 reacted specifically with Lys51 in the basic region of Tat(37–72) peptide [38]. Site-specific photo-crosslinking experiments on Tat–TAR complex using 4-thiouracil as a photoactive nucleoside showed that Tat interacts with U23, U38, and U40 in the major groove of TAR RNA [39]. In a recent study, 6-thio-G was incorporated at specific sites in TAR RNA sequence and Tat–TAR photo-crosslinking experiments were performed [40]. Results of these experiments provide direct evidence that, during RNA–protein recognition, Tat is in close proximity to O⁶ of G21 and G26 in the major groove of TAR RNA. Taken together, these studies establish that Tat binds TAR RNA at the trinucleotide bulge region and interacts with two base pairs above and below the bulge in the major groove of RNA.

The basic regions of Tat proteins are directly involved in RNA binding. Short basic peptide mimics of this region bind TAR RNA in the bulge region [31–34, 41]. A number of studies determined the sequence requirements within the basic region of Tat for RNA binding. *In vivo* experiments using full-length Tat and *in vitro* studies with synthetic peptides indicate that conservation of overall positive charge, including several arginines, is essential for TAR RNA recognition. For example, Tat–TAR interactions are not affected by interchanging the basic region sequences of Tat and Rev [42]. Amino acid substitutions that recreate the consensus sequence of the RNA-binding region (R/KXXRRXRR, where R is arginine, K is lysine, and X is any amino acid) reconstitute a functional Tat protein. These results are inconsistent with *in vitro* peptide studies showing that high-affinity TAR RNA-binding requires three out of four arginines within the RXXRR stretch, although any one arginine can be substituted suggesting some redundancy [41].

4.4

RNA as a Small Molecule Drug Target

Protein–nucleic acid interactions are involved in many cellular functions, including transcription, RNA splicing, and translation. Readily accessible synthetic molecules that can bind with high affinity to specific sequences of single- or double-stranded nucleic acids have the potential to interfere with these interactions in a controllable way, making them attractive tools for molecular biology and medicine. Successful approaches used thus far include duplex-forming (antisense) [43] and triplex-forming (anti-gene) oligonucleotides [44–46], peptide nucleic acids (PNA) [47], and pyrrole-imidazole polyamide oligomers [48, 49]. Each class of compounds employs a readout system based on simple rules for recognizing the primary or secondary structure of a linear nucleic acid sequence. Another approach employs carbohydrate-based ligands, calicheamicin oligosaccharides, which interfere with the sequence-specific binding of transcription factors to DNA and inhibit transcription *in vivo* [50, 51]. While antisense oligonucleotides and PNA employ the familiar Watson–Crick base-pairing rules, two others, the triplex-forming oligonucleotides and the pyrrole-imidazole polyamides, take advantage of straightforward rules to read the major and minor grooves, respectively, of the double helix itself.

In addition to its primary structure, RNA has the ability to fold into complex tertiary structures consisting of such local motifs as loops, bulges, pseudoknots, and turns [52, 53]. It is not surprising that, when they occur in RNAs that interact with proteins, these local structures are found to play important roles in protein–RNA interactions (Fig. 4.4). This diversity of local and tertiary structure, however, makes it impossible to design synthetic agents with general, simple-to-use recognition rules analogous to those for the formation of double- and triple-helical nucleic acids. Since RNA–RNA and protein–RNA interactions can be important in viral and microbial disease progression, it would be advantageous to have a general method for rapidly identifying synthetic compounds for targeting specific RNA structures. A particular protein-binding RNA structure can be considered as a molecular receptor not only for the protein with which it interacts but also for synthetic compounds, which may prove to be antagonists of the protein–RNA interaction.

Two examples of such interactions are the Tat–TAR and Rev–RRE recognition, which are essential elements in the mechanism of HIV-1 gene expression. The following sections will describe recent developments in the identification of ligands for inhibition of Tat–TAR interactions.

4.5

Ligands for TAR RNA

4.5.1

TAR RNA Bulge Binders

As discussed above, Tat protein binds a trinucleotide bulge sequence in TAR RNA. Therefore, it is obvious that a Tat antagonist should be able to recognize the bulge

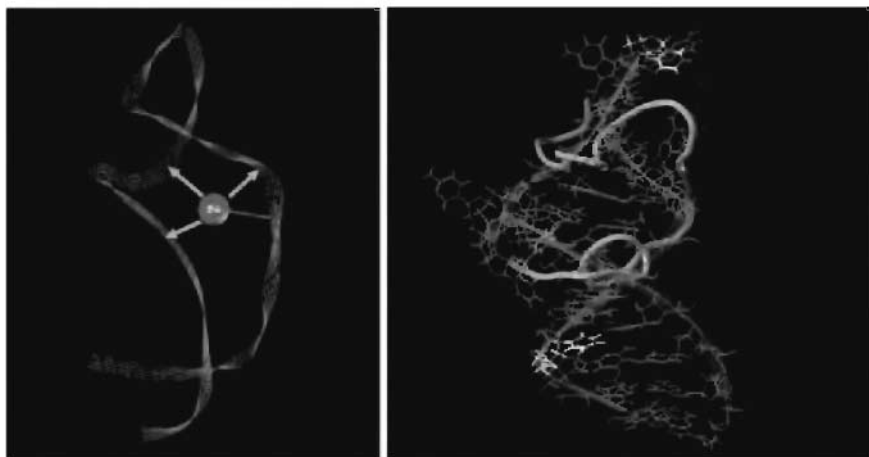


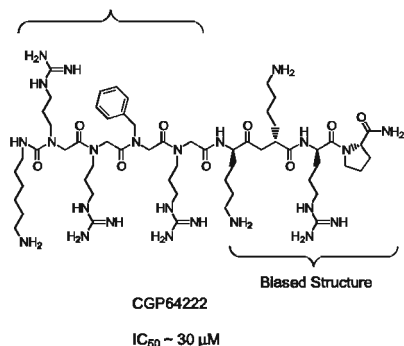
Fig. 4.4. TAR RNA folding and Tat–TAR interactions. (a) Tertiary folding of TAR RNA probed by RNA self-cleavage method using a tethered iron chelate [131]. Iron(II) is covalently attached at a specific site in the RNA sequence and arrows show the regions of RNA cleaved by the production of localized hydroxyl radicals. (b) Proposed model for Tat peptide–TAR interactions showing protein orientation and the proximity of various nucleotides to the peptide in the RNA–protein complex. Ribbon structure of TAR RNA is shown in yellow lines and nucleotides in red

[55]. Ribbon structure of the Tat peptide (ribbon/tube) and the N-terminal Phe38 are shown in cyan color. Ribbon structure of the Tat peptide is drawn from Tat protein structure [132]. Orientation of the Tat peptide is based on previous photo-crosslinking results indicating that Lys41 and Arg57 are close to U42 and U31, respectively [66,130]. Lys41 and Arg57 side chains are shown in green. U42 is shown in yellow and U31 in atom-by-type colors. Structures of RNA and protein were visualized using Insight II software on an IRIS workstation.

structure and, ideally, should be able to bind TAR bulge with affinities higher than that of Tat protein. Hamy and co-workers identified a peptidic compound, CGP64222, that was able to bind TAR RNA with high affinities [54]. NMR studies suggested that CGP64222 binds the bulge region of TAR and induces conformational change in TAR resulting in a structure very similar to that of a Tat–peptide-bound TAR RNA [54, 55]. This nine-residue oligomer, a hybrid peptide/peptoid, was screened and identified by a deconvolution combinatorial library method. The Tat activity in a cellular Tat-dependent *trans*-activation assay was inhibited with 10–30 μ M CGP64222. The structure of CGP64222 is shown in Fig. 4.5.

In another study, Hamy *et al.* [56] reported the identification of low-molecular weight Tat antagonists, their affinities for TAR RNA, and biological activities. A series of compounds on the basis of published structural data of the molecular interactions between TAR and Tat-derived peptide was synthesized. This new class of Tat antagonists contains two different functional motifs, a polyaromatic motif for stacking interactions with TAR RNA and a polycationic anchor for contacts with the phosphate backbone of RNA. A varying linker to connect the stacking motif with the RNA-binding motif was used. The most active compound competed with Tat–TAR complex formation with a competition dose CD_{50} of 22 nM *in vitro* and

The structure of CGP64222 screened by the deconvolution of combinatorial libraries



Positive charges for phosphate backbone interaction

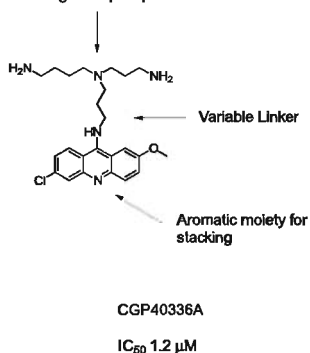


Fig. 4.5. Structures of TAR RNA bulge-binding molecules that inhibit Tat–TAR interactions [54, 56].

blocked Tat activity in a cellular system with an IC₅₀ of 1.2 μM. Figure 4.5 shows the structure of the active compound. From structure–activity relationship studies, two new features of Tat–TAR inhibitors became clear: (1) Modification of the linker length has a mild effect on activity and the structure of polyamine moiety is critical for Tat–TAR inhibition. (2) The position of the polyaromatic ring for a substitution of the linker is important and the type of chemical bond between the linker and the polyaromatic motif is also crucial for activity.

4.5.2

Targeting Multiple Sites in TAR RNA

To identify small organic molecules that inhibit HIV-1 replication by blocking Tat–TAR interactions, Mei *et al.* [57] screened their research compound libraries and reported three inhibitors of Tat–TAR interactions that target TAR RNA and not the protein. These three Tat–TAR inhibitors include neomycin, quinoxaline, and aminoquinoxaline. Chemical structures of these compounds, IC₅₀, and their binding sites on TAR RNA are outlined in Fig. 4.6. Each of these inhibitors recognizes a different structural region in TAR RNA such as the bulge, lower stem, and the loop sequence.

In another attempt to discover Tat–TAR inhibitors, Mei *et al.* [58] screened their corporate compound library containing approximately 150,000 compounds. Selective Tat–TAR inhibitors were screened by *in vitro* high-throughput screening assays and inhibitory activities were determined by gel mobility shift assays, scintillation proximity assays, filtration assays, and electrospray ionization mass spectrometry (ESI-MS). After *in vitro* assays, Tat-activated reporter gene analyses were employed to investigate the cellular activities of the primary Tat–TAR inhibitors. Approximately 500 Tat–TAR inhibitors were selected from *in vitro* assays and 50 compounds exhibited dose-dependent cellular activities with IC₅₀ values ≤ 50 μM.

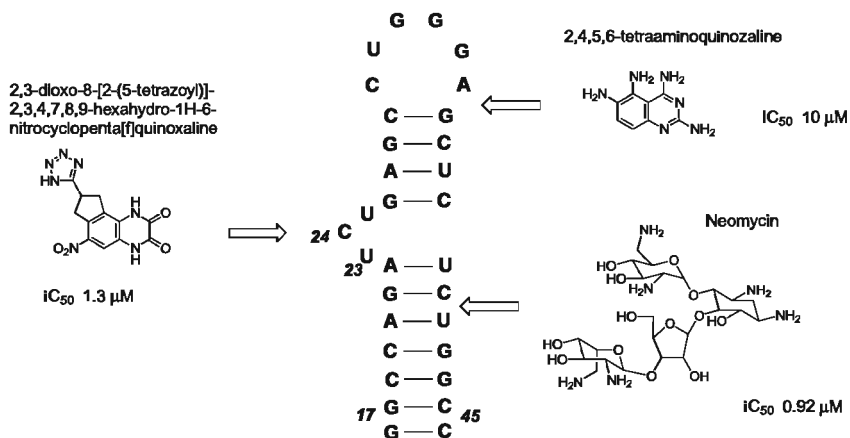


Fig. 4.6. Structures and IC_{50} values of three TAR RNA ligands. Putative RNA-binding sites are indicated [57].

Among them, approximately 20 compounds were relatively non-toxic (therapeutic index, TC_{50}/IC_{50} , ≥ 5) and considered selective for Tat-dependent transcription.

4.5.3

Targeting RNA with Peptidomimetic Oligomers

4.5.3.1 Backbone modification

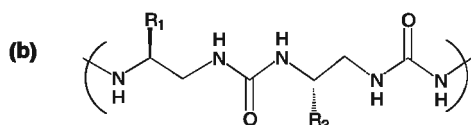
We have recently begun to examine TAR RNA recognition by unnatural biopolymers [59, 60]. We synthesized oligocarbamates and oligourea containing the basic arginine-rich region of Tat by solid-phase synthesis methods, and tested for TAR RNA binding. The oligocarbamate backbone consists of a chiral ethylene backbone linked through relatively rigid carbamate groups [61]. Oligoureas have backbones with hydrogen-bonding groups, chiral centers, and a significant degree of conformational restriction. Introducing additional side chains at the backbone NH sites can further modify biological and physical properties of these oligomers (Fig. 4.7).

A Tat-derived oligourea binds specifically to TAR RNA with affinities significantly higher than the wild-type Tat peptide. To synthesize Tat-derived oligourea on solid support, we used activated *p*-nitrophenyl carbamates and protected amines in the form of azides, which were reduced with $SnCl_2$ -thiophenol-triethylamine on solid support [62, 63]. After HPLC purification and characterization by mass spectrometry, the oligourea was tested for TAR RNA binding. The Tat-derived oligourea was able to bind TAR RNA and failed to bind a mutant TAR RNA without the bulge residues [60].

Equilibrium dissociation constants of the oligourea-TAR RNA complexes were measured using direct and competition electrophoretic mobility assays. Dissociation constants were calculated from multiple sets of experiments which showed

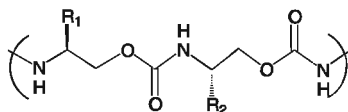
(a) ⁴⁸Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-⁵⁷

RNA-binding Tat Peptide



Structure of Oligourea Backbone

(c)



Structure of Oligocarbamate Backbone

Fig. 4.7. (a) The Tat-derived peptide, amino acids 48–57, contains the RNA-binding region of Tat protein. Structure of the oligourea (b) and oligocarbamate (c) backbone. Sequence of the oligourea and oligocarbamate correspond to the Tat peptide shown in (a), except the addition of an L-Tyr amino acid at the C-terminus of oligourea [59, 60].

that the oligourea binds TAR RNA with a K_D of $0.11 \pm 0.07 \mu\text{M}$. To compare the RNA-binding affinities of the oligourea to natural peptide, we synthesized a Tat-derived peptide (Tyr47 to Arg57) containing the RNA-binding domain of Tat protein. Dissociation constants of the Tat peptide–RNA complexes were determined from multiple sets of experiments under the same conditions used for oligourea–TAR RNA complexes. These experiments showed that the Tat peptide (47–57) binds TAR RNA with a K_D of $0.78 \pm 0.05 \mu\text{M}$. A relative dissociation constant (K_{REL}) can be determined by measuring the ratios of wild-type Tat peptide to the oligourea dissociation constants (K_D) for TAR RNA. Our results demonstrate that the calculated value for K_{REL} was 7.09, indicating that the urea backbone structure significantly enhanced the TAR-binding affinities of the unnatural biopolymer, and this difference in K_D values could be more dramatic because gel mobility shift methods severely underestimate absolute peptide–RNA-binding affinities [41].

Site-specific photo-crosslinking and competition experiments showed that a small Tat-derived oligourea binds TAR RNA specifically with high affinity and interacts in the major groove of TAR RNA similar to Tat peptides. Due to the difference in backbone structure, oligoureas may differ from peptides in hydrogen-bonding properties, lipophilicity, stability, and conformational flexibility. Moreover, oligoureas are resistant to proteinase K degradation. These characteristics of oligoureas may be useful in improving pharmacokinetic properties relative to peptides. RNA recognition by an oligourea provides a new approach for the design of drugs, which will modulate RNA–protein interactions.

4.5.3.2 D-Peptides

Due to the difference in chirality, D-peptides are resistant to proteolytic degradation and cannot be efficiently processed for major histocompatibility complex class II-restricted presentation to T helper cells (T_H cells). Consequently, D-peptides would not induce a vigorous humoral immune response that impairs the activity of L-peptide drugs [64]. The D-peptide ligands may provide useful starting points for the design or selection of novel drugs.

Can D-peptides recognize naturally occurring nucleic acid structures? To test this hypothesis, we synthesized a D-Tat peptide, Tat(37–72), containing the basic arginine-rich region of Tat by solid-phase peptide synthesis methods. After HPLC purification and characterization by mass spectrometry, the D-Tat peptide was tested for TAR RNA binding. Similar to L-Tat, the D-Tat peptide was able to bind TAR RNA and failed to bind a mutant TAR RNA without the bulge residues. Equilibrium dissociation constants of the D-Tat–TAR RNA complexes were measured using direct electrophoretic mobility shift assays [65, 66]. Dissociation constants were calculated from eight sets of experiments which showed that the D-Tat peptide binds TAR RNA with a K_D of 0.22 μ M. Under similar experimental conditions, L-Tat(37–72) binds TAR RNA with a K_D of 0.13 μ M [67].

To test the effects of D-Tat peptide on HIV-1 transcription in a cell-free system, *in vitro* transcription reactions were performed using HeLa cell nuclear extract and linearized HIV-1 DNA template [68]. Tat produced a large increase in the synthesis of correctly initiated 530 nucleotide runoff RNA transcripts [69]. Tat stimulated transcription at concentrations ranging from 50 ng to 300 ng per 10 μ L reactions. Quantitation revealed that 100 ng of Tat per reaction produced a 10–12-fold stimulation of HIV-1 transcription. Control experiments showed that Tat did not significantly increase transcription from an HIV-1 promoter with a mutated TAR element either in the stem region (G26 to C26) or in the loop sequence (U31 to G31). Increasing amounts of D-Tat resulted in a significant decrease in Tat-mediated transcriptional activation. In the presence of 1 μ g D-Tat (approximately equal to 3 times the wild-type Tat), more than 80% Tat *trans*-activation was inhibited [69]. The amount of recovered transcripts and the efficiency of transcription were normalized by including a labeled RNA not originating from HIV-1 LTR. To determine the specificity of *trans*-activation inhibition by D-Tat, a mutant D-Tat peptide, Gly44–Gln72, where all Arg residues in the RNA-binding region were substituted with Ala, was synthesized. The mutant D-Tat was unable to bind TAR RNA in electrophoretic mobility shift experiments and did not inhibit Tat *trans*-activation *in vitro*. These results indicate that D-Tat is able to specifically inhibit Tat *trans*-activation *in vitro*.

It has been previously established that Tat peptides containing the basic domain are taken up by cells within less than 5 minutes and accumulate in the cell nucleus [70]. Since the D-Tat peptide also contains the basic domain of Tat, we reasoned that this peptide would be rapidly taken up by HeLa cells and accumulate in the nucleus. Once D-Tat peptide reaches the nucleus, it would compete with Tat for TAR binding and lead to inhibition of Tat function. To test this hypothesis, we added D-Tat during transfection of pSV2-Tat [71] and pAL [72] plasmids into HeLa

cells containing an integrated LTR-CAT reporter [73]. Plasmids pSV2Tat and pAL express the first exon of Tat protein and luciferase enzyme, respectively. Transfection of pSV2Tat enhanced transcription as determined by CAT activity. Increasing amounts of the Δ -Tat resulted in a decrease of CAT activity while luciferase activity was not affected [69]. Tat *trans*-activation was inhibited more than 60% by 5 μ g (approx. 0.5 μ M) Δ -Tat peptide. Further addition of Δ -Tat did not further inhibit Tat *trans*-activation, probably because maximum peptide uptake efficiency is reached at 5 μ g of Δ -Tat. To rule out the possibility that the observed inhibition of *trans*-activation is due to some non-specific toxicity of the Δ -peptide or reduction of the pSV2Tat plasmid uptake, transcription of luciferase gene was monitored. Transcription of luciferase gene was not affected by Δ -Tat peptide as measured by luciferase enzymatic activity assays. Cell viability assays showed that cells were not killed by Δ -Tat treatment. Specificity of the inhibition was tested by adding a mutant Δ -Tat peptide, Gly44–Gln72, where all Arg residues in the RNA-binding region were substituted with Ala during transfection of plasmids and analyzing the CAT and luciferase activities as described above for Δ -Tat. This mutant Δ -Tat peptide did not inhibit Tat *trans*-activation. Thus, these results indicate that the Δ -Tat peptide specifically inhibits *trans*-activation by Tat protein *in vivo* [69].

These findings show that a small Tat-derived Δ -peptide binds TAR RNA and selectively inhibits Tat *trans*-activation. It remains to be determined whether a broad range of RNA–protein interactions can be selectively targeted. These results present an example of the application of Δ -peptides as artificial regulators of cellular processes involving RNA–protein interactions *in vivo*.

4.6

Combinatorial Library Approach in the Discovery of Small Molecule Drugs Targeting RNA

4.6.1

Combinatorial Chemistry

The demand for a variety of chemical compounds for identifying and optimizing new drug candidates has increased dramatically. In the past, traditional mass screening of natural products from plants, marine organisms, and synthetic compounds has been successful in identifying a lead chemical structure. In order to support this demand, chemists have developed new methodologies that are accelerating the drug discovery process. Combinatorial synthesis is one of the most promising approaches to the synthesis of a large collection of diverse molecules because vast libraries of molecules having different chemical identities are synthesized in a short period of time [74–79]. There is considerable evidence to show that combinatorial chemistry plays an important role in the lead discovery process. For example, a variety of biological targets such as proteases [63, 80, 81], protein kinases [82–84], cathepsin D [85], and SH3 domain [86–88] have been screened with this new technology.

Combinatorial synthesis has been primarily facilitated by the application of solid-phase synthesis [89, 90]. Each substrate is linked to a solid support (a polymer bead), and it is possible to synthesize a variety of products that are spatially separated, and thus reagents and by-products not bound to the beads may be removed simply by filtration. In short, the combinatorial synthesis is faster, and thus more efficient and much cheaper than classical organic synthesis, and can give up to thousand or even million of products simultaneously.

The combinatorial drug discovery process has three major parts: (1) The generation of a large collection of diverse molecules, known as combinatorial libraries, by systematic synthesis of a variety of building blocks. (2) Screening of such libraries with biological targets to identify novel lead compounds. (3) Determining the chemical structures of active compounds. Therefore, the combinatorial discovery process requires not only the rational design and synthesis of combinatorial libraries of molecular diversity, but also the development of screening methodologies for library evaluation.

A variety of libraries have been prepared by synthetic and biological methods such as phage display, polysomes, and plasmids [91, 92]. Two major synthetic methods are the iterative deconvolution approach [93, 94] and the one-bead one-compound method [95]. First, iterative deconvolution is a chemical method in which the chemical structure of the active compound in a combinatorial library is characterized in an iterative manner. It involves screening of combinatorial library pools, identification of the active sublibrary pool, resynthesis of sublibraries, and rescreening of the resynthesized sublibraries. This method has the advantage that it affords fully characterizable, non-modified structure, solution-phase libraries which afford more realistic interaction results than solid-support bound libraries. However, the time-consuming nature of having to resynthesize and reassay sublibraries and the potential inconsistencies during resynthesis have led to a search for alternative combinatorial methods. Recently, Hamy *et al.* [54, 56] have used this method to identify inhibitors of HIV-1 replication. The one-bead-one-compound method is based on the fact that the combinatorial bead library contains single beads displaying only one type of compound although there may be up to approx. 100 pmol (approx. 10^{13} molecules) of the same molecules on a single 90- μ m-diameter polymer bead.

This approach has several unique features: (1) A large combinatorial library is synthesized by a split synthesis method. (2) Each library member (compound) is spatially separated in the solution and all the library compounds can be screened independently at the same time. (3) Once active beads are screened, the chemical structure of the active beads may be determined directly by using NMR, mass spectrometry, and HPLC [96, 97] or by an encoding method [98, 99].

4.6.2

Split Synthesis

The beauty of split synthesis is that it is a simple and very efficient synthetic method (Fig. 4.8) [100]. A sample of support material (bead) is divided into a

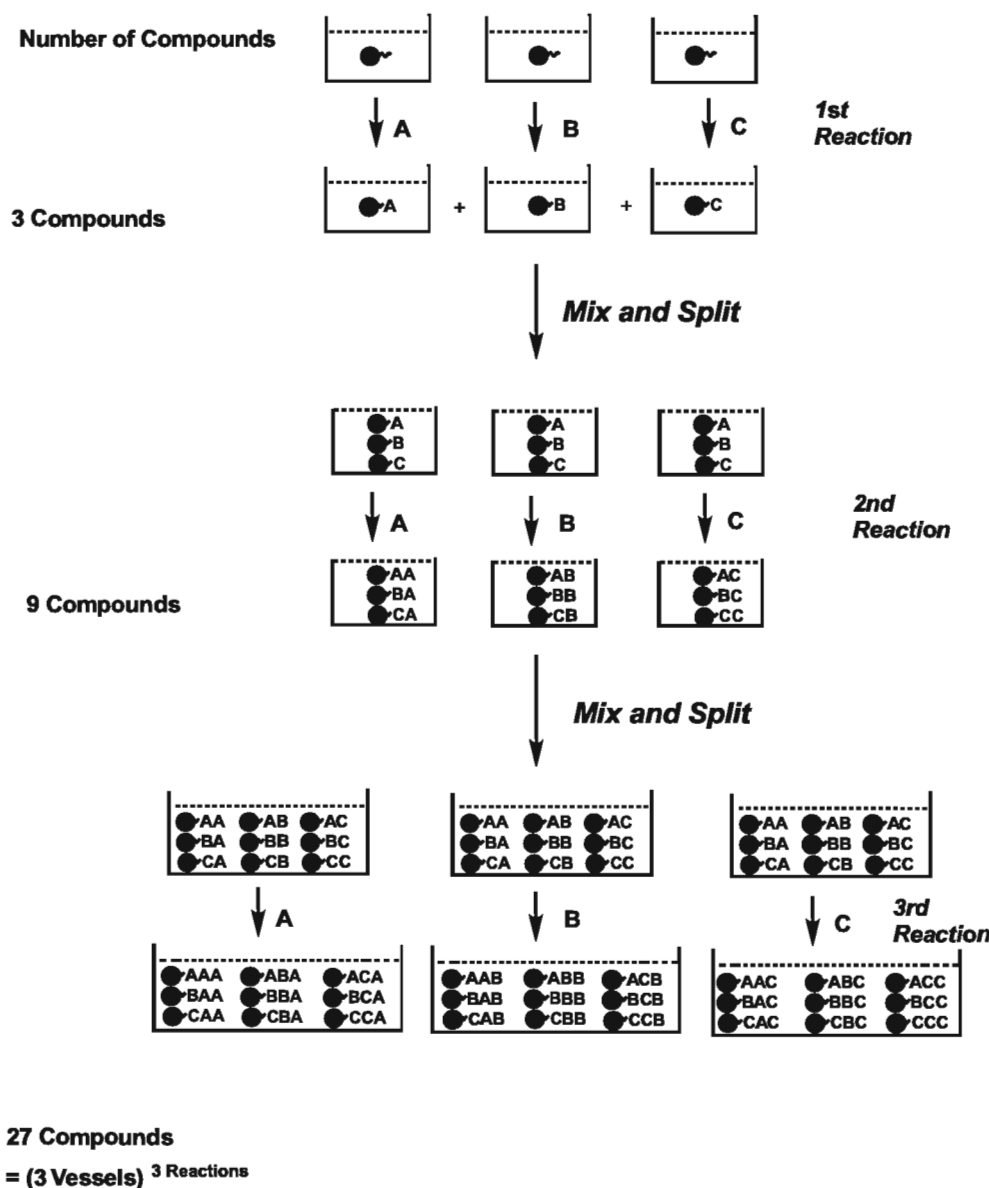


Fig. 4.8. Split synthesis using three monomers would produce as many as 27 different timers.

number of equal portions (n) and each reaction vessel is individually reacted with a specific substrate. After completion of the substrate reaction, it is subsequently washed to remove by-products and excess reagent. The individual reaction products are recombined, the whole is thoroughly mixed, and divided again into portions. Further reaction with a set of reagents gives complete sets of possible dimer

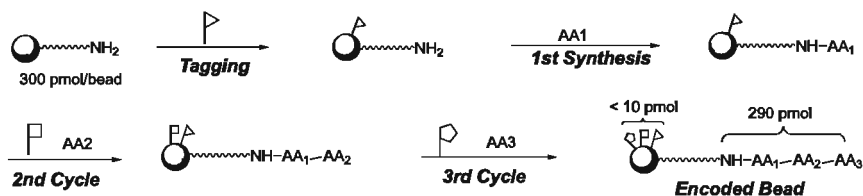


Fig. 4.9. Schematic representation of the encoding process.

combinatorial libraries, and this whole process may then be repeated as necessary (total x times). After this split synthesis, the number of library compounds obtained arises from the exponential increase in molecular diversity, in this case n to the power of x (n^x). For example, split synthesis using 20 different D and L amino acids at each site of a pentapeptide would produce as many as $20^5 = 3,200,000$ different compounds.

4.6.3

Encoding

The idea of encoding synthetic information with a chemical tag was first proposed by Brenner and Lerner [101]. Encoding involves attaching unique arrays of readily analyzable chemical tags to each bead that designate the particular set of reagents used in the split synthesis of that specific bead [99, 102]. Thus analyzing any bead for its tag content yields the history for the synthesis of that specific bead (Fig. 4.9). Although one could use a different tag for each reagent, it is much simpler to use a mixture of tags because tag mixtures of N different tags can encode 2^N different reactions. For example, only six tags are needed to encode as many as 64 different reactions. While almost any kind of chemical can be used for encoding, there are practical problems because tags need to be chemically inert to library synthesis and reliably analyzed in picogram quantity from a single positive bead. Numerous tagging methods have been developed such as oligonucleotide, peptide, and halophenyl tag approaches [74, 102, 103]. Among these methods, halophenyl tagging was chosen because it fulfills the following requirements:

1. An assurance of fast structural determination by GC/ECD (gas chromatography using electron capture detector). In early stages of encoding strategies, biopolymer tags were used [104]. The problems of molecular structure determination using biopolymers are the stability of the tag to the vigorous organic reaction conditions and the amount of compound on one bead may not allow for characterization by Edman degradation or PCR. This problem can be solved using photocleavable halophenyl tags that can afford the structural determination in 10 minutes using femtomole quantity of tags on the bead.
2. Affordability of a variety of tagging compounds. Using different spacers and halophenyl compounds, 30–40 tagging compounds are easily obtained with commercially available starting materials.

3. Simplicity of the tag cleavage reactions. The photocleavable halophenyl tag is cleaved by UV irradiation and it does not require any chemical reactions.
4. Sensitivity of halophenyl derivatives. A few femtomole quantity of halophenyl functionality has been detected with GC/ECD analysis.

4.6.4

On-bead Screening and Identification of Structure-specific TAR-Binding Ligands

Previous studies using combinatorial chemistry to identify new ligands to block the TAT-TAR interaction have relied on a variety of complex methods that are labor intensive or require expensive robotics equipment [58]. For the most part, these methods originated in the study of individual protein–nucleic acid interaction experiments. Moreover, in some cases time-consuming deconvolution strategies are also needed to identify the individual compounds responsible for the properties found in a mixture of compounds tested together [54, 56]. We decided to investigate on-bead screening methods that have been previously used with success on small organic receptors. This entailed covalently attaching the dye Disperse Red to the TAR RNA (Fig. 4.10) and incubating it in a suspension of library beads made from the split synthesis method. Diffusion of low-molecular-weight receptors into a bead of TentaGel resin is known to be rapid, whereas one might expect that a macromolecule such as a protein or large nucleic acid might be excluded from the bead interior where the bulk of the peptide is displayed. Nevertheless, we have found that the dye–TAR conjugate was able to enter the beads and bind in a structure-dependent manner. Peptides specific for portions of TAR other than the bulge region were blocked by using a relatively large concentration of an unlabeled TAR analog lacking the natural 3-nucleotide bulge. A small amount of detergent and using a low RNA concentration (250 nM) also minimizes non-specific binding. Another advantage to our method is the use of chemically encoded beads [105].

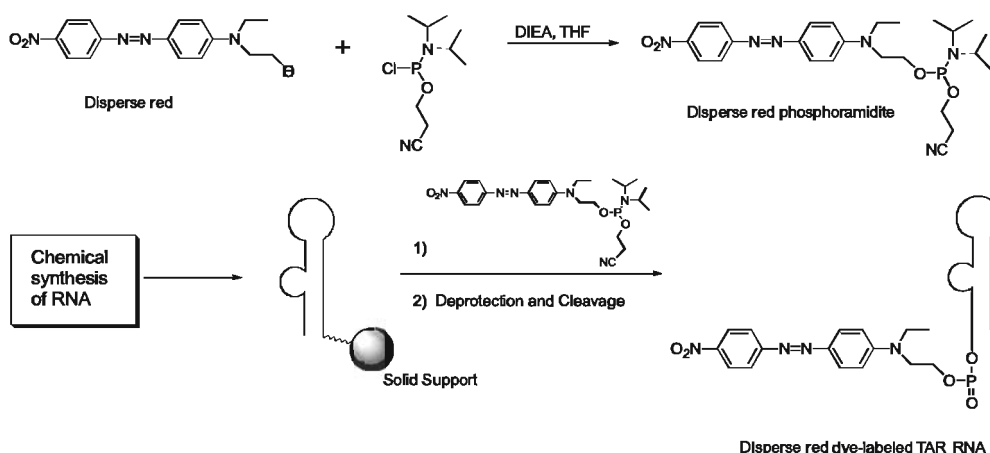


Fig. 4.10. Covalent attachment of the dye Disperse Red to the TAR RNA.

Tab. 4.1. RNA-binding ligands.

ID no.	Structures	Frequency	Color	K_D (nM) ^a	K_{REL} ^b
1	NH ₂ -(1)Lys-(D)Lys-(1)Asn-OH	2	Red	420 ± 44	1.73
2	NH ₂ -(1)Lys-(D)Lys-(D)Asn-OH	1	Pink	4173 ± 208	0.17
3	NH ₂ -(1)Lys-(1)Lys-(1)Asn-OH	2	Pink	3224 ± 183	0.23
4	NH ₂ -(1)Arg-(D)Lys-(1)Ala-OH	1	Pink	2640 ± 219	0.28
5	NH ₂ -(1)Arg-(D)Lys-(D)Val-OH	1	Pink	10,434 ± 594	0.07
6	NH ₂ -(1)Arg-(D)Lys-(1)Arg-OH	1	Pink	878 ± 80	0.83
7	NH ₂ -(D)Thr-(D)Lys-(1)Asn-OH	1	Pink	564 ± 80	1.29
8	NH ₂ -(D)Thr-(D)Lys-(1)Phe-OH	1	Pink	2087 ± 244	0.35

^a K_D values were determined from four independent experiments.
^b $K_{REL} = K_D$ of a basic Tat peptide (727 ± 74 nM)/ K_D of inhibitor.
The bead library peptides were all *N*-acetylated. Peptides used for *in vitro* and *in vivo* experiments were not *N*-acetylated and contained free *N*-termini.
Adapted from Ref. [106].

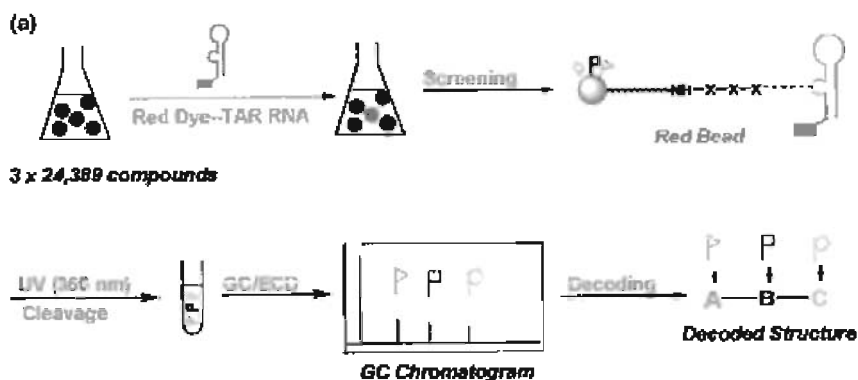
Once a dye-stained bead is selected, the identification of the RNA-binding small molecule is rapid and straightforward (Fig. 4.11). Although many binding experiments are conducted simultaneously, the compounds remain discrete, each in its own assay vessel, the bead produced by the split-synthesis method [106].

4.6.5
Ligand Sequence Analysis

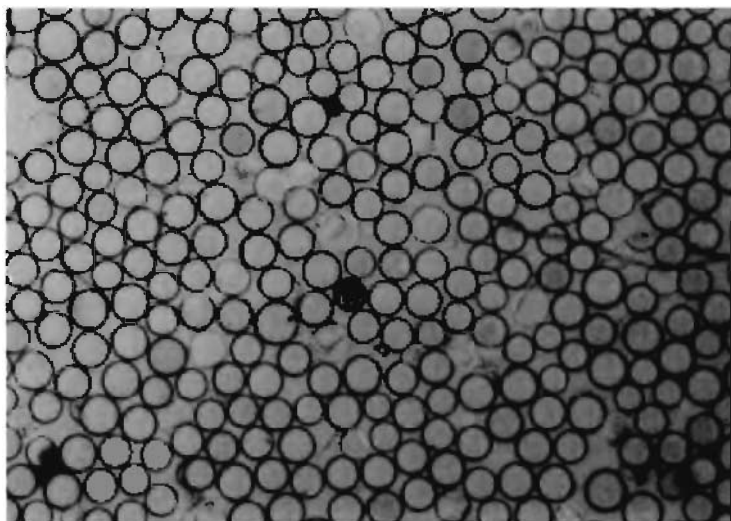
Upon incubating the dye–TAR conjugate with the library we picked a set of beads and decoded the structure of RNA-binding ligands (Tab. 4.1). To verify that our assay reflected RNA–trimer interaction and to determine the affinity of these trimer ligands for TAR RNA, we resynthesized trimer peptides 1–8 (Tab. 4.1) and measured their dissociation constants with wild-type TAR RNA. The results in Tab. 4.1 confirm that the on-bead assay mimics RNA binding because ligand 1 has the highest affinity for TAR RNA ($K_D = 441$ nM). To compare the RNA-binding affinities of eight ligands to natural Tat peptide, we synthesized a Tat-derived peptide (Gly48 to Arg57) containing the RNA-binding region of Tat protein. Dissociation constants of the Tat peptide–RNA complexes were determined under the

Fig. 4.11. (a) Schematic presentation of screening and decoding of the combinatorial library. TAR RNA was labeled with a red dye, Disperse Red, at its 5' end by chemical synthesis and incubated with the trimer library. (b) A portion of the beads in the library after incubating with red-dye-labeled TAR RNA. The dark bead in the center was identified as ligand 1. (c) The equilibrium interaction between dye-labeled TAR RNA and a tripeptide tethered to beads. A suspension of beads containing ligand 1 in Tris-HCl buffer (400 μL) was incubated with dye-labeled TAR RNA (1 μM) at 4°C for 5 h. Beads were stained red upon TAR RNA binding (left). Red beads became colorless when excess of unlabeled TAR RNA (middle) or ligand 1 (right) was added, indicating the displacement of red–TAR RNA from the beads. (Reprinted from Hwang *et al.* [106].)

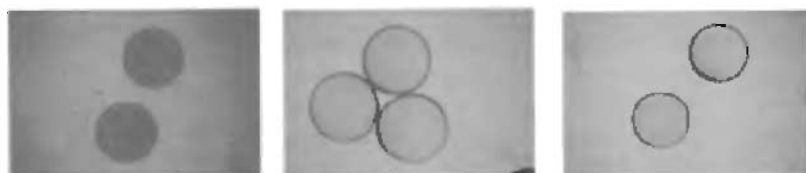
same conditions used for trimer ligand–TAR RNA complexes. These experiments showed that the Tat peptide (48–57) binds TAR RNA with a K_D of 698 nM. A relative dissociation constant (K_{REL}) can be determined by measuring the ratios of the Tat peptide to trimer ligand dissociation constants (K_D) for TAR RNA. These re-



(b)



(c)



sults are shown in Tab. 4.1. Ligand 1 binds TAR RNA with affinities higher than that of the wild-type Tat peptide. These results indicate that selection frequency reflects ligand activity and, if a large enough library sample is used, could be used as an indicator of ligand affinity for RNA [106].

It was interesting that ligands 1 and 7 were the tightest-binding tripeptides found, suggesting a consensus sequence of X-(D)Lys-(L)Asn. Furthermore, two diastereomers of ligand 1, peptides 2 and 3, were found in the assay, 3 being the only homochiral sequence. RNA–peptide binding measurements revealed that the dissociation constants for these two diastereomeric sequences were approximately 7 times higher than for the strongest sequence, 1. This sharp loss of binding energy among diastereomers indicates that the binding interaction is highly stereospecific and not merely the result of a non-specific lysine-phosphate backbone attraction. Another interesting feature of these results is that all but one of the TAR-binding sequences found were heterochiral and would have been missed by other techniques such as phage display which only use the proteinogenic amino acids. Use of D- and L-amino acids together yields a richer stereochemical variety of ligands, in addition to the diversity imparted by using the alpha-amino acids.

4.6.6

Heterochiral Small Molecules Target TAR RNA Bulge

The interaction sites of our trimer ligand 1 on TAR RNA were determined by NMR experiments [106]. NMR spectra of free TAR and TAR complex with ligand 1 were recorded. Due to the spectral overlap, it was impossible to follow all but a few well-isolated resonances by conventional one-dimensional experiments. Therefore, we carried out two-dimensional NOESY and TOCSY experiments. All spectra were recorded on a Bruker AMX-500 NMR spectrometer operating at 500 MHz for ^1H equipped with triple resonance probe and H-broadband inverse detection probes. The 1D and 2D ^1H spectra were recorded at approximately 1–2 mM RNA and ligand concentrations in 5 mM phosphate buffer (pH 5.5), with up to 100 mM NaCl. All other conditions were the same as described earlier by Aboul-ela *et al.* [55]. We obtained a set of complete TAR RNA assignments. Increasing amounts of ligand 1 were added to TAR RNA and the spectral changes were monitored by two-dimensional TOCSY experiments. The TOCSY spectrum contains a region where only pyrimidine H5–H6 resonances are found and this region has a well-dispersed 2D spectrum. Resonances in the free RNA and RNA–ligand complexes were assigned by NOESY experiments. Results of our TOCSY experiments are shown in Fig. 4.12. Resonances in the bulge region, U23 and C24, were shifted. All other resonances were not affected significantly by the addition of the ligand.

To address the question whether spectral changes at U23 and C24 were due to specific ligand 1 binding or the result of perturbation by a non-specific exogenous ligand, we performed NMR experiments in the presence of a basic tripeptide containing L-Lys amino acids. Our results showed that the Lys-peptide did not cause shift of resonances in the bulge region including U23 and C24 (data not shown), indicating that ligand 1 specifically interacts with TAR RNA at the bulge region.

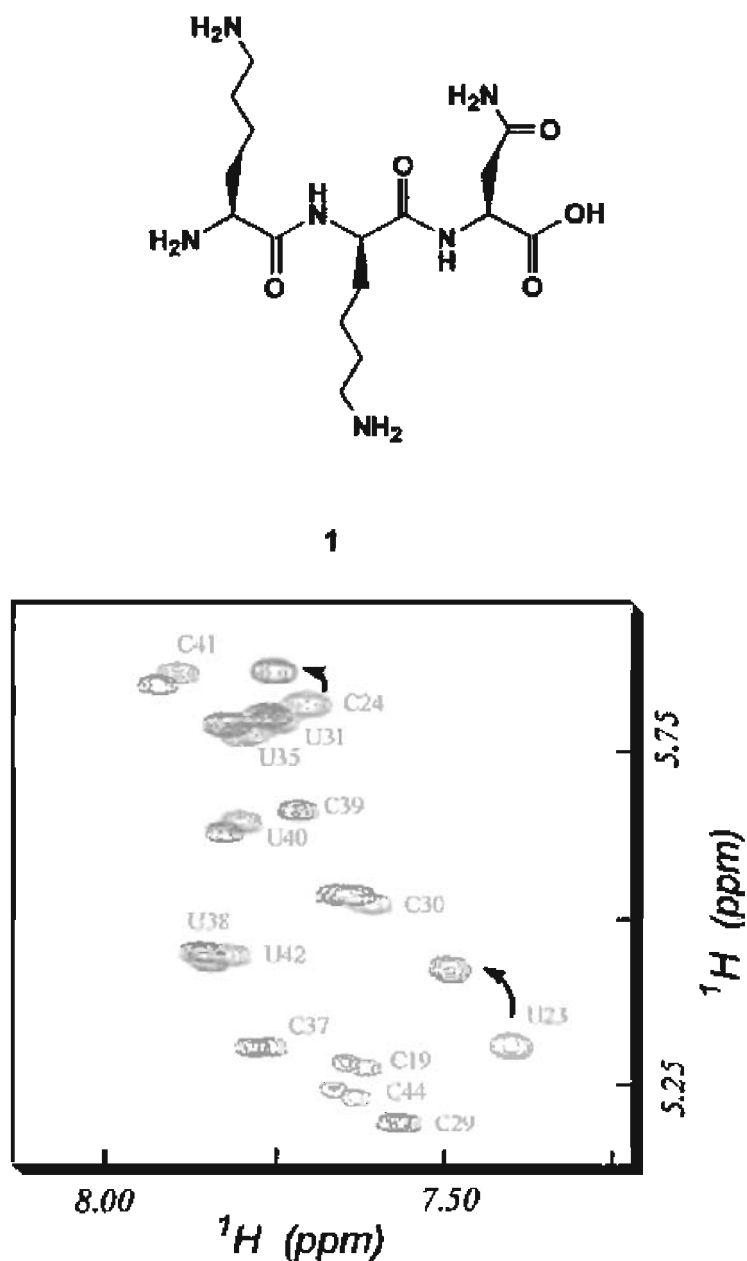


Fig. 4.12. Titration of wild-type TAR RNA by increasing amounts of the ligand **1**, (L) Lys-(D) Lys-(L) Asn (Upper). (Lower) Superposition of TOCSY spectra at increasing concentration of

1 (free RNA, pink and 1:1 ligand to RNA ratio, blue) shows that only resonances at bulge U23 and C24 are affected by the ligand **1** binding. Adapted from Hwang *et al.* [106].

Interestingly, ligand 1–TAR RNA interactions are different from TAR RNA–peptide or TAR–Arg complexes reported in previous NMR studies [55, 107] because there were no detectable interactions with G26 and A27 regions as observed in previous studies. Another TAR-binding ligand, CGP64222, which contains Arg side chains in its sequence also causes conformational change in TAR and creates an RNA structure that is similar to TAR–peptide structure [54]. These results indicate that the ligand 1 is the first ligand that binds specifically to the bulge of TAR RNA in a manner different from previously reported TAR ligands and Tat peptides. These findings suggest an intriguing possibility that small molecules that interact with TAR RNA and induce a conformational change in TAR resulting in a structure different from that of Tat–TAR complex could be used to lock RNA in a non-functional structure.

4.6.7

Inhibition of Tat *trans*-Activation *in vivo*

To test whether this small molecule–RNA interaction could be used to control HIV-1 gene expression *in vivo*, we used HL3T1 cells, a HeLa cell line derivative containing an integrated HIV-1 LTR promoter and CAT reporter gene [73]. We added different amounts of ligand 1 during transfection of pSV2–Tat [71] and pAL [72] plasmids into HL3T1 cells. Plasmids pSV2Tat and pAL express the first exon of Tat protein and luciferase enzyme, respectively. Luciferase reporter gene provides an internal control. Transfection of HeLa cells with pSV2Tat enhanced transcription as determined by CAT activity. Increasing amounts of the ligand 1 resulted in a decrease of CAT activity while luciferase activity was not affected. In the presence of 700 nM concentrations of ligand 1, more than 90% of Tat *trans*-activation was inhibited [106]. To rule out the possibility that the observed inhibition of *trans*-activation could be due to some non-specific toxicity of the ligand 1 or reduction of the pSV2Tat plasmid uptake, transcription of luciferase gene was monitored. Transcription of luciferase gene was not affected by the ligand 1. Cell viability assays showed that ligand 1 treatment was not toxic to the cells. Further control experiments showed that weaker TAR RNA-binding ligands such as L-argininamide and a scrambled Tat peptide containing D-amino acids had no inhibitory effect on Tat *trans*-activation [69].

4.7

Cyclic Structures as RNA-targeting Drugs

Efforts to develop cyclic peptide-based drugs increased manifold after the antibiotic gramicidin S was found to be a cyclic decapeptide. Many antibiotics and toxins are also known to be cyclic amino acid sequences. Cyclization of amino acid sequences results in increased metabolic stability, potency, receptor selectivity, and bioavailability [108–110]. Cyclic peptides have been used as synthetic immunogens [111], transmembrane ion channels [112], potent vaccine for diabetes [113], antigens for Herpes Simplex Virus [114], inhibitor against alpha-amylase [115], pancreatic trip-

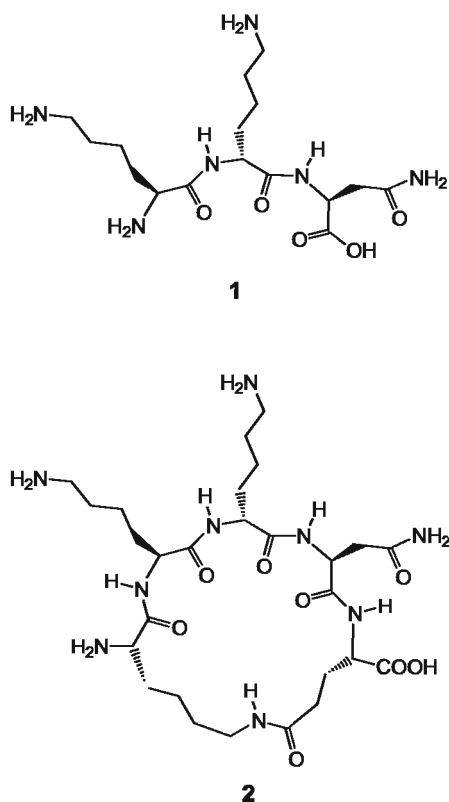


Fig. 4.13. The structure of tripeptide (**1**) and cyclic peptide (**2**).
Reprinted from Tamilarasu *et al.* [127].

sin [116], integrin $\alpha_v\beta_3$ [117], and as protein stabilizer [118]. Side chain to side chain lactamization has been utilized to improve receptor selectivity in enkephalins [119], cholecystokinin [120], melanotropin [121], tachykinin [122], RGD-dependent adhesion proteins [123], and many other biological systems [124, 125]. If designed carefully without causing drastic changes in the conformation of active peptides, the rigid geometry of the cyclic peptides enhances the binding affinity towards a selected target molecule compared to their linear counterparts.

To improve pharmacokinetic properties of the ligand 1 described above, we planned to synthesize a cyclic peptide derivative (Fig. 4.13) based on the ligand 1 structure. In general, homodetic cyclic peptides are made by head-to-tail, N-terminal to side chain or side chain to side chain coupling methods on solid supports [126]. Our modeling studies suggested that the cyclic peptide derived from side chain to side chain coupling method would least affect the conformation of our linear ligand 1, therefore, we designed a synthetic method based on this strategy [127]. This cyclic peptide inhibited transcriptional activation by Tat protein in human cells with an IC_{50} of approximately 40 nM. Cyclic peptides that can target

specific RNA structures provide a new class of small molecules that can be used to control cellular processes involving RNA–protein interactions *in vivo*.

4.8

Summary and Perspective

Tat and Rev proteins and other auxiliary factors intricately control regulation of HIV-1 gene expression. The relevance of RNA structure and RNA–protein interactions to the regulation of gene expression suggests the design of drugs that specifically target regulatory RNA sequences. There has been a considerable effort to discover specific inhibitors of Tat–TAR and Rev–RRE interactions over the past few years and a number of high-affinity RNA ligands have been identified. Combinatorial chemical synthesis is one of the most promising approaches to the synthesis of large collections of diverse molecules. The use of encoded combinatorial chemistry to screen ligands for specific RNA targets would allow the discovery of novel molecules containing diverse structures. To avoid the problem of rapid hydrolysis by host enzymes, peptides and drug molecules containing unnatural linkages can be designed. Structural and activity analysis of the lead molecules will provide new insights into designing drugs with improved properties. The discovery of highly selective and cell permeable inhibitors of RNA–protein interactions would greatly assist us in understanding the functional significance of RNA–protein interactions *in vivo*.

An important consideration in identification of inhibitors of RNA–protein interactions, which is often overlooked, is that the inhibitor does not always have to bind RNA at the binding site of the protein. Although it is true that flexibility of the RNA structure makes it difficult to design molecules for specific RNA sequences, however, this flexibility of RNA folding can be exploited to lock an RNA into a non-functional structure. For example, a TAR ligand can bind the sequences below or above the bulge, or loop region and cause conformational change in TAR RNA that is not recognized by Tat and cellular proteins involved in transcriptional activation. Similarly, TAR bulge-binding ligands can be identified that interact with the RNA in a manner different from that of Tat. Locking RNA into a non-functional structure could also be kinetically more favorable than a ligand–RNA interaction that competes with protein for the same site.

With the increasing wealth of knowledge being generated in the field of RNA–protein recognition and identification of new RNA targets, it would be exciting to see how chemists and biologists use this information to discover drugs that target specific RNA structures and manipulate RNA–protein interactions to control biological processes.

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5

DNA and RNA Recognition and Modification by Gly-Gly-His-Derived Metallopeptides

Eric C. Long and Craig A. Clausseri

5.1

Introduction

5.1.1

General Considerations

Despite their central position in the evolution of DNA- and RNA-binding proteins and certain natural products, peptides containing the 20 ribosomally translated amino acids represent a relatively untapped resource with respect to the design of low-molecular-weight nucleic acid-binding molecules. This status is due, in part, to the general lack of defined three-dimensional structure within most short, 3–5 amino acid peptides in aqueous solution [1]. The above consequence is unfortunate given the molecular-recognition capabilities of amino acids. Peptides contain guanidinium, amide, amine, planar hydrophobic moieties, and a host of other side chain functional groups that can be harnessed to interact with a nucleic acid structure through hydrogen bonding, electrostatics, hydrophobic, and van der Waals forces [2]. Indeed, this potential was recognized in previous studies of amino acid- and peptide-DNA association phenomena [3–7].

Through the above interactions, and in a fashion complementary to the DNA-binding properties of distamycin, netropsin [8–10], and linear or hairpin polyamides [11–14], designed peptides of the appropriate three-dimensional architecture theoretically have the wherewithal to recognize DNA or RNA via not only their nucleotide sequences, but also their groove structures, stacked Watson–Crick base pairs, phosphodiester backbone or some combination of these features. Importantly, the potential for an appropriately structured peptide to target a nucleic acid may lead to strategies to augment the activity of existing DNA- or RNA-binding agents or to develop lead compounds resulting in wholly organic structures with biological properties. Peptides have a distinct advantage in the development of nucleic acid-binding compounds through their relative ease of individual or combinatorial synthesis [15–17], the ability to alter readily the chirality of select α -carbon stereo-centers, and their potential transformation into peptidomimetic [18] or biosynthetic agents. Overall, the key to exploiting amino acids and peptides in the

design of low-molecular-weight nucleic acid-binding molecules appears to be the inclusion of strategies which enable a peptide to be structured appropriately in solution and to also contain a judicious choice of amino acid side chain functional groups.

5.1.2

Metallopeptides in the Study of Nucleic Acid Recognition

With the goal of exploring the nucleic acid recognition capabilities of peptides containing naturally occurring amino acids, ongoing efforts from this laboratory employ transition metal ions to generate low-molecular-weight peptides with well-defined three-dimensional architectures [19, 20]. In a fashion that is analogous to other metal complexes that often display selective DNA [21–26] and RNA [25, 27] binding and, at times, antitumor properties [28–34], the use of transition metal ions in the generation of structured, nucleic acid-binding peptides provides several distinct advantages including: (1) the ability to construct complexes with well-defined geometries and (2) the inclusion of metal-centered redox properties enabling the final complex to mark a location of binding through an act of DNA or RNA strand scission [21, 22]. Thus, similar to the zinc-finger strategy that has evolved naturally in transcription factors [35], metallopeptides create well-defined peptides that present functional groups to a nucleic acid target leading to selective or perhaps specific binding interactions.

As described in the subsequent sections of this chapter, the metal binding properties of simple tripeptides of the form $\text{NH}_2\text{-Xaa-Xaa-His-CONH}_2$ (where Xaa is an α -amino acid) create a core scaffold upon which to present amino acids and their pendant side chains to a nucleic acid target [20]. Systems of this type permit metal ligation throughout the entire peptide sequence defining rigidly in space the position of all function groups and minimizing structurally undefined regions. The long-term goal of this experimental strategy is to understand, at the level of a low-molecular-weight complex, details concerning preferred amino acid- and peptide-DNA/RNA contacts, groove preferences, and how particular spatial arrangements of amino acid side chains can influence the efficiency and selectivity of nucleic acid binding. In this way, knowledge pertaining to protein- and low-molecular-weight agent-nucleic acid recognition principles can be gained and perhaps exploited in future generations of designed DNA- or RNA-targeted agents [36–40].

5.2

Interactions of Gly-Gly-His-Derived Metallopeptides with DNA

5.2.1

Natural Occurrence and Metal-binding Properties

Peptides of the general form $\text{NH}_2\text{-Xaa-Xaa-His-CONH}_2$, or in their least substituted form, Gly-Gly-His, represent the consensus sequence of the N-terminal

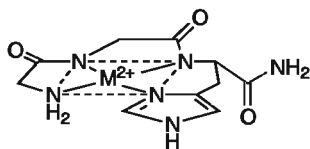


Fig. 5.1. The structure of $M(II)\cdot Xaa\text{-}Xaa\text{-}His$ metallopeptides.

$Cu(II)$ or $Ni(II)$ chelating domain of the serum albumins [41]. In addition to the albumins, this sequence also appears in neuromedins C and K, human sperm protamine P2a, and the histatins [41]. At or above physiological pH, Gly-Gly-His and other Xaa-Xaa-His tripeptides bind avidly to $Cu(II)$ or $Ni(II)$ with dissociation constants of 10^{-16} – 10^{-17} M [41, 42]. Metal ion chelation by the tripeptide occurs (Fig. 5.1) through the terminal amine, two intervening deprotonated amide nitrogens and the histidine imidazole nitrogen, resulting in distorted square planar or square planar complexes with $Cu(II)$ [42] and $Ni(II)$ [43], respectively. While this mode of binding indicates that $Cu(II)/Ni(II)\cdot Xaa\text{-}Xaa\text{-}His$ complex formation should not be overly influenced by the amino acid side chains found at either the first or second peptide positions, as noted previously [41], some substitutions actually assist in increasing the metal-binding affinity of the core peptide [44].

Importantly, for purposes of nucleic acid recognition, using the peptide bond as a basis for metal ion ligation, as described above, allows the side chain functional groups contained within a chosen peptide to be presented at the periphery of the total complex and available for interaction with a nucleic acid target. Inversion of chirality at individual α -carbon stereocenters also enables key side chain functional groups to be projected from different locations of the core metallopeptide. Thus, like the “metallopeptide” portion of the antitumor agent bleomycin, which is responsible for the 5'-GC and 5'-GT selectivity exhibited by the drug [29, 45], $M(II)\cdot Xaa\text{-}Xaa\text{-}His$ metallopeptides have the ability to present a well-defined core structure that permits interactions to occur between the amino acid side chains presented by the complex and a nucleic acid target. In addition, these metallopeptides do not form isomers at the metal center, eliminating the need to resolve individual species prior to DNA recognition studies.

5.2.2

Development as a DNA-Cleavage Agent

The study of Xaa-Xaa-His metallopeptides with nucleic acids began with a report of the activity of Gly-Gly-His in the presence of $Cu(II)$ + ascorbate against Ehrlich ascites tumor cells [46] and a further study [47] that demonstrated DNA strand scission by $Cu(II)\cdot Gly\text{-}Gly\text{-}His$ + ascorbate (Fig. 5.2). DNA strand scission in these cases was attributed to the generation of hydroxyl radicals by the $Cu(II)$ center in a reaction that actually is attenuated by the peptide–ligand [48, 49]. Following these initial reports, the DNA-cleavage activity of $Cu(II)\cdot Gly\text{-}Gly\text{-}His$ was harnessed in the generation of an N-terminal affinity cleavage appendage to the DNA-binding

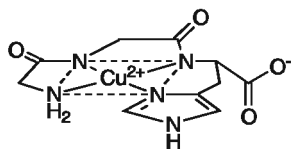


Fig. 5.2. The structure of Cu(II)-Gly-Gly-His containing a carboxylated-terminus.

domain of Hin recombinase [Gly-Gly-His(Hin 139–190), synthesized via modified *t*Boc protocols] (Fig. 5.3). Cu(II)-Gly-Gly-His(Hin 139–190) + sodium ascorbate/hydrogen peroxide [50] and Ni(II)-Gly-Gly-His(Hin 139–190) + magnesium monoperoxyphthalic acid (MMPP), iodosobenzene, or hydrogen peroxide [51, 52] were observed to induce DNA strand scission at the site of Hin recombinase binding, as directed by the Hin 139–190 portion of this conjugate. Observations made with Cu(II)/Ni(II)-Gly-Gly-His(Hin 139–190) indicated that the metallopeptide portion likely resided in the DNA minor groove and generated a non-diffusible oxidant resulting in deoxyribose modification.

Importantly, Ni(II)-Gly-Gly-His(Hin 139–190) was found to be considerably more efficient than the corresponding Cu(II) complex in producing a highly focused oxidation of the sugar–phosphate backbone. Freely diffusing radical intermediates akin to those generated by Fe(II)-EDTA systems [21, 22] were not evident, suggesting the formation of a ligand- or metallopeptide-based radical that permitted the chemistry to be directed to the DNA backbone. In addition, the use of Ni(II) as the activating metal in the presence of exogenous chemical oxidants avoided complicating background DNA damage produced by the radical generating ability of free Cu(II) + ascorbate [47] in the absence of a chelating peptide.

With the report of the above activity, further examples of Cu(II) and Ni(II) complexes of Xaa-Xaa-His-conjugated proteins have been generated with the aim of using the metallotripeptide portion as an affinity cleavage appendage. These systems include N-terminal Gly-Gly-His-modified Sp1 [53] and Gly-Lys-His-modified Fos [54], both of which were generated through biosynthetic protocols. In each of these examples, and Gly-Gly-His(Hin 139–190), the metallo-Gly-Gly-His appendage is passive with respect to DNA recognition and association by the much larger protein portion. Activation of the metallopeptide moiety of the conjugate resulted in DNA cleavage at the protein-binding site. Along with these examples of engineered protein conjugates, studies with a truncated peptide model of human

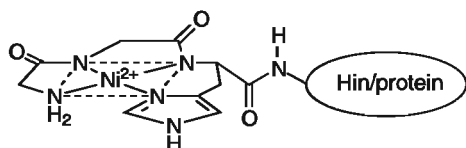


Fig. 5.3. The structural arrangement within Ni(II)-Gly-Gly-His(Hin 139–190) and other N-terminal Gly-Gly-His-modified proteins for DNA affinity cleavage.

protamine P2, a protein that contains an N-terminal Xaa-Xaa-His motif in its native sequence [55], found that Cu(II) or Ni(II) binding actually promoted the association of this peptide mimic with DNA and in the presence of hydrogen peroxide resulted in oxidative DNA damage [56, 57]. These studies suggested a plausible mechanism for the carcinogenicity and toxicity of Ni(II).

In addition to the study of DNA-binding proteins, the Cu(II)/Ni(II)-Xaa-Xaa-His motif has also found utility in the development and examination of other nucleic acid binding and cleaving molecules. Examples include Cu(II)-Gly-Gly-His-modified netropsin [58], low-molecular-weight peptides [59], anthraquinone [60], peptide nucleic acids (PNAs) [61], oligonucleotides [62, 63], and naphthalene diimides [64]. Along with their ability to induce nucleic acid strand scission, Ni(II)-Gly-Gly-His and its conjugates have the wherewithal to carry out directed protein cleavage [65] and to modify and cross-link proteins [66–71].

In light of the utility of Cu(II)/Ni(II)-Gly-Gly-His-containing systems and their ability to generate a non-diffusible oxidant, this laboratory sought early on to extend the capabilities of this motif through the development of a means to place it at any location within a synthetic polypeptide strand [72, 73]. In its native form, the positioning of a Gly-Gly-His motif is limited to the N-termini of natural polypeptide structures due to the required terminal amine functional group as a fourth nitrogen donor. To circumvent this requirement (Fig. 5.4): (1) a synthetic Gly-His-(Xaa)_n-resin-bound peptide can be coupled to an orthogonally protected Orn residue (*N*^δ-Boc-*N*^α-Fmoc-protected Orn in the case of *t*Boc peptide synthesis protocols) followed by (2) selective deprotection of the Orn side chain and continued solid phase peptide synthesis via the δ -amine of Orn to generate (Xaa)_n-(δ)-Orn-Gly-His-(Xaa)_n peptides. After final deprotection, this connectivity permits the α -amino group of Orn and the adjacent Gly and His residues to bind metal ions in a fashion identical to an N-terminal Gly-Gly-His regardless of its location in a synthetic peptide. It was found also that the DNA-cleavage properties of this modified motif were essentially identical to an N-terminal Gly-Gly-His model peptide [72].

Resulting from studies of model peptides containing the δ -Orn-Gly-His sequence, this laboratory noticed their ability to mediate *selective* DNA strand scission in the presence of Ni(II). These observations suggested that the core M(II)-Xaa-Xaa-His structure, even in the absence of a defined DNA-binding domain, exhibited DNA site-selectivity thus presenting an opportunity to explore peptide-DNA interactions in a systematic fashion.

5.2.3

DNA Binding and Modification by Ni(II)-Xaa-Xaa-His Metallopeptides

5.2.3.1 Selective minor groove recognition and binding

Given the structural and chemical properties of the Ni(II)-Xaa-Xaa-His motif, this laboratory chose to examine the DNA recognition potential of these metal-lotriptides as independent, low-molecular-weight complexes [74]. In these and subsequent studies, Ni(II) complexes were chosen over those containing Cu(II) due to their reported ability to produce an efficient, non-diffusible oxidizing equiv-

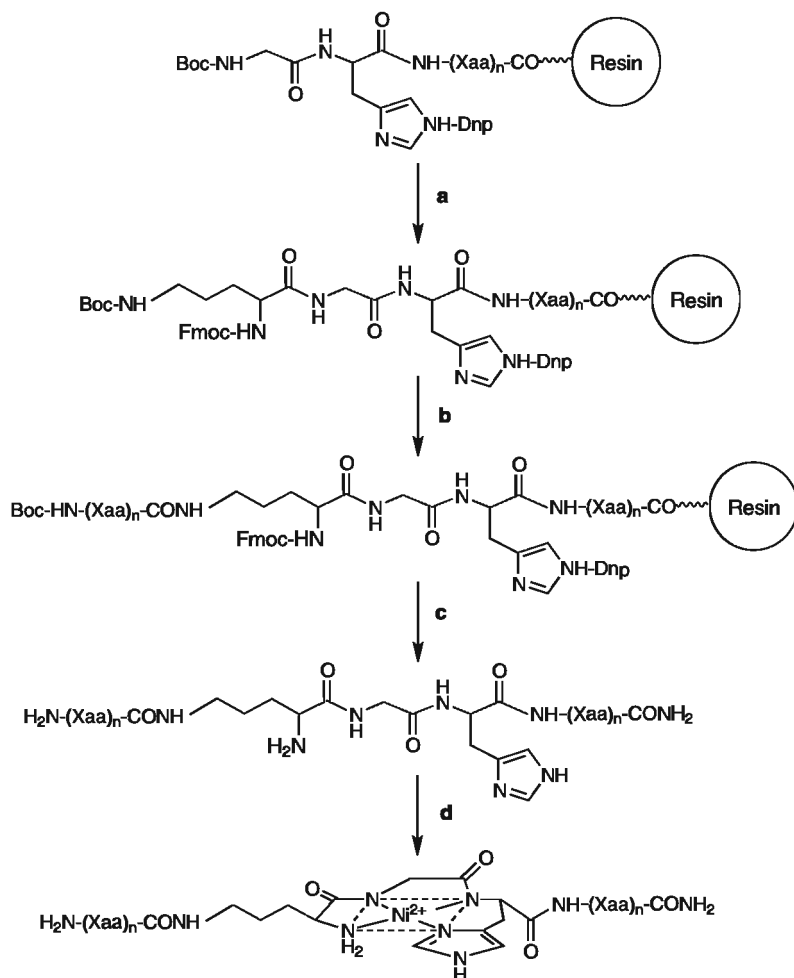


Fig. 5.4. Stepwise solid-phase peptide synthesis of Orn-containing metal-binding oligopeptides. Synthesis involves: (a) coupling of Boc-Orn(Fmoc) to resin-bound peptides terminating in Gly-His; (b) additional cycles

of Boc-benzyl peptide synthesis via the side chain of Orn; (c) side chain deprotection and cleavage from the resin; (d) HPLC purification and addition of $\text{Ni}(\text{OAc})_2$.

alent [51, 52] and also due to the lack of reactivity of $\text{Ni}(\text{II})$ versus $\text{Cu}(\text{II})$ in the absence of a coordinated tripeptide–ligand. Additionally, in light of one previous examination of $\text{Ni}(\text{II})$ -Gly-Gly-His containing the carboxylate analog of the peptide–ligand (i.e. NH_2 -Gly-Gly-His- COOH), which did not reveal significant amounts of DNA modification [75], we chose to examine carboxamide peptide–ligands in these and all subsequent experiments (i.e. NH_2 -Xaa-Xaa-His- CONH_2). Carboxamide peptide–ligands create overall charge-neutral complexes in the case of $\text{Ni}(\text{II})$ -Gly-Gly-His (due to the coordination of two deprotonated amides) or posi-

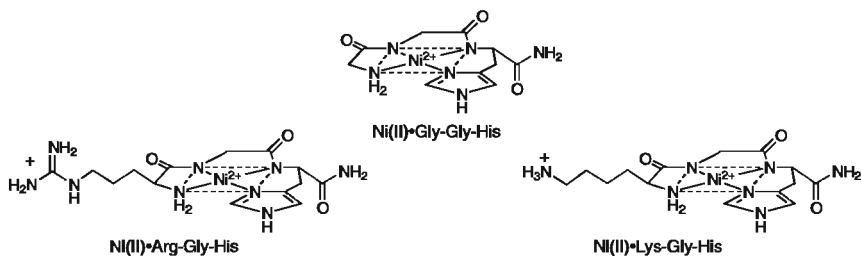


Fig. 5.5. The structures of $\text{Ni(II)}\cdot\text{Gly-Gly-His}$, $\text{Ni(II)}\cdot\text{Arg-Gly-His}$, and $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ -bearing carboxamide termini.

tively charged complexes upon inclusion of Arg or Lys within $\text{NH}_2\text{-Xaa-Xaa-His-CONH}_2$. As a further factor in this choice, carboxamide-containing peptide ligands do not decarboxylate [43] in the presence of metal ions, thus creating a more stable ligand to support the redox activity of the metal center.

Initially, we chose to examine the DNA site-selectivity of three $\text{Ni(II)}\cdot\text{Xaa-Xaa-His}$ metallotriptides (Fig. 5.5) containing carboxamide termini. $\text{Ni(II)}\cdot\text{Gly-Gly-His}$, $\text{Ni(II)}\cdot\text{Lys-Gly-His}$, and $\text{Ni(II)}\cdot\text{Arg-Gly-His}$ [74]. This choice of amino acid substitutions stemmed from the desire to compare the activity of an unsubstituted, charge-neutral species with two overall positively charged metallopeptides containing amino acid substitutions found commonly in DNA-binding proteins [2]. Using ^{32}P end-labeled restriction fragments and polyacrylamide gel electrophoresis (PAGE), the selectivities of the above complexes were compared in 10 mM sodium cacodylate buffer, pH 7.5, containing 50 μM calf thymus carrier DNA. Upon activation in the presence of equimolar amounts of KHSO_5 (with respect to [metallopeptide]) for one minute without a post-reaction chemical work-up, direct and selective DNA strand scission was observed (Fig. 5.6). In the case of $\text{Ni(II)}\cdot\text{Gly-Gly-His}$, the DNA-cleavage patterns revealed a preference for AT-containing regions of the DNA duplex, while with Lys or Arg, the overall cleavage intensity was increased due to the positively charged nature of these complexes and highly directed to AT-rich regions. The DNA-binding affinities of the positively charged complexes, determined through distamycin competition experiments and ethidium displacement assays, were estimated later [76] to be in the $\sim 10^6\text{--}10^7\text{ M}^{-1}$ range with $\text{Ni(II)}\cdot\text{Gly-Gly-His}$ being approximately 2- to 3-fold lower in affinity. While the observation of an AT-selective interaction parallels other known DNA-binding molecules targeted to the minor groove [77–79], the level of discrimination exhibited by these metallopeptides nonetheless was surprising given their relatively small size. Overall, these studies revealed for the first time that the $\text{M(II)}\cdot\text{Xaa-Xaa-His}$ motif alone was capable of exhibiting site-selective DNA strand scission.

In conjunction with the initial study described above, the influence of amino acid stereochemistry was also probed through substitutions of two D-amino acids into the Xaa-Xaa-His framework [74]; D-His was substituted for L-His in the C-terminal peptide position of $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ and L/D-Asn residues were substituted for the Gly residues in $\text{Ni(II)}\cdot\text{Gly-Gly-L/D-His}$. These alterations were

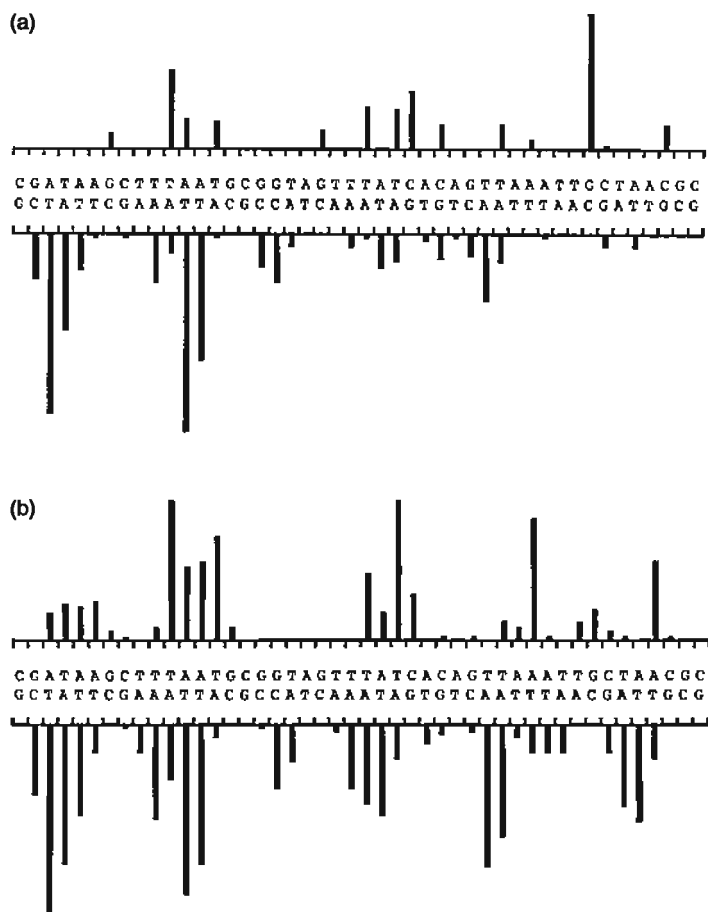


Fig. 5.6. Histogram illustrating the typical site-selectivity of DNA cleavage induced by Ni(II)-Gly-Gly-His (a) and Ni(II)-Arg-Gly-His (b) in a DNA restriction fragment substrate.

Bar lengths are proportional to the intensity of direct DNA strand scission observed. Essentially identical results are obtained for Ni(II)-Arg-Gly-His and Ni(II)-Lys-Gly-His.

chosen to place an amide functionality, also found in protein-DNA interactions [2], at various locations around the metallopeptide periphery. Upon $\iota \rightarrow \text{D}$ -His substitution within Ni(II)-Lys-Gly-His, a noticeable decrease in the focused AT-selectivity was observed in experiments that employed restriction fragment substrates. DNA cleavage was now observed to occur at mixed GC- and AT-rich regions in addition to the stronger AT-sites observed with the corresponding ι -His diastereomer. A potential basis for this decrease in selectivity was suggested through molecular modeling: D -His substitutions re-position the C-terminal amide functionality to be co-planar with the bulk of the metallopeptide creating a more flattened structure (Fig. 5.7). Relative to metallopeptides containing ι -His, this structural rearrangement might be accommodated more easily by additional minor groove



Fig. 5.7. Molecular models illustrating the structural changes that occur upon L $\rightarrow$ D-His substitution within an Ni(II)-Xaa-Xaa-His metallopeptide; the metallopeptides are oriented edge-on with the C-terminal amide to

the left of each structure (darkened). The metallopeptide on the left contains an L-His residue, the metallopeptide on the right contains a D-His residue.

sequences. Interestingly, substitution of D-His to create Ni(II)-Gly-Gly-D-His(Hin 139–190) also resulted in an alternative pattern of DNA cleavage at the binding site of Hin recombinase, suggesting some form of diastereofacial selectivity upon metallopeptide activation or DNA association [51, 52].

In the case of Asn substitutions within Ni(II)-Gly-Gly-His, two of the resulting charge-neutral metallopeptides, Ni(II)-Asn-Gly-His and Ni(II)-Gly-Asn-His, resulted in DNA-cleavage patterns similar to the selectivity and intensity produced by Ni(II)-Gly-Gly-His [74]. Also, consistent with earlier observations with Ni(II)-Lys-Gly-D-His, Ni(II)-Gly-Asn-D-His appeared somewhat less selective. In contrast, however, to the other Asn-substituted metallopeptides, Ni(II)-Gly-D-Asn-His exhibited a noticeable selectivity for several 5'-CCT sites and a less pronounced selectivity for a 5'-CCA and 5'-CCG site within the restriction fragment substrate used. Interestingly, these sites were avoided by the other metallopeptides examined in these early studies and occurred at junctures between two AT-rich regions. Again, molecular modeling suggested that in contrast to the other metallopeptides in this series, Ni(II)-Gly-D-Asn-His was unique in its projection of two amide functionalities toward the same face of the planar metallopeptide. As suggested at the time [74], this arrangement may lead to an overall metallopeptide shape or pattern of hydrogen bond donor/acceptors that permitted increased discrimination amongst available binding sites presented by the DNA helix.

Along with demonstrating the selective DNA recognition of Ni(II)-Xaa-Xaa-His metallopeptides, the results obtained through these initial experiments also revealed several important aspects of their DNA-binding and -modification properties under our stated conditions: (1) Direct DNA strand scission was observed at preferred regions *and* isolated examples of all four available nucleobase sites (Fig. 5.6) without a post-reaction chemical work-up, supporting the previous suggestion that Ni(II)-metallopeptide activation results in a non-diffusible oxidant of the deoxyribose moiety without a particular nucleobase reactivity [51, 52]. (2) Although not necessary to reveal a pattern of DNA modification, the intensity of DNA cleavage at a site of metallopeptide interaction can be slightly enhanced by post-reaction *n*-butylamine or piperidine treatment pointing to the generation of direct strand breaks *and* the formation of alkaline-labile lesions reminiscent of those generated by bleomycin through deoxyribose C4'-hydroperoxidation and C4'-hydroxylation, respectively [28–32]. (3) While alternating or mixed AT-rich regions are preferred, homopolymeric AT sites (i.e. poly(dA)-poly(dT) sequences) are avoided, suggesting

that the narrowing of the minor groove widths at such sites inhibits metallopeptide binding [80]. (4) Cleavage of both complementary strands of a duplex DNA substrate revealed a distinct 3'-asymmetric cleavage pattern (Fig. 5.6) indicative of minor groove residence in other systems [21, 22]. (5) Metallopeptide-induced DNA cleavage can be inhibited in the presence of the well-documented minor groove binding agent distamycin [8–10], implying a competition for similar binding sites.

Overall, the above studies revealed that Ni(II)-Xaa-Xaa-His metallopeptides, as independent low-molecular-weight complexes, are capable of selectively recognizing DNA in a fashion that is influenced by the nature of the peptide ligand, the side chain functional groups present, and their chirality and placement around the periphery of the total metal complex. In addition, the recognition properties and characteristics of the DNA damage observed supported the notion that Ni(II)-Xaa-Xaa-His metallopeptides associate with the minor groove of the DNA helix, exhibit sensitivity to minor groove structural features, and produce DNA strand scission through an interaction with the deoxyribose positions accessible from this groove location.

Recently, studies with Cu(II)-Gly-Gly-His, Cu(II)-Lys-Gly-His, and Cu(II)-Arg-Gly-His have also been performed in an attempt to probe directly the binding and orientation of these metallopeptides when they associate with DNA [81]. In this study, the metallopeptides were examined by DNA fiber EPR spectroscopy and molecular modeling. DNA fiber EPR spectroscopy can determine the positioning and orientation of a DNA-bound, EPR-active Cu(II) center through the angular dependence of their EPR line shapes with respect to an oriented DNA fiber.

Initially, it was confirmed that all the above-named metallopeptides exhibited EPR values in frozen solution or in frozen DNA pellets that were consistent with identical, planar tetradentate structures as also found with the corresponding Ni(II)-coordinated metallopeptides. Subsequently, EPR spectra of the Cu(II)-Xaa-Gly-His metallopeptides and the Cu(II) complex of $\text{NH}_2\text{-Gly-Gly-His-COOH}$ bound to B-form DNA-fibers at room temperature showed a conspicuous and consistent angular dependence to their line shapes, indicating that the nature of the Xaa amino acid does not influence the orientation of the DNA-bound species. Examination of actual and simulated spectra indicated that the angular dependence of the EPR spectra fit well assuming that the $g_{\parallel}$ axes of the Cu(II) complexes were tilted about 50° from the DNA fiber axis. This finding indicates that the mean coordination planes of these metallopeptides were oriented approximately 40° relative to the DNA helical axis, suggesting that the metallopeptides were stereospecifically oriented with respect to a DNA groove.

In contrast to B-form DNA fibers, the EPR spectra of the metallopeptides bound to A-form DNA fibers suggested that the complexes were randomly oriented with respect to the DNA helix. This lack of orientation with A-form DNA is not surprising due to the transformation of the distinct narrow and deep minor groove found in B-form DNA to a wide and shallow feature as found in the A-form [82].

In light of the results described above, models were constructed using B-form DNA oligonucleotides and the crystal structure of Cu(II)-Gly-Gly-His [42] with appropriate amino acid substitutions. As illustrated for Cu(II)-Arg-Gly-His (Fig. 5.8)

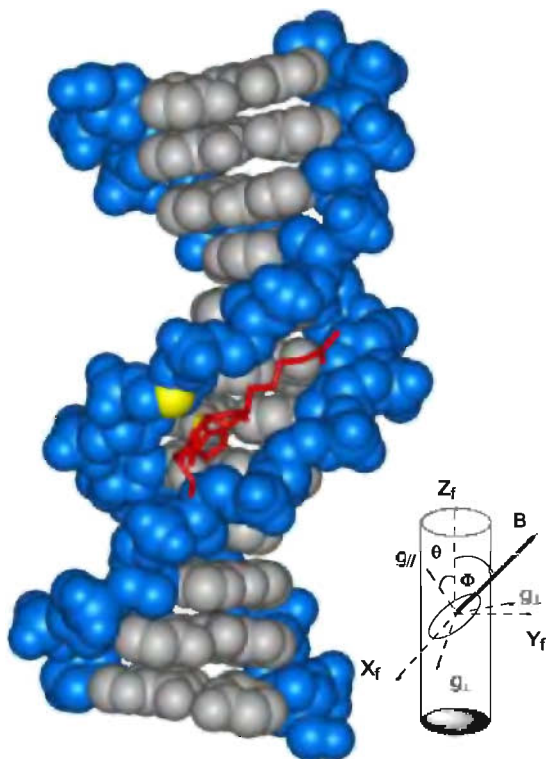


Fig. 5.8. A molecular model of Cu(II)-Arg-Gly-His (red) docked in the minor groove of B-form DNA with the $g_{\parallel}$ axis of the complex oriented 50° relative to the DNA helix axis. With this orientation, the mean plane of the

metallopeptide is oriented 40° relative to the helix axis. The metallopeptide is easily accommodated by the minor groove and in this orientation the metal center (yellow) is positioned close to a C4' H (yellow).

with the His imidazole and N-terminal amine inserted into the minor groove of B-form DNA, based on evidence provided by the study of the corresponding Ni(II)-Xaa-Xaa-His metallopeptides [74, 83], the width of the minor groove can accommodate well and actually direct a bound metallopeptide to orient its coordination plane approximately 40° relative to the DNA helical axis. With such a positioning, the metallopeptide is well constrained between the walls of the minor groove and permits the His imidazole functional group to interact with the floor of the minor groove via hydrogen bonding (e.g., with the O2 of thymine or the N3 of adenine [14]). Evidence for this type of His interaction is derived from the following: (1) Ni(II)-Xaa-Xaa-His metallopeptides containing a His N3 methyl substituent do not cleave DNA [83]; (2) the N3 pyrrole nitrogen of His is a very good hydrogen bond donor upon metal complexation [84]; and (3) earlier DNA fiber EPR investigations have indicated that His residues were a key factor in DNA binding [85]. The orientation described above also permits the N-terminal amino acid side chain to interact with the floor and walls of the minor groove and the second amino acid

in the peptide–ligand to interact with features of the exterior surface of the DNA such as the phosphate backbone. Importantly, with the metallopeptides docked as described within the B-form minor groove, the metal center is positioned to interact with the C4' H of a deoxyribose ring of a proximal nucleotide residue.

5.2.3.2 Minor groove-directed deoxyribose oxidation

To further confirm the location of the Ni(II)·Xaa-Xaa-His metallopeptide–DNA association and the mechanism of direct DNA strand scission observed under the conditions used to examine their site-selective binding, this laboratory carried out an analysis of the products formed upon direct DNA strand scission [86]. Previously, mechanistic investigations carried out with Ni(II)·Gly-Gly-His(Hin 139–190) activated with MMPP, iodosobenzene, or hydrogen peroxide suggested that the metallopeptide appendage resided in the minor groove at the site of Hin recombinase binding, generating, upon chemical activation, a non-diffusible oxidant that produced deoxyribose-centered damage [51, 52]. Evidence for deoxyribose damage obtained at that time included: (1) the formation of cleaved DNA fragments bearing 3'- and 5'-phosphorylated termini; (2) a kinetic isotope effect ($k_H/k_D = 1.6$) upon C4'-deuteration of a target nucleotide; and (3) the observation that DNA strand scission was enhanced upon post-reaction chemical work-up with *n*-butylamine reminiscent of the documented behavior of the C4'-hydroxylated deoxyribose lesion generated by Fe(II)·bleomycin.

With two representative metallotriptides, Ni(II)·Gly-Gly-His and Ni(II)·Lys-Gly-His, our laboratory examined the nature of the direct DNA strand scission products formed under the reaction conditions used in earlier investigations of their site-selectivities (10 mM sodium cacodylate, pH 7.5) [86]. By employing duplex substrates with 5'- or 3'-³²P end-labeling of the *same* DNA restriction fragment strand, cleavage analyses by PAGE permitted identification of the new 3'- and 5'-termini present at a site of metallopeptide-induced DNA fragmentation, respectively. These experiments were performed in parallel with Fe(II)·bleomycin-induced cleavage of the same labeled restriction fragments to allow a direct comparison to be made with the products from an authentic, well-characterized C4'-oxidant [28–32].

The termini identified at the sites of Ni(II)·Gly-Gly-His- and Ni(II)·Lys-Gly-His-induced cleavage, when activated with KHSO₅, MMPP, or hydrogen peroxide, were all found to be consistent with deoxyribose C4' H abstraction and included: (1) 5'-phosphorylated termini and 3'-phosphoroglycolate termini, indicating the intermediacy of a C4'-hydroperoxide lesion and (2) 3'-termini that were phosphorylated, or upon post-reaction work-up with NaOH or NH₂NH₂ contained deoxyribose-derived fragmentation products characteristic of the breakdown of keto-aldehydic abasic lesions resulting from C4'-hydroxylation of a target nucleotide (Fig. 5.9) [28–32]. In addition to these DNA termini-attached products, monomeric products representing the remaining fragments of a targeted deoxyribose ring released into solution were also identified and quantitated: (1) free nucleobases and nucleobase propenals (detected as thiobarbituric acid-reactive components) were detected and found to correlate well with the extents of keto-aldehydic abasic site and 3'-

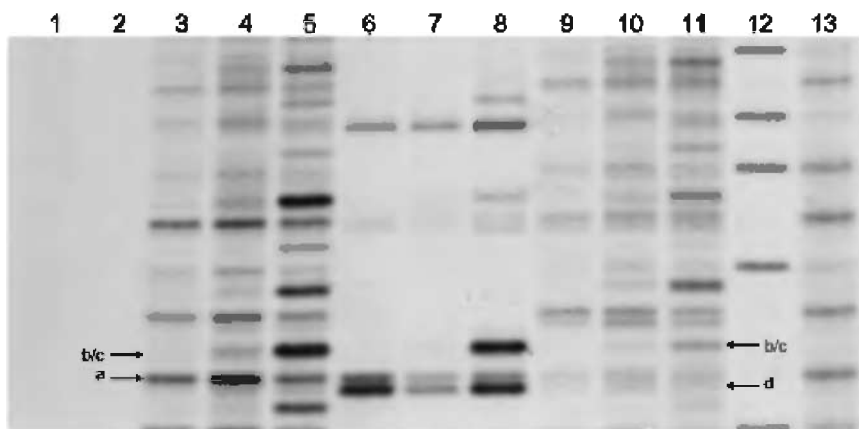


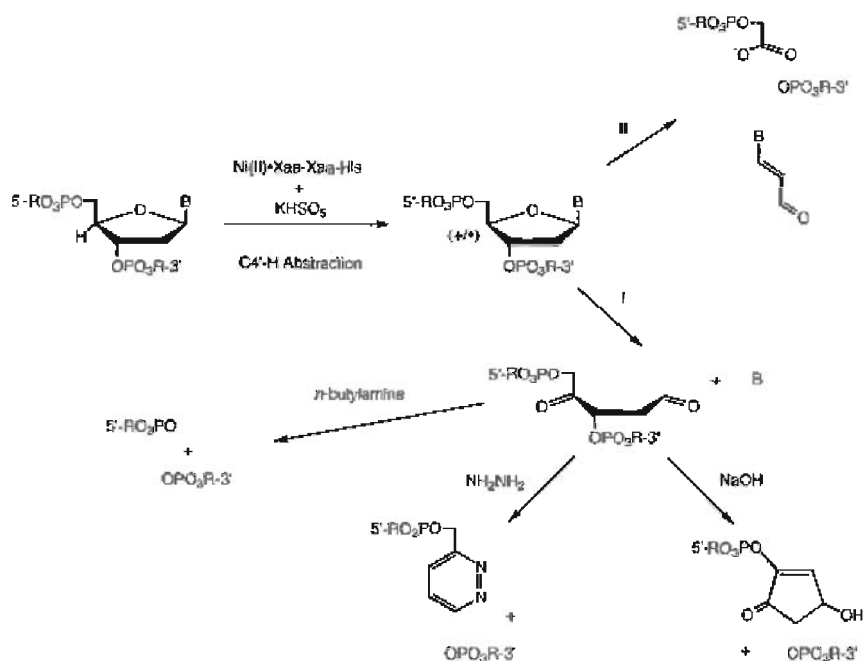
Fig. 5.9. Portion of an autoradiogram of a high-resolution denaturing polyacrylamide gel analyzing the new 3'-termini produced from a 5'-³²P end-labeled restriction fragment cleaved by Ni(II)-Lys-Gly-His (lanes 3–5) or Ni(II)-Gly-Gly-His (lanes 9–11) + KHSO₅ compared to Fe(II)-bleomycin (lanes 6–8). Lane 1, intact DNA; lane 2, reaction control, metal + KHSO₅

alone; lanes 12 and 13, Maxam-Gilbert G + A, and T + C reactions, respectively. Labeled bands illustrate: (a) 3'-phosphorylated termini; (b/c) closely migrating NaOH-induced 3'-termini and 3'-phosphoryridazine termini (lanes 4/5, 7/8, and 10/11, respectively); and (d) 3'-phosphoglycolate termini (lanes 3–11).

phosphoroglycolate lesion formation, respectively, for a given metallopeptide and (2) the identity of the nucleobases released also paralleled the site-selectivity of the metallopeptides. It should be noted, however, that free guanine was found to be partially degraded by the action of Ni(II)-Xaa-Xaa-His + KHSO₅, making its quantitation difficult in comparison to the remaining three nucleobases [86]. In addition to the above, experiments designed to trap intermediates produced from metallopeptide interactions with deoxyribose sites other than the C4' H failed to detect their presence [86].

The results described above indicate that Ni(II)-Gly-Gly-His and Ni(II)-Lys-Gly-His (activated with KHSO₅, MMPP, or hydrogen peroxide) associate with the DNA minor groove under the conditions employed and, like Fe(II)-bleomycin, mediate C4' H abstraction to generate C4' radicals or C4' cations leading to two distinct mechanistic pathways to final product formation (Scheme 5.1): (1) C4'-hydroperoxide lesions are formed upon O₂ addition to a C4' radical resulting in direct DNA strand scission with the release of 5'-phosphorylated termini, 3'-phosphoroglycolate termini, and nucleobase propenals and (2) C4'-hydroxylated lesions are generated upon solvent addition to a C4' cation leading to keto-aldehydic abasic sites and a concomitant loss of free nucleobases. These abasic lesions can subsequently be trapped with NaOH or NH₂NH₂ in a fashion consistent with the characterized alkaline-labile lesion produced by Fe(II)-bleomycin [28–32].

Along with defining the locations of binding and the chemistry of direct DNA strand scission under our conditions, these mechanistic experiments also revealed



Scheme 5.1. Summary of DNA cleavage products observed and proposed pathways of deoxyribose-based DNA strand scission by Ni(II)-Xaa-Gly-His metallopeptides activated with KHSO₅, MMPP, or H₂O₂. Pathway I involves the intermediate formation of a C4'-OH modified deoxyribose and release of free nucleobases, resulting in keto-aldehydic

alkaline-labile lesions that can be further modified with NaOH, hydrazine, or *n*-butylamine while pathway II occurs through an intermediate C4'-OOH resulting in direct DNA strand scission and the formation of 3'-phosphoroglycolate termini and nucleobase propenals.

key differences between the activities of the individual complexes examined. While Ni(II)-Gly-Gly-His and Ni(II)-Lys-Gly-His generated the same products resulting from C4'-H abstraction, indicating an identical underlying mechanism, they did so with differing ratios of C4'-OH to C4'-OOH lesions (Fig. 5.9). In the case of the charge-neutral complex Ni(II)-Gly-Gly-His, which binds DNA less tightly than the positively charged complexes, the C4'-OOH lesion appeared to a greater extent in comparison to Ni(II)-Lys-Gly-His (as monitored through 3'-phosphoroglycolate and nucleobase propenal formation). This observation suggests that relatively tight metallopeptide-DNA binding can limit the rate of diffusion of O₂ to the site of C4'-H abstraction decreasing the formation of 3'-phosphoroglycolate lesions (i.e. direct strand breaks) while with weaker binding metallopeptides, O₂ can add readily to the radical lesion formed at the C4' carbon. Interestingly, these results imply that a lower DNA binding affinity, or careful consideration of association/dissociation rates of complex formation, may actually improve the efficiency of direct strand scission by a cleavage agent that uses similar pathways to final product formation.

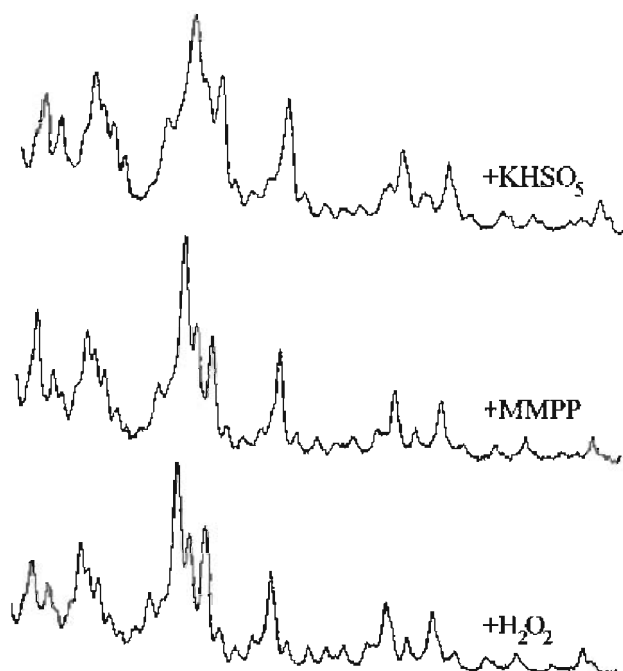
Additionally, with Ni(II)-Gly-Gly-His the ratio of C4'-OH to C4'-OOH lesions appears also to be affected by the particular nucleotide sequence cleaved. Thus, as observed previously with Fe(II)-bleomycin [87] and synthetic diimine complexes of Rh(III) [88], the microheterogeneity of the DNA helix, and its influence on the DNA-binding affinity of a targeted agent, alters the partitioning between two available mechanistic pathways to final product formation. The above may also explain the lack of observed 3'-phosphoroglycolate termini in studies of Ni(II)-Gly-Gly-His(Hin 139–190) [52]. The protein portion of this conjugate mediates tight DNA association, limiting, perhaps also in conjunction with the specific nucleotide sequence cleaved, the accessibility of O₂ to the initial C4' radical lesion resulting predominantly in C4' hydroxylation.

5.2.3.3 Nature of the intermediate involved in deoxyribose oxidation

Given the above, it appears that Ni(II)-Xaa-Xaa-His metallopeptides associate selectively with the DNA minor groove resulting, upon activation, in abstraction of the C4' H prominently located there. As stated earlier, activation of the Ni(II)-Xaa-Xaa-His metallopeptides can be supported by KHSO₅, the organic peracid MMPP, or hydrogen peroxide (albeit with differing efficiencies, ratios of activating agent to metallopeptide, and reaction times) [86]. Worthy of note is the fact that the final site-selectivities observed, identity and ratios of products released, and the mechanisms of action employed are identical for a given metallopeptide when these three rather structurally diverse activating reagents are used suggesting the formation of a common activated intermediate (Fig. 5.10) [86]. Certainly, the generation of individual activated metallopeptide species that differ with each of these supporting reagents would likely lead to differences in their final site-selectivities or mechanisms/ratios of product formation.

To probe the nature of the activated Ni(II)-Xaa-Xaa-His metallopeptide responsible for C4' H abstraction and direct DNA strand scission, the effects of commonly employed radical scavengers on Ni(II)-Lys-Gly-His-induced conversion of supercoiled form I DNA to relaxed form II DNA were compared upon activation of this metallopeptide with KHSO₅, MMPP, and hydrogen peroxide [86]. Under the assay conditions used, which monitored direct DNA strand scission without subsequent chemical workup, the scavengers ethanol, *tert*-butyl alcohol, DMSO, and mannitol had little to no effect on plasmid conversion from form I to form II DNA while parallel control experiments showed substantial inhibition of Fe(II)-EDTA-induced DNA cleavage by hydroxyl radicals.

These data indicate that the “activated” metallopeptide formed by KHSO₅, MMPP, and hydrogen peroxide not only produced identical site-selective DNA cleavage and deoxyribose fragmentation products, but also behaved similarly in the presence of four well-established radical scavengers supporting the notion of some common activated intermediate. These results, in total, also indicate that the activated Ni(II)-Xaa-Xaa-His metallopeptide formed does not produce direct, deoxyribose-centered strand scission through the generation of diffusible hydroxyl radicals, as noted by others [66]. Also, these data indicate that the active intermediate involved in direct DNA strand scission via deoxyribose modification is not a diffu-



3'-TACTGTAATTGGATATTTTTATCC GCA T A G T G C-5'-[P]

Fig. 5.10. Densitometric analyses of autoradiograms from high-resolution denaturing polyacrylamide gels illustrating the similarities in site-selectivities and product (termini) formation that occurs upon metallopeptide activation with KHSO_5 , MMPP, or hydrogen peroxide.

sible or metal-bound sulfate radical ($\text{SO}_4^{\bullet-}$) when KHSO_5 is employed in activation [86, 89]. While these results apparently rule out the involvement of the above diffusible radical species in the mechanism of deoxyribose-centered strand scission, as noted at the time, they do not preclude their formation and involvement in other pathways of DNA modification such as nucleobase modification that does not result in direct DNA strand scission [90].

In light of the above evidence, it appears that C4' H abstraction of the deoxyribose ring occurs through a common activated metallopeptide formed in the presence of KHSO_5 , MMPP, or hydrogen peroxide. Given the characteristics of this intermediate, we have proposed [86] that this species may be a high valent, peptide-bound $\text{Ni(III)-O}\cdot$ or Ni(IV)=O , effectively a Ni-bound hydroxyl radical equivalent, which may be formed through the heterolytic splitting of the oxygen-oxygen bond contained within KHSO_5 , MMPP, and hydrogen peroxide (Fig. 5.11). While yet to be proven definitively, indirect support for this intermediate is provided by several lines of evidence: (1) There are observed differences in the relative reactivities of the three activating reagents used ($\text{KHSO}_5 > \text{MMPP} \gg \text{H}_2\text{O}_2$) which parallel closely the acidity of their leaving groups upon oxygen-oxygen bond het-

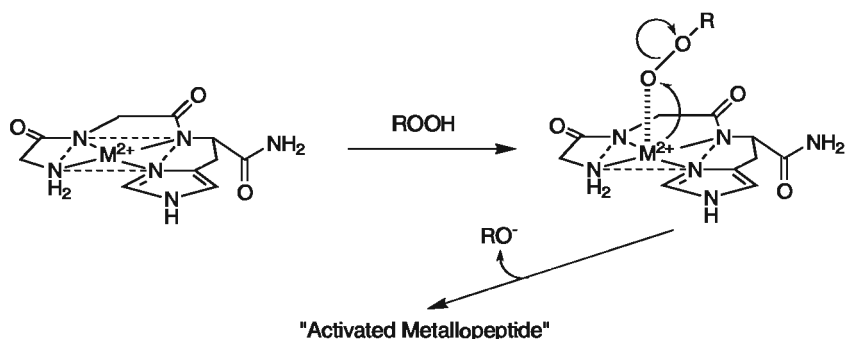


Fig. 5.11. Proposed mechanism of Ni(II)-Xaa-Xaa-His activation through Ni-promoted heterolytic cleavage of a peroxide/peracid oxygen-oxygen bond.

erolysis ($H_2SO_4 > COOHCH_6H_4COOH \gg H_2O$), a feature that is known to influence the ability of a peroxide functional group to act as an oxygen atom donor to a metal center [91, 92]. (2) Upon activation of Ni(II)-Lys-Gly-His in the absence of a DNA substrate, a UV-visible absorption shift occurs from 425 nm to a more intense peak at 375 nm indicative of a structural change of the metal complex from square-planar to an oxidized, higher valent Ni(III) or Ni(IV) species of octahedral geometry [93]. (3) Ni(II)-Gly-Gly-His has also been shown to catalyze alkene epoxidations [51, 52], suggesting that, like other Ni complexes where a ligand-bound Ni(III)-O- or Ni(IV)=O has been proposed [94, 95], a similar intermediate may be generated with Ni(II)-Xaa-Xaa-His metallopeptides. Certainly, such an activated intermediate associated with a minor groove-bound metallopeptide would be capable of abstracting the C4' H, leading to the observed profile of deoxyribose fragmentation products.

5.2.3.4 Generation of minor groove binding combinatorial libraries

Having developed a working model of their minor groove binding, this laboratory is pursuing a systematic examination of Ni(II)-Xaa-Xaa-His metallopeptide-DNA interactions through the development of combinatorial libraries [76]. As a test case, it was determined initially if an "optimized" Ni(II)-Xaa-Xaa-His metallopeptide could be developed to target B-form DNA through the synthesis of positional scanning combinatorial libraries [15–17]. These libraries were designed to examine nearly all possible combinations of naturally occurring L- α -amino acids within the Xaa positions of the Xaa-Xaa-His tripeptide-ligand, excluding Cys and Trp residues to prevent disulfide bond formation, as confirmed [96], and the partial intercalation of DNA [5–7], respectively. Using standard *t*-Boc synthesis protocols [97], two main libraries were generated in which the first and second peptide positions were varied systematically (Fig. 5.12). These libraries were verified by quantitative amino acid analyses to contain a uniform representation of all the amino acids included in the split-and-mix synthesis.

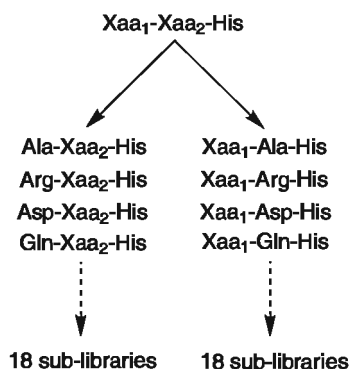


Fig. 5.12. Generation of combinatorial Xaa₁-Xaa₂-His ligand-peptide libraries.

Initially, the DNA-cleavage efficiencies of the individual members of these libraries were assayed for their ability to convert directly supercoiled, form I plasmid DNA to nicked-circular form II DNA via the chemistry described earlier (using KHSO₅ or MMPP as activating agents) [76, 86]. This assay provides a reasonably high-throughput means to determine the relative activities of each member of the synthetic libraries but does not select for any particular site-selectivity. For both libraries it was found that DNA-cleavage efficiency was highly dependent upon the identity of the amino acid found at a given Xaa position (Fig. 5.13). With the library that systematically varied the N-terminal Xaa₁ position, it was found that the DNA-

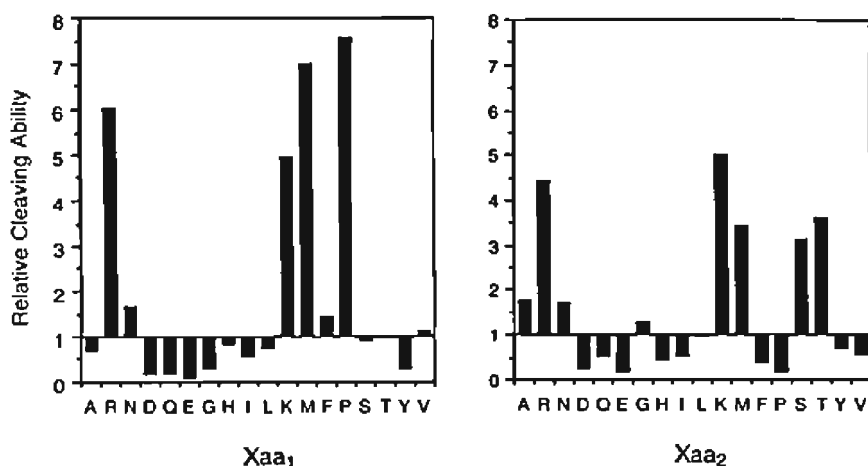


Fig. 5.13. Quantitation of direct DNA strand scission activity by combinatorial libraries of Ni(II)-Xaa₁-Xaa₂-His metallopeptides relative to Ni(II)-Gly-Gly-His (1 on relative scale). Xaa₁ denotes the identity of the amino acid

(in single-letter code) present at the N-terminal peptide position, while Xaa₂ denotes the identity of the amino acid present at the second peptide position.

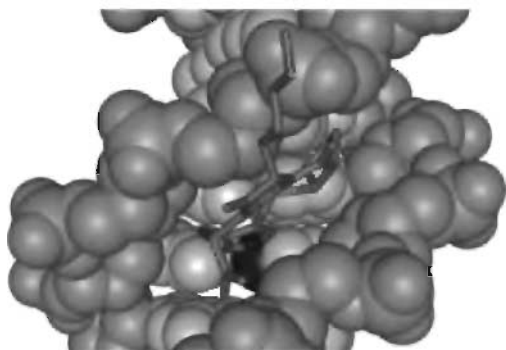


Fig. 5.14. Molecular model of Ni(II)-Pro-Lys-His (red) bound to the minor groove of B-form DNA with the His imidazole H-bonded to the N3 of adenine (green): (or O2 of thymine) and the Ni(II) center (yellow) in close proximity to a C4' H (yellow).

cleavage efficiency was enhanced between 5- and 8-fold relative to Ni(II)-Gly-Gly-His, which served as a baseline for comparison, when Arg, Lys, Met, or Pro were present (Pro > Met > Arg > Lys). In the case of the second Xaa₂ peptide position, DNA-cleavage efficiency was enhanced between 3- and 5-fold when Arg, Lys, Met, Ser, or Thr were present (Lys > Arg > Met/Ser/Thr). In both the N-terminal and second peptide positions, the remaining amino acids included in the library syntheses exhibited cleavage efficiencies that were either similar to, or slightly reduced in comparison to Ni(II)-Gly-Gly-His, most notably Glu, Asp, Gln, Tyr, and Phe (Fig. 5.13).

The results of the above experiments are important in that they: (1) predict that Ni(II)-Pro-Lys-His, containing the most active amino acids in their respective positions, would substantially increase direct DNA strand scission relative to Ni(II)-Gly-Gly-His and (2) allow a visualization of the contribution of nearly all naturally occurring amino acids and their side chain functionalities within the Ni(II)-Xaa-Xaa-His framework on the cleavage of B-form DNA. In the former case, independent synthesis of Ni(II)-Pro-Lys-His verified its ability to bind metal ions and cleave DNA an order of magnitude better than Ni(II)-Gly-Gly-His, thus indicating that the substitutions had nearly an additive effect [76].

Overall, it appears that for B-form DNA the positively charged amino acids Lys and Arg can favorably influence metallopeptide–DNA binding in either the first or second peptide positions with a preference for Arg in the first position. Lys was, however, most active in the second peptide position. These preferences can be attributed to an increased electrostatic attraction between the metallopeptide and the polyanionic DNA backbone. In addition to electrostatic interactions, however, it was surprising to find that Pro was the most active amino acid in the N-terminal peptide position, increasing the direct DNA strand scission activity of the metallopeptides to an even greater extent than Lys and Arg. As expected, however, Pro in the second position prevented metal binding and exhibited a lack of DNA re-

activity. It was found also that Thr and Ser residues, containing side chain alcohol functionalities, contributed to the DNA-cleavage activity of the metallopeptides only when located in the second peptide position while Met residues in the first or second peptide positions also contributed positively to the reactivity of these metallopeptides. In the case of Met residues, under the oxidative conditions employed to activate the metallopeptides, it is likely that Met sulfones or sulfoxides were present.

To probe the possible basis for the increased direct strand scission activity of the combinatorially selected metallopeptides, further experiments were performed [76]. It was found that: (1) the overall metal-binding abilities of individual members of the metallopeptide libraries were equivalent, with 1:1 complexes being formed (except with Xaa-Pro-His peptides); (2) the DNA-binding affinities of the selected metallopeptides were found to be similar within a factor of 2–4 in comparison to Ni(II)-Gly-Gly-His; (3) the cleavage efficiencies of ^{32}P end-labeled restriction fragments by individual library members paralleled their activities in the initial activity screen; (4) while subtle differences between individual active library members were observed, their DNA site-selectivities maintained an underlying selectivity for AT-rich regions, as expected given the nature of the initial activity screen employed and the absence of D-amino acid substitutions [74]; and (5) minor groove binding and the mechanism of direct deoxyribose-centered strand scission via C4' H abstraction remained unaltered. Given that cleavage enhancements for the selected metallopeptides were up to 10-fold greater than that exhibited by Ni(II)-Gly-Gly-His, these findings suggested that particular structural features of the selected metallopeptides, beyond their simple 2- to 4-fold increase in equilibrium binding affinity, may be responsible for the observed enhancement in cleavage activity. It is possible that the selected combinations of amino acids in the metallopeptide framework result in an increased anchoring/rigid binding interaction in the minor groove or an advantageous positioning relative to the C4' H position of a target nucleotide, leading to the observed increased in B-form DNA strand scission activity.

To examine the possible structural basis for the increased activity of select members of the metallopeptide libraries, molecular modeling was performed using the metallopeptide with the highest selected activity, Ni(II)-Pro-Lys-His (Fig. 5.14, see p. 106) [76]. In light of modeling and structures [43] described earlier, and given that the common structural element of the metallopeptide that supports AT-selectivity is the His imidazole ring, models were constructed in which the N3 nitrogen of the His imidazole was hydrogen bonded to either the N3 of adenine or the O2 of thymine located on the floor of the minor groove. With this interaction, the β , γ , and δ carbons of the Pro ring are positioned deeply in the minor groove and make van der Waals contacts with the deoxyribose sugars which form the walls of this groove. The narrow structure of AT-rich regions complements well the width of the metallopeptide, suggesting a stabilized interaction that would be lacking with only a Gly substitution [14, 77, 82]. With the insertion of the first and third amino acids into the minor groove, the Lys residue contained within the second peptide position is exposed at the surface of the DNA helix and poised to allow

contact between the ϵ -amino group of its side chain and phosphate groups proximal to the bound metallopeptide. The side chain alcohol functional groups of Ser and Thr would be capable of similar interactions with the DNA when included at this peptide position.

The above models suggest that for the direct strand scission of B-form DNA, Ni(II)-Xaa-Xaa-His metallopeptides, in addition to the role of the His imidazole, can be optimized through the placement of hydrophobic features via a Pro residue in the first peptide position and either positively charged residues (Lys/Arg) or polar residues (Ser/Thr) in the second, exterior-facing, peptide position. As found in earlier models, this overall structural orientation with relationship to a B-form DNA helix allows the metal center to be poised to interact (3–4 Å) with the C4' H of an adjacent deoxyribose ring. Using this model as a guide, ongoing investigations are attempting to refine its accuracy and to examine details of this peptide–DNA interaction through high-resolution methods.

The studies described above have verified the utility of combinatorial methodologies, as applied in other systems [98, 99], toward the discovery and development of Ni(II)-Xaa-Xaa-His metallopeptides with select properties. While this initial study did not attempt to screen for select binding sites, ongoing studies involving expanded libraries containing natural and select unnatural amino acids will screen these libraries for their ability to target particular linear sequence-selectivities and anomalous structural features within DNA. In addition to the above, it is also important to note that the majority of the amino acids selected through the above-described library screening procedure, and found to enhance the minor groove interaction of the metallopeptide framework, are those included in several important classes of minor groove-binding protein motifs. Examples of these proteins include: Hin recombinase [100, 101]; *E. coli* integration host factor [102]; the repeating Ser-Pro-Lys/Arg-Lys/Arg motifs found in sea urchin histones H1 and H2B [103]; the AT-hook motif found in mammalian high mobility group (HMG-I) chromosomal proteins [104, 105]; and DNA architectural proteins [106]. This finding suggests that the activity of Ni(II)-Xaa-Xaa-His metallopeptides is influenced by and can serve to model the fundamental amino acid–minor groove interactions that nature has chosen to use in protein–minor groove recognition. Thus, a systematic examination of Ni(II)-Xaa-Xaa-His systems may provide insight into protein–DNA interactions while also providing information that assists in the development of low-molecular-weight DNA-binding agents.

5.2.3.5 Guanine nucleobase modification/oxidation

Along with their ability to site-selectively recognize and bind to the minor groove and to induce direct DNA strand scission through deoxyribose-centered oxidation, Ni(II)-Xaa-Xaa-His metallopeptides can be induced to oxidize guanine nucleobases in a KHSO_5 or sulfite/ O_2 -dependent reaction [107]. In an initial study of their reactivity in comparison with other Ni(II) complexes that mediate guanine nucleobase modification, however, Ni(II)-Gly-Gly-His (containing a carboxylated terminus) was found to exhibit little DNA modification of a ^{32}P end-labeled oligonucleotide substrate in the presence of 10 mM phosphate, pH 7.0, 100 mM NaCl and

100 μM KHSO_5 [75]. This result can be attributed to the overall negative charge of this complex and its tendency to decarboxylate [43] under oxidative conditions.

Subsequent to our report of the direct DNA strand scission activity of $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ containing a C-terminal amide [74], further studies of this same metallopeptide indicated that $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ was capable of guanine nucleobase oxidation in the presence of KHSO_5 (or through the autooxidation of sulfite) [107] in a reaction reminiscent of other Ni(II) complexes that were studied as probes of DNA structure [24]. The conditions employed in these reactions involved 10 μM $\text{Ni(II)}\cdot\text{Lys-Gly-His}$, 100 mM NaCl , and 10 mM sodium phosphate, pH 7.0, activated with 100 μM KHSO_5 or 50–100 μM NaSO_3 . The reactions were incubated for 1 hour prior to a post-reaction chemical work-up (involving 1 M piperidine/90°C/30 min) that was necessary to induce DNA polymer strand scission. It was further reported that the DNA modification observed was consistent with the formation of an intermediate guanine radical cation [90] resulting in guanine nucleobase oxidation products and ultimately DNA strand scission upon hot piperidine treatment. The reactivity of guanine residues found within a restriction fragment substrate paralleled their reported sequence-dependent ionization potentials [108]. Importantly, this reaction suggested a potential mechanism for the toxicity associated with sulfite exposure especially in light of the binding of Ni(II) by proteins such as histones [107]. At that time, it was proposed that $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ catalyzed the formation of a Ni(III) -bound sulfate radical [107]. The redox cycling of this system was examined further [109], supporting the intermediacy of a Ni(III) -peptide species.

Further analysis of the guanine nucleobase modification capabilities of $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ supported the findings discussed above (Fig. 5.15) [86]. It was found

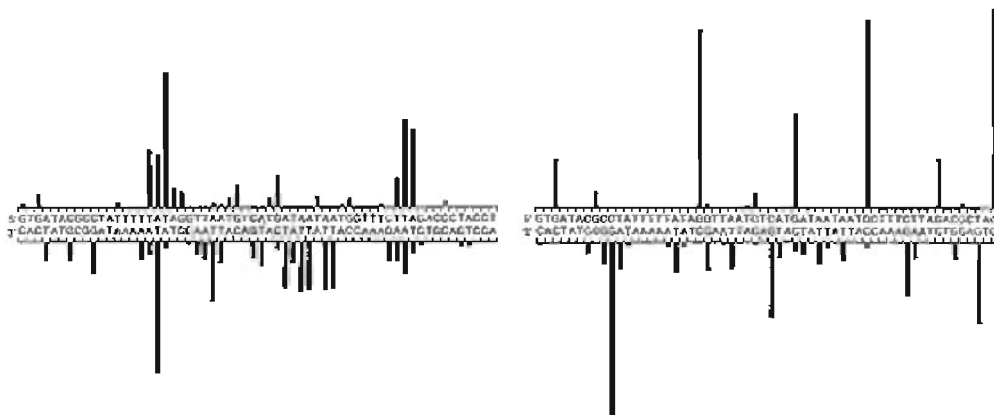


Fig. 5.15. Histograms generated upon densitometric analysis of 3'- ^{32}P end-labeled restriction fragment cleavage by $\text{Ni(II)}\cdot\text{Lys-Gly-His}$ under low ionic strength/low KHSO_5

conditions (left) and high ionic strength/high KHSO_5 conditions (right). Both cleavage reactions illustrated received a post-reaction work-up with piperidine/90°C.

that Ni(II)-Lys-Gly-His produced guanine nucleobase modification of restriction fragment substrates when the reactions included 100 μ M KHSO₅, 100 mM NaCl, 10 mM phosphate, pH 7.0, for 30 min followed by a post-reaction work-up involving piperidine/90°C/30 min. Gel analyses of reaction aliquots of these samples prior to piperidine treatment revealed slightly less direct strand scission products at the expected AT-rich regions reported previously [74]. However, when compared in parallel to the reaction conditions initially employed [74] in an examination of Ni(II)-Xaa-Xaa-His sequence-selectivity (10 mM sodium cacodylate, pH 7.5, one equivalent of KHSO₅), Ni(II)-Lys-Gly-His induced the expected intensity of direct strand scission of DNA at AT-rich regions with or without a post-reaction piperidine treatment [86]. No appreciable guanine nucleobase modification was observed under these low salt/low KHSO₅ conditions.

In order to determine if the guanine nucleobase chemistry induced by Ni(II)-Lys-Gly-His occurred as a function of the activating agent employed, further experiments were carried out using MMPP and hydrogen peroxide as supporting reagents [86]. It was found that Ni(II)-Lys-Gly-His activated with MMPP or hydrogen peroxide in 10 mM sodium cacodylate, pH 7.5, produced the expected pattern of deoxyribose-centered direct strand scission products at AT-rich regions [74]. Importantly, however, even when aliquots of these same reactions were treated with piperidine/90°C, guanine nucleobase modification was not observed [86]. Additionally, it was reported that Ni(II)-Lys-Gly-His could not be activated to produce guanine nucleobase oxidation with MMPP or hydrogen peroxide even under the conditions used to produce this activity with KHSO₅ (100 mM NaCl, 10 mM phosphate, pH 7.0, for 30 min followed by a post-reaction work-up involving piperidine/90°C/30 min) [86].

These findings indicate that guanine nucleobase modification [107] by Ni(II)-Lys-Gly-His occurs most readily as a function of metallopeptide activation with KHSO₅ under specific conditions (excess KHSO₅ relative to metallopeptide, 100 mM NaCl, 10 mM phosphate, pH 7.0, for 30 min followed by a post-reaction work-up involving piperidine/90°C/30 min) while deoxyribose-centered oxidation via C4' H abstraction can occur upon metallopeptide activation with one equivalent of KHSO₅ or MMPP/hydrogen peroxide under relatively low ionic strength conditions [74, 86]. These studies suggest that Ni(II)-Lys-Gly-His may be capable of forming two distinctly different "activated" species when KHSO₅ is used as a supporting reagent under different reaction conditions: (1) An oxygenated Ni(III)-bound activated species when one equivalent of KHSO₅ is used, in common with the activated metallopeptides also produced by MMPP and hydrogen peroxide, that is capable of recognizing and binding to the minor groove of DNA resulting in specific C4' H abstraction under low ionic strength conditions [74, 86]. (2) A sulfate radical-derived species that forms under conditions of excess KHSO₅ in the presence of 100 mM NaCl resulting in predominant guanine nucleobase modification via a guanine radical cation intermediate [107]. While the interaction of species similar to the latter with DNA have been postulated as proceeding through the intermediate metallation of the N7 position of the guanine nucleobase [24],

recent investigations with other Ni(II) complexes also support the potential for an outer-sphere interaction with the DNA helix [110].

5.2.4

DNA Strand Scission by Co-Xaa-Xaa-His Metallopeptides

5.2.4.1 Activation via ambient O₂ and light

While activation systems involving Ni(II) or Cu(II) complexes of Xaa-Xaa-His peptides have aided our understanding of their DNA recognition, binding, and modification properties, the need for exogenous chemical reductants or oxidants during the course of their activation often limit investigations to systems tolerant of these co-reactants and can alter the observed products of a reaction. Thus, the development of strategies to activate Xaa-Xaa-His-derived metallopeptides with a minimal number of co-reactants may facilitate their study and application in further biochemical analyses.

Towards the above goal, this laboratory has examined the activation and DNA-recognition/cleavage properties of Co(II/III)-Xaa-Xaa-His metallopeptides [111, 112]. The use of cobalt as the metal center in these second-generation systems was prompted by the established reactivity of complexes of this metal with ambient O₂ [113–115] and upon light activation [21, 23, 116]. Indeed, Co(III) derivatives of bleomycin and analogs of this drug exhibit light- and O₂-dependent DNA strand scission [117–119]. In addition to considerations of reactivity, cobalt has been established to bind Xaa-Xaa-His peptides through equatorial coordination of the peptide terminal amine, two deprotonated amides and the His imidazole at alkaline pH values to generate exchange-inert Co(III)-Xaa-Xaa-His metallopeptides similar to those generated through Ni(II) or Cu(II) binding (Fig. 5.16) [120]. Importantly, with regards to the study of metallopeptide–nucleic acid recognition, Co(III)-Xaa-Xaa-His systems increase the dimensionality of the overall metallopeptide in comparison to those containing Ni(II) or Cu(II) by providing axial coordination sites for further nitrogen- or oxygen-based ligands, resulting in an octahedral metallopeptide. This increased dimensionality may provide additional points of contact between the metallopeptide and a target nucleic acid, thus expanding their molecular recognition capabilities.

Initially, it was reported that irradiated admixtures of Co(II) + Gly-Gly-His in 5 mM sodium borate buffer, pH 8.0, resulted in the direct conversion of supercoiled form I DNA to nicked-circular form II DNA [111]. These experiments included a

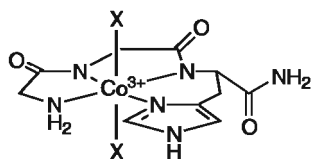


Fig. 5.16. The structure of Co(III)-Xaa-Xaa-His metallopeptides containing axial (X) nitrogen- or oxygen-bearing ligands.

comparison of: (1) Co(II)-Gly-Gly-His + ambient O₂ + *hν*; (2) Co(II)-Gly-Gly-His + ambient O₂ without irradiation; and (3) preformed (NH₃)₂Co(III)-Gly-Gly-His + *hν*. Of these reactions, it was found that Co(II)-Gly-Gly-His + ambient O₂ + *hν* was the most active, being able to totally convert the available form I DNA into form II while the remaining two systems converted <50% of the available substrate. This comparison suggested that DNA cleavage by Co(II)-Gly-Gly-His + ambient O₂ + *hν* was not simply due to the Fenton-like generation of radicals nor excitation of the ligand field bands of these complexes alone. Curiously, however, this comparison did reveal the activity of Co(II)-Gly-Gly-His + O₂ in the absence of irradiation. Subsequent reactions also examined the activity of Co(II) + Lys-Gly-His under the conditions described above [111]. It was found that this metallopeptide, now containing a positively charged Lys residue, increased the level of DNA cleavage activity in comparison to Co(II)-Gly-Gly-His due to considerations of electrostatics. This observation also suggested the specific delivery of an active metal complex to the DNA as opposed to the generation of radicals in bulk solution.

Given its increased level of DNA cleavage activity, Co(II) + Lys-Gly-His + ambient O₂ + *hν* was employed in an examination of the mechanism of this reaction [111]. It was reported that: (1) hydroxyl radical scavengers (mannitol and DMSO) diminished DNA cleavage activity by ~10%; (2) the reaction was unenhanced in D₂O, indicating the absence of singlet oxygen sensitization; and (3) DNA cleavage was unaffected by superoxide dismutase. Given these findings, and in the light of the reported activity of Co(III)-bleomycin and its analogs [117–119], it was suggested that DNA cleavage by Co(II) + Xaa-Xaa-His + ambient O₂ + *hν* may occur through the intermediate formation of an oxygenated Co(III)-peptide that generates hydroxyl radicals in close proximity to a DNA substrate.

In support of an intermediate oxygenation step, control experiments found that anaerobic Co(II) + peptide admixtures were unable to cleave DNA upon irradiation [111]. Further analysis of the interaction of O₂ with Co(II)-Lys-Gly-His by UV-visible spectroscopy (Fig. 5.17) indicated the rapid formation of μ -peroxo dimers [113–115] of these metallopeptides that decompose over the course of an hour (Fig. 5.18) in a fashion analogous to the oxygenation chemistry of Co(II)-bleomycin [121]. This reactivity results in oxygenated or aquated Co(III)-Xaa-Xaa-His metallopeptides that mimic the DNA cleavage activity of the Co(III)-bleomycin “brown” complex [117, 119]. Importantly, as suggested at the time, these systems decouple peptide metallation and activation thus allowing the purification and storage of a Co(III)-containing metallopeptide (or a protein modified for affinity cleavage) for later use and photochemically induced nucleic acid strand scission.

5.2.4.2 Highly selective DNA cleavage via ambient O₂ activation

As a follow-up to the DNA cleavage observed with Co(II)-Gly-Gly-His + ambient O₂ without irradiation and the ability of Co(II)-Xaa-Xaa-His metallopeptides to form μ -peroxo dimers, our laboratories pursued a further investigation of the aerobic activation of Co(II)-containing metallopeptides in the presence of DNA [112]. In the area of DNA recognition and modification, synthetic compounds that activate O₂ to induce nucleic acid modification are of keen interest due to the physio-

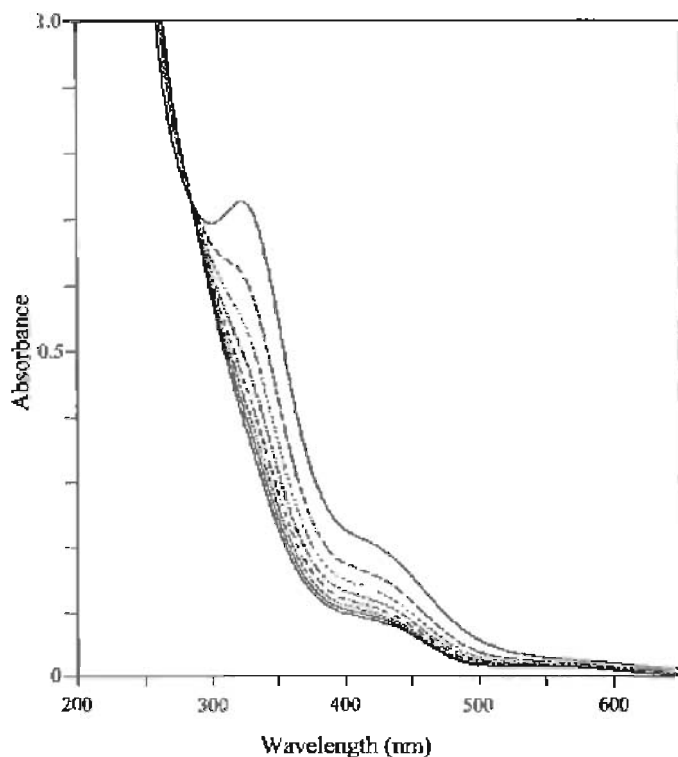


Fig. 5.17. UV-visible spectra of Co(II) + Lys-Gly-His in the presence of ambient dioxygen (top scan, $t = 0$; bottom scan, $t = 10$ min; the spectrum continues to decrease steadily for up to 1 h).

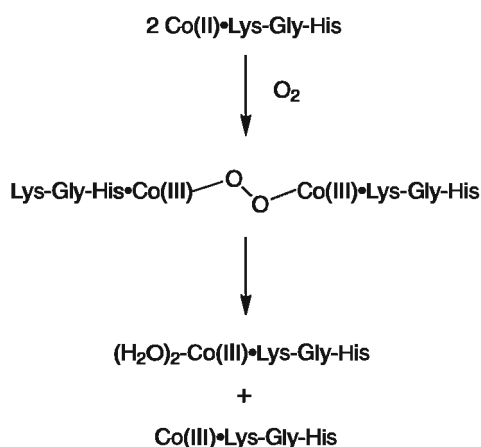


Fig. 5.18. Illustration of the oxygenation of Co(II) + Lys-Gly-His resulting in the intermediate formation of a μ -peroxo dimer and final monomeric Co(III)-Lys-Gly-His metallopeptides.

logical availability of molecular oxygen. Such systems could lead to agents that function *in vivo* [122, 123].

To examine the above, admixtures of Co(II) in the presence of one equivalent of Lys-Gly-His were allowed to react with ^{32}P end-labeled restriction fragment substrates in the presence or absence of ambient O_2 [112]. It was found that Co(II) + Lys-Gly-His induced the O_2 -dependent modification of a single thymine residue within the sequence 5'-AGGTGG (Fig. 5.19). The chemistry of the initial DNA lesion formed was found to be labile to a post-reaction work-up with piperidine (1 M piperidine/ $90^\circ\text{C}/30$ min). Mild NaOH treatment (0.1 M NaOH/ $60^\circ\text{C}/5$ min) did not induce strand breakage, suggesting that DNA modification proceeds

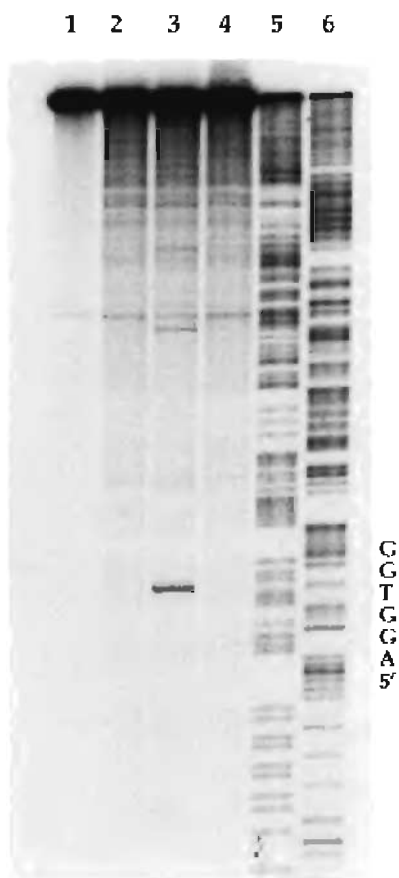


Fig. 5.19. Autoradiogram of a denaturing polyacrylamide gel illustrating the O_2 -dependent modification of 5'-AGGTGG sites within ^{32}P end-labeled DNA restriction fragments. All lanes received a post-reaction work-up with piperidine: lane 1, reaction

control, intact DNA; lane 2 reaction control, 100 μM Co(II); lane 3, 100 μM Co(II) + 100 μM Lys-Gly-His + O_2 ; lane 4, 100 μM Co(II) + 100 μM Lys-Gly-His under anaerobic conditions; lanes 5 and 6, Maxam-Gilbert G + A and T + C reactions, respectively.

through a mechanism involving thymine nucleobase modification and not through deoxyribose oxidation [90].

Additional analysis of this reaction revealed the high level of selectivity of Co(II)-Lys-Gly-His for the 5'-AGGTGG site [112]: (1) Other restriction fragments containing this same 5'-AGGTGG sequence were similarly targeted. (2) While a lower level of DNA modification is observed at 5'-ATGAGA sites, modification does not occur at nested subsets of the main target sequence nor the very similar sequence 5'-CGGTGG. (3) Restriction fragments bearing ^{32}P end-labeled strands containing the sequence complementary to the targeted 5'-AGGTGG site (i.e. 5'-CCACCT) do not become modified, supporting the involvement of a non-diffusible active species as also suggested by radical scavenger experiments. (4) While other metallopeptides, including Gly-Gly-His, Arg-Gly-His, Gly-Asn-His, and Pro-Lys-His are capable of generating μ -peroxo dimers in the presence of Co(II) + O₂, and mediating the relaxation of supercoiled DNA, they did not target 5'-AGGTGG sites nor other sites contained within the restriction fragments examined. (5) To assess the reactivity and accessibility of the T residue within 5'-AGGTGG sites, restriction fragments containing this sequence were also treated with KMnO₄ [124]. This sequence was found to exhibit a low reactivity to this reagent, suggesting that the T residue within the 5'-AGGTGG site was efficiently stacked within the DNA double helix.

Overall, the above evidence supported the notion that a specific binding interaction occurs between the active metallopeptide and the targeted 5'-AGGTGG sequence. To test the potential involvement of the μ -peroxo dimer formed through aerobic oxidation of two Co(II)-Lys-Gly-His metallopeptides, modification of the 5'-AGGTGG sequence was monitored as a function of μ -peroxo dimer decomposition [112]. These experiments revealed that the intensity of T modification in the target sequence diminished in parallel with the decomposition of the μ -peroxo dimer. At early time points in the reaction when the μ -peroxo dimer concentration is high, T nucleobase modification is most intense; as the dimer decomposes, T nucleobase modification also diminishes. This observation supported strongly the notion that the μ -peroxo dimer was responsible for T nucleobase modification within the 5'-AGGTGG sequence.

In addition to the above, indirect support for the participation of the μ -peroxo dimer in the targeting of the 5'-AGGTGG site was derived from the examination of molecular models [112]: (1) It was found that the size of the μ -peroxo dimer of two Co(III)-Lys-Gly-His metallopeptides was capable of interacting with most of the six-base-pair recognition site, whereas a metallopeptide monomer would only be capable of interacting with two to three base pairs. (2) Considering that the recognition site contains two 5'-XGG subsequences and the known targeting of the major groove by other Co complexes [125–127], if each metallopeptide half of the μ -peroxo dimer were to interact with one of the available 5'-GG sites, the dimer could deliver its oxidizing peroxy bridging ligand to the 5–6 double bond of the T residue located in the middle of the recognition sequence. Oxidation of the 5–6 double bond of thymine would result in a piperidine-labile nucleobase lesion [90], as observed.

Overall, edit μ -peroxo dimers of Co(II)-Xaa-Xaa-His metallopeptides may lead to reactive, extended metallopeptide structures capable of recognizing and delivering a reactive oxygen equivalent derived from molecular oxygen to select DNA sites. Further study of this strategy will seek to investigate details of the chemistry of the DNA lesion(s) formed and will also attempt to harness this chemistry, in conjunction with combinatorial peptide–ligand libraries [76], to deliver a reactive O_2 equivalent to alternative DNA sites.

5.3

Recognition and Cleavage of RNA by Ni(II)-Xaa-Xaa-His Metallopeptides

Along with their ability to selectively recognize and induce DNA strand scission, Ni(II)-Xaa-Xaa-His metallopeptides also mediate selective RNA modification and cleavage [128]. As demonstrated for DNA, Ni(II)-Xaa-Xaa-His metallopeptides have the ability to increase our knowledge of fundamental RNA molecular recognition events by providing various amino acid contacts and overall complex architectures to complement the wealth of tertiary structures found in folded RNAs [27].

As an initial test of their abilities, Ni(II)-Gly-Gly-His, Ni(II)-Arg-Gly-His, and Ni(II)-Lys-Gly-His, again containing side chain functional groups often found in RNA–protein interactions [129, 130], were reacted with two unique RNA structures: 3'- ^{32}P end-labeled tRNA^{Phe} and the TAR RNA of HIV-1 [128]. As shown (Fig. 5.20), treatment of these RNAs with the metallopeptides listed above followed by the addition of one equivalent of $KHSO_5$ resulted in predominant modification within the loop structures of these RNA substrates upon post-reaction work-up with aniline-acetate: (1) in tRNA^{Phe}, the metallopeptides were observed to modify the D, anticodon, and the T Ψ C loops and (2) with TAR, RNA modification was observed to occur primarily in the apical loop of this hairpin structure. Unique from their reactivity with DNA, the Ni(II)-Xaa-Gly-His metallopeptides employed in this experiment produced identical patterns of RNA damage regardless of the nature of the terminal Xaa amino acid [128].

Given the requirement for an aniline-acetate post-reaction work-up, it is likely that the metallopeptides target these RNA structures through an initial act of nucleobase modification that may be similar to other reported Ni complexes [24, 131]. However, with their apparent selectivity for loop structures, the variety of nucleobases targeted, and their lack of reactivity with single-stranded regions containing similar nucleobases, it is possible that RNA modification is preceded by an act of RNA recognition and binding.

While their exact mode of RNA recognition and modification remains undefined, it is interesting to note that within the RNA loops targeted, each metallopeptide appeared to produce a pattern of damage mainly toward their 3'-halves, suggesting an RNA-induced orientation of the metallopeptide that directs the modification of accessible nucleobases within each loop. This observation: (1) differentiates the metallopeptides from several other reagents that interact and modify single-stranded RNA, often based on nucleobase modification alone [22, 27,

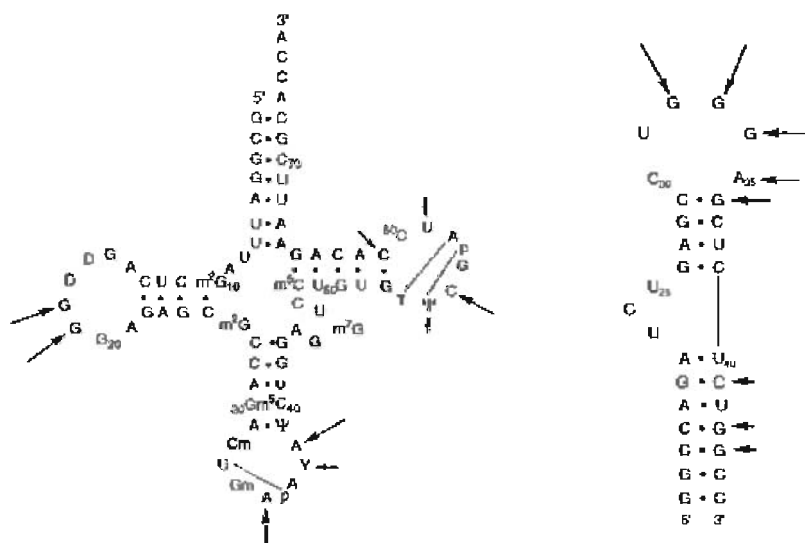


Fig. 5.20. Sites of RNA modification (arrows) induced by Ni(II)-Xaa-Gly-His metallopeptides activated with KHSO_5 . The secondary structure of tRNA^{Phe} is shown on the left (dashed lines indicate intra-loop hydrogen bonding) and the secondary structure of the TAR RNA of HIV-1 is shown on the right.

132] and (2) suggests that the metallopeptides may behave in a fashion that mimics RNA-binding protein elements that often selectively recognize loop structures [129, 130].

At the time, speculation as to their mode of interaction resulted in the suggestion that RNA loops may create binding pockets that complement the overall structure of Ni(II)-Xaa-Gly-His metallopeptides. Given the similar patterns of RNA loop damage observed for all the metallopeptides tested, it is possible that, as with the minor groove of DNA, the His imidazole may insert into the loop region and sense its local environment, resulting in the oxidative modification of the nucleobases present. Thus, Ni(II)-Xaa-Gly-His metallopeptides may sense intra-loop structural features such as the “uridine turns” present in the anticodon and T Ψ C loops of tRNA^{Phe} where the U residues of these loops are hydrogen bonded to a phosphate on the opposite side of the loop [133]. Curiously, examination of the metallopeptide-induced RNA cleavage patterns within these loops suggested that the metallopeptides avoided the nucleotides immediately adjacent to the phosphates involved in these “U-turns”. In comparison, with the apical loop of the TAR RNA, which lacks intra-loop hydrogen bonding [134], metallopeptide-induced modification occurs unimpeded throughout its 3'-half.

While the above study of RNA recognition by Ni(II)-Xaa-Gly-His metallopeptides tested only two substrates out of many unique RNA structures, it did indicate the ability of these metallopeptides to selectively modify RNA, allowed insight as to the chemistry of this nucleic acid modification, and provided a basis and incentive for

future experiments. As with studies being carried out with DNA, the availability of combinatorial libraries of Ni(II)-Xaa-Xaa-His metallopeptides will allow many more amino acid–RNA interactions to be tested, perhaps allowing the discovery of unique RNA-targeted three-dimensional structures [27, 135].

5.4

Summary

Metallopeptides derived from Ni(II), Cu(II), or Co(III) complexes of Gly-Gly-His-derived peptides create unique and reactive agents that impact our understanding of nucleic acid structure and recognition through the development of affinity cleavage strategies and as stand-alone, low-molecular-weight complexes. As activated metallotriptides, these complexes have the ability to recognize DNA and RNA structures resulting in selective strand scission and modification thus permitting details of amino acid– and peptide–nucleic acid recognition to be revealed. Information gathered by studies of metallopeptide–nucleic acid recognition and chemistry, through the use of combinatorial peptide–ligand libraries, their available well-defined three-dimensional shapes, and the potential for ambient O₂ activation, may eventually permit the generation of molecules with unique biological properties while also providing fundamental insight into protein–nucleic acid interactions.

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6

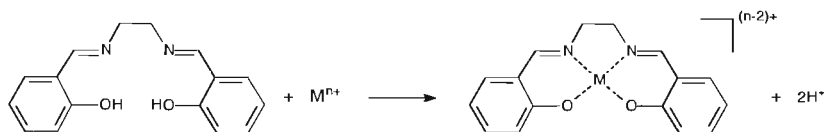
Salen–Metal Complexes

S. E. Rokita and C. J. Burrows

6.1

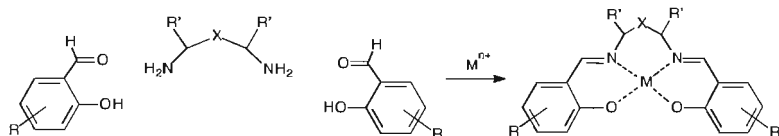
Introduction

The distinct properties and ready availability of metal “salens” have inspired their use in numerous disciplines, including inorganic, organic, and biological chemistry. The salen ligand (bis(salicylaldehyde)ethylenediimine) provides a relatively rigid tetradentate environment that significantly influences the steric and electronic properties of their transition metal complexes in a predictable manner [1, 2]. The two phenolic hydrogens dissociate upon metal binding and help to create a strongly electron-donating environment in conjunction with the two neutral Schiff base imines (Scheme 6.1). Much of the recent interest in these systems originates in part from the efficiency of their manganese and nickel derivatives to catalyze olefin epoxidation in the presence of terminal oxidants such as iodosylbenzene, peracids, and hypochlorite (common bleach) [3–5]. A wide array of metal and salen combinations have since been examined for a variety of chemical and biochemical activities. Two notable examples illustrating the diversity of these efforts include development of a manganese–di(*tert*-butylated)salen that promotes asymmetric epoxidation [6] and a cationic nickel–salen that covalently couples to accessible guanine residues in DNA [7].



Scheme 6.1. Metal coordination by salen.

Salen derivatives are easily prepared in a single procedure by condensing two equivalents of a salicylaldehyde with a diamine (Scheme 6.2). This assembly usually occurs spontaneously [8] and may also be promoted by addition of a metal or other templates [9, 10]. Alternative stepwise condensation allows for incorporation of non-identical salicylaldehyde components [11]. Most of the early salens were



Scheme 6.2. Assembly of a metal–salen complex.

designed to form neutral complexes with dicationic metals for study in organic solvents, but an anionic ligand had also been developed for aqueous application [12]. Neither of these were very appropriate for reaction with polyanionic nucleic acids under physiologically compatible conditions and, in one case, the anionic substituents changed the coordination geometry of the metal [12]. Our laboratories consequently synthesized a salen with two pendent quaternary ammonium groups to provide solubility in water and general electrostatic affinity for nucleic acids [7]. Cationic salen–metal complexes are also now being examined for use in protein [13, 14] and lignin [15] oxidation.

Commercial availability of numerous diamines and salicylaldehydes supports rapid and facile construction of many individual salen derivatives [8, 16] and may be adapted for modern combinatorial methods in the future [17, 18]. By varying the metal and ligand substituents, salen complexes can be shown to either cleave the phosphoribose backbone of DNA or alkylate the nucleobase guanine within DNA and RNA under control of a range of different redox partners and with various structural specificities as described in this chapter. Many of these salen derivatives additionally offer practical alternatives to the most popular probes of nucleic acids that are based on metal complexes such as FeEDTA [19], Cu(1,10-phenanthroline)₂ [20], NiCR [21, 22], and those containing rhodium [23] or ruthenium [24]. The ease of functionalizing and assembling salens has similarly expedited their conjugation to biologically active compounds and incorporation into chimeric metal complexes.

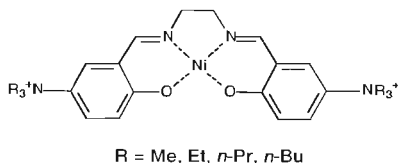
6.2

Reversible Binding of Simple Metal–Salen Complexes

Metal–salen complexes are most often adopted for biochemical application based on their ability to modify nucleic acids covalently. Consequently, few studies have focused expressly on their non-covalent interactions, although such studies on binding are necessary if the independent contributions of electrostatics, groove recognition, and intercalation are to be measured. A sequence- or site-specificity determined by the covalent reactivity of a salen should not necessarily be considered synonymous with its binding specificity since only a limited number of orientations may support efficient reaction. Reversible association between salen complexes and nucleic acids are best identified through chemical footprinting [25, 26] and a variety of physical methods [9]. The results of these studies can often be

used to rationalize existing data on reaction specificity and help when designing subsequent generations of salen-based reagents.

Both the metal and the ligand affect the interactions of their complex with nucleic acids by defining the net charge, coordination number, and solubility, as well as steric and hydrophobic properties. For example, Cr(III) and Mn(III) form water-soluble complexes with the parent salen ligand (Scheme 6.1) because of their net (+1) charge but their interactions with DNA can vary due to the square-pyramidal geometry of the pentadentate manganese complex with one axially ligated water and the octahedral geometry of the hexadentate chromium complex with two axially waters [27]. Ni(II) and Cu(II) form neutral complexes with the parent salen ligand and thus require charged substituents on the ligands to achieve water solubility. The M(II) oxidation state of these metals also form square-planar geometries and do not readily gain axially ligands unless converted to their M(III) states. Only one study has directly addressed the competing influence of steric bulk and hydrophobicity, and this relied only on a series of Cu(II)–salen derivatives (Scheme 6.3) [28]. Sterics dominated this example since the affinity for DNA decreased as the size of the ligand increased (R: Me $\Rightarrow$ Bu).



Scheme 6.3. Systematic variation of the steric and hydrophobic properties of a Cu(II) salen.

Most other characteristics of binding between metal–salens and nucleic acids also derive from studies on DNA and cationic copper complexes. No doubt the net positive charge of these derivatives confers a favorable electrostatic affinity to the polyanionic phosphoribose backbone of DNA. Whether further association is based on groove binding or intercalation is somewhat more controversial. ESR spectroscopy indicated that the salen illustrated in Scheme 6.3 (R = Me) orients with respect to DNA fibers in a manner most consistent with binding in the major groove [29]. In contrast, replacing the ethylene diimine with a phenylene diimine unit shifted the salen to an almost perpendicular orientation with respect to the fiber as expected for an intercalative mode of binding [29]. Interestingly, the ethylene derivative ($K_a = 1.0 \times 10^3$ M) bound approximately 50-fold more tightly than the phenylene derivative ($K_a = 53 \times 10^3$ M) to calf thymus DNA [28].

Another salen analog containing a cationic alkylamine-substituted ethylene bridge readily displaced ethidium from calf thymus DNA and appeared by electric linear dichroism to bind perpendicular to the helical axis of DNA much like the previous phenylene derivative [9]. However, this copper–salen did not induce helical unwinding as expected for intercalation and consequently major groove binding was proposed as an alternative to be considered equally [9]. A preference for bind-

ing at AT base pairs or AT-rich regions was also detected by its greater affinity for poly(dA-dT) versus poly(dG-dC) [9]. This result suggests that coordination between guanine N7, the strongest DNA ligand for copper [30, 31], and the salen complex did not control binding although it does control the reaction selectivity of nickel–salen as described in Section 6.4. Perhaps the wider major groove formed by AT base pairs relative to GC base pairs stabilizes interactions with Cu(II)–salen. Interestingly, a series of Mn(III)–salen derivatives expressed a reaction specificity for regions of DNA with high AT content in parallel to the binding selectivity expressed by the copper derivatives. But in this case, target selectivity was ascribed to the minor groove characteristics of AT-rich regions [32]. Further experiments will be needed to distinguish between these possibilities since sequence-dependent deviations from the canonical Watson–Crick duplex induce commensurate changes in both major and minor groove dimensions [33]. Regardless of the mode by which metal–salens bind to DNA, their selectivity can be easily manipulated by conjugating them to drugs such as distamycin and ellipticine with known affinity for DNA [25, 26].

Reversible binding of nickel–salens to nucleic acids has not been examined although these complexes covalently couple to unpaired guanines of DNA and RNA with high specificity in the presence of a peracid such as monoperoxyphthalic acid (MMPP) or monoperoxysulfate (HSO_5^-) [7, 34–37]. This ability to detect unusual secondary structures in nucleic acids is unique to the nickel derivatives and may originate from a transient Ni(III) species that coordinates preferentially to the N7 position of highly accessible guanine residues [7, 21, 35]. Such a possibility was recently demonstrated through distance-dependent effects of a paramagnetic complex of nickel as detected by the proton NMR spectra of duplex DNA containing an unpaired guanine [38, 39].

6.3

Nucleic Acid Strand Scission Induced by Simple Metal–Salen Complexes

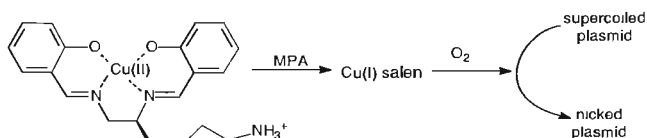
Metal–salen complexes screened for reactivity with nucleic acids have typically been found to induce oxidative scission of the phosphoribose backbone leading to direct strand fragmentation. This net process can involve a variety of different reactive intermediates and sites of phosphoribose oxidation [40]. A limited number of complexes have the ability to react spontaneously but most require the presence of an oxygen donor and, in certain cases, a reductant. The nature of both the ligand and bound metal govern which conditions are necessary to support covalent reaction of nucleic acids. These requirements in turn delineate the range of applications suitable for each derivative. The reductive activation required by copper–salens is particularly attractive since it avoids use of strong oxidants and has the potential for use *in vivo*. Most other metal–salens require oxidants ranging from hydrogen peroxide to peracids, although some need only molecular oxygen. Each system that is competent at direct strand scission is reviewed in more detail below (Sections 6.3.1–6.3.4). Typical assays used to screen for this type of activity include

nicking of supercoiled plasmid DNA and scission of polynucleotide restriction fragments (see below for example, Figs. 6.1 and 6.2). Unfortunately, other types of nucleic acid modification are not detected by these techniques and certain modes of reaction such as covalent adduct formation can proceed without detection unless alternative methods are used for product analysis.

6.3.1

Metal–Salens Activated by Reductants for Strand Scission

In analogy to $\text{Cu}(\text{1,10-phenanthroline})_2$ [20], copper–salens are also easily reduced from their $\text{Cu}(\text{II})$ to $\text{Cu}(\text{I})$ forms. These in turn react readily with ambient levels of molecular oxygen to generate intermediates which drive substrate oxidation [9, 41]. For example, a copper–salen containing a single alkylamine substituent ($50\ \mu\text{M}$) converted a plasmid (1 hour, 37°C) entirely from its supercoiled (form I) to nicked (form II) conformation by strand cleavage in the presence of 2-mercaptopropionic acid (MPA, $2.5\ \text{mM}$) and air-saturated buffer (Scheme 6.4, Fig. 6.1) [9]. The efficiency of plasmid nicking increased moderately after the DNA-binding agents anthracenedione and ellipticine were conjugated through the alkylamine of the copper–salen [25, 41]. Also, MPA was observed to be more effective than dithio-



Scheme 6.4. Plasmid strand scission mediated by a copper–salen complex.

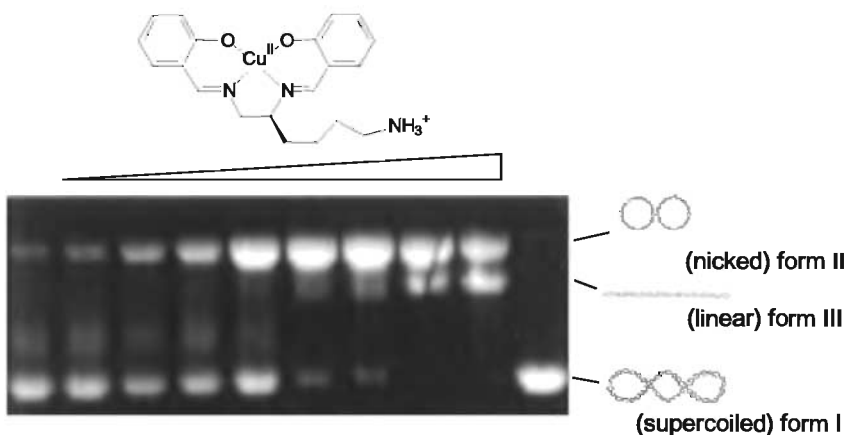


Fig. 6.1. Agarose gel electrophoresis used to detect nicking of supercoiled plasmid DNA in the presence of MPA and varying concentrations of the copper–salen illustrated in Scheme 6.4 under aerobic conditions. Adapted from Ref. [9].

threitol or hydrogen peroxide at promoting these processes. Surprisingly, an equivalent distamycin conjugate acted on a restriction fragment without sequence selectivity despite the preferential binding of distamycin to AT-rich regions [26]. The origins of this result are not yet clear since the ultimate oxidant for at least $\text{Cu}(1,10\text{-phenanthroline})_2$ and other copper-containing complexes is thought to be non-diffusible and hence be retained within the site of DNA recognition [40, 42]. Even the diffusible hydroxyl radical generated by iron–EDTA affinity conjugates primarily acts within the local vicinity of its DNA target [43, 44].

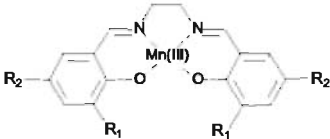
6.3.2

Metal–Salens Activated by Peracids for Strand Scission

The majority of metal–salens rely on the presence of an added oxidant to initiate DNA scission or nicking although exact requirements depend on the nature of the salen and its bound metal. Some derivatives effect scission only in the presence of strong oxidants such as a peracid (MMPP or KHSO_5) while others need only molecular oxygen. One of the very first studies reported a reaction between a salen derivative and plasmid DNA involving a $\text{Ni}(\text{II})$ complex of the parent salen, bis(salicylaldehyde)ethyl enediimine, along with oxidants MMPP and iodosylbenzene alternatively [45]. Both conditions supported strand scission but their efficiencies could not be easily determined due to the low solubility of the neutral $\text{Ni}(\text{II})$ –salen and iodosylbenzene. In addition, the high molecular weight of the polynucleotide products prevented detection of the unique ability of nickel–salen to couple covalently to its target sites of DNA [7].

A $\text{Mn}(\text{III})$ complex of the same salen parent has the advantage of a net (+1) charge that enhanced its solubility in aqueous media and provided electrostatic affinity to DNA. Reaction of this complex in the presence of MMPP was characterized in part by direct strand scission of a 517-base-pair restriction fragment (Fig. 6.2) [32]. This allowed for product identification at nucleotide resolution and revealed a selectivity for AT-rich regions [32]. The observed selectivity was attributed to preferential binding and phosphoribose oxidation in the minor groove of DNA by a $\text{Mn}(\text{V})\text{oxo}$ –salen intermediate or a species with equivalent activity. In this case, association in the minor groove was implied by a characteristic 3' offset in the profile of scission products formed by the complementary strand. These results were also reminiscent of the reaction and target selectivity of a cationic $\text{Mn}(\text{III})$ porphyrin derivative in the presence of KHSO_5 [46]. An extensive series of $\text{Mn}(\text{III})$ –salens were subsequently characterized to establish a structural basis for reaction efficiency and target recognition [16]. Despite considerable perturbation of the aromatic ring's electronic and steric properties and the diimine linker's composition, the position of DNA scission remained relatively constant. In contrast, reaction efficiency varied by over 10-fold. Significant differences in efficiency and even some variation in product profiles were also detected for enantiomers created by asymmetric substitution on the diimine linker [16].

Alternative salen derivatives based on the presence of two pendant quarternary amines were developed to improve the solubility properties of otherwise neutral



— G H H H tBu R₁
H H CH₃ Cl Br R₂

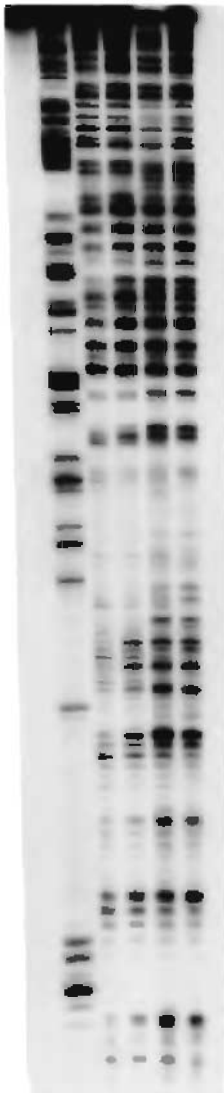
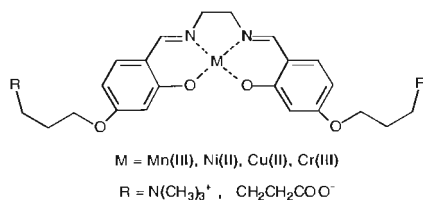


Fig. 6.2. Denaturing polyacrylamide gel electrophoresis used to detect direct strand scission of DNA by a series of manganese salens in the presence of MMPP. Adapted from Ref. [32].

M(II)–salens and to create an electrostatic attraction for nucleic acids [7, 47]. One of these ligand systems was then used to compare the activity of Mn(III), Ni(II), Cu(II), Co(II) and Cr(III) derivatives in the presence of MMPP (Scheme 6.5). Plasmid cleavage assays confirmed the expected high reactivity of the Mn(III)– and Ni(II)–salens (1–50 μ M) and also revealed a lack of reactivity of Cu(II)–, Co(II)– and Cr(III)–salens [47, 48]. The importance of electrostatics in designing salens was concurrently illustrated by the inability of a Ni(II)–salen complex containing an anionic appendage (Scheme 6.5) to either oxidize or reversibly bind DNA in a detectable manner. The need to match reaction conditions with each type of salen complex was also highlighted by the ability of the Co(II)–salen to nick plasmid DNA in the absence, rather than presence, of MMPP (see discussion below on spontaneous activation of molecular oxygen, Section 6.3.4). Similarly, the copper–salen complex was unable to induce strand scission in the presence of an oxidant such as MMPP but was quite proficient at scission in the presence of molecular oxygen and a reductant such as MPA [9].



Scheme 6.5. Cationic and anionic metal salens.

Primer extension assays were used in further studies to compare the same series of metal–salens and did indeed verify the reactivity of the Ni(II)– and Mn(III)–salens in a qualitative manner. This assay relies on the inability of DNA polymerase to replicate past sites of nucleobase or phosphoribose damage [49]. Unlike the plasmid-nicking assay, primer extension is very useful for identifying the exact sites of nucleotide modification and hence the sequence selectivity of reaction. However, this assay cannot distinguish between strand scission and many types of base modification. Thus, reaction efficiencies measured by the plasmid-nicking assay should be considered independently from those of primer extension. Similarly, the observable end points of the two assays are quite different since 100% nicking of plasmid DNA is equivalent to approximately one oxidation event per >8600 nucleotides (pBR322), whereas primer extension may detect only one event per ~200 nucleotides. If oligonucleotide systems are used, even more abundant reaction might be necessary for its detection [7]. Whatever type of modification was ultimately detected with the Ni(II)– and Mn(III)–salens, the former expressed a preference for GC sequences and the latter expressed a preference for AT sequences [47, 48]. These results are generally consistent with the nickel–salens described in Section 6.4 on nucleic acid adduct formation as well as with other nickel and manganese complexes not based on salens [16, 21, 32, 46, 49, 50].

6.3.3

Metal Salens Activated by Hydrogen Peroxide for Strand Scission

Metal salens that are active with hydrogen peroxide rather than a stronger oxidant such as MMPP offer milder conditions for nucleic acid reaction. However, these types of systems have not been widely explored nor applied in biochemical studies. Their modes of action are often difficult to characterize since hydrogen peroxide has the potential to act as a radical precursor, a reductant and an oxidant. A Mn(III)–salen had originally been shown to promote double-strand cleavage of plasmid DNA with the same specificity, whether MMPP or hydrogen peroxide was present [32]. Manganese derivatives also exhibit the unusual ability to react under diverse conditions ranging from ascorbate to KHSO_5 [51]. Hydrogen peroxide only very weakly supports reaction of a copper–salen–anthracenedione conjugate in comparison to its reaction in the presence of the reductant MPA [41] and would not be expected to support reaction of the nickel–salens. In contrast, hydrogen peroxide-dependent reactions based on iron are ubiquitous, and thus efficient plasmid nicking by hydrogen peroxide and an iron–salen complex was not surprising [52]. An Ru(III)–salen derivative containing two pendant cationic groups also promotes hydrogen peroxide-dependent oxidation of DNA [53]. The resulting products of this reaction did not undergo spontaneous strand scission but instead required subsequent treatment with hot piperidine for scission. All nucleotides were susceptible to modification and modest selectivity for non-helical DNA, including bulge and hairpin structures, was observed. The basis for selectivity of the Ru(III)–salen has not yet been identified, but intercalation of the salen complex appears unlikely [53].

6.3.4

Metal–Salens Activated by Molecular Oxygen for Strand Scission

Recent efforts have begun to focus on metal–salen complexes that may act spontaneously in the absence of any co-reactant or at least in the mere presence of molecular oxygen. Such complexes have the potential for broad application and, in particular, may be used for characterizing nucleic acid conformation and dynamics *in vivo* [54]. The ability of Co(II)–salens to bind molecular oxygen reversibly has been known for over six decades and has already been subject to intensive investigation [8, 55, 56]. A Co(II)–salen containing two cationic appendages (Scheme 6.5) has also been shown capable of nicking plasmid DNA under standard aerobic conditions [57]. Reaction was inhibited by the presence of the oxidant MMPP but greatly stimulated by the reductant dithiothreitol (DTT) (Scheme 6.6). Together, these and other data suggest formation of a complex between molecular oxygen and Co(II)–salen which serves as a crucial intermediate for nucleic acid oxidation. Primer extension analysis of duplex DNA treated with the Co(II)–salen in the presence and absence of added DTT revealed a slight preference for reaction at guanine residues but the exact products formed under these conditions have not yet been identified [57].



Scheme 6.6. Oxygen activation by Co(II)–salen.

A Ni(II)–salen complex equivalent to the Co(II) complex above was incapable of inducing plasmid scission in the presence of only molecular oxygen [57]. Model studies in organic solvents indicate that standard Ni(II)– and Cu(II)–salens neither bind nor activate molecular oxygen under ambient conditions [58, 59]. Reduction of the salen diimine to a diamine, however, generates a ligand that still coordinates to Ni(II) and now enables the resulting complex to undergo oxidation in the presence of molecular oxygen [58, 59]. Derivatives of Ni(II)–tetrahydrosalen have since been constructed for aqueous solubility but not yet applied to nucleic acid analysis [60]. In these cases, the reduced ligand might serve as the sacrificial reductant of molecule oxygen. Sulfite (SO_3^{2-}) has similarly been used in this manner during a process involving nickel–salen-dependent reduction of molecular oxygen (see Section 6.5).

Unlike the Ni(II) derivatives, a Cu(II)–tetrahydrosalen remained inert to molecular oxygen [59]. Copper(II)–dioxygen chemistry was alternatively stimulated by use of salen derivatives containing hydroxy groups on the aromatic rings [61]. A derivative containing bis(*para*-hydroxy) substituents most readily formed oxygen radicals in the presence of molecular oxygen and similarly expressed greatest proficiency at nicking plasmid DNA when compared to its bis(*ortho*-hydroxy) analog [61]. In contrast, the *meta* isomer did not detectably produce such radicals and was unable to cause plasmid nicking.

A similar trend was observed for the iron complexes of these same bishydroxy-substituted salens [62]. The *para*-substituted derivative (50 μ M) nicked 75% of the available supercoiled DNA in 3.5 h under aerobic conditions (37°C), whereas the *ortho* and *meta* derivatives nicked no more than 6% of the DNA. Iron complexes of the parent salen lacking the hydroxy groups and the reduced tetrahydrosalen exhibited little or no reaction. The *para* derivative also bound reversibly to DNA with surprisingly much greater affinity than any of its analogs as indicated by its ability to stabilize helical DNA and significantly increase the T_m values for poly(dA-dT) and calf thymus DNA [62]. The basis for this strong association has not yet been identified and does not influence or guide reaction to any particular nucleotide sequence. Direct polynucleotide fragmentation is generated by this iron–salen without sequence specificity in a manner reminiscent of iron–EDTA and, like iron–ETDA, the iron–salen has been applied to mapping DNA–drug interactions [62].

Finally, Cr(V)–salens may yet serve as the epitome of a self-activated reagent since no additional oxidant or reductant was necessary to achieve plasmid nicking [63] and base oxidation [64].

6.4

Covalent Coupling between Simple Nickel–Salen Complexes and Nucleic Acids

Each assay frequently used to identify and define nucleic acid modification is typically capable of observing only one type of product and hence provides only a limited perspective on the overall reaction. As described above, plasmid nicking is exquisitely sensitive to strand fragmentation but offers no information on the sites of fragmentation or on other types of reaction. Primer extension alternatively detects the sites of reaction but cannot distinguish between base modification and strand fragmentation. Examining the full range of products from a particular set of reaction conditions then necessitates a number of complementary approaches. Without a series of oligonucleotide-based studies, the unique ability of nickel–salen derivatives to attach covalently to nucleic acids in a nucleotide- and conformation-specific pattern would not have been readily apparent [7, 35]. This activity has since provided an important alternative to the more commonly observed strand scission and may ultimately allow for examining systems of great complexity.

The efficiency and type of oxidation promoted by nickel complexes was shown very early to be highly sensitive to its ligand environment. Tetraazamacrocycles forming square-planar complexes with nickel (see, for example, NiCR, Fig. 6.3) were most proficient at inducing oxidation of guanine residues exhibiting high solvent accessibility using a peracid such as MMPP or KHSO_5 as oxidant [21, 65, 66]. In contrast, a pentaazamacrocyclic complex of nickel required only molecular oxygen and appeared to oxidize both guanine and adenine residues [21, 67]. Both nickel complexes were capable of nicking plasmid DNA, and nucleobase oxidation was evident after hot piperidine treatment induced the diagnostic strand scission (see, for example, lane 7, Fig. 6.3). When a cationic nickel–salen, Ni(II)–TMAPES, was tested in the presence of KHSO_5 , a distinct DNA product migrating more slowly than the parent oligonucleotide was detected by gel electrophoresis (lane 2, Fig. 6.3) [7]. This product was unique to the salen complex and was not observed under the same conditions with the tetraazamacrocyclic complexes of nickel nor a diimine complex in which salicylaldehyde was replaced by the non-aromatic acetylacetone (NiTMAPAA, lane 8, Fig. 6.3). This slowly migrating product was first proposed to be a covalent adduct between DNA and Ni(II)–TMAPES, and this was later confirmed by mass spectroscopy [7, 35]. Some heterogeneity in the type of salen–DNA linkage was also originally considered since not all of the slowly migrating product fragmented under standard hot piperidine conditions (30 min) (lane 4, Fig. 6.3). However, extended treatment with piperidine fully cleaved the modified DNA and no heterogeneity of reaction sites is now suspected [35, 49].

The mechanism of Ni(II)–TMAPES coupling to DNA most likely involves formation of a ligand center that couples to the C8 position of guanine, a typical site for radical addition (Scheme 6.7) [68–70]. Such a proposal is consistent with the inhibition of coupling observed for derivatives containing methyl and chloro substituents at the *ortho* and *para* positions of the phenolic ring that sterically hinder the centers with most radical character [35]. Salen ligand radicals were also previously invoked to explain oxidative polymerization of nickel–salens during elec-

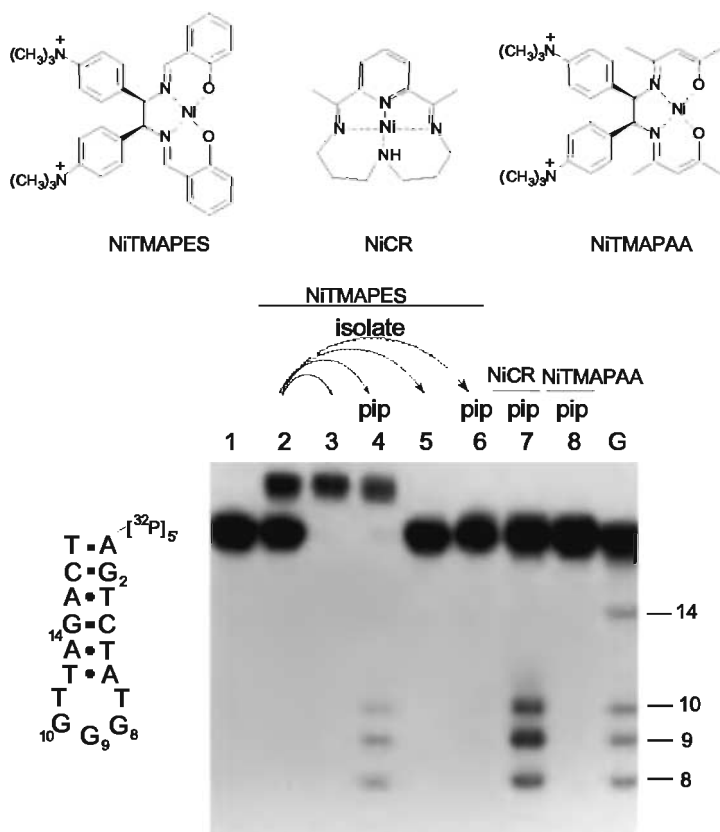
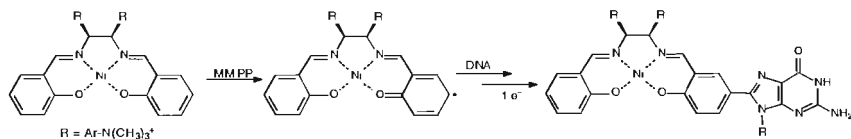


Fig. 6.3. Denaturing polyacrylamide gel electrophoresis used to detect covalent coupling and piperidine-induced strand scission of a hairpin forming oligonucleotide in the presence of KHSO_5 and NiTMAPES versus other nickel complexes. Adapted from Ref. [7].



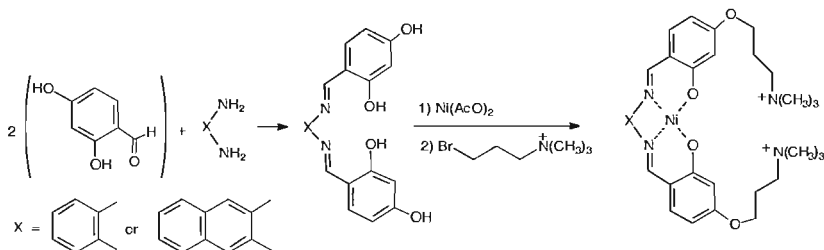
Scheme 6.7. Proposed mechanism of coupling between NiTMAPES and an accessible guanine residue.

trochemical analysis [71, 72]. Certain nickel protein and peptide complexes similarly appear to generate related tyrosyl radicals in the presence of a peracid and induce a coupling reaction as indicated by subsequent protein–protein cross-linking [73, 74].

The conformational selectivity of Ni(II) –TMAPES was first demonstrated by its

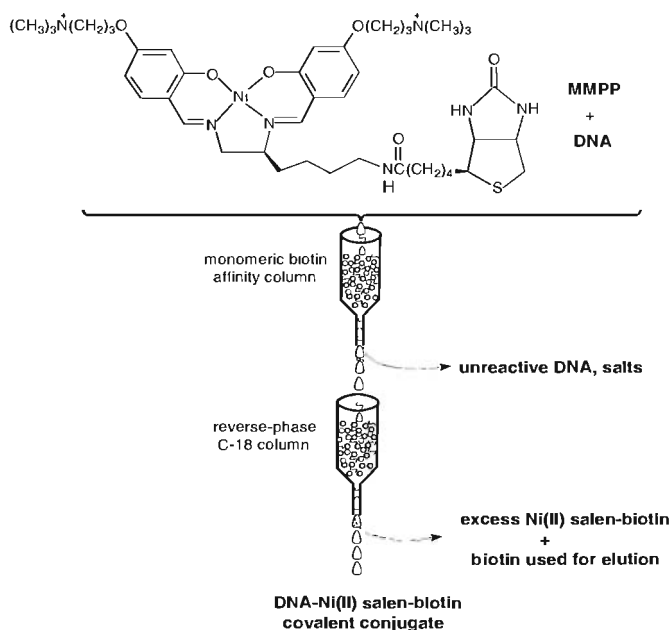
coupling to any of three extrahelical guanines within a hairpin loop (lane 4, Fig. 6.3) [7]. No reaction was evident for the two helical guanines even though one is only a single base pair from the helix terminus. The conformational dependence of modification is equivalent to that previously described for a macrocyclic complex of nickel NiCR [21, 22] (lane 7, Fig. 6.3) and subsequently expanded to include another nickel–salen [37]. Target recognition for at least NiCR seems to correlate with access to guanine N7, an intrinsic site for nickel coordination [21, 38, 39, 75] and likely the other complexes act similarly. However, an advantage of the Ni(II)–salen derivatives is their ability to add bulky substituents rather than oxidatively degrade the reactive guanine residues. This became apparent from the strong signals detected by primer extension when mapping RNA and DNA structure with Ni(II)–TMAPES [34, 35]. Ni(II)–TMAPES has since been used to characterize folding intermediates of self-splicing RNA [76] and junctions of B- and Z-helical DNA [77].

Widespread application of Ni(II)–TMAPES has been limited in part by its difficult preparation although a relatively efficient synthesis has recently been published [7, 78, 79]. Still, alternative salens retaining both their solubility under aqueous conditions and their ability to couple with nucleic acids can now be prepared in three steps from commercially available starting materials (Scheme 6.8) [35, 48]. Many analogous derivatives may also be envisioned to perform equally well as structural probes of DNA and RNA.



Scheme 6.8. Efficient synthesis of cationic salens.

An especially versatile salen ligand was constructed with an alkylamine appendage that has the ability to either confer water solubility to an otherwise neutral M²⁺ complex or act as a convenient attachment for DNA-directing reagents (Scheme 6.4) [9, 25, 26, 41]. An analogous nickel–salen–biotin conjugate has since been generated from combining components of two complementary salen ligands, the alkylamine containing ethylenediamine derivative illustrated in Scheme 6.4 and the quarternary amine containing salicylaldehyde derivative illustrated in Scheme 6.5 [36]. The resulting salen maintained its selectivity for coupling to guanine residues and provided an expedient method of biotinylating DNA in a conformation selective manner (Scheme 6.9). This reagent may now provide entry into the myriad of biotin-derived kits available for molecular biology and has already supported isolation of modified DNA by avidin-based affinity chromatography and its chemiluminescent detection by streptavidin–alkaline phosphatase conjugates.



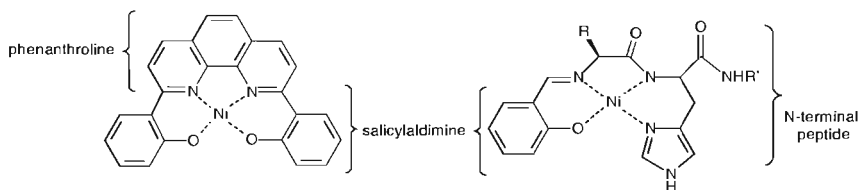
Scheme 6.9. A conjugate of Ni(II) salen and biotin allows for selective reaction and product isolation.

6.5

Chimeric Metal–Salen Complexes

The simplicity and adaptability of metal ligands based on salen have just begun to inspire design of chimeric (or hybrid) derivatives that combine the beneficial properties of salicylaldimine with other known coordination systems. The breadth of opportunity presented by this strategy has the potential to create many predictable, yet new and significant, activities. Both classical and combinatorial approaches may once again be applied to such development as practiced earlier with salen itself.

1,10-Phenanthroline has garnered much attention in biological chemistry due to extensive use of its copper complex for mapping nucleic acid and protein structure [80]. This ligand controls the redox activity of the bound copper as well as the interaction of the complex with biomolecules. Features of phenanthroline and salen have recently been integrated to form a novel ligand with a metal-dependent affinity for DNA (Scheme 6.10) [51]. The cobalt complex significantly stabilized duplex DNA by enhancing its T_m by as much as 10°C. The corresponding nickel and manganese complexes exhibited weaker stabilization and the copper complex expressed only a minimal effect. The cobalt complex also induced plasmid nicking in the presence of a thiol reductant (MPA) and molecular oxygen in analogy to that observed with the earlier salen-based system. However, the equivalent copper



Scheme 6.10. Salen-derived chimeric complexes.

complex was surprisingly unreactive with plasmid DNA under the same conditions that had previously supported nicking of DNA by the copper salen. Reaction of the nickel complex was similarly suppressed and no covalent coupling to DNA was observed [51]. Identifying the source of these divergent characteristics should ultimately help distinguish the relative influence of target association and ligand chemistry on the modes and selectivity of nucleic acid modification.

Peptides and proteins with N-terminal sequences containing histidine at position 3 offer another intriguing ligand system for combination with salen on account of their ability to coordinate and activate metals for protein crosslinking [73, 81] and DNA oxidation [82–84]. Each of these activities has been successfully used to detect protein–protein and protein–DNA interactions. Replacing the terminal amino acid with salicylaldehyde creates an alternative chimera (Scheme 6.10) that may share the reactivity of salen but exhibit target selectivity directed by an attached (poly)peptide. Initial investigations on a related Cu(II)–salicylidene-glycylglycine complex suggested possible coordination to cytidine as indicated by paramagnetic line broadening of its ^1H NMR signals [85]. More extensive analysis has been published on a Ni(II)–salicylidene diamino acid complex containing an arginine for water solubility and a histidine to support tetradentate coordination (Scheme 6.10) [86]. Reaction of the peptide–salen chimera was initiated by addition of KHSO_5 or alternatively by autoxidation of sulfite. This additional activity appears to generate HSO_5^- *in situ* through a process that is catalyzed by nickel–peptide complexes including that formed by the salen–peptide hybrid [87]. Consistent with the nickel and salen components of the chimera, covalent coupling between a guanine residue and the complex was observed and apparently formed through an intermediate phenolic radical reminiscent of that generated by Ni(II)–TMAPES [86]. When desired, the DNA–metal adduct could be partially disassembled by treatment with EDTA. Removal of the nickel induced hydrolysis of the ligand’s imine linkage and released the peptide component. The net effect of this chimera consequently allows for delivery of a salicylaldehyde equivalent to DNA and hence the introduction of a unique aldehyde group that is available for subsequent conjugation and detection.

6.6

Conclusion

By the appropriate choice of metal and salen components, coordination complexes may be constructed to promote most every type of nucleic acid oxidation that has

been achieved collectively from a broad range of individual metal systems including (1) direct strand scission via oxidation of sugar residues, (2) base modification leading to piperidine-sensitive cleavage, and (3) oxidative adduct formation. The conformational selectivity exhibited by certain nickel–salens provides useful probes of nucleic acid structure while the lack of selectivity exhibited by certain iron and manganese derivatives provides reagents for footprinting nucleic acid interactions with proteins and small molecules. Metal–salen complexes have also been designed to function under reductive, oxidative and ambient conditions, thus supporting numerous applications including the potential for study *in vivo*. Simultaneous control of both target recognition and type of modification has been rather challenging but may become more common with designs based on salen conjugates and chimeras. Recent use of DNA as a template for assembling a salen–oligonucleotide conjugate illustrates a particularly exciting strategy for tailoring the function of a salen derivative directly to its intended target [10].

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7

Charge Transport in DNA

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7.1

Introduction

Double-helical DNA contains within its interior an extended π -stacked array of aromatic, heterocyclic base-pairs. As such, the DNA double helix represents, almost uniquely, a well-defined molecular π -stack. Solid-state π -stacked materials, particularly when doped, can be effective conductors and semiconductors [1, 2]. In the years since the delineation of the DNA structure by Watson and Crick [3], by analogy, it has been asked whether double-helical DNA might similarly display properties associated with molecular conductivity [4, 5]. Indeed, whether double-helical DNA, owing to its π -stacked structure, might be an effective conduit for charge transport (CT) has intrigued physicists, chemists, and biologists for over 40 years.

Early studies of DNA charge transport generated significant interest and extensive debate. Physicists carried out varied measurements of electron transport in DNA, resulting in DNA being described by some as an insulator and others as a quantum wire [6–8]. Based upon pulse radiolysis studies, radiation biologists suggested charge could migrate over as short a distance as 3 base pairs [9] or as long a distance as 200 base pairs [10]. More recent studies applied more sophisticated techniques in measurements of DNA conductivity. Differential conductivity depending upon DNA orientation was seen in studies of aligned DNA films [11], a large current that increased linearly with applied voltage was found when the DNA molecules were oriented perpendicular to the electrode, and in contrast, no current was detected with a parallel alignment. Direct conduction measurements were carried out on small collections of DNA duplexes arranged into 600-nm-long DNA ropes, and these studies revealed semiconductor behavior, with resistivity values that were comparable to those of other conducting or semiconducting materials [12].

Furthermore, single-molecule conduction measurements on dry poly(G)-poly(C) showed no conduction at low voltages, but at higher voltages, DNA seemed to support large currents [13]. While some consensus may now have been reached that DNA possesses properties of a wide band gap semiconductor [14], varied conductivity measurements on dry DNA samples at low temperatures continue to ap-

pear consistent with DNA being everything from an insulator to a superconductor [15]. Likely the variability in DNA structure and integrity in these physical measurements adds to the variability in conclusions obtained.

Chemists first focused on CT in DNA in the context of earlier studies of protein electron transfer [16], and *analogously* to protein systems, well-defined oligonucleotide duplexes containing pendant donors and acceptors were constructed so as to measure electron transfer rates and yields as a function of distance on DNA duplexes in solution. The ability of the DNA π -stack to serve as a medium for long-range electron transport was quantified utilizing β , which, from Marcus theory, is a measurement of the exponential decay with distance of electronic coupling of the medium [17]. For σ -bonded systems, including proteins, the value of β was found to be $\sim 1.0 \text{ \AA}^{-1}$; for comparison, a β value of $\sim 0.1 \text{ \AA}^{-1}$ is found for conjugated polymers [18]. In measurements of electron transfer on DNA assemblies, β was found to range from $= 0.2 \text{ \AA}^{-1}$ [19, 20] to $\sim 0.6\text{--}0.7 \text{ \AA}^{-1}$ [21], to as high as $1.4 \text{ \AA}^{-1}$ [22]. Recent experiments have demonstrated the complexity of experimental evaluation of β for DNA, and have suggested that the apparent disparity in CT distance dependence may arise because different experimental assemblies might operate within different regimes of a mechanistic continuum between one-step superexchange and multi-step hopping [23]. Hence β , in the context of non-adiabatic tunneling models, may not be the most appropriate parameter to characterize DNA-mediated CT.

Studies in our research group have focused on using DNA assemblies with intercalators or modified bases serving as donors and acceptors. These studies using intercalators as direct probes of the π -stack have revealed particularly shallow distance dependences in CT, underscoring the importance of stacking to effective CT in DNA.

Here we focus on these studies of DNA CT using well-stacked probes of the DNA duplex. This discussion is therefore intended not as an exhaustive review of the literature but instead as an illustrative description of what we have learned through a mix of biochemical, spectroscopic, and electrochemical studies in our laboratory. We have found CT in DNA to be exquisitely sensitive to π -stacking of the donor, the acceptor, and the intervening base pair array. As a result, CT may be most usefully applied as a probe of nucleic acid structure and structural dynamics.

7.2

DNA Metallointercalators

In characterizing the π -stack as a medium for DNA-mediated CT, it is important to have the probes directly coupled into the π -stack. Therefore, our laboratory has utilized a number of metallointercalators to study long-range electron transfer in DNA, particularly, dipyrrophenazine (dppz) complexes of ruthenium(II) (e.g. $[\text{Ru}(1,10\text{-phenanthroline (phen)})(4'\text{-methylbipyridine-4-butyl acid (bpy')})(\text{dppz})]^{2+}$ and 9,10-phenanthrenequinone diimine (phi) complexes of rhodium(III) (e.g. $[\text{Rh}(\text{phi})_2\text{bpy}']^{3+}$) [24]. These ligands afford the octahedral ruthenium and rho-

dium metal complexes tight DNA binding via intercalation ($K \geq 10^6 \text{ M}^{-1}$), and the DNA interactions of these intercalators have been extensively characterized [25].

7.2.1

Phenanthrenequinone Diimine Complexes of Rhodium

Studies of phi complexes of rhodium have shown that these complexes bind avidly to DNA ($K \geq 10^6 \text{ M}^{-1}$) by intercalation of the phi ligand [26, 27]. The sequence specificity of these complexes can be varied by altering the ancillary, non-intercalating ligands. High-resolution NMR studies initially provided insight into the binding mode of these phi complexes [28]. The studies revealed that the complexes bound DNA from the major groove, and that intercalation provided a means by which the functional groups on the ancillary ligands and those contained in the major groove could interact. In fact, NMR studies of the complex, $\Delta\alpha\text{-[Rh}(R,R\text{-dimethyltrien})\text{phi}]^{3+}$ (where R,R -dimethyltrien is $2R,9R$ -diamino-4,7-diazadecane), which was designed to recognize the sequence $5'\text{-TGCA-}3'$, revealed site-specific binding of the complex to a decamer that centrally contained a $5'\text{-TGCA-}3'$ sequence and delineated specific, rationally designed hydrogen bonding and methyl-methyl contacts [29]. Recently, a $1.2\text{-\AA}$ crystal structure was obtained for the $\Delta\alpha\text{-[Rh}(R,R\text{-dimethyltrien})\text{phi}]^{3+}$ complex, intercalated into a DNA octamer (Fig. 7.1) [30]. The structure revealed that intercalation of the complex occurs via the major groove, and, based upon five independent views of the intercalator, minimal perturbation of the π -stack is observed; the phi ligand inserts into and essentially serves as an additional base pair within the π -stack. In the context of our studies of DNA CT, this intercalation does not appear to perturb the base stack either globally or locally.

The rich photochemistry of phi complexes of rhodium enables demarcation of binding sites of the metal complexes in addition to providing potent, intercalating photooxidants [27]. Hence, irradiation of the complexes bound to DNA at different wavelengths induces different photochemical reactions [31]. Irradiation of the phi complexes with ultraviolet light ($\lambda = 313 \text{ nm}$) leads to direct strand scission of the DNA sugar-phosphate backbone with products consistent with abstraction of the C3' hydrogen from the sugar that is near the activated phi ligand at the intercalation site. This chemistry then marks the site of binding. If, instead, the phi complexes are irradiated with visible light bound to DNA ($\lambda \geq 365 \text{ nm}$), oxidative damage to the DNA bases results. These phi complexes of rhodium, when photo-excited, appear to be sufficiently potent to promote oxidation of all of the nucleotides.

7.2.2

Dipyridophenazine Complexes of Ruthenium

The dipyridophenazine complexes of ruthenium(II) have been shown to possess remarkable photophysical properties when bound to DNA [32, 33]. Irradiation of these complexes with visible light leads to a metal-to-ligand charge transfer excited

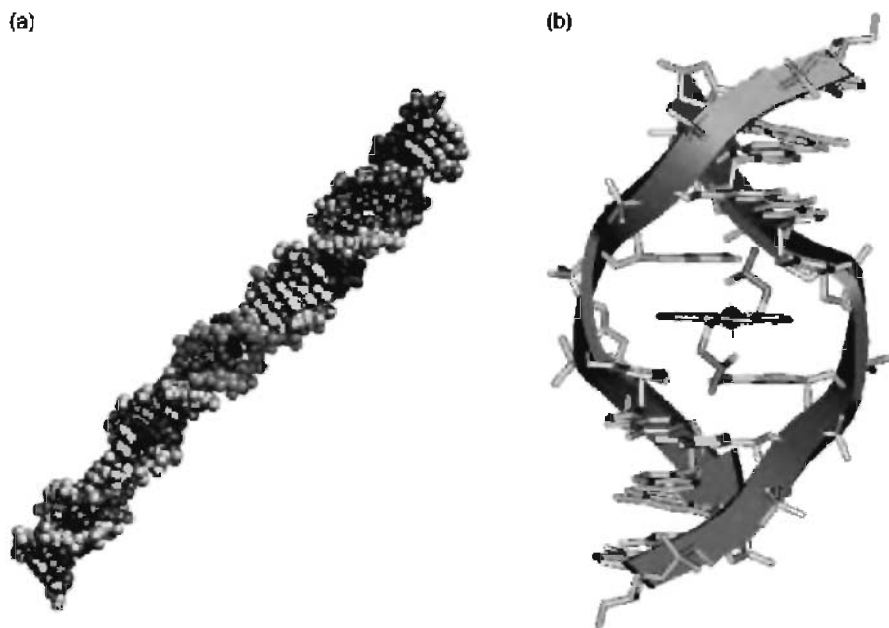


Fig. 7.1. (a) Five $\Delta\text{-}\alpha\text{-}[\text{Rh}(\text{R},\text{R}\text{-dimethyltrien})\text{ph}]^{3+}$ -DNA octamer complexes stacked end to end in the asymmetric cell of the crystal. (b) A major groove view of intercalation of $\Delta\text{-}\alpha\text{-}[\text{Rh}(\text{R},\text{R}\text{-dimethyltrien})\text{ph}]^{3+}$ bound to

5'-G-dIU-TGCAAC-3'. As shown in the figure, minor perturbation of the π -stack is observed. The intercalator is inserted as an additional base-pair step. Adapted from [30].

state, which is localized on the dppz ligand. This localization of the charge on the dppz ligand has been particularly valuable in probing DNA-mediated CT, because excitation of intercalated dipyrrophenazine complexes directs the charge transfer directly into the π -stack, not onto an ancillary ligand [34].

Perhaps more important is the interesting differential luminescence quenching seen with the complex when free in aqueous solution compared with bound to DNA. Luminescence of the dppz complex of ruthenium is easily detected in organic solvents; however, none is observed in aqueous solution. This quenching by water has been attributed to proton transfer from the solvent to the nitrogen atoms of the phenazine [35]. Upon intercalation of the dppz into DNA, however, the luminescence is maintained, for the DNA π -stack protects the phenazine nitrogen atoms from the solvent. Hence dppz complexes of ruthenium serve as sensitive “light switches” for DNA.

This “light switch” characteristic of the dipyrrophenazine complexes has proven to be a valuable spectroscopic tool in delineating how the intercalated dppz ligand is bound to DNA [36]. NMR studies of partially deuterated $[\Delta\text{-Ru}(\text{phen})_2\text{dppz}]^{2+}$ bound to a hexamer revealed that the dppz ligand prefers to intercalate via the major groove and that two binding orientations (e.g. symmetrical and asymmetrical) were identified [37]. In the symmetrical mode, the phenazine nitrogens are

protected from the solvent, whereas in the asymmetrical mode, the phenazine nitrogens are more solvent exposed. Other studies have suggested that some dppz complexes may bind to DNA also from the minor groove side [38]. A DNA intercalator that possesses both major and minor groove orientations has not been established previously. It would therefore be valuable to establish whether both binding orientations occur through intercalation and what truly determines access of the dppz complex to the base pair stack.

7.3

Photophysical Studies of Electron Transport in DNA

7.3.1

Electron Transport between Ethidium and a Rhodium Intercalator

Our early finding of long-range electron transfer quenching in an assembly containing tethered metallointercalators provided an intriguing introduction to studies of CT through DNA [19]. These early studies underscored clear differences between CT in DNA versus protein systems [39]. To further probe the distance dependence of CT in DNA and to begin to delineate the importance of π -stacking to the CT process, the classic organic intercalator, ethidium, was employed as the photoexcited electron donor and $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ as the acceptor (Fig. 7.2) [40]. Both the donor and acceptor molecules were tethered to duplexes of variable length

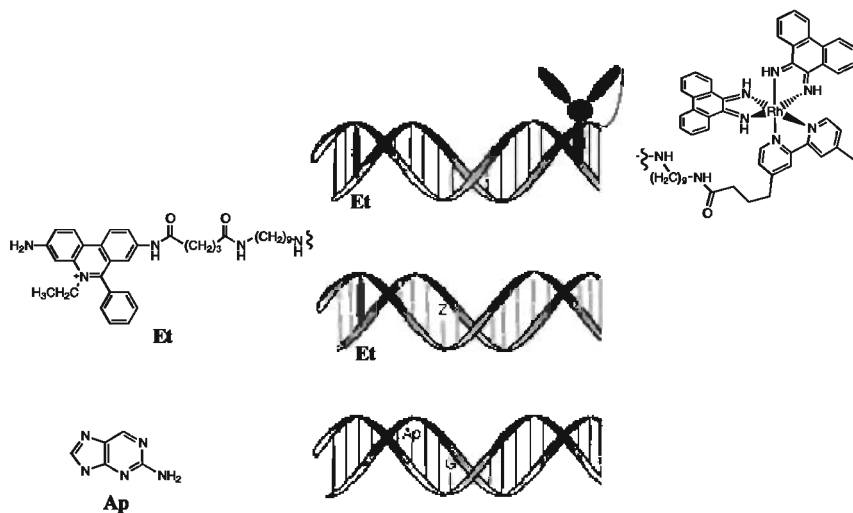


Fig. 7.2 Schematic illustration of some DNA assemblies used to probe photoinduced charge transfer. Shown (top to bottom) are duplexes containing donors and acceptors as two pendant intercalators [20], an intercalator and modified base [23, 41], and two bases [42, 44].

to permit systematic investigation of the electron transfer over defined donor–acceptor distances. Photoinduced quenching of the ethidium excited state was monitored for a series of duplexes which were 10–14 base pairs in length and had donor–acceptor separations that ranged from 24 to 38 Å. Fluorescence quenching provided a measure of electron transfer efficiency. Interestingly, a shallow distance dependence in the quenching yield was observed for this system. Moreover, although the quenching variations appeared to reflect an attenuation in the yield of electron transfer, time-resolved measurements showed that dramatic variations in the electron transfer rate were not apparent. Thus we proposed that electron transfer through the base pair stack was faster than our ability to detect it ($>10^9 \text{ s}^{-1}$) and that electron transfer through DNA was sensitive to π -stacking, so that the distance dependence in quenching yield observed reflected the increased probability of a stacking defect with increasing donor–acceptor distance, not a significant change in rate.

To further probe the sensitivity of the electron transfer process to π -stacking, a CA mismatch, which is known to cause local perturbations in base-stacking but not global structural distortions, and a GA mismatch, which continues to be well stacked in the DNA due to increased aromatic surface area, were introduced into the DNA assemblies. Substantial electron transfer quenching was still evident in the duplex containing the GA mismatch, which had a quenching yield similar to that of the Watson–Crick paired duplex. However, a significant reduction in quenching yield was observed in the presence of the CA mismatch. This result provided a clear demonstration of the sensitivity of the electron transport process to perturbations in the intervening base pair stack. Moreover this experiment established unambiguously that the path of CT is through π -stacked bases.

7.3.2

Ultrafast Charge Transport in DNA: Ethidium and 7-Deazaguanine

Having observed photoinduced long-range electron transport between metallo-intercalators, we also wanted to examine assemblies in which a DNA base could serve as the reactant. The guanine base analog 7-deazaguanine possesses an oxidation potential that is $\sim 300 \text{ mV}$ below that of guanine. Hence, in assemblies containing tethered ethidium as the photoexcited donor, selective oxidative quenching by this analog but not guanine is possible within a mixed DNA sequence [41].

Fluorescence measurements of the ethidium-deazaguanine system (Fig. 7.2) first revealed that electron transport could occur over a range of donor–acceptor separations (6–24 Å) and that the transport occurred on a subnanosecond time scale, with a quenching yield that also exhibited a shallow distance dependence. Furthermore, as in the earlier study, upon the incorporation of mismatches into the ethidium/7-deazaguanine duplexes, the quenching yield was significantly diminished. Hence, results were consistent with fast electron transport between the intercalated ethidium and deazaguanine mediated by the DNA π -stack.

The critical importance of DNA dynamics in attenuating the CT process was then underscored in time-resolved studies on the femtosecond time scale of the

photooxidation of deazaguanine in a series of tethered ethidium duplexes [23]. Again the measurements indicated that it was primarily the yield of electron transfer that varied with distance rather than the rate. Transient absorption measurements revealed a 5-ps component in the electron transfer decay, which we assigned to direct electron transfer from deazaguanine, and a 70-ps component, which corresponded to the time scale for motion of the ethidium within its binding site. With an increase in donor–acceptor separation, we observed that these components decreased in yield but not significantly in their decay times. Hence, we understood these results in the context of a model in which the initial population of duplexes that were properly stacked and aligned to permit effective coupling were represented by the 5-ps component, yielding fast long-range CT, while CT on the longer 70-ps time scale was gated by the motion of the ethidium within its intercalation site, requiring motion and realignment of the ethidium to permit long-range CT. Interestingly, these studies provided the first direct observation of electron transfer rates through DNA and showed the sensitivity of electron transfer to dynamical motions within the base pair stack.

7.3.3

Base–Base Charge Transport

While metalintercalators have been useful in probing the DNA-mediated CT process, we were also interested in developing systems through which we might examine base–base electron transfer directly. This was accomplished by exploiting the fluorescence properties of 2-aminopurine and ethenoadenine, analogs of adenine, as well as 7-deazaguanine.

A systematic investigation of DNA-mediated photooxidation of guanine by ethenoadenine and 2-aminopurine was first performed using 12-base-pair duplexes with donor–acceptor separations ranging from 3.4 Å to 13.6 Å (Fig. 7.2) [42]. The studies revealed fast CT over a range of distances in duplexes containing guanine and 2-aminopurine; the rates for interstrand base–base electron transfer were $\sim 10^8 \text{ s}^{-1}$ with $\beta = 0.1 \text{ Å}^{-1}$. These rates indicated efficient coupling among the donor, acceptor, and intervening bases. Notably, however, in duplexes containing ethenoadenine, a bulkier fluorescent analog of adenine, and guanine, a steeper distance dependence was observed ($\beta = 1.0 \text{ Å}^{-1}$). Analogously, slow electron transfer kinetics and steep distance dependences were also observed in a system using stilbene-bridged DNA hairpins ($\beta = 0.6 \text{ Å}^{-1}$) [21, 43].

The base–base electron transfer studies also allowed us to differentiate between interstrand and intrastrand electron transfer [42]. In B-form duplexes containing 2-aminopurine, the electron transport kinetics for the intrastrand reactions were monitored on the femtosecond time scale and were seen to be $>10^3$ times faster than the interstrand reactions [44]. This difference again is consistent with the path for charge transport being through the stacked bases. In the case of the interstrand reaction. However, because the B-form duplex maintains primarily intra-strand stacking, interstrand electron transfer requires transfer across a hydrogen-bonded base pair.

Thus, coupling of the donor, acceptor, and intervening base moieties into the π -stack is paramount for effective CT. In systems that have a well-stacked π -way, lower β values are expected, whereas in systems that do not have significant base stacking, β values approaching those observed in proteins ($\beta \sim 1.0 \text{ \AA}^{-1}$) are expected. From these studies, it can be concluded that fast CT is mediated by an intrastrand, π -stacked pathway.

7.4

DNA-mediated Electron Transport on Surfaces

7.4.1

Characterization of DNA-modified Surfaces

The remarkable ability of DNA to form self-assembled monolayers on gold surfaces by way of aliphatic alkanethiol linkers has permitted us to utilize also electrochemical techniques to study DNA-mediated charge transport processes that involve ground state reactants [45]. Our laboratory has constructed and extensively characterized the assembly of DNA films on gold surfaces (Fig. 7.3) [46]. To im-

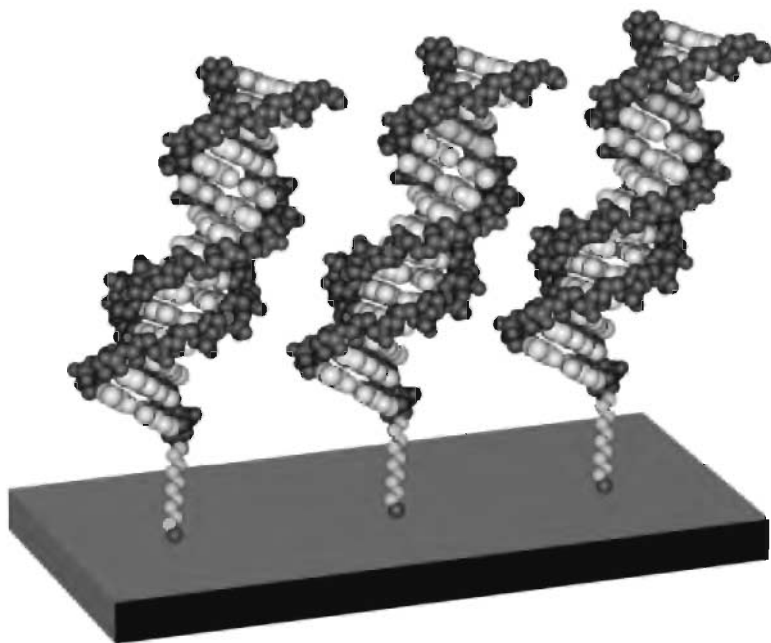


Fig. 7.3. Schematic illustration of alkane thiol-functionalized DNA duplexes, immobilized on a gold electrode. An intercalator (shown in blue), bound near the top of the DNA monolayer, is reduced by electron transfer from the gold surface through the DNA π -stack.

mobilize a high density of DNA duplexes on the surface (e.g. close-packing), the DNA is deposited on the surface in the presence of high concentrations of magnesium ion. Direct quantitation of the surface density is achieved by ^{32}P radioactive labeling. Utilizing atomic force microscopy (AFM), a 45° orientation of the duplexes relative to the gold surface was also determined. Moreover, in AFM studies as a function of applied potential, we found that application of a positive potential caused the DNA, if not closely packed, to lie down upon the surface, hence compressing the monolayer (~ 20 Å), whereas application of a negative potential induced an increase in monolayer thickness (~ 50 Å), consistent with the DNA 15-mer duplexes standing upright, perpendicular to the surface.

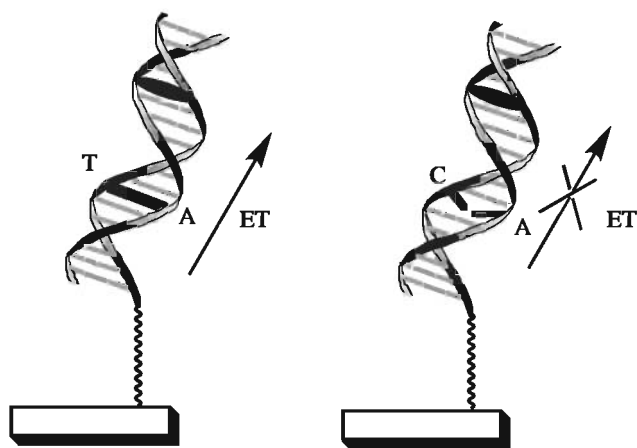
7.4.2

Electrochemical Probe of Redox Reactions of Intercalators

Early electrochemical experiments performed in our laboratory involved methylene blue, an aromatic heterocycle that binds to DNA by way of intercalation [47]. The reduction of methylene blue at micromolar concentrations could be easily monitored on an electrode modified to contain densely packed DNA duplexes. The binding affinity of the methylene blue to the DNA film was found to be comparable to that seen with DNA in solution; the binding stoichiometry, however, was significantly lower, consistent with methylene blue having access only to sites near the top of the film. Because of the large intervening distance expected between the gold and the film-bound methylene blue, these first studies suggested fast rates of DNA-mediated electron transport. As with other methods of analysis, however, well-defined systems were needed to remove the ambiguity of intercalator binding within the DNA-modified surfaces.

A redox active antitumor intercalator, daunomycin, was chosen as a covalently bound intercalator in studies designed to investigate systematically CT as a function of distance on the DNA-modified surfaces [48]. Daunomycin also undergoes a reversible reduction within the reduction window of the monolayer. Additionally, upon treatment with formaldehyde, the intercalator can crosslink to the 2-amino group of guanine [49] to form an adduct which has been crystallographically characterized [50]. Hence, thiol-derivatized duplexes were constructed containing a single guanine–cytosine base step to probe electrochemically the effects of distance on long-range electron transport [48]. Structural characterization of the DNA films did not reveal a difference between those films containing crosslinked daunomycin duplexes from those that did not contain the crosslinked moiety.

Interestingly, regardless of the position of the daunomycin crosslinked within the 15-mer duplex, reduction of DNA-bound daunomycin was observed and at a comparable rate. Rates of electron transfer through the film could be estimated in measurements where the scan rate was varied, and these showed slow rates of 10^2 s^{-1} , irrespective of the position of the intercalator; in fact similar rates were seen with other redox-active probes simply attached to the alkane-thiol linker we employed. These observations suggested to us that the rate-limiting step of the process was tunneling through the alkane-thiol linkage; in this context, then, CT



Effective Electron Transport (ET) **Attenuated Electron Transport (ET)**

Fig. 7.4. Illustration of the effect of a CA mismatch on electron transport through DNA films. As indicated by the schematic, the introduction of a mismatch, intervening between the gold electrode and intercalator bound near

the top of the film, (right) interrupts base-pair stacking locally and effectively attenuates reduction of the intercalator. In the well-stacked duplex (left), in contrast, reduction of the intercalator is efficient.

through 40-Å distances in DNA was necessarily faster. Again to determine explicitly the path of CT in these films, a CA mismatch was introduced at a position between the daunomycin and the gold (Fig. 7.4). With this intervening mismatch, we found no detectable reduction of the daunomycin. The path for CT is therefore through the base stack, and interruptions in the stack within the films also sensitively attenuate CT.

7.4.3

Sensing Mismatches in DNA

While the CA-mismatch experiment represented an important control in our first studies, establishing the path for CT, it also provided a demonstration that a single-base mismatch in DNA could be detected electrochemically. Current techniques for mismatch discrimination commonly rely upon differential hybridization, thermodynamic measurements of the small and sequence-dependent differences between well-matched and mismatched duplexes. Hence, we considered that the sensitivity of long-range CT to stacking might also be exploited in the development of a new class of DNA-based diagnostic tools [51, 52].

To amplify the sensitivity of mismatch detection using this strategy, an electrocatalytic cycle was coupled to the electron transport in well-packed DNA-modified films, magnifying signal differences that arise from small stacking perturbations. The cycle begins with the reduction of the methylene blue intercalator, non-covalently bound near the top of the DNA monolayer. The reduced methylene blue,

in turn, reduces ferricyanide, that is contained in solution and repelled by the anionic DNA-modified electrode, hence allowing for further reduction of methylene blue. The catalysis is limited only by the amount of ferricyanide contained in the solution. Using chronocoulometry, integrating the current at the methylene blue potential, all of the possible single-base mismatches (e.g. GA, CA, etc.), including the difficult purine–purine mismatches, in addition to naturally occurring DNA lesions (e.g. A_{ox}:T), could be detected using electrocatalysis [52]. We have also found, perhaps not surprisingly, that mismatches are also sensitively detected in DNA/RNA hybrids (E.M. Boon, unpublished results). Moreover, this technology can be applied in probing how nucleic acid analogs, such as those utilized for antisense applications, affect duplex stacking [53].

We have also applied this assay in tests of physiologically important sequences where mutations cause disease [52]. For example we examined hotspots for mutations in the *p53* gene. These mutations have been implicated in a variety of human cancers. We have also fabricated DNA chips, prepared using gold surfaces of variable size on silicon wafers. We found a linear response in charge accumulation with decreasing electrode size (30–500 μm) and with high signal-to-noise. Given the dimensions of the gold surfaces, assuming close-packing of the DNA, this corresponds to detection of 10^8 DNA molecules on the chip. The technology, therefore, offers promise as a sensitive, new diagnostic tool to detect mutations and single-nucleotide polymorphisms.

7.5

Long-range Oxidative Damage to DNA

Within the cell, radical damage to DNA is known to occur. Given our studies demonstrating long-range CT through DNA, we considered whether CT processes might be an issue physiologically. In other words, can damage to a DNA site arise from a distance through DNA-mediated CT?

7.5.1

Long-range Oxidative Damage at 5'-GG-3' Sites by a Rhodium Intercalator

Of the four DNA bases, guanine has been shown to have the lowest oxidation potential ($E^\circ \sim +1.3$ V versus normal hydrogen electrode (NHE)) [54]. Experimental studies and *ab initio* molecular orbital calculations have revealed that the 5'-G of 5'-GG-3' doublets is preferentially oxidized because the highest occupied molecular orbital (HOMO) lies on the 5'-G of a 5'-GG-3' doublet [55, 56]. The oxidation of guanine in DNA leads to piperidine-sensitive base lesions, which are revealed as strand breaks [57]. Hence, this damage at the 5'-G of 5'-GG-3' doublets has become a hallmark for electron transfer chemistry.

Oxidative damage to DNA at a distance was first demonstrated in DNA assemblies containing the tethered, intercalating photooxidant $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$, spatially separated from two 5'-GG-3' sites (Fig. 7.5) [58]. With binding non-covalently

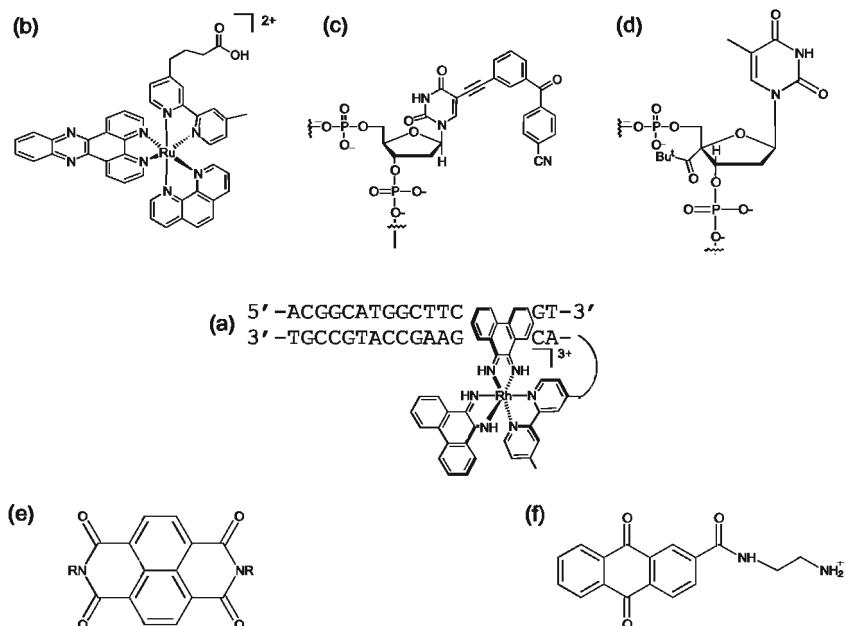


Fig. 7.5. Photooxidants used to probe long-range oxidative damage to DNA. (a) DNA assembly, first used to probe long-range oxidative damage to DNA, containing $[Rh(\phi)_2bpy']^{3+}$ spatially separated from two 5'-GG-3' doublets;

(b) $[Ru(phen)(dppz)(bpy')]^{2+}$; (c) cyanobenzophenone-modified deoxyuridine; (d) 4-pivavoyl-modified deoxythymine; (e) naphthalene diimide; (f) a substituted anthraquinone.

of the sequence-neutral intercalator, $[Rh(\phi)_2(DMB)]^{3+}$ ($DMB = 4,4'$ -dimethylbpy) and irradiation at 365 nm, oxidation of the 5'-G of 5'-GG-3' was evident; irradiation at higher energy (313 nm) leads instead to direct strand scission, marking the site(s) of metal complex binding. When the rhodium complex was covalently tethered to the duplex, photolysis at 313 nm yielded direct strand breaks near the terminus to which the rhodium complex was tethered. However, with irradiation instead at 365 nm and piperidine treatment, it was found that the two 5'-GG-3' sites were damaged at distances 17 Å and 34 Å away from the bound metallointercalator.

To examine systematically the distance dependence of long-range oxidative damage, a series of 28 base pair duplexes was constructed that contained proximal and distal 5'-GG-3' sites [59]. The proximal guanine doublet remained at a fixed distance from the rhodium intercalator, while the distal guanine doublet was marched out in two base-pair increments relative to the position of the intercalator. This incremental increase in distal location allowed for exploration of the importance of helical phasing (e.g. stacking of the intercalator and guanine doublet on the same or opposite side of the helix) in charge transport in addition to the distance dependence of the process. The ratio in yield of oxidative damage at the 5'-G

of 5'-GG-3' doublets provides a measure of the relative efficiency of the charge transport process.

Paralleling our spectroscopic studies, the yield of oxidative damage was not found to be significantly attenuated over a distance of 75 Å. Moreover, the helical phasing of metal complex and the distal guanine site did not appear to be a pertinent parameter. Also, similar to spectroscopic experiments, the long-range CT chemistry was found to be quite sensitive to stacking, both of the oxidant and of the intervening base pairs. Introduction of base bulges (e.g. 5'-ATA-3') in a DNA assembly containing tethered rhodium between 5'-GG-3' sites positioned distal and proximal to the rhodium, for example, caused a dramatic diminution in the distal/proximal ratio of oxidative damage [60].

To probe further the distance dependence of the electron transport process, 63 base-pair duplexes containing either tethered $[\text{Rh}(\text{phi})_2\text{bpy}']^{3+}$ or $[\text{Ru}(\text{phen})(\text{dppz})(\text{bpy}')]^{2+}$ and six 5'-GG-3' sites, located 31–197 Å from the metal-ligand intercalator, were constructed [59]. Ruthenium(III) complexes of dppz, generated *in situ* with quenching of the ruthenium(II) excited state by a non-intercalating quencher (e.g. methyl viologen) had also been shown to promote oxidative damage to DNA from a distance [61]. Remarkably, in the large assemblies, both excited Rh(III) and ground state Ru(III) intercalating oxidants were found to substantially damage DNA over a distance of 200 Å. Therefore, these studies demonstrated charge migration through DNA to effect damage over a biologically significant distance regime.

Oxidative damage to DNA from a distance does not require a metal-ligand intercalator as oxidant. Since our first study establishing oxidative damage from a distance, a variety of oxidants have been used to carry out this long-range chemistry. Figure 7.5 illustrates some of the different photooxidants employed. These include ethidium [62], a cyanobenzophenone-modified deoxyuridine [63], 4-pivaloyl-modified deoxythymine [64], naphthalene diimide [65, 66], and anthraquinone [67]. Indeed studies with the anthraquinone moiety bound at a discrete site on long oligonucleotide duplexes confirmed that long-range oxidative damage can be generated from a 200-Å distance [68]. Many of these photooxidants are currently being employed in experiments to explore DNA CT mechanistically.

7.5.2

Models for Long-range DNA Charge Transport

Once it became clear that oxidative damage to DNA from a distance through CT was general and not a unique property associated with metal-ligand intercalators, new theoretical models were proposed to account for the long-range chemistry that had been delineated. These models focused on descriptions of long-range CT through a combination, to varying extents, of hopping and tunneling mechanisms. In a tunneling mechanism, the orbitals of the donor and acceptor molecules are significantly below that of the DNA bridge. Therefore, the charge is considered to tunnel through the DNA bridge without forming transient species along the path. The DNA base-pair stack forms a “virtual” bridge of communication between

donor and acceptor, and the rate of CT is expected to show an exponential dependence on the distance separating donor and acceptor. At the other extreme, in the hopping mechanism, the donor and acceptor orbitals are energetically close to or above the bridge states. Hence charge is considered to transiently occupy the bridge. If the rate of charge migration through the DNA bridge is faster than irreversible trapping of the transient radical, then the charge would be able to migrate long distances with a quite shallow distance dependence [69, 70].

Based upon measurements of oxidative yield, Geise, Jortner, and co-workers proposed that CT through DNA occurs by a mixture of hopping and tunneling mechanisms [71]. Specifically, they proposed that hopping occurs primarily among guanine sites, the lower sites energetically, while tunneling is preferred through TA steps. This proposal was based first upon the observation of little oxidative damage when a 5'-TATA-3' intervened between guanine sites [63, 71]. Schuster also described CT through DNA in the context of a hopping model, but one involving phonon-assisted polaron formation [68, 72]. In this model, he considered that charge might be delocalized over a polaron within the DNA bridge, formed in response to the electrostatic perturbations associated with transport of the charge; propagation of the polaron through the DNA would be assisted by phonon interaction. In reconciling our own photophysical and oxidative damage results, a hopping mechanism seemed appealing to consider. The probability of all the bases being properly stacked and aligned to permit charge transfer over 200 Å was vanishingly small. Thus we considered a "domain hopping" model, in which charge hopping proceeds among domains, where delocalization within a given domain depends upon sequence-dependent dynamics and structure [59].

It is likely, however, that the description is far more complicated still. In the case of photophysical studies with intercalators bound to DNA, the donor and acceptor orbitals energetically can lie close to the bridge states and are extremely well coupled with the base-pair stack. Conversely, in the case of our electrochemical studies, the redox intercalator potential lies far below that of the DNA bridge. Do these different experiments lie at different ends of a mechanistic continuum or can they be considered together? Heller and co-workers introduced the importance of longitudinal polarizability as a factor in considering these results [73]. If the DNA is polarized along the direction of the helix axis, CT is expected to be facilitated. Photoexcitation or the presence of an applied potential within the base-pair stack could provide the means to generate a polarized stack.

Additional mechanistic insights will clearly be needed to reconcile all these results. Irrespective of the mechanism, however, it is clear that stacking within the DNA duplex is a critical factor governing and distinguishing long-range CT.

7.5.3

Sequence Dependence of DNA Charge Transport

To systematically investigate the sequence dependence of charge transport, a series of 21 base-pair oligonucleotides, functionalized with the metallointercalator, $[\text{Rh}(\phi)_2\text{bpy}]^{3+}$, was designed containing guanine doublets located proximal and

distal to the photooxidant [74]. The surrounding sequence of the guanine doublet sites remained constant, while the intervening sequence, containing adenines and thymines, was varied. This family of assemblies was prepared to test directly the proposal of guanine hopping and AT tunneling.

We found that having adenines intervening between the guanine doublet sites led to consistently higher yields of oxidative damage as compared to having either thymines or alternating adenine and thymines between the G-doublet sites (Tab. 7.1). The assemblies containing runs of adenines, with their extensive stacking overlap, would be expected to have the most efficient charge transport based upon simple stacking arguments. Similarly, having thymines in the intervening medium between the guanine doublets would be expected to provide the poorest conduits for charge transport, with AT-tracts yielding an intermediate level of damage. Importantly, and in contrast, based upon an AT tunneling proposal [71], poor transport, if any, would be expected for the assemblies containing a short run of adenines and thymines, with an exponentially decreasing oxidative yield expected as the length of the AT run increased. In fact, increasing the number of adenines or thymines between guanine doublets did not result in marked decreases in long-range oxidative damage [74]. Instead, we observed an *increase* in oxidative yield for all assemblies with increasing oligonucleotide length. Also, inconsistent with guanine hopping, we found that the insertion of a guanine-cytosine step into the otherwise AT sequence actually *decreased* the efficiency of charge transport to the distal guanine site (Tab. 7.1).

These studies certainly illustrate a sequence-dependence in DNA CT, but not with as simple a picture as had been proposed. For example, studies have shown that A-tracts, once nucleated by a sufficient stretch of adenines, readily adopt conformations that differ from that of canonical B-form DNA [75], and this nucleation was used to explain the increase in oxidative damage yield with increasing length. Similarly, many studies have indicated a significant flexibility associated with the sequence 5'-TATA-3' [76], and this can be used to explain the low efficiency of CT across a 5'-TATA-3' site. Hence we consider CT in the context of hopping among domains defined by their sequence-dependent conformations [59, 74]. The results, therefore, underscore and reflect the sensitivity of CT to dynamical stacking and to sequence-dependent structure and flexibility.

7.5.4

The Effects of Ion Distribution on Long-Range Charge Transport

Charge transport through well-stacked DNA appears to be much faster than the trapping of the resultant guanine radical by O_2 and H_2O . Hence, one might expect the oxidative yields at guanine doublets contained on the same strand of a duplex to be similar if their thermodynamic potentials are equal. However, using our rhodium(III) and ruthenium(II) intercalators, we observed distal/proximal oxidative damage ratios that were significantly higher than one. A possible explanation for this observation was that the high cationic charge on the metallointercalator

Tab. 7.1. DNA assemblies used to investigate long-range oxidative damage to DNA

Sequence	Distal 5'-G/proximal 5'-G oxidation ratio ^a
 AC^{Rh} GAGCCGTTTTCGCGTAT-3' 3'-TGCTCGGC AAAACGGCATA-5' *^b	2.5 (2) ^c
 AC^{Rh} GAGCCGTTTTTTTTTTCGCGTAT-3' 3'-TGCTCGGC AAAAAAAAAACGGCATA-5' *^b	1.2 (1) ^c
 AC^{Rh} GAGCCGAAAAGCCGTAT-3' 3'-TGCTCGGCTTTTCGGCATA-5' *^b	0.9 (1) ^c
 AC^{Rh} GAGCCGAAAAAAAAAGCCGTAT-3' 3'-TGCTCGGCTTTTTTTTCGGCATA-5' *^b	0.4 (3) ^c
 AC^{Rh} GAGCCGTATAGCTATAGCCGTAT-3' 3'-TGCTCGGCATATCGATATCGGCATA-5' *^b	0.6 (1) ^c
 AC^{Rh} GAGCCGTTTTCGCGTAT-3' 3'-TGCTCGGC AAAACGGCATA-5' *^b	5.2 (4) ^{c,d}
 AC^{Rh} GAGCCGTTTTCGCGTAT-3' 3'-TGCTCGGC AAAACGGCATA-5' *^b	0.4 (1) ^d
 ACG^{Rh} ATGCCGACAGTGTCGGAT-3' 3'-TGCATACGGCTGTGACACGGCTA-5' *^b	1.7 (2) ^c
 ACG^{Rh} ATGCCGACATTGTGCCGAT-3' 3'-TGCATACGGCTGTACACGGCTA-5' *^b	0.2 (1) ^c

^a Measured damage yields at the 5'-G of 5'-GG-3' sites. The statistical error in the last digit is listed in parentheses.

^b * indicates location of ³²P end-labeling.

^c Data taken from study of the dependence of CT on sequence.

^d Data taken from study of the dependence of CT on charge distribution.

^e Data taken from study of the effects of intervening mismatches.

bound near the terminus of the duplex was sufficient to attenuate the potential of the guanine doublet proximal to the intercalator.

To examine how ion distribution on the DNA helix affects charge transport, we assessed oxidative damage yields upon photooxidation of assemblies covalently tethered with $[\text{Rh}(\text{phi})_2\text{bpy}']^{3+}$ and ^{32}P -end-labeled either at the 5' end, which introduces two negative charges at the terminus or at the 3' end, which introduces one negative charge [77]. Using an assembly containing an intervening A_6 -tract, we found that changing the ^{32}P -label from the 5' end of the duplex to the 3' end resulted in a dramatic decrease in charge transport to the distal guanine site (Tab. 7.1). Furthermore, upon the introduction of an unlabeled phosphate at the 5' end of the duplex and maintenance of the 3'- ^{32}P end-label, an intermediate increase in oxidative yield was observed. Analogous behavior was also seen with mixed DNA sequences.

We have proposed that these results reflect a change in the oxidation potential at the distal guanine site relative to the proximal site due to change in charges at the termini of the oligomer. Coarse calculations of the internal dielectric of DNA based upon these data suggested particularly high values, an indication of the high polarizability of the DNA π -stack. Such results certainly require consideration in developing mechanisms for DNA CT. Indeed the high longitudinal polarizability of DNA offers an attractive means to reconcile photophysical, biochemical, and electrochemical measurements that have been carried out [75].

7.5.5

Mismatch Influence on Long-range Oxidative Damage to DNA

Just as we had seen both in photophysical and electrochemical experiments that contain intervening mismatches, which locally perturbed the DNA base-pair stack and sensitively modulated DNA CT, we were also interested in determining the effect of intervening mismatches on long-range oxidative damage [78]. To investigate this systematically, 22 base-pair duplexes, containing intervening mismatches between guanine doublet sites and functionalized with the metallointercalator, $[\text{Ru}(\text{phen})(\text{dppz})(\text{bpy}')]^{2+}$, were assembled. This resulted in a total of 16 possible base-pair and mismatch combinations. As in other experiments, the efficiency of charge transport was quantified through measurements of the ratio in yield of 5'-G oxidative damage at guanine doublets located distal versus proximal to the intercalating oxidant. ^1H NMR measurements of base-pair opening lifetimes were also performed to probe mismatch dynamics.

Overall, the trend in oxidative damage yields varied in the order $\text{GC} \sim \text{GG} \sim \text{GT} \sim \text{GA} > \text{AA} > \text{CC} \sim \text{TT} \sim \text{CA} \sim \text{CT}$. Generally the purine-purine mismatches did little to attenuate the amount of oxidative damage observed at the distal guanine site, while introduction of a pyrimidine-pyrimidine mismatch resulted in much lower oxidative yields (Tab. 7.1). The extent of distal/proximal guanine oxidation in different mismatch-containing duplexes was compared with the helical stability of the duplexes, electrochemical data for intercalator reduction on different mismatch-containing DNA films, and base-pair lifetimes for oligomers containing

the different mismatches derived from ^1H NMR measurements of the imino proton exchange rates. While a clear correlation was evident both with helix stability and the electrochemical data, monitoring reduction of an intercalator through DNA films, it was interesting to observe that damage ratios correlated most closely with base-pair lifetimes. Competitive hole trapping at the mismatch site, which had been proposed by others [79], did not appear to be a key factor governing the efficiency of transport through the mismatch. Because of the close correlation with dynamical measurements of DNA, these results served once again to underscore the importance of base dynamics in modulating long-range CT through the DNA base-pair stack [78]. Indeed, it has become increasingly clear that measurements of DNA CT may offer a new and sensitive route to assess sequence-dependent base dynamics within the DNA duplex.

7.6

Using Charge Transport to Probe DNA–Protein Interactions and DNA Repair

Given the efficiency of DNA CT that was documented over biologically relevant distance regimes, it became important to consider roles and consequences of DNA CT within the cell. With that aim in mind, we have begun to explore how proteins modulate DNA CT, how nucleosomal packaging affects DNA CT damage, how DNA repair can be promoted through CT, and even whether long-range CT through DNA occurs within the nucleus of the cell.

7.6.1

DNA-Binding Proteins as Modulators of Oxidative Damage from a Distance

Proteins that bind DNA do so utilizing a range of structural motifs. Some modes of DNA binding involve significant perturbations in base-pair structure. Given the sensitivity of DNA CT to such structural perturbations, we were interested in determining how protein binding through different motifs would affect long-range DNA CT.

DNA assemblies were constructed that incorporated specific protein binding sites between two 5'-GG-3' sites that were spatially separated from the 5'-tethered photooxidant $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ [80]. Again, we assessed the efficiency of DNA charge transport as a function of protein binding through measurements of the ratio of damage yield at the 5'-GG-3' site distal to the photooxidant versus that at the proximal 5'-GG-3' site. The proteins utilized included methyltransferase *Hha* I, the TATA-binding protein (TBP), the restriction endonuclease *Pvu*II, and the Antennapedia homeodomain protein (ANTP), proteins which bind DNA through different, structurally well-characterized modes.

We focused first on perturbations in long-range oxidative damage as a result of binding the base-flipping enzyme methyltransferase *Hha* I [81]. This enzyme recognizes the sequence 5'-G*CGC-3' and methylates the target cytosine (*C) after completely flipping the base out of the duplex stack [82]. The π -cavity that is

created is filled by insertion of a glutamine side chain (Gln237) from the enzyme; the glutamine essentially serves as a “bookmark” to hold the place for the cytosine. From the perspective of CT chemistry, the binding of methyltransferase *Hha* I to DNA provided an ideal system to study how the insertion of a σ -bonded residue within the π -stack would affect long-range DNA CT. What we observed was that the yield of oxidative damage at the distal 5'-GG-3' site decreased significantly with protein binding. We also tested the effects of binding of a mutant methyltransferase *Hha* I which inserts a π -stacking tryptophan residue (Q237W) into the cavity; in this case, oxidative damage to the distal site was restored.

Studies of long-range oxidative damage in the presence of other DNA-binding proteins yielded consistent results [80]. For example, crystallographic studies show that DNA binding of TBP, which plays an important role in transcription initiation, to the sequence 5'-TATAAA-3', induces a 90° kink at either end of the already flexible binding site [83, 84]. With this DNA substrate, owing to the flexible 5'-TATAAA-3' site, a low damage ratio was evident even without protein. In the presence of TBP, however, a significant decrease in charge transport to the distal guanine site was also observed as a function of increasing protein concentration. In general, then, it was observed that yields of long-range oxidative damage correlated directly with the nature of the nucleoprotein interaction. Interactions that disturb the DNA π -stack inhibit DNA CT. Alternatively, interactions that promote no helix distortion, such as *Pvu*II and ANTP, but, as a result of tight packing, may rigidify the π -stack, serve instead to enhance the ability of the DNA base stack to provide a conduit for CT. Thus, we observed that protein binding to DNA modulates long-range CT both negatively and positively, depending upon the specific protein/DNA interactions in play.

7.6.2

Detection of Transient Radicals in Protein/DNA Charge Transport

We were interested also in determining if proteins can participate directly in DNA CT chemistry. Can electrons or electron holes be transferred effectively between the DNA and protein within the macromolecular assembly? Both spectroscopic and biochemical techniques were employed to monitor transient radical intermediates as a result of CT through DNA–protein assemblies [85]. To facilitate spectroscopic studies, assemblies containing a methyltransferase *Hha* I-binding site were constructed with the tethered, intercalator [Ru(phen)(dppz)(bpy')]²⁺, and the flash-quench technique [61] was employed to generate the Ru(III) oxidant *in situ*, triggering the long-range oxidation chemistry. Because we could monitor the same assemblies using both biochemical measurements of oxidative damage yield and transient absorption measurements of rates of formation of radical intermediates in the CT process, we could correlate the two directly.

Upon irradiation of an assembly containing the mutant methyltransferase *Hha* I (Q237W)-bound 14 base pairs from the site of ruthenium intercalation, extensive oxidative damage was observed to the guanine located 5' to the tryptophan intercalation site; no similar damage was evident with binding of the wild-type protein

[85]. Parallel transient absorption experiments also revealed the formation of a radical species. Interestingly, the full transient absorption spectrum of this intermediate contained features characteristic of both the guanine and tryptophan radicals. It should be noted that the oxidation potential of the tryptophan is ~ 1.0 V versus NHE [86]. Hence hole transport proceeded through DNA to the inserted tryptophan from the protein and to the 5'-guanine, where irreversible oxidative damage occurred.

To examine the distance dependence of the rate of formation of the tryptophan and guanine radicals, a series of ruthenium-tethered duplexes was prepared containing methyltransferase *Hha* I-binding sites, located at distances varying from 24 to 51 Å away from the ruthenium intercalator [85]. The yield of oxidative damage observed at the 5'-position to the inserted tryptophan diminished little with increasing distance. Transient absorption measurements also showed no variation in the rate of radical formation with increasing distance; the rate constant for radical formation was $>10^6$ s $^{-1}$ in each assembly. Hole transport through DNA over this 50-Å distance regime, therefore, was not rate limiting. In fact these kinetic results complemented well our earlier findings of base-base electron transfer rates on the order of 10^{10} s $^{-1}$ [44]. More recently, we have carried out analogous experiments using a methylindole moiety incorporated directly into the DNA as an artificial base [87]. These studies of CT yielded comparable results and, in fact, set the lower limit for CT through the stack as $>10^7$ s $^{-1}$, coincident with formation of the Ru(III) oxidant through diffusional quenching.

7.6.3

Electrical Detection of DNA–Protein Interactions

Our electrochemical assay on DNA-modified electrodes provided another means to probe protein/DNA interactions by monitoring the effect of protein binding on the DNA base stack [88]. This assay yielded results wholly complementary to our measurements of oxidative damage, despite the fact that here reduction of a bound intercalator was being measured, while in the biochemical experiments photo-induced DNA oxidation chemistry was being probed. Practically, however, in the electrochemistry experiment, the measurement could be made in real time and without gel electrophoresis.

Functionalized with a thiol-terminated linker and with daunomycin crosslinked to a guanine residue near the duplex terminus, DNA assemblies were constructed that contained the binding sites for either methyltransferase *Hha* I, uracil-DNA glycosylase (UDG), TBP, or *Pvu*II. Measures were taken to prevent enzyme turnover (e.g. using 2'-fluorouracil as opposed to uracil in binding UDG), to insure that binding of the native enzyme to DNA could be studied. The self-assembly of the functionalized DNA monolayer on the gold surface was performed in the absence of magnesium ion, to allow for loose packing of the film and binding of protein within the monolayer. Any remaining exposed gold surface was then back-filled with mercaptohexanol. Tab. 7.2 provides a summary of the electrochemical responses obtained by chronocoulometry in the presence of the different proteins.

Tab. 7.2. Electrochemical detection of DNA–protein interactions using tethered daunomycin^a

Sequence ^b	Assay	DNA association	Integrated charge ^c
H-5' -AIAIATICAIATCC T-3' 3' -TCTCTACICITCTAGG A-5'	Without protein		High
	M. <i>Hha</i> I	Base-flipping, inserts Q	Low
	M. <i>Hha</i> I (Q237W)	Base-flipping, inserts W	High
H-5' -AIAIATICAIATCC T-3' 3' -TCTCTAC CITCTAGG A-5'	Without protein		High
	M. <i>Hha</i> I	Base-flipping, inserts Q	Low
	M. <i>Hha</i> I (Q237W)	Base-flipping, inserts W	High
H-5' -AICTIAATCAITCC T-3' 3' -TCIACTUAITCAGG A-5'	Without protein		High
	UDG	Base-flipping	Low
H-5' -IAIATATAAAICACC T-3' 3' -CTCTATATTCITGG A-5'	Without protein		High
	TBP	Kinks DNA by 90°	Low
H-5' -TCTTCAICTTIAIACC T-3' 3' -ATAAITMIACTCTGG A-5'	Without protein		High
	R. <i>PvuII</i>	No structural perturbation	High

^aSummary of results taken from [88].

^bSequence used to form DNA film.

^cBased on chronocoulometry.

^dI. *Hha* I, methyltransferase *Hha* I; UDG, uracil-DNA glycosylase; TBP, TATA-binding protein; R. *PvuII*, restriction endonuclease *PvuII*.

As in the biochemical experiments, electrochemistry revealed that binding of native methyltransferase *Hha* I, the base-flipping enzyme that inserts a glutamine into the π -stack, significantly attenuated the electrochemical response. Consistent with transport through a π -way, binding of the mutant protein methyltransferase *Hha* I (Q237W), which inserts a tryptophan into the π -stack, showed little attenuation of electrochemical response. We carried out a parallel series of experiments on DNA-modified electrodes containing an abasic site within the protein recognition site. In this case, only a small electrochemical signal was evident either without protein or with native protein. Interestingly, however, binding of the mutant here as well filled the π -stack and thus yielded a high electrochemical response. UDG, a base excision repair enzyme that extrudes uracil upon binding, was also probed using this assay. Analogous to the results found upon binding of the native methyltransferase *Hha* I, UDG also showed a marked decrease in electrochemical response upon protein binding. Furthermore, introducing a gross structural distortion, as that found upon binding of TBP, which significantly kinks DNA, also led to an attenuation in the electrochemical signal.

To determine whether this novel assay could also be useful in monitoring enzymatic reactions with DNA, a DNA film was prepared to study the endonuclease activity of *PvuII*. As a function of incubation with the protein, a decrease in signal was evident, and the kinetics, monitored by this signal reduction, paralleled closely that determined by gel assay of restriction fragmentation. We are currently interested in determining whether these assays might be sufficiently sensitive to allow

us to follow the real time fluctuations in base-pair motions associated with protein binding and reaction.

These data further illustrate the exquisite sensitivity of DNA electron transport to stacking perturbations and offer a novel assay through which to structurally probe how different proteins interact with DNA. This assay also offers a new opportunity to apply CT chemistry in directly probing DNA-protein interactions in real time.

7.6.4

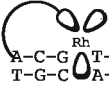
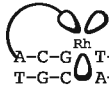
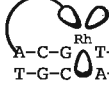
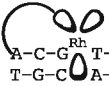
Repair of Thymine Dimers

In addition to probing oxidative damage to DNA from a distance, we became interested in exploring other reactions on DNA that might be promoted at long-range through CT chemistry. We determined that DNA CT can also be utilized to promote the repair of thymine dimers in DNA [89]. Thymine dimers, the most common photochemical lesions in DNA, result from the photoinduced [2 + 2] cycloaddition reaction between adjacent thymines contained on the same polynucleotide strand to form the cyclobutane dimer [90]. This lesion is removed in eukaryotic cells by the excision of the dimer. However, in bacteria, the photolyase enzyme promotes repair using electron transfer chemistry; with irradiation in the visible region, a reduced flavin cofactor adds an electron to the cyclobutane dimer, triggering repair of the lesion and release of the electron back to the enzyme. In model systems, repair of the cyclobutane dimer has been triggered both oxidatively and reductively [91, 92]. Hence, with our potent rhodium intercalating photooxidant ($E^\circ \sim +2.0$ V versus NHE), we considered that thymine dimer repair might be promoted within the DNA duplex triggered by oxidation [89].

To perform these studies, 16 base-pair duplexes containing the tethered photooxidant $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$ and a thymine dimer located 16–26 Å away from the metallointercalator, were constructed. Upon irradiating the duplexes at 400 nm, a high-performance liquid chromatography assay revealed a substantial amount of thymine dimer repair with increasing irradiation time (Tab. 7.3). Analogous to our studies of oxidative damage, we also found that this repair was insensitive to increasing the distance between the metallointercalator and the thymine dimer (16–26 Å). Furthermore, the repair efficiency was also found to be sensitive to π -stacking perturbations; a decrease in repair efficiency was seen with the introduction of base bulges between the site of rhodium intercalation and the thymine dimer. Hence, not only is DNA able to promote long-range oxidative damage to DNA from a remote site through CT chemistry, but CT chemistry with a similar sensitivity to the intervening base stack can also be harnessed to trigger thymine dimer repair from a distance.

The long-range repair of thymine dimers is not a unique property of the rhodium intercalator. Other potent photooxidants can also promote the repair of thymine dimers [93]. A series of duplexes were constructed containing the thymine dimer lesion and various intercalating photooxidants, including anthraquinone derivatives, naphthalene diimide, $[\text{Rh}(\text{phi})_2\text{bpy}]^{3+}$, and N-8-glycyl ethidium, were probed. Analogous to our rhodium intercalator, substantial repair from a distance

Tab. 7.3. Long-range thymine dimer repair using the tethered photooxidant $[\text{Rh}(\text{phi})_2(\text{bpy}')]^3 \cdot$

DNA assembly	Distance (Å) ^a	Repair (%)
 A-C-G ^{Rh} T-C-T-C-A-A-C-T-C-A-C-G-T T-G-C ^{Rh} A-G-A-G-T-G-A-G-T-G-C-A-5'	19	79 ^b
 A-C-G ^{Rh} T-C-T-C-A-A-C-T-C-A-C-G-T T-G-C ^{Rh} A-G-A-G-T-G-A-G-T-G-C-A-5'	26	100 ^b
 A-C-G ^{Rh} T-C-T-C-A-A-C-T-C-A-C-G-T ^c T-G-C ^{Rh} A-G-A-G-T-G-A-G-T-G-C-A-5'	—	47 ^b
 A-C-G ^{Rh} T-A-T-G-C-C-G-T-C-A-A-C-T T-G-C ^{Rh} A-T-A-C-G-G-C-A-G-T-T-G-A-5'	36	70 ^d

^aDistances between the rhodium intercalator, as shown schematically, and the cyclobutane ring of the dimer, which are based upon 3.4 Å base-pair separation.

^bRepair is expressed as the percentage of thymine dimer repaired, as determined by HPLC analysis, after 6 h of irradiation at 365 nm. Data taken from [89].

^cThe thymine dimer is shown in bold with a $\wedge$. The extruding bases schematically show the position of the base bulges.

^dData taken from [95] and describes the amount of repaired dimer after 3 h of irradiation at 365 nm.

was observed using the organic intercalator naphthalene diimide, which has a reduction potential of +1.9 V versus NHE. However, repair was not observed using either ethidium or anthraquinone derivatives as non-covalently bound intercalators. In the case of ethidium, it is understandable that the organic intercalator cannot perform repair, since the reduction potential of the ethidium is sufficiently below that of the dimer. Surprisingly, the anthraquinone was also unable to repair the dimer [94]. The anthraquinone derivatives have a reduction potential of ~ 2.0 V versus NHE from the excited triplet state and therefore should have sufficient driving force for repair. We have attributed the lack of repair with the anthraquinone moiety to the fact that its excited singlet state is particularly short-lived [93]. In fact, anthraquinone derivatives that do not undergo rapid conversion to the excited triplet state can promote thymine dimer repair. Interaction with the singlet state may therefore be critical for effective repair.

Studies were also performed to examine the competition between oxidative damage and thymine dimer repair, since oxidation of guanine sites should be favored thermodynamically [95]. Duplexes were constructed containing either a 5'-GG-3' site, a thymine dimer, or both moieties, and the duplexes were co-

valently modified with a metallointercalating oxidant (either $[\text{Rh}(\text{phi})_2\text{bpy}']^{3+}$ or $[\text{Ru}(\text{phen})(\text{dppz})(\text{bpy}')^{2+}]$). Both thymine dimer repair and guanine oxidation were then systematically examined. Interestingly, the data revealed that thymine dimer repair proceeds efficiently in the presence of potential thermodynamic traps provided by guanine doublets (Tab. 7.3). Although it is thermodynamically favorable to perform guanine oxidation, it appears kinetically preferable to repair thymine dimers. This competition is useful to consider in the context of generally assessing reactions that may proceed within the cell based upon CT chemistry. Indeed other DNA-mediated CT reactions, perhaps initiated by proteins bound to DNA, could conceivably be carried out along the duplex without irreversible reaction at guanines as long as these reactions are similarly kinetically favored.

7.6.5

Oxidative Damage to DNA in Nucleosomes

In eukaryotic cells, DNA is packaged within nucleosome core particles [96]. In this unit, ~ 150 base pairs of DNA are wrapped around an octamer of histone proteins, to which the DNA is associated by non-specific, electrostatic interactions. It has been thought that this packaging of nucleosomal DNA serves to protect it from damage. The DNA is dynamically restricted and less accessible to solution-borne damaging agents. However, oxidative damage through CT chemistry does not require solution accessibility. On the other hand, the restricted motion and overall bending of the DNA within the nucleosome would not be expected to favor long-range CT. The question of whether DNA CT proceeds through the nucleosome core particle was therefore interesting to consider.

To investigate long-range oxidative damage in nucleosomes, several 146 base-pair palindromic duplexes were constructed, containing 14 5'-GG-3' sites [97]. These duplexes were modeled after that crystallographically characterized within the nucleosome core particle [96]. Nucleosomes were then formed using these duplexes and their structural integrity was confirmed using biochemical methods [97].

First damage within the nucleosome was probed using non-covalently bound $[\text{Rh}(\text{phi})_2\text{DMB}]^{3+}$. Upon irradiation of the rhodium-bound assemblies, damage was evident at the 5'-G of 5'-GG-3' sites. Little specific binding of the rhodium complex was evident. Perhaps not surprisingly, the nucleosomal DNA cannot unwind to accommodate the intercalator, and hence, intercalator binding is inhibited. Nonetheless, despite the poor binding, significant oxidative damage was observed. Given the poor binding, the results suggested that this damage had arisen through long-range CT within the nucleosome.

To establish long-range CT within the nucleosome definitively and to probe the distance over which charge can migrate, the metallointercalator $[\text{Rh}(\text{phi})_2\text{bpy}']^{3+}$ was covalently tethered to the 5' ends of the palindromic 146 base-pair duplex and nucleosomes were formed. Upon irradiation of the duplex, selective 5'-G oxidative damage at 5'-GG-3' sites located 8–24 base pairs away from the site of intercalation was revealed (Fig. 7.6, see p. 171). Notably, guanine oxidation was not observed at

sites located further away from the site of rhodium intercalation either in the core particle or even in the absence of histones. It is likely that the highly bent DNA structure utilized to obtain consistent nucleosome phasing for these experiments also interfered with long-range CT. Nonetheless, over a significant distance regime (>75 Å), long-range CT was established within the nucleosome core particle.

These data hold implications for damage to and repair of the genome *in vivo*, where much of the DNA is bound within nucleosome core particles. It has been considered that histones, in addition to packaging and regulating DNA, also function to protect DNA, since the histones reduce binding by a variety of potentially dangerous small molecules. In fact, binding of DNA to histone proteins to form nucleosome core particles does reduce the ability even of the rhodium complex to intercalate into the DNA. However, and importantly, packaging of DNA as nucleosomes does not protect it from long-range damage through CT through the base-pair stack. Indeed, such damage generated by long range CT within the nucleosome may persist preferentially and lead to the formation of permanent mutations.

7.6.6

DNA Charge Transport within the Nucleus

Many of our studies lead us to consider the physiological ramifications of DNA-mediated CT. Since DNA is the genetic material encoding all of the information within the cell, it becomes important to ask whether in the cell there are mechanisms to protect DNA from oxidative damage at a distance and additionally perhaps also to exploit DNA CT chemistry. A first step in exploring these issues involves establishing whether, indeed, DNA CT can proceed within the cell nucleus.

Towards that end, our rhodium intercalators were incubated with HeLa cell nuclei and irradiated to promote photooxidation [98]. After isolation of the DNA, and treatment with base excision repair enzymes to reveal base damage through strand breaks, the damaged DNA was amplified using the ligation-mediated polymerase chain reaction. Using this protocol, we probed exon 5 of the *p53* gene as well as a transcriptionally active promoter within the phosphoglycerate kinase gene (*PGKI*). Oxidative damage studies on exon 5 of the *p53* gene revealed clearly the preferential damage at the 5'-G of 5'-GGG-3', 5'-GG-3', and 5'-GA-3' sites, a signature of electron transfer damage to DNA. Thus the rhodium complex, with photoactivation, can promote oxidative damage to DNA within the nucleus. More interesting still were the experiments in which damage on the *PGK* promoter was probed. Here, oxidative damage was found at protein-bound sites which are inaccessible to rhodium. Thus, on transcriptionally active DNA within the cell nucleus, DNA-mediated CT can promote base damage from a distance. Importantly, then, even within the nucleus, direct interaction of an oxidant is not necessary to generate a base lesion at a specific site. These observations set the stage for studies directed at probing *in vivo* applications of DNA CT. Certainly these data require consideration in understanding cellular mechanisms for DNA damage and repair.

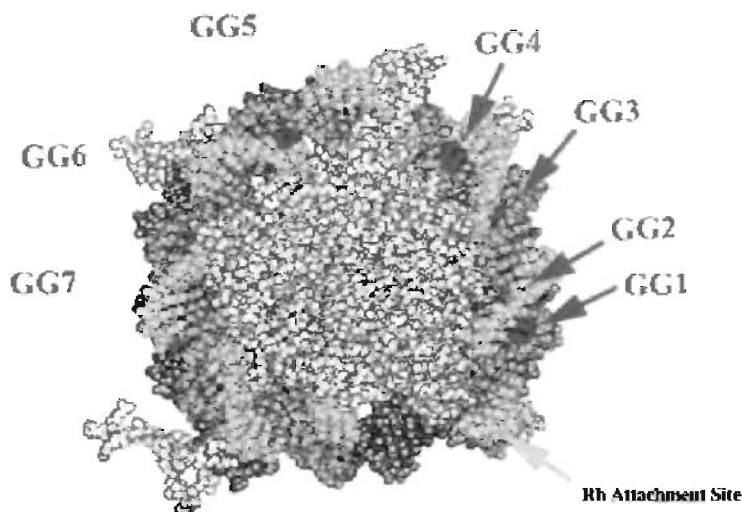


Fig. 7.6. Schematic illustration of long-range charge transport in a nucleosome core particle. The nucleosome was assembled containing $[\text{Rh}(\phi)_2\text{bpy}]^{3+}$ covalently tethered to the terminus (indicated by the purple arrow). Efficacious charge transport leading to oxida-

tive damage at a site 24 base pairs away (GG4) from the site of rhodium intercalation was evident [97]. The nucleosome structure shown here is based upon that determined crystallographically [96] and shows DNA in blue and histones in gray.

7.7

Conclusions

The range of studies described here, including spectroscopic, electrochemical, and biochemical measurements, all have pointed to a remarkable chemistry that depends upon the π -stacked structure of DNA. It is this dependence on stacking that distinguishes CT in nucleic acids from that through other macromolecular assemblies. We continue to be surprised by the exquisite sensitivity of DNA CT to the dynamical π -stacked structure of nucleic acids.

We can now begin to harness this sensitivity to π -stacking in a variety of applications. Electrochemistry on DNA films offers a novel and sensitive route for the diagnosis of mutations in DNA. These electrochemical methods may offer also strategies to analyze protein–DNA interactions and to screen for new drugs that inhibit such interactions. Moreover, CT chemistry may provide a completely new means to probe and characterize the sequence-dependent dynamics and flexibilities of nucleic acids. From the experiments already carried out, it is clear that the dynamical motions within DNA, dependent on sequence, local conformation, and protein interaction, modulate sensitively CT through the base-pair stack.

Perhaps most intriguing to consider is whether this chemistry is harnessed within the cell. CT through DNA proceeds over biologically significant distances. Perhaps then some sequences in DNA must be insulated and protected from long-

range CT damage. Other sites within the genome may instead be hot spots to which CT damage is funneled. The modulation of CT by sequence, conformation, and DNA-binding proteins clearly also offers a means of physiological control and regulation within the cell. Indeed, given that proteins can participate in DNA CT chemistry, perhaps they also utilize the DNA stack in long-range signaling or in repair. The remarkable sensitivity we have observed in detecting mutations and lesions in DNA could conceivably be exploited within the cell.

Our studies of DNA CT chemistry has therefore certainly provided a rich treasure of questions and ideas to explore. These experiments have provided us with a new perspective through which to view double-helical DNA. CT chemistry through DNA offers new challenges to exploit and unravel.

Acknowledgements

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8

DNA Interactions of Novel Platinum Anticancer Drugs

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8.1

Introduction

Platinum compounds constitute a discrete class of anticancer agents, which are now widely used in the clinic. The interest in antitumor platinum drugs has its origin in the 1960s, with the serendipitous discovery by Rosenberg of the inhibition of division of bacterial cells by platinum complexes [1]. The first platinum anticancer drug in clinical use was cisplatin (*cis*-diamminedichloroplatinum(II)), which is a very simple and purely inorganic molecule (Fig. 8.1). In spite of its simplicity, it is one of the most potent drugs available for anticancer chemotherapy [2–4]. It is highly effective for the treatment of testicular and ovarian cancer and is used in combination regimens for a variety of other carcinomas, including bladder, small lung tumors and those of head and neck [5]. However, cisplatin toxicity in tumor cells is coupled with several drawbacks (such as nephrotoxicity, neurotoxicity, and emetogenesis) [2]; hence there has been strong interest in the development of improved platinum drugs and consequently in understanding the details of the molecular mechanisms underlying the biological efficacy of the platinum compounds.

There is a large body of experimental evidence that the success of platinum complexes in killing tumor cells mainly results from their ability to form various types of adducts on DNA [6, 7]. Hence, extensive research has been carried out on DNA modifications by platinum antitumor drugs (for reviews, see, for example, Refs [8–10]). This chapter reviews a recent development in the study of DNA modifications by some novel classes of platinum anticancer compounds in cell-free media, which are compared with the effects of cisplatin.

8.2

Modifications by Cisplatin

8.2.1

Adducts and Conformational Distortions

The development of new classes of platinum antitumor drugs is often based on comparisons with the effects of cisplatin. Therefore, in this section the most im-

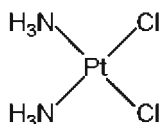


Fig. 8.1. Structure of cisplatin, the first platinum antitumor drug used in the clinic.

portant milestones in understanding DNA interactions with cisplatin are briefly addressed. Cisplatin reacts with DNA in the cell nucleus, where the concentration of chlorides is markedly lower than in extracellular fluids. The drug loses its chloride ligands in media containing low concentrations of chloride to form positively charged mono aqua and diaqua species (Fig. 8.2). It has been shown [11] that only these aquated forms bind to DNA.

Cisplatin is administered intravenously. Hence, before it reaches DNA in the nucleus of tumor cells it may interact with various compounds including sulfur-containing molecules (Fig. 8.2). These interactions are generally believed to play a role in mechanisms underlying tumor resistance to antitumor platinum drugs, their inactivation, and side effects [12, 13]. Generally, sulfur-containing biomolecules, such as those containing thiols are known to be highly reactive toward cisplatin so that they inactivate this drug. On the other hand, biomolecules containing thioethers (L-methionine (L-HMet)), including isolated L-HMet molecules, also form platinum–sulfur adducts which have been postulated to be a drug reservoir

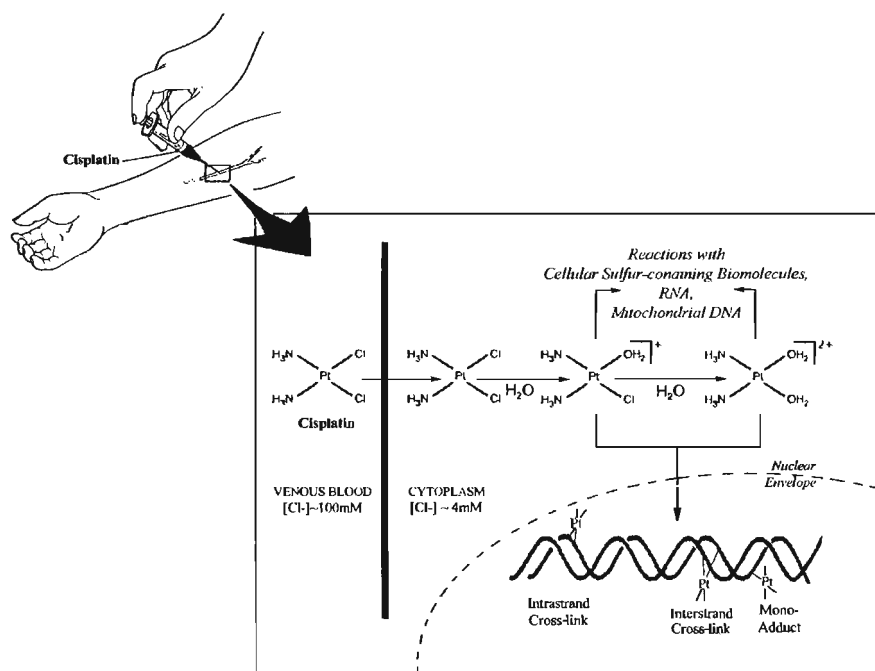


Fig. 8.2. Administration and *in vivo* chemistry of the anticancer drug cisplatin.

for platination of DNA. Thus, it has been proposed that the complexes formed between cisplatin and biomolecules containing thioethers may act as an activated form of platinum compounds with slowly hydrolyzing leaving groups. In other words, it has been suggested [14, 15] that these complexes (or adducts) may function as intermediates that may be transformed into platinum–DNA adducts. The reactions of L-HMet with cisplatin have been examined frequently. The major products of these reactions have been found to contain *S,N*-chelated L-Met, such as $[\text{Pt}(\text{L-Met-S,N})(\text{NH}_3)_2]$ and $[\text{Pt}(\text{L-Met-S,N})_2]$ [16, 17]. In addition, it has been demonstrated [14] that L-HMet increases the rate of reaction of cisplatin with monomeric guanosine 5'-monophosphate (GMP). Thus, these results seem to support the view that methionine residues could mediate the transfer of platinum onto DNA. However, in contrast to these expectations it has been demonstrated (O. Vrana, V. Brabec, unpublished results) that L-HMet decreases the rate of reaction of antitumor cisplatin with base residues in natural, high-molecular-mass DNA (such as plasmid or calf thymus DNA).

It has also been shown convincingly that the formation of the complex of cisplatin with L-HMet makes coordination of base residues in a polynucleotide chain by the platinum atom in cisplatin markedly more difficult than that of bases in monomeric regions of nucleic acids. Hence, the possibility proposed earlier [14, 15] that platinum can be transferred onto DNA via displacement of methionine *S*-bound to cisplatin by guanine residues in polymeric DNA appears less likely. This also implies that these results do not support the view [12, 13] that cisplatin bound to monomeric methionine or to methionine residues in peptides or proteins could potentially act as a drug reservoir available for platination of DNA in the nucleus of tumor cells. In this respect, thioethers seem to behave like thiols that inactivate cisplatin.

Bifunctional cisplatin binds to DNA in a two-step process, first forming monofunctional adducts preferentially at the guanine residues, which subsequently close to intrastrand crosslinks between adjacent purine residues (~90%) (1,2-GG or 1,2-AG intrastrand crosslinks) [18, 19]. Other minor adducts are 1,3-GXG intrastrand crosslinks ($X = A, C, T$), interstrand crosslinks (~6% in linear DNA) preferentially between guanine residues in the 5'-GC/5'-GC sequence [20] and monofunctional lesions. In all adducts, cisplatin is coordinated to the N7 atom of purine residues. The percentage of the 1,2-intrastrand adducts formed by cisplatin is larger than statistically expected, so this crosslink has generally been assumed to be the important adduct for anticancer activity and has therefore been most extensively investigated.

The adducts formed by cisplatin in DNA affect its secondary structure. The formation of major 1,2-GG or 1,2-AG intrastrand crosslinks of cisplatin leads to marked conformational alterations in DNA (Fig. 8.3a) [7, 21]. These adducts induce a roll of 26–50° between the platinated purine residues, displacement of platinum atom from the planes of the purine rings, a directional, rigid bend of the helix axis toward the major groove and a local unwinding of 9–11°. In addition, severe perturbation of hydrogen-bonding within the 5'-coordinated GC base pair, widening and flattening of the minor groove opposite the cisplatin adduct, creation

of a hydrophobic notch, global distortion extending over 4–5 base pairs and additional helical parameters characteristic of A-form of DNA have also been reported.

The interstrand crosslink, which is preferentially formed by cisplatin between opposite guanine residues in the 5'-GC/5'-GC sequence [22], also induces several irregularities in DNA (Fig. 8.3b) [20, 23, 24]. The crosslinked guanine residues are not paired with hydrogen bonds to the complementary cytosines, which are located outside the duplex and not stacked with other aromatic rings. All other base residues are paired, but distortion extends over at least four base pairs at the site of the crosslink. In addition, the *cis*-diammineplatinum(II) bridge resides in the minor groove and the double helix is locally reversed to a left-handed, Z-DNA-like form. This adduct induces the helix unwinding by 76–80° relative to B-DNA and also the bending of 20–40° of the helix axis at the crosslinked site toward the minor groove. More detailed descriptions of structures of crosslinks of cisplatin may be found in other recent reviews [7, 20, 21, 25].

The 1,3-GXG intrastrand crosslink formed by cisplatin bends the helix axis toward the major groove by ~30° and locally unwinds DNA (by ~19°) [26, 27]. In addition, DNA is locally denatured and flexible at the site of the adduct.

The monofunctional adducts of cisplatin (preferentially formed at the guanine sites) also distort DNA in a sequence-dependent manner [28, 29]. These distortions disturb stacking interactions in double-helical DNA and make bases around the adduct more accessible. In addition, the monofunctional adduct also unwinds the duplex (unwinding angle is ~6° [30]). On the other hand, no intrinsic bending is induced in DNA by these monofunctional adducts [28].

8.2.2

Effects on DNA Replication and Transcription

DNA replication and transcription are essential processes in rapidly proliferating tumor cells so that their inhibition should result in cytostatic effects. As various adducts formed on DNA are capable of inhibiting DNA replication or transcription by DNA-dependent DNA or RNA polymerases, the effects of platinum adducts on these processes have been thoroughly investigated.

Cisplatin inhibits DNA synthesis by prokaryotic and eukaryotic DNA polymerases both *in vitro* and *in vivo* [31–33]. Inhibition of DNA synthesis occurs mainly at GG sites consistently with the fact that these sites are preferential binding sites of cisplatin. The monofunctional adducts of cisplatin affect replication only negligibly. It has been demonstrated in the studies of replication of DNA modified by cisplatin [33] that the inhibition of DNA elongation is not complete; the polymerases are able to bypass the adducts. The frequency of replication bypass varies for the different polymerases. The ability of DNA polymerases to bypass the lesions of platinum compounds is consistent with the view that these compounds are mutagenic through such a replication bypass. The mutagenic properties of drugs used in the clinic are important factors to consider since mutagenicity may lead to the development of secondary tumors. Interestingly, the 1,2-GG intrastrand crosslinks of cisplatin are considerably less mutagenic than 1,2-AG crosslinks in *E. coli*,

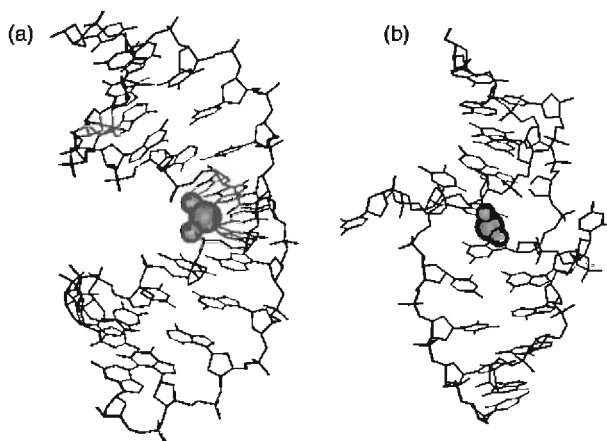


Fig. 8.3. Crystal structures of DNA duplexes containing an 1,2-GG intrastrand crosslink (a) and interstrand crosslink (b) of cisplatin. Images of the duplexes (a) 5'-d(CCTCTG*G*TCTCC)/5'-d(GGAGACCAGAGG) [191] and (b) 5'-d(CCTCG*CTCTC)/5'-d(GAGAG*CGAGG)

[24], where the asterisks represent the sites involved in the crosslinks of cisplatin. Colors: black, thymine; orange, adenine; green, cytosine; red, guanine; yellow, platinum atom; blue, nitrogen atom.

whereas controversial mutagenicity has been reported for the 1,3-GXG intrastrand crosslink [34, 35]. Although there is no doubt that inhibition of DNA replication is an important part of the mechanism underlying the antitumor effects of cisplatin, it becomes evident that it cannot fully explain the antitumor efficiency of this drug [36] so that this mechanism is more complex [37, 38].

Another essential function of the cellular metabolism affected by lesions formed on DNA by cisplatin is DNA transcription. Transcription by eukaryotic and prokaryotic RNA polymerases (RNA polymerase II and SP6 or T7 RNA polymerases, respectively) of DNA modified by cisplatin *in vitro* has been investigated in detail [22, 39–42]. The RNA polymerases react differently at the various platinum adducts. Bifunctional adducts of cisplatin strongly inhibit transcription of platinated DNA by RNA polymerases. The RNA polymerases entirely bypass the monofunctional adducts of cisplatin [22, 41, 43]. It has been suggested [43] that the platinum adducts which block the RNA polymerases not only constitute a physical barrier to the progress of the enzymes on the template but also specifically alter the properties of transcription complexes as a consequence of the specific conformational changes that they induce in template DNA.

8.2.3

Cellular Resistance, Repair

The limitations of using cisplatin in cancer chemotherapy are also associated with intrinsic and acquired resistance of tumor cells to this drug. Resistance to cisplatin is multifactorial and in general it may consist of mechanisms either limiting

the formation of DNA adducts and/or operating downstream of the interaction of cisplatin with DNA to promote cell survival. The formation of DNA adducts by cisplatin can be limited by reduced accumulation of the drug, enhanced drug efflux and cisplatin inactivation by coordination to sulfur-containing biomolecules including metallothioneins (see Section 8.2.1 and Fig. 8.2), whose production may be increased as a consequence of cisplatin treatment. The second group of mechanisms includes enhanced repair of DNA adducts of cisplatin and increased tolerance of the resulting DNA damage.

In human cells, cisplatin intrastrand adducts are removed from DNA mainly by the nucleotide excision repair (NER) mechanism. It has been found using cell-free extracts or a reconstituted NER system that 1,2- and 1,3-intrastrand crosslinks of cisplatin are efficiently repaired [44]. Importantly, this repair of 1,2- but not 1,3-intrastrand crosslinks is blocked upon addition of an HMG-domain protein (HMG = high mobility group) (see Section 8.2.4.1).

An *in vitro* excision repair of a site-specific cisplatin interstrand crosslink has also been studied using mammalian cell-free extracts containing HMG-domain proteins at levels that are not high enough to block excision repair of the 1,2-intrastrand adducts [44]. Excision of the interstrand crosslinks formed by cisplatin has not been detected. DNA interstrand crosslinks pose a special challenge to repair enzymes because they involve both strands of DNA and therefore cannot be repaired using the information in the complementary strand for resynthesis.

There is also evidence that another cellular repair mechanisms, such as recombination or mismatch repair (MMR), can affect antitumor efficiency of cisplatin. The role of recombination in survival of the cells treated with cisplatin has been also demonstrated, but mainly in prokaryotic cells [45]. The sensitivity of many recombination-deficient mutants to cisplatin has been examined and almost all of the strains are very sensitive to cisplatin, in comparison with the corresponding wild-type cells. In addition, mutants deficient in both recombination repair and NER are more sensitive to cisplatin than single mutants. This may imply that the role of recombination in protecting cells from cisplatin toxicity is independent of NER. On the other hand, it cannot be entirely excluded that DNA lesions in prokaryotic cells treated with cisplatin are repaired or tolerated by a mechanism which is different from those effective in eukaryotic cells [46].

Recent observations support the view that MMR mediates the cytotoxicity of cisplatin in tumor cells [47, 48] and that dysfunction of this type of DNA repair may result in the resistance of tumor cells to cisplatin or in drug tolerance [49]. The function of the MMR is to scan newly synthesized DNA and remove mismatches that result from nucleotide incorporation errors made by DNA polymerases. To explain cisplatin tolerance, it is assumed that replication bypass of DNA adducts of cisplatin leads to mutations. During MMR, the strand to be corrected is nicked, a short fragment containing the mismatch is excised, and new DNA fragment is synthesized. The MMR system always replaces the incorrect sequence in the daughter strand, which would leave the cisplatin adduct unrepaired. This activity initiates a futile cycle. During DNA synthesis to replace the excised short fragment, the DNA polymerases would repeatedly incorporate mutations, followed by

attempts to remove them. The repeated nicks in DNA formed at each ineffective cycle of repair could trigger a cell death response. Thus, MMR recognition of cisplatin adducts on DNA may trigger a programmed cell death pathway rendering MMR-proficient cells more sensitive to DNA modification by cisplatin than MMR-deficient cells.

8.2.4

Recognition of the Lesions by Cellular Proteins

It is generally believed that antitumor activity of cisplatin is mediated by the recognition of its DNA adducts by cellular proteins. Several classes of these proteins have been identified and mechanisms have been proposed as to how they mediate the antitumor effects of cisplatin. Extensive reviews addressing these questions have been published recently [50–52]. Therefore, in this section only some selected aspects of recognition of DNA adducts formed by cisplatin by damaged DNA-binding proteins are described.

8.2.4.1 HMG-domain proteins

HMGB1 and HMGB2 proteins belong to a group of architectural chromatin proteins that play some kind of structural role in the formation of functional higher order protein–DNA or protein–protein complexes or as signaling molecules in genetically regulated repair pathways. These structure-specific proteins (which recognize DNA with little sequence-specificity and have a high affinity for some non-canonical DNA structures including a kinked DNA) bind selectively to the 1,2-GG or AG adducts of cisplatin, but not to its 1,3-intrastrand crosslinks.

The full-length HMGB1 protein contains two tandem HMG-box domains, A and B, and an acidic C-tail [53]. The domains A and B in HMGB1 protein (HMGB1a and HMGB1b) are linked by a short lysine-rich sequence (containing seven amino acids, A/B linker region). Each “minimal” domain A or B alone specifically recognizes 1,2-GG intrastrand crosslink of cisplatin [54]. The affinity of HMGB1a for DNA containing a 1,2-GG intrastrand crosslink is generally higher than that of HMGB1b and it has been proposed that HMGB1a is the dominating domain in the full-length HMGB1 that binds to the site of the intrastrand crosslink, while HMGB1b facilitates binding providing additional protein–DNA interactions. Interestingly, the binding affinity of HMGB1a and HMGB1b with the duplex containing the site-specific 1,2-GG intrastrand crosslink is modulated by the nature of the bases that flank the platinum lesion [54, 55].

The HMG domain binds to the minor groove of the DNA double helix opposite the platinum 1,2-intrastrand crosslink located in the major groove [56]. Distortions such as prebending, unwinding at the site of platination and preformation of a hydrophobic notch as a consequence of the 1,2-intrastrand crosslink formation are important for the recognition and affinity of the platinum-damaged DNA-binding proteins. Moreover, the A/B linker region in the full-length HMGB1 protein attached to the N-terminus of the domain B markedly enhances binding of the B domain to DNA containing the site-specific 1,2-GG intrastrand crosslink [57].

These studies have been extended to DNA interstrand crosslinks produced by cisplatin or transplatin at the sites where these adducts are formed preferentially [58]. Mammalian HMGB1 protein binds to the interstrand crosslink of cisplatin with a slightly lower affinity, as to the 1,2-GG intrastrand crosslink. Our most recent analysis has revealed that isolated HMGB1a has no affinity to the duplex interstrand crosslinked by cisplatin, whereas the isolated HMGB1b containing the A'/B-linker region attached to its N-terminus binds with a noticeable affinity although lower than is that of the "minimal" HMGB1a to 1,2-GG intrastrand crosslink (J. Kasparkova, M. Stros, O. Delalande, J. Kozelka, V. Brabec, unpublished results).

1,3-GXG intrastrand crosslink of cisplatin is not recognized by HMGB1, in spite of the fact that bending and unwinding induced in DNA by this adduct are rather similar to those induced by 1,2-GG intrastrand adduct. This suggests that there are other factors which control the recognition of platinum adducts by DNA-binding proteins [20, 58].

There are a variety of proteins other than HMGB1 and HMGB2 that contain HMG boxes, a conserved 80-amino-acid domain found in a variety of eukaryotic DNA-binding proteins, including a number of transcription factors. Many of these proteins (for instance HMG-D, human and *Drosophila* SSRP1, Cmb1, hUBF, Ixr1, mtTFA, LEF1, SRY, T160, tsHMG, human FACT factor) recognize and bind to DNA modified by cisplatin and several recent reviews describing these binding studies are available [7, 50, 51, 59].

8.2.4.2 Proteins without an HMG domain

The TATA-binding protein (TBP) [60] is essential for transcription initiation in eukaryotes. TBP recognizes and binds to the minor groove of a consensus sequence, TATAAA, known as the TATA box. It binds selectively to and is sequestered by cisplatin-damaged DNA [61, 62]. It has been shown that cisplatin turns the GG sequence into a potential site for TBP and it has been suggested that TBP binds selectively to a specific three-dimensional structure [62]. Another protein lacking an HMG domain that has been shown to bind to cisplatin-modified DNA is Y-box binding protein 1 [63]. A very abundant chromatin protein, the linker histone H1, also binds strongly to DNA modified by cisplatin [64, 65].

A relatively large number of proteins that specifically bind to cisplatin adducts are DNA repair proteins. The repair proteins that recognize cisplatin-modified DNA are mainly those involved in the first step of the repair pathway, i.e. in damage recognition. The repair proteins that have probably attracted the most attention in the studies of recognition of DNA modified by platinum drugs are those that are absent in patients with the NER deficiency characteristic of the disease xeroderma pigmentosum (XP). The minimal factors necessary for removal of damaged nucleotides also include XPA protein and replication protein A (RPA), which are among the major damage-recognition proteins involved in the early stage of NER. XPA (32-kDa protein) and RPA (composed of 70-, 34- and 14-kDa subunits) are able to bind damaged DNA independently, although RPA interaction stimulates XPA binding to damaged DNA. It has been suggested recently [66] that XPA in

conjunction with RPA constitutes a regulatory factor that monitors DNA bending and unwinding to verify the damage-specific localization of NER complexes or control their three-dimensional assembly.

The proteins involved in the MMR pathway also display affinity for cisplatin-modified DNA. For instance, human MutS α and its component of MSH2 exhibit an enhanced affinity to DNA containing 1,2-GG intrastrand crosslink [67–69].

DNA photolyase, which is involved in the repair of cyclobutane pyrimidine dimers [70], and Ku autoantigen [71], which takes part in recombination and double-strand break repair, are further examples of proteins lacking an HMG domain that bind to cisplatin-modified DNA.

The human 3-methyladenine DNA glycosylase involved in the base excision repair can also tightly bind to intrastrand crosslinks of cisplatin [72]. Although this protein is unable to release any of these adducts from DNA it has been suggested that it can facilitate their NER.

Another repair protein that has also been thoroughly tested as to whether it specifically recognizes DNA adducts of cisplatin is T4 endonuclease VII, an enzyme with deoxyribonuclease activity [58, 73].

It has been hypothesized that sensitivity of tumor cells might also be associated with the processes involving tumor suppressor protein p53 [74, 75]. The tumor suppressor protein p53 is a nuclear phosphoprotein consisting of 393 amino acids and containing four major functional domains (Fig. 8.4a) [76]. The protein is involved in the control of cell cycle, DNA repair and apoptosis. Hence, p53 is a potent mediator of cellular responses against genotoxic insults [77] and exerts its effect through transcriptional regulation. Upon exposure to genotoxic compounds, p53 protein levels increase due to several post-transcriptional mechanisms.

Interestingly, on average, cells with mutant p53 are more resistant to the effect of cisplatin [78]. Sequence-specific DNA-binding activity of p53 protein is crucial for its tumor suppressor function. Active p53 binds as a tetramer to over 100 different response elements naturally occurring in the human genome (but only approximately 50 show functionality [79]). Free DNA in the segments corresponding to the consensus sequence is already intrinsically bent toward the major groove and the bends are mostly localized at two C(a/t)|(a/t)G tetramers [80, 81]. This localized intrinsic bending has been suggested to contribute in a fundamental way to the stability of the tetrameric p53-DNA complex [80].

DNA interactions of active wild-type human p53 protein with DNA modified by cisplatin has been investigated [82]. DNA adducts reduce the binding affinity of the consensus DNA sequence to p53 whereas the adducts of clinically ineffective *trans* isomer of cisplatin do not. This result has been interpreted to mean that the precise steric fit required for formation and stability of the tetrameric complex of p53 with the consensus sequence in DNA (Fig. 8.4b) cannot be attained as a consequence of additional conformational perturbances induced by cisplatin adducts in the consensus sequence. The results also demonstrate an increase of the binding affinity of p53 to DNA lacking the consensus sequence and modified by cisplatin, but not by transplatin. In addition, major 1,2-GG intrastrand crosslinks of cisplatin are only responsible for this enhanced binding affinity of p53, which is, however,

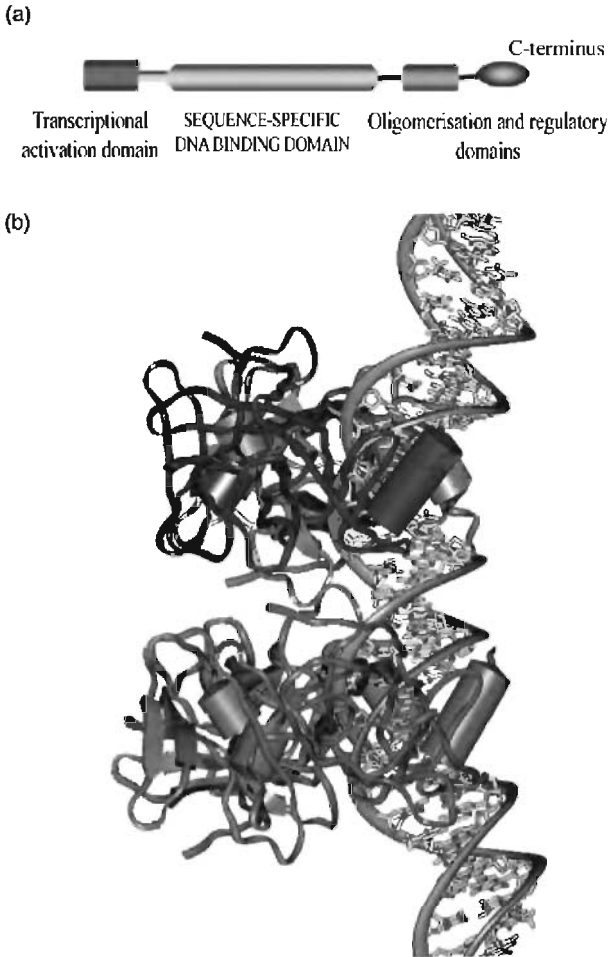


Fig. 8.4. (a) Schematic representation of active p53 molecules. The p53 sequence-specific DNA-binding domain (blue) binds to the p53 consensus DNA sequence. At the p53 C-terminus, another sequence-non-specific DNA-binding site occurs which coincides with a negative regulatory segment. The latter

segment inhibits DNA-binding functions of the core domain in non-modified full-length p53 protein. (b) Three-dimensional model for the complex of tetrameric p53 bound to DNA bent towards the major groove. The single monomeric p53 subunits are shown in different colors. Adapted from Ref. [80] with permission.

markedly lower than the affinity of p53 to unplatinated DNA containing the consensus sequence.

The distinctive structural features of 1,2-intrastrand crosslinks of cisplatin have been suggested to play a unique role for this adduct in the binding of p53 to DNA lacking the consensus sequence [82]. This intriguing scenario for the mechanism by which cisplatin adducts modulate biological effects of p53 might be some man-

ifestation of the hijacking model (see Section 8.2.5). In addition, comparison of the binding of active, latent and activated p53 to DNA fragments modified by cisplatin has been performed (M. Fojta, H. Pivonkova, M. Brazdova, E. Palecek, S. Pospisilova, B. Vojtesek, J. Kasparkova, V. Brabec, unpublished results). Modifications of DNA lacking consensus sequence by cisplatin enhanced affinity of all three forms of p53. The difference in the binding affinity to platinated and unmodified DNA is even more pronounced in the case of the latent form in comparison with the active form. Consistently with the latter observation, activation of the latent form reduces its preference for platinated DNA. In addition, the p53 core domain appears to be the primary site of sequence-specific binding of non-modified DNA in active p53, while the C-terminus of p53 (Fig. 8.4a) interacts selectively with platinated DNA. These findings suggest that interactions of p53 with platinated DNA may have implications in cell responses to cisplatin treatment.

The cisplatin-damaged DNA-binding proteins apparently occur in nature for purposes other than for specific recognition of platinum adducts in DNA, since platinum compounds do not occur naturally. The affinity of DNA modified by cisplatin to DNA-binding proteins, which may have a fundamental relevance to the antitumor activity of cisplatin and its simple antitumor analogs, is probably a coincidence when some platinum adducts in double-helical DNA adopt a structure that mimics the recognition signal for these proteins.

8.2.5

Mechanism of action of cisplatin

It is generally believed that the key intracellular pharmacological target for platinum compounds is DNA. The adducts formed by cisplatin distort DNA conformation, but it still remains uncertain which of these adducts is the most important in terms of producing anticancer effects. DNA adducts of cisplatin inhibit replication and transcription, but they are also bypassed by DNA or RNA polymerases. In addition, cisplatin adducts are removed from DNA mainly by NER. They are, however, also recognized by a number of proteins which could block DNA adducts of cisplatin from damage recognition needed for repair (Fig. 8.5a). The other hypothesis, based on the observation that a number of proteins recognize cisplatin-modified DNA, is that cisplatin-DNA adducts hijack proteins away from their normal binding sites, thereby disrupting fundamental cellular processes (Fig. 8.5b). Experimental support for these hypothetical aspects of the mechanism underlying the antitumor activity of cisplatin or resistance of some tumors to this drug has been recently thoroughly reviewed [7, 9, 21, 49, 51, 75, 83].

Initially, inhibition of DNA replication was considered to be a process very likely relevant to antitumor efficiency of cisplatin [31]. However, later studies have indicated that cisplatin inhibits tumor cell growth at doses which are considerably lower than those needed to inhibit DNA synthesis [36]. Subsequent observations have revealed that cisplatin can trigger G₂ cell cycle arrest and programmed cell death (apoptosis) [37, 38] exposing a mechanism of cytotoxicity of this drug. However, since apoptosis is a very complex process, a number of possible pathways

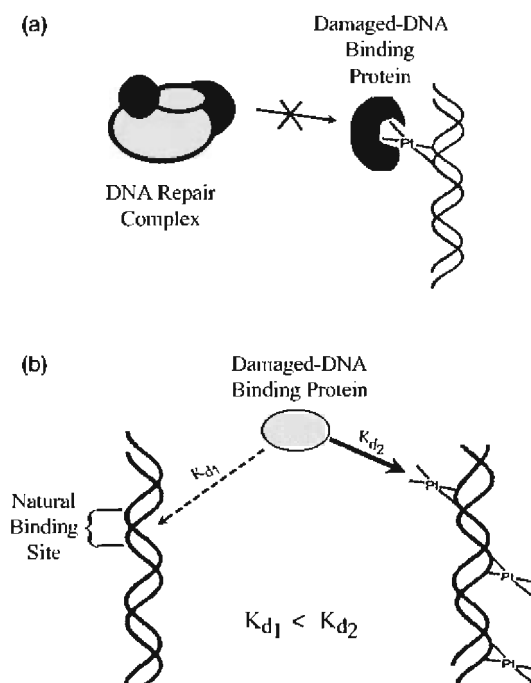


Fig. 8.5. Molecular mechanisms of antitumor activity of cisplatin. (a) The repair shielding model. If the protein recognizing 1,2-GG or AG intrastrand crosslink (for instance HMG-domain protein) binds to these lesions it can inhibit their repair by physically blocking the access to other repair proteins. The crosslinks of cisplatin persist in DNA and potentiate their toxicity. (b) Hijacking model. If the protein

recognizing 1,2-GG or AG intrastrand crosslink (for instance transcription factor, p53, TBP) binds to these lesions with a higher affinity than to their natural binding sites ($K_{d1} < K_{d2}$), the proteins could be titrated away from their usual (for instance transcriptional regulatory) function. This may disrupt processes critical for cell survival.

have to be still explored for a complete understanding of the mechanism by which cisplatin triggers apoptosis [84].

8.3

Modifications by Antitumor Analogs of Cisplatin

8.3.1

Carboplatin

The search for an agent less toxic than cisplatin led to the development of carboplatin (*cis*-diamminocyclobutanedicarboxylatoplatinum(II)) (Fig. 8.6), which has achieved routine clinical use. The leaving group of carboplatin is a cyclobutanedicarboxylato ligand which undergoes a slower rate of aquation than the chlorides in

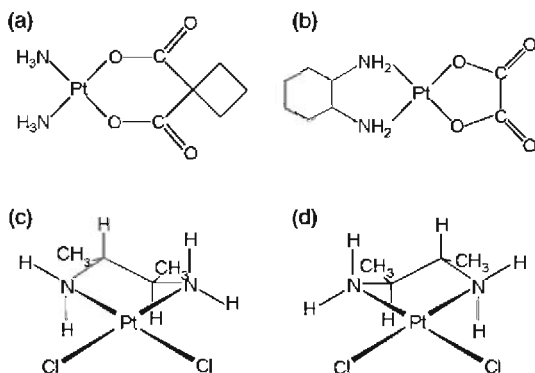


Fig. 8.6. Structures of direct bifunctional analogs of cisplatin: (a) carboplatin, (b) oxaliplatin, (c) PtCl₂(*R,R*-DAB), and (d) PtCl₂(*S,S*-DAB).

cisplatin. Carboplatin forms a similar spectrum of DNA adducts as cisplatin with a slightly different sequence preference [85]. The concentrations of carboplatin approximately two orders of magnitude higher are needed to obtain DNA platination levels equivalent to cisplatin. Increased DNA-binding affinity of carboplatin has been observed in the presence of nucleophiles, such as those present in human breast cancer cell cytoplasmic extracts, thiourea or glutathione [86]. Conformational alterations induced in DNA by carboplatin in cell-free media have been characterized by circular dichroism and differential pulse polarography [87]. The changes of non-denaturational character similar to those observed for cisplatin at the same level of DNA platination have been found. Although less toxic than cisplatin, carboplatin is still only active in the same range of tumors as cisplatin [88].

In spite of some interesting and promising results describing DNA modifications by carboplatin, as yet no substantial advantages over cisplatin have been demonstrated. Carboplatin is more easily administered and is less toxic at standard doses, but exhibits the similar efficacies in most solid tumors and is administered intravenously [89]. From a mechanistic DNA-binding point of view, this is not too surprising since the adducts produced on DNA by cisplatin and carboplatin are similar, although they differ in their relative rates of formation.

8.3.2

Oxaliplatin

Another cisplatin analog that has received approval for use in Asia, Europe, and Latin America is oxaliplatin ((1*R*,2*R*-diamminocyclohexane)oxalatoplatinum(II)) (Fig. 8.6b). In terms of clinical activity, oxaliplatin is the first platinum antitumor drug to demonstrate activity in colon and ovarian cancers [89–91]. Importantly, oxaliplatin has been shown to be active in combination with 5-fluorouracil and leucovorin for the treatment of colorectal cancer, a disease in which cisplatin and carboplatin show little activity [92–94]. These important results lend substantial

support to the hypothesis that structural modifications of the carrier ligand in cisplatin may alter spectrum of antitumor activity, and so overcome resistance.

In order for its reaction with DNA to occur, the parent compound must become aquated. The hydrolysis of oxaliplatin to form reactive species, 1,2-DACH diaqua platinum(II) ($[\text{Pt}(\text{R},\text{R}\text{-DACH})(\text{H}_2\text{O})_2]^{2+}$) (DACH = 1,2-diaminocyclohexane), is a slower process than the hydrolysis of cisplatin, but it is facilitated by HCO_3^- and H_2PO_4^- ions [95]. Site- and region-specificity of lesions induced by oxaliplatin in DNA have been determined [96]. The sites of oxaliplatin adducts and their spectrum [97, 98] are nearly identical to the situation when DNA is modified by cisplatin. On the other hand, oxaliplatin is inherently less able than cisplatin to form DNA adducts [99]. Oxaliplatin adducts are repaired with kinetics similar to those of cisplatin adducts. Oxaliplatin, however, is more efficient than cisplatin per equal number of DNA adducts in inhibiting DNA chain elongation. Despite lower DNA reactivity, oxaliplatin exhibits similar or greater cytotoxicity in several human tumor cell lines. Thus, oxaliplatin requires fewer DNA lesions than cisplatin to achieve cell growth inhibition [99].

The conformation of the major DNA adduct formed by oxaliplatin (1,2-GG intrastrand crosslink) has been studied first by using molecular modeling [100]. These studies suggest that the overall conformational alterations induced in DNA by the 1,2-GG intrastrand crosslink of cisplatin and oxaliplatin are similar. The bulky DACH ring of the oxaliplatin adduct fills much of the DNA major groove, making it narrower and less polar at the site of the crosslink. Quite recently, the crystal structure of the 1,2-GG intrastrand crosslink of oxaliplatin in a dodecamer duplex has been reported [101, 102] and confirmed similarity of the overall conformational alterations induced in DNA by the 1,2-GG intrastrand crosslink of cisplatin and oxaliplatin. The crosslink formed by oxaliplatin bends the double helix by $\sim 30^\circ$ toward the major groove. A novel feature of this structure is the presence of a hydrogen bond between pseudoequatorial NH hydrogen atom of the R,R-DACH ligand and the O6 atom of the 3'-G of the platinated d(GG) site. This finding has provided structural evidence for the importance of chirality in mediating the interaction between oxaliplatin and double-stranded DNA and a new kind of chiral recognition between an enantiometrically pure metal complex and the DNA double helix.

The subtle differences in overall conformation of 1,2-GG intrastrand crosslinks of cisplatin and oxaliplatin have been suggested [103] to influence further processing of the oxaliplatin crosslink in the cell. Full-length HMGB1 protein and mismatch repair proteins have been found to have a slightly lower affinity to the crosslink of oxaliplatin than to that of cisplatin [47, 103]. In addition, DNA lesions generated by oxaliplatin are also repaired *in vitro* by the mammalian NER pathway with kinetics similar to those of the lesions of cisplatin [104]. Another interesting finding is that eukaryotic DNA polymerases β , γ , ξ , and η bypass 1,2-GG intrastrand crosslink of cisplatin less readily than the same crosslink of oxaliplatin [103, 105–107]. In addition, the misincorporation frequency of DNA polymerase β is slightly greater with 1,2-GG intrastrand crosslink of oxaliplatin than with that of cisplatin on primed single-stranded DNA. Thus, it may be possible that differences in the

conformation of 1,2-intrastrand crosslinks of cisplatin and oxaliplatin can be responsible for differences exhibited by these compounds in both the fidelity and efficiency of translesion synthesis by DNA polymerases.

Also interestingly, the MMR protein complex binds to DNA globally modified by cisplatin (see also Section 8.2.4.2), but not to that by oxaliplatin [108]. In addition, *E. coli* mismatch repair protein MutS recognizes the oxaliplatin-modified DNA with 2-fold lower affinity than the cisplatin-modified DNA [109]. Perhaps steric hindrance by the non-polar DACH ligand may prevent the MMR complex or proteins from recognizing the lesion. The latter findings may also be related to the observation that MMR-deficient cells are slightly more resistant to cisplatin but not to oxaliplatin [47, 110].

Besides $[\text{Pt}(\text{R,R-DACH})]^{2+}$ (directly derived from oxaliplatin), other enantiomeric forms of this complex exist. The biological activity of platinum complexes with enantiomeric amine ligands such as $[\text{Pt}(\text{R,R-DACH})]^{2+}$ and $[\text{Pt}(\text{S,S-DACH})]^{2+}$ and other enantiomeric pairs has been investigated intensively [111–114]. For instance, the DACH carrier ligand has been shown to significantly affect the ability of platinum–DNA adducts to block essential processes such as replication and transcription [115]. Also importantly, $[\text{PtCl}_2(\text{N-N})]$ complexes with $\text{N-N} = \text{DACH}$, 2,3-diaminobutane (DAB) (Fig. 8.6c,d) or 1,2-diaminopropane, having an S configuration at the asymmetric carbon atoms, are markedly more mutagenic toward several strains in *Salmonella typhimurium* than their R isomers [116]. Even more importantly, oxaliplatin (having λ -gauche conformation of the chelate ring) exhibits higher activity toward various cancer cell lines than the S,S-enantiomer, so that oxaliplatin and not its S,S-enantiomer has been approved for clinical use [117]. Hence, although the asymmetry in the amine ligand in these platinum complexes does not involve the coordinated nitrogen atom but rather an adjacent carbon atom, a dependence of the biological activity on the configuration of the amine is observed.

Recently, modifications of DNA by analogs of antitumor cisplatin containing enantiomeric amine ligands, such as $\text{PtCl}_2(\text{R,R-DAB})$ and $\text{PtCl}_2(\text{S,S-DAB})$ (Fig. 8.6c,d), have also been studied [118]. The major differences resulting from the modification by the two enantiomers consist in the thermodynamical destabilization and conformational distortions induced in DNA by the major 1,2-GG intrastrand crosslinks. The crosslink formed by $\text{PtCl}_2(\text{S,S-DAB})$ is more effective in inducing overall destabilization of the duplex and global conformational alterations than that formed by $\text{PtCl}_2(\text{R,R-DAB})$. Bending toward major groove and unwinding angles due to the crosslink of $\text{PtCl}_2(\text{S,S-DAB})$ (24° and 15° , respectively) are smaller than those due to the crosslink of $\text{PtCl}_2(\text{R,R-DAB})$ (35° and 20° , respectively). However, the overall destabilization of the duplex due to the crosslink of $\text{PtCl}_2(\text{S,S-DAB})$ is greater than that due to the crosslink of $\text{PtCl}_2(\text{R,R-DAB})$. In addition, the duplex containing the crosslink of $\text{PtCl}_2(\text{R,R-DAB})$ shows considerably more pronounced distortion of the base pair adjacent to the crosslink on its 3' side. In contrast, the duplex containing the crosslink of $\text{PtCl}_2(\text{S,S-DAB})$ shows larger distortion of the base pair on the 5' side of the crosslink.

Interestingly, the intrastrand crosslinks of the $\text{PtCl}_2(\text{R,R-DAB})$ bind to HMGB1a

and HMGB1b with a similar affinity as the same crosslink of cisplatin (J. Malina, J. Kasparkova, G. Natile, V. Brabec, unpublished results). In contrast, the crosslink of $\text{PtCl}_2(\text{S},\text{S}-\text{DAB})$ binds to the HMGB1a protein with a noticeably lower affinity, whereas the affinity of HMGB1b to the crosslink of $\text{PtCl}_2(\text{S},\text{S}-\text{DAB})$ is only slightly lower than that to the crosslinks of $\text{PtCl}_2(\text{R},\text{R}-\text{DAB})$ or cisplatin. As the intrastrand crosslink of the $\text{PtCl}_2(\text{S},\text{S}-\text{DAB})$ thermodynamically destabilizes DNA more than the crosslink of the $\text{PtCl}_2(\text{R},\text{R}-\text{DAB})$ [118], this result supports the previously established correlation [119] that the increasing thermodynamical destabilization due to the 1,2-GG intrastrand crosslink of cisplatin lowers its affinity to HMG-domain proteins.

The 1,2-GG intrastrand crosslinks of both enantiomers are efficiently removed from DNA by NER in the *in vitro* assay using mammalian or rodent cell-free extracts capable of removing the 1,2-intrastrand crosslinks of cisplatin (J. Malina, J. Kasparkova, G. Natile, V. Brabec, unpublished results). Consistent with the different affinity of the intrastrand crosslinks of both enantiomers to HMG-domain proteins, addition of HMGB1a only blocks the repair of the crosslinks of $\text{PtCl}_2(\text{R},\text{R}-\text{DAB})$, whereas the repair of the crosslinks of $\text{PtCl}_2(\text{S},\text{S}-\text{DAB})$ remains unaffected. Similar results have been also obtained with DNA modified by $\text{PtCl}_2(\text{DACH})$ enantiomers.

It has been suggested that during replication, translesion synthesis past platinum adduct catalyzed by DNA polymerases may be error prone and result in mutations. No data which would allow comparison of translesion synthesis past 1,2-GG intrastrand crosslink of $\text{Pt}(\text{R},\text{R}-\text{DACH})$ and $\text{Pt}(\text{S},\text{S}-\text{DACH})$ are available. On the other hand, it has been shown [103, 120] that translesion synthesis past 1,2-GG intrastrand crosslink of cisplatin or oxaliplatin is markedly or entirely inhibited if this crosslink is bound to HMGB1 protein. Hence, it is reasonable to expect that HMGB1 proteins can modulate the efficiency of DNA polymerases to bypass the 1,2-GG intrastrand crosslink also formed by Pt-DACH compounds and consequently affect their mutagenicity. Thus, considerably lower mutagenic effects of $\text{Pt}(\text{R},\text{R}-\text{DACH})$ or $\text{Pt}(\text{R},\text{R}-\text{DAB})$ in comparison with *S,S*-enantiomers [116] could be due to markedly tighter binding of HMG-domain proteins to the major 1,2-GG intrastrand crosslink of *R,R*-enantiomers than to the crosslink of *S,S*-enantiomers: this tighter binding would inhibit translesion synthesis past the crosslink more efficiently and consequently would reduce its mutagenic effects.

Thus, these results suggest that very fine structural modifications, such as those promoted by enantiomeric ligands in bifunctional platinum(II) compounds, can modulate the “downstream effects” such as specific protein recognition by DNA-processing enzymes and other cellular components. More specifically, as a consequence of enantiomorphism of carrier amine ligands of cisplatin analogs their major DNA crosslinks not only exhibit different conformational features, but also are processed differently by cellular components. It has also been suggested that these differences are associated with a different antitumor and other biological activities of the two enantiomers. As such, the results expand our knowledge of how stereochemistry of the carrier amine moiety in antitumor platinum(II) compounds influences some crucial processes underlying their toxicity toward cancer cells

and provide a rational basis for the design of new platinum antitumor drugs and chemotherapeutic strategies.

Additional carrier ligands with aliphatic cyclic components have been also tested. One compound, which continues in phase II clinical trials and for which DNA reactions have been described, is lobaplatin (1,2-diaminomethylcyclobutane-platinum(II) lactate). This platinum drug is active in ovarian tumors in patients previously treated with cisplatin and/or carboplatin [121, 122]. Like cisplatin and oxaliplatin, lobaplatin also forms predominantly 1,2-GG and 1,2-AG intrastrand crosslinks in DNA [98]. Lobaplatin forms in cell-free media about four times less crosslinks than cisplatin. However, this difference is smaller in cells, suggesting enhancement of adduct formation by certain cellular mechanisms and/or compounds.

8.3.3

Other Analogs

One way to affect biological activity of cisplatin is to target this platinum compound to DNA differently by the attachment of the platinum moiety to a suitable carrier [123, 124]. Compared with untargeted analogs, such compounds may exhibit a different DNA-binding mode including sequence selectivity [125, 126]. Importantly, this change of the DNA-binding selectivity has already been shown to result in good *in vitro* and *in vivo* activity in tumors resistant to conventional agents. Thus, the objective of the research focused on this type of cisplatin analog is to design multifunctional molecules that bind DNA in predictable ways and that may have novel pharmacological activities.

8.3.3.1 Bidentate analogs

One class of platinum complexes targeted to DNA involves cisplatin linked to an intercalator. An example is (1,2-diaminoethane)dichloroplatinum(II) linked to acridine orange by trimethylene or hexamethylene chains (Fig. 8.7a) [127]. In the reaction with DNA, the platinum residues crosslink two base residues, while acridine orange is intercalated between the base pairs at a distance of one to two base pairs from the platinum-binding site.

The poor oral bioavailability properties of cisplatin or carboplatin were the impetus for a search for oral activity in platinum drugs. One compound selected for clinical evaluation from this class is also a bifunctional analog of cisplatin AMD473 [*cis*-amminedichloro(2-methylpyridine)platinum(II)] (Fig. 8.7b). AMD473 is less reactive toward the sulfur-containing molecules, such as methionine and thiourea [128]. The DNA-binding properties of AMD473 differ from those of cisplatin. On naked DNA, several adducts unique to AMD473 have been observed [128]. Within cells, AMD473 forms interstrand crosslinks much more slowly than cisplatin. An interesting finding is that antibodies elicited against DNA modified by AMD473 do not recognize DNA modified by cisplatin.

The idea of developing an orally available platinum drug has led to the design of antitumor platinum(IV) analogs [129, 130]. In addition, being inert to substitution,

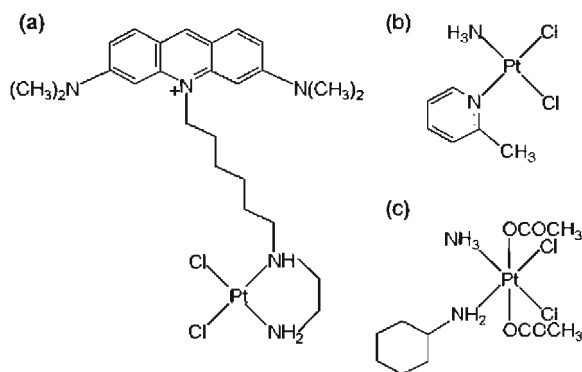


Fig. 8.7. Structures of targeted bifunctional analogs of cisplatin: (a) (1,2-diaminoethane)dichloroplatinum(II) linked to acridine orange by hexamethylene chain, (b)

cis-amminedichloro(2-methylpyridine) platinum(II) (AMD473), and (c) *bis*-acetatoamminedichloro(cyclohexylamine)platinum(IV) (JM216).

platinum(IV) complexes theoretically have the advantage of demonstrating fewer side effects than their platinum(II) counterparts. The oxidation state of the platinum atom in platinum coordination compounds determines the steric configuration of the molecule. Platinum(II) structures are planar molecules, while platinum(IV) derivatives assume an octahedral shape.

Octahedral platinum(IV) complexes undergo ligand substitution reactions that are slow relative to those of their platinum(II) analogs. They have been considered the compounds which are unable to react directly with DNA. The antitumor activity of platinum(IV) compounds has been suggested to require *in vivo* reduction to the kinetically more labile, and therefore reactive, platinum(II) derivatives [131–133]. Thus, platinum(IV) complexes are frequently designated as pro-drugs that have to be first, after their administration, activated by a reaction with reducing agents present in body liquids. On the other hand, mechanisms other than reduction to active platinum(II) species may be important for cytostatic efficiency of platinum(IV) drugs [134]. Several platinum(IV) analogs of cisplatin covalently bind simple nucleic acid bases or their monomeric derivatives [135, 136] or an isolated DNA [87, 137] directly without addition of any reducing agent. In contrast to cisplatin, however, the rate of the reaction of platinum(IV) complexes with DNA is markedly lower [87]. In addition, at the same level of the modification in cell-free media, thermal stability, renaturation and conformational alterations in DNA modified by the platinum(II) and platinum(IV) analogs are different [87].

One of the tetravalent analogs of cisplatin is also JM216 [*bis*-acetatoamminedichloro(cyclohexylamine)platinum(IV)] (Fig. 8.7c), the first orally administrable platinum complex that has entered clinical trials. The DNA-binding properties of JM216 on naked DNA or within tumor cells are similar to those of cisplatin, although there are some differences in the nature of the adducts [138]. JM216 forms interstrand crosslinks. As regards major intrastrand crosslinks, some differ-

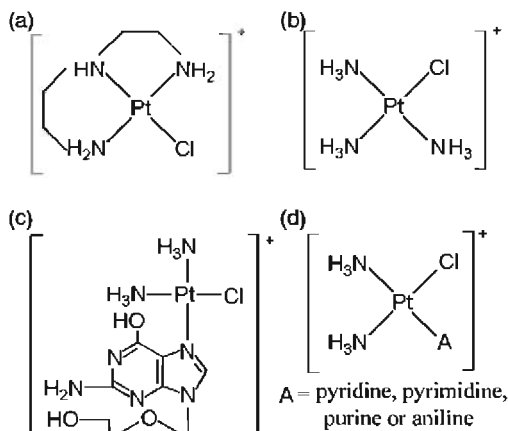


Fig. 8.8. Structures of platinum(II) triamine cations: (a) $[\text{PtCl}(\text{dien})]^+$, (b) $[\text{PtCl}(\text{NH}_3)_3]^+$, (c) $\text{cis-}[\text{PtCl}(\text{NH}_3)_2(\text{N7-ACV})]^+$, and (d) antitumor trisubstituted platinum(II) compounds containing pyridine, pyrimidine, purine, or aniline ligand.

ences between these adducts formed by JM216 and cisplatin may be deduced from the analysis by using antibodies elicited against DNA modified by cisplatin.

8.3.3.2 Monodentate analogs

Exploring new structural classes of platinum antitumor drugs has also resulted in the discovery of new platinum(II) complexes including those of formula $\text{cis-}[\text{PtCl}(\text{NH}_3)_2(\text{A})]^+$ (where A is a heterocyclic amine ligand) (Fig. 8.8d). Thus, these formally monofunctional complexes are analogs of cisplatin only containing one leaving chloride group, similar to closely related and simpler, but inactive, platinum–triamine complexes, such as $[\text{PtCl}(\text{dien})]\text{Cl}$ or $[\text{PtCl}(\text{NH}_3)_3]\text{Cl}$ (Fig. 8.8a,b). The adducts formed between double-helical DNA and $[\text{PtCl}(\text{dien})]\text{Cl}$ comprise mainly monofunctional adducts at N7 atoms of guanine residues [139]. These monofunctional adducts distort DNA and reduce its thermal stability in a sequence-dependent manner [28, 29, 140]. The most pronounced effects are observed if the platinated guanine residue is flanked by single pyrimidines on both 3' and 5' sides. Also importantly, the conformational distortion is always more pronounced in the flanking base pairs containing the base on the 5' site of the monofunctional adduct. These distortions disturb stacking interactions in double-helical DNA and make bases around the adduct more accessible. In addition, the monofunctional adduct also unwinds the duplex [30] – the $[\text{PtCl}(\text{dien})]\text{Cl}$ –DNA structure exhibits an unwinding angle of 6° , but no bending [28].

The DNA-binding mode of the new antitumor monodentate platinum compounds has been also intensively investigated. An interesting example of this class of platinum compounds is the trisubstituted platinum(II) compounds in which A = pyridine, pyrimidine, purine, piperidine or aniline (Fig. 8.8d) [141, 142]. These compounds have demonstrated activity against a number of murine tumors

and human tumor cell lines. These trisubstituted platinum(II) compounds form on DNA monofunctional adducts which have been suggested to be responsible for the antitumor activity of these agents. The monofunctional adducts of these compounds are capable of blocking DNA replication *in vitro* almost as efficiently as the major adducts of cisplatin, but are not recognized by cellular damaged DNA recognition proteins. The principal sites at which the replication is blocked are single guanines. Interestingly, the compound in which A = 4-methyl pyridine distorts double-helical DNA in the base pairs flanking the adduct on its 5' side due to significant contacts of the 4-methyl pyridine ring with the backbone of the DNA on the 5' side of the adduct, but there is no indication for the intercalation of the pyridine ring [143, 144]. Taken together, the platinum-triamines have been characterized as a new class of platinum anticancer agents which modify DNA differently from cisplatin. These differences have been proposed to be associated with different biological effects of these monofunctional compounds in comparison with cisplatin.

In the search for new, therapeutically more effective platinum drugs, platinum(II) compounds containing, as a part of the coordination sphere, certain selected antiviral nucleosides have been synthesized as well [145, 146]. Several compounds of this type exhibit similar or enhanced antiviral activities *in vitro* and in many instances are less toxic to normal cells than either component. A novel compound, $cis-[PtCl(NH_3)_2(N7-ACV)]^+$ has been synthesized [146] which contains antiviral acyclovir (ACV) in the coordination sphere of cisplatin (Fig. 8.8c). It exhibits activity against various herpes viruses and has been found to be as effective as cisplatin when equitoxic doses are administered *in vivo* to P388 leukemia-bearing mice. It has also been found to be active against a cisplatin-resistant subline of the P388 leukemia. $cis-[PtCl(NH_3)_2(N7-ACV)]^+$ binds to DNA forming monofunctional adducts preferentially at guanine sites. These adducts are capable of terminating DNA and RNA synthesis *in vitro* [147] and affect some structural and other physical properties of DNA in a way that is similar to those produced by the major adduct of cisplatin. It has been suggested that the ACV ligand itself interacts with DNA in a non-covalent manner, producing certain structural features similar to those of the major adduct of cisplatin. These could be recognized and further processed by some components of tumor cells in a fundamentally different way from other monofunctional platinum adducts such as $[PtCl(dien)]Cl$.

Whatever the detailed mechanism of biological activity of this new class of monodentate antitumor platinum(II) agents, these compounds are interesting from a mechanistic point of view and, therefore, worthy of additional testing.

8.4

Modification by Antitumor Analogs of Clinically Ineffective Transplatin

Since the discovery of the antitumor activity of cisplatin, none of the structural analogs of cisplatin which have advanced to clinical trials are likely to display novel clinical properties in comparison to the parent drug. Therefore, the search con-

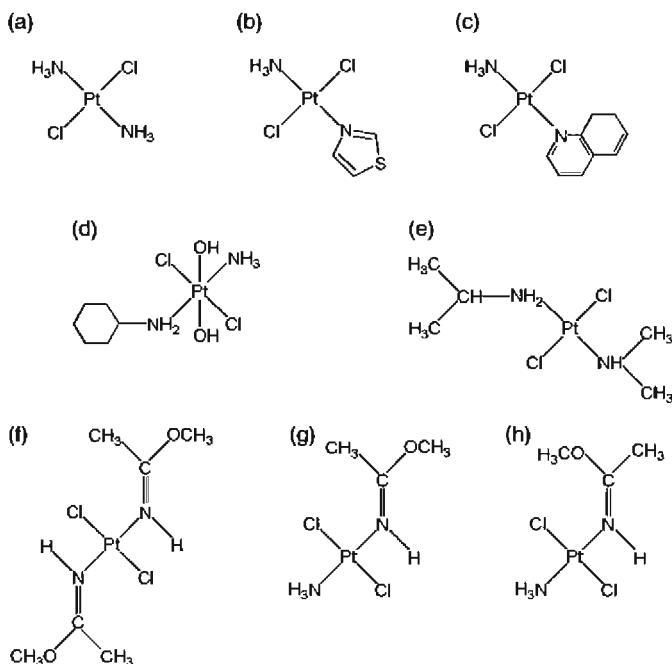


Fig. 8.9. Structures of transplatin and its antitumor analogs: (a) transplatin, (b) *trans*-[PtCl₂(NH₃)(thiazole)], (c) *trans*-[PtCl₂(NH₃)(quinoline)], (d) *trans*-amine(cyclohexylamine) dichlorodihydroxoplatinum(IV) (JM335), (e)

trans-[PtCl₂(dimethylamine)(isopropylamine)], (f) *trans*-[PtCl₂(*E*-iminoether)₂], (g) *trans*-[PtCl₂(NH₃)(*E*-iminoether)], and (h) *trans*-[PtCl₂(NH₃)(*Z*-iminoether)].

tinues for an improved platinum antitumor agent, motivated by the desire to design a less toxic compound that is non-cross-resistant with cisplatin or carboplatin. In this search the hypothesis that platinum drugs that bind to DNA in a fundamentally different manner to cisplatin will have altered pharmacological properties has been tested. This concept has already led to the synthesis of several new unconventional platinum antitumor compounds that violate the original structure–activity relationships. The clinical inactivity of transplatin (Fig. 8.9a) is considered a paradigm for the classical structure–activity relationships of platinum drugs [8], but to this end several new analogs of transplatin which exhibit a different spectrum of cytostatic activity, including activity in tumor cells resistant to cisplatin, have been identified.

The antitumor *trans*-platinum complexes whose DNA-binding mode will be reviewed in this section include three distinct series (Fig. 8.9): (1) analogs containing iminoether groups of general formula *trans*-[PtCl₂(*E*-iminoether)₂]; (2) analogs containing planar amine ligand of general structure *trans*-[PtCl₂(NH₃)(L)], where L = planar amine such as quinoline or thiazole; (3) analogs with asymmetric aliphatic ligands and *trans*-[PtCl₂(NH₃)(L)], where L = cyclohexylamine (CHA). As the DNA-binding mode of these new antitumor transplatin analogs is compared

with that of the parent inactive compound, we will summarize briefly the fundamental features of DNA modified by transplatin first.

8.4.1

Modifications by Transplatin

Structure–activity studies often employ inactive compounds such as transplatin (Fig. 8.9a). It has been widely used to investigate the mechanism of action of cisplatin. In this approach, differences between active and inactive compounds that may be responsible for the pharmacological effect are searched for. Hence, the clinical inactivity of the *trans* isomer of cisplatin is considered a paradigm for the classical structure–activity relationships of platinum drugs.

Transplatin–DNA adducts are mainly interstrand crosslinks (~12% in linear DNA) preferentially formed between guanine and complementary cytosine residues and a relatively large proportion (~50%) of adducts remain monofunctional [41, 148]. Intrastrand crosslinks between non-adjacent guanine residues or between guanine and either adenine or cytosine residues have been also deduced from the results of the analyses of isolated DNA incubated with transplatin at a relatively high extent (one or more Pt atoms per 100 nucleotides). However, it has been suggested [25, 149] that in cells transplatin forms only a small amount of interstrand crosslinks because of the slow closure of the monofunctional adducts coupled to their trapping by intracellular sulfur nucleophiles.

Transplatin also forms in DNA various types of adducts which affect DNA conformation less severely than the crosslinks of cisplatin. Monofunctional lesions, which are the major lesions, affect DNA conformation very little, like the adducts of [PtCl(dien)]Cl (see Section 8.3.3.2). The conformational alterations induced by the interstrand crosslinks are, however, much less severe than those induced by the crosslinks of cisplatin. The duplex is only slightly distorted on both sides of the crosslink but all bases are still paired and hydrogen bonded. The interstrand crosslink of transplatin unwinds the double helix by ~12° and induces a slight, flexible bending of ~20° of its axis toward minor groove [150–152]. The 1,3-intrastrand crosslink of transplatin, which is a less likely lesion in cells [149], unwinds the double helix by 45°, bends the DNA by 26°, and locally denatures, imparting a flexibility to the DNA which acts like a hinge joint without producing a directed bend [26, 153].

8.4.2

Analogs Containing Iminoether Groups

One class of the *trans*-platinum complexes that exhibit antitumor activity comprises analogs of transplatin in which NH₃ is substituted by iminoether ligands. The *trans*-[PtCl₂(*E*-iminoether)₂] complex (Fig. 8.9f) is not only more cytotoxic than its *cis* congener, but is also endowed with significant antitumor activity [154, 155] (iminoether = HN=C(OCH₃)CH₃; it can have either *E* or *Z* configuration depending on the relative position of OCH₃ and N-bonded Pt with respect to the C=N

double bond, *cis* in the *Z* isomer and *trans* in the *E* isomer). These results strongly imply a new mechanism of action for *trans*-[PtCl₂(*E*-iminoether)₂]. By analogy with the diamminedichloroplatinum(II) isomers, the inhibition of DNA synthesis by *trans*-[PtCl₂(*E*-iminoether)₂] implies a role for DNA binding in the mechanism of action. The presence of the iminoether group may result in altered hydrogen bonding and steric effects affecting the kinetics of DNA binding, the structures, and/or the stability of the adducts formed and resulting local conformational alterations in DNA. Bifunctional *trans*-[PtCl₂(*E*-iminoether)₂] preferentially forms stable monofunctional adducts at guanine residues in double-helical DNA even when DNA is incubated with the platinum complex for a relatively long time (48 h at 37°C in 10 mM NaClO₄) [156]. The random modification of natural DNA in cell-free media results in the non-denaturational alterations in the conformation of DNA similar to the modification by cisplatin, but differently from the modification by transplatin, which produces denaturational distortions in DNA [87]. The most striking feature of the lesions of *trans*-[PtCl₂(*E*-iminoether)₂] is that they prematurely terminate RNA synthesis at sites and with an efficiency similar to those of major DNA adducts of cisplatin [157]. This is a very interesting finding since the prevalent lesions formed on DNA by *trans*-[PtCl₂(*E*-iminoether)₂] are monofunctional adducts at guanine residues and monofunctional DNA adducts of other simpler platinum(II) complexes (such as those of [PtCl(dien)]Cl, cisplatin, transplatin) do not terminate RNA synthesis [22, 41]. In addition, it is generally accepted that monofunctional DNA adducts of cisplatin or transplatin are not relevant to the cytostatic effects of these metal complexes.

Recently, the short duplex containing the single, central monofunctional adduct of *trans*-[PtCl₂(*E*-iminoether)₂] at the guanine residue has been analyzed by NMR spectroscopy [158]. This analysis has yielded the model from which it is possible to roughly estimate the bending induced by the monofunctional adduct of *trans*-[PtCl₂(*E*-iminoether)₂]. The bending angle estimated in this way was ~45° toward the minor groove, i.e. in a direction opposite to that of the major 1,2-GG intrastrand crosslink of cisplatin. This result has been confirmed by studying the bending induced by single, site-specific monofunctional adduct of *trans*-[PtCl₂(*E*-iminoether)₂] in the oligodeoxyribonucleotide duplexes (19–22 bp) using electrophoretic retardation as a quantitative measure of the extent of planar curvature (O. Novakova, J. Kasparkova, J. Malina, G. Natile, V. Brabec, unpublished results). The bending angle estimated by this phasing assay was ~20° toward the minor groove. Hence, the distortion induced in DNA by the monofunctional adduct of *trans*-[PtCl₂(*E*-iminoether)₂] is apparently distinctly different from that produced in DNA by major 1,2-GG intrastrand crosslink of antitumor cisplatin. Then, it is also not surprising that HMG-domain proteins have no affinity to the monofunctional adduct of *trans*-[PtCl₂(*E*-iminoether)₂]. On the other hand, this adduct is removed from DNA by NER with an efficiency similar to that of 1,2-GG intrastrand crosslink of cisplatin. However, on the basis of the analogy with the mechanism of antitumor activity of cisplatin, the NER of *trans*-[PtCl₂(*E*-iminoether)₂] monofunctional adducts should be blocked, allowing the damage to persist.

As HMG-domain proteins do not bind to *trans*-[PtCl₂(*E*-iminoether)₂] lesion, it

is reasonable to expect that a pathway different from that including non-covalent binding of damaged DNA-binding proteins is effective in blocking NER of the *trans*-[PtCl₂(*E*-iminoether)₂] lesions. Importantly, monofunctional adducts formed by *trans*-[PtCl₂(*E*-iminoether)₂] on DNA can crosslink various types of proteins (including histone H1) and this crosslinking markedly enhances the efficiency of monofunctional adducts of *trans*-[PtCl₂(*E*-iminoether)₂] to terminate DNA and RNA synthesis *in vitro* (O. Novakova, J. Kasparkova, J. Malina, G. Natile, V. Brabec, unpublished results). In addition, the crosslinking of proteins to DNA monofunctional adducts of *trans*-[PtCl₂(*E*-iminoether)₂] blocks NER of these lesions, suggesting a different mechanism for “repair shielding” of genotoxic *trans*-[PtCl₂(*E*-iminoether)₂] adducts. Unique properties of monofunctional DNA adducts of *trans*-[PtCl₂(*E*-iminoether)₂] and mainly their ability to crosslink proteins might be of fundamental importance in explaining the anticancer activity of this class of *trans*-platinum(II) complexes.

Recently, in order to contribute further to our understanding of the DNA-binding mode of *trans*-platinum complexes with iminoether ligands, DNA modifications by the complexes only containing one iminoether group have been examined [159]. These complexes were *trans*-[PtCl₂(NH₃)(*Z*-iminoether)] and *trans*-[PtCl₂(NH₃)(*E*-iminoether)] (mixed *Z* and mixed *E*, respectively) (Fig. 8.9g,h). In a panel of human tumor cell lines, both mixed *Z* and mixed *E* show a cytotoxic potency higher than that of transplatin and mixed *Z* is more active and less toxic than mixed *E*. In the reaction with DNA in a cell-free medium, mixed *Z* forms monofunctional adducts that do not evolve into intrastrand crosslinks but close slowly into interstrand crosslinks between complementary guanine and cytosine residues. These interstrand crosslinks behave as hinge joints, increasing the flexibility of the DNA double helix. These data demonstrate that several features of DNA-binding mode of the antitumor-active mixed *Z* are very similar to those of transplatin, which suggests that the clinical inactivity of transplatin does not depend only on its specific DNA-binding mode.

8.4.3

Analogues Containing Planar Amine Ligand

The *trans*-platinum compounds of this class that have been studied can be grouped into three distinct series of the general structure *trans*-[PtCl₂(L)(L')]: (1) L = L' = pyridine or thiazole; (2) L = quinoline and L' = substituted sulfoxide R'R''SO, R' = CH₃, CH₂Ph, Ph; and (3) L = quinoline, thiazole, or pyridine and L' = NH₃ [160]. There are two major chemical differences between NH₃ and a planar ligand. Steric hindrance by the appropriately positioned H atoms of the ring system reduces chemical reactivity and surface and stacking interactions become more pronounced over the hydrogen bonding associated with NH₃ groups. In this section the results of several biophysical, biochemical, and molecular modeling studies of DNA modifications by antitumor *trans*-[PtCl₂(NH₃)(quinoline)] or *trans*-[PtCl₂(NH₃)(thiazole)] (Fig. 8.9b,c) are described as examples [161, 162].

trans-[PtCl₂(NH₃)(quinoline)] or *trans*-[PtCl₂(NH₃)(thiazole)] bind monofunctionally to DNA with a rate similar to that of transplatin. The overall rate of the rearrangement to bifunctional adducts is also similar to that observed in the case of DNA modification by transplatin, i.e. it is relatively slow (after 48 h about 34% of adducts remain monofunctional). In contrast to transplatin, however, its analogs containing the planar quinoline or thiazole ligand form considerably more inter-strand crosslinks after 48 h (~30%) with a much shorter half-time (~5 h) (~12% for transplatin, $t_{1/2} > 11$ h [20, 41]). In addition, the planar ligand in all or in a significant fraction of DNA adducts of these transplatin analogs, in which platinum is coordinated by base residues, is well positioned to interact with the duplex.

The adducts of the transplatin analogs containing the planar quinoline or thiazole ligand terminate *in vitro* RNA synthesis preferentially at guanine residues. Interestingly, DNA modified by the *trans*-platinum compounds containing the planar ligand is recognized by the antibodies that specifically recognize DNA modified by cisplatin, which suggests that these transplatin analogs behave in some respects like cisplatin. Models for both monofunctional adducts and bifunctional interstrand crosslinks have been proposed. Computer-generated models show that the combination of monofunctional covalent binding and a stacking interaction between the planar ligand and the DNA bases can produce a kink in the duplex which is strongly suggestive of the directed bend produced by the major cisplatin-DNA adduct (1,2-GG intrastrand crosslink).

Further investigations have been focused on the analysis of short duplexes containing the single site-specific adduct of *trans*-[PtCl₂(NH₃)(thiazole)] (Fig. 8.9b) (J. Kasparkova, N. Farrell, V. Brabec, unpublished results). The monofunctional adduct creates a local conformational distortion, which is localized to five base pairs around the adduct and includes a stable curvature (34° toward major groove) and unwinding (13°). Hence, this distortion is very similar to that produced in DNA by major 1,2-GG intrastrand crosslink of antitumor cisplatin. In addition, these monofunctional adducts are recognized by HMGB1-domain proteins with an affinity similar to that of the 1,2-GG intrastrand crosslink of cisplatin. The effective removal of these adducts from DNA by NER has been also observed, but it is inhibited if the NER system is supplemented by HMGB1a or full-length HMGB1 proteins. Structural properties, recognition by HMG-domain proteins and NER of monofunctional adducts of [PtCl(dien)]Cl or *trans*-[PtCl₂(NH₃)(thiazole)] on the one hand and 1,2-GG intrastrand crosslink of cisplatin on the other are compared in Tab. 8.1.

Additional work has shown [162], using Maxam–Gilbert footprinting, that *trans*-[PtCl₂(NH₃)(quinoline)] and *trans*-[PtCl₂(NH₃)(thiazole)] preferentially form DNA interstrand crosslinks between guanine residues at the 5'-GC/5'-GC sites. Thus, DNA interstrand crosslinking by transplatin analogs containing the planar ligand is formally equivalent to that by antitumor cisplatin, but different from clinically ineffective transplatin which preferentially forms these adducts between complementary guanine and cytosine residues [41].

These results have shown for the first time that the simple chemical modification of structure of an inactive platinum compound alters its DNA-binding mode

Tab. 8.1. Comparison of the structural properties, recognition by HMG-domain proteins and NER of monofunctional adducts of [PtCl(dien)]Cl and *trans*-[PtCl₂(NH₃)(thiazole)] with 1,2-GG intrastrand crosslink of cisplatin (see Figs 8.1, 8.8a, and 8.9b for structures of the platinum complexes)

Properties	Monofunctional adduct of [PtCl(dien)]Cl	Monofunctional adduct of <i>trans</i>-[PtCl₂(NH₃)(thiazole)]^d	1,2-GG intrastrand crosslink of cisplatin
Bending	No ^b	34° toward major groove	32–34° toward major groove ^{f,g}
Unwinding	6° ^c	12° ⁱ	13° ^{d,h}
Recognition by:			
HMGB1a ^a	No ^d	Yes, $K_{D(\text{app})} \sim 38.5 \text{ nM}^c$	Yes, $K_{D(\text{app})} \sim 30.8 \text{ nM}^a$
HMGB1b ^a	No ^d	Yes, $K_{D(\text{app})} \sim 2.1 \text{ }\mu\text{M}^c$	Yes, $K_{D(\text{app})} \sim 1.9 \text{ }\mu\text{M}^a$
Mammalian NER	No ^d	Yes	Yes ^{a,i}

^aThe results reported here were obtained with the same samples of the domain A and domain B of HMGB1 protein under identical conditions [178].

^bBrabec *et al.*, 1992 [28].

^cKeck and Lippard, 1992 [30].

^dKasparkova, J., Farrell, N., Brabec, V., unpublished results.

^eApparent dissociation constants, $K_{D(\text{app})}$, were estimated in the same way as described [186].

^fRice *et al.*, 1988 [187].

^gBellon *et al.*, 1990 [26].

^hBellon *et al.*, 1991 [188].

ⁱZamble *et al.*, 1996 [44].

into that of an active drug and that the processing of the monofunctional DNA adducts of these *trans*-platinum analogs in tumor cells may be similar to that of the major bifunctional adducts of “classical” cisplatin.

8.4.4

Other Analogs

Recently, the DNA-binding mode of a new cytotoxic *trans*-platinum compound containing different aliphatic amines (*trans*-[PtCl₂(dimethylamine)(isopropylamine)]) (Fig. 8.9c) has been evaluated in cell-free media [163]. *trans*-[PtCl₂(dimethylamine)(isopropylamine)] readily forms DNA interstrand crosslinks. The number of these crosslinks is considerably higher than the number formed under the same conditions by transplatin or cisplatin. In addition, the compound exhibits a preferential binding affinity to alternating purine–pyrimidine sequences in DNA, forms the adduct that inhibits the B–Z transition in DNA, and blocks DNA synthesis *in vitro* more efficiently than the adducts of cisplatin. These particular DNA-binding properties have been proposed to be related to the efficiency of *trans*-[PtCl₂(dimethylamine)(isopropylamine)] in inducing apoptosis and some selective killing in an *H-ras* overexpressing cell line [163–165].

The search for a clinically useful orally available antitumor platinum drug also led to the design of several platinum(IV) compounds with *trans* geometry of leaving ligands [129]. An example is JM335 [*trans*-amine(cyclohexylamine) dichlorodihydroxoplatinum(IV)] (Fig. 8.9d). Mechanistic studies with JM335 in carcinoma cell lines [138] have revealed DNA-binding properties different from those of cisplatin. JM335 induces single-strand breaks in DNA (formation of this DNA lesion is cell line-dependent) and interstrand crosslinks. Interestingly, DNA extracted from cells treated with JM335 is not recognized by an antibody elicited against DNA modified by cisplatin. In addition, the kinetics of apoptosis is more rapid for JM335 than for its *cis* isomer. However, JM335 is generally less cytotoxic than cisplatin, probably due to its inactivation by thiols, so that it has not been selected for clinical trials. Nevertheless it remains one of the more interesting and active *trans*-platinum complexes.

8.5

Modifications by Polynuclear Platinum Antitumor Drugs

Polynuclear platinum compounds comprising di- or trinuclear platinum centers linked by variable length diamine chains constitute another new class of antitumor drugs with chemical and biological properties different from cisplatin. The first drug of this class which already entered phase II clinical trials in cancer patients is the trinuclear compound [$\{trans\text{-PtCl}(\text{NH}_3)_2\}_2\mu\text{-trans-Pt}(\text{NH}_3)_2\{\text{H}_2\text{N}(\text{CH}_2)_6\cdot\text{NH}_2\}_2\}^{4+}$] (designated as BBR3464) (Fig. 8.10b). Notable chemical features of this

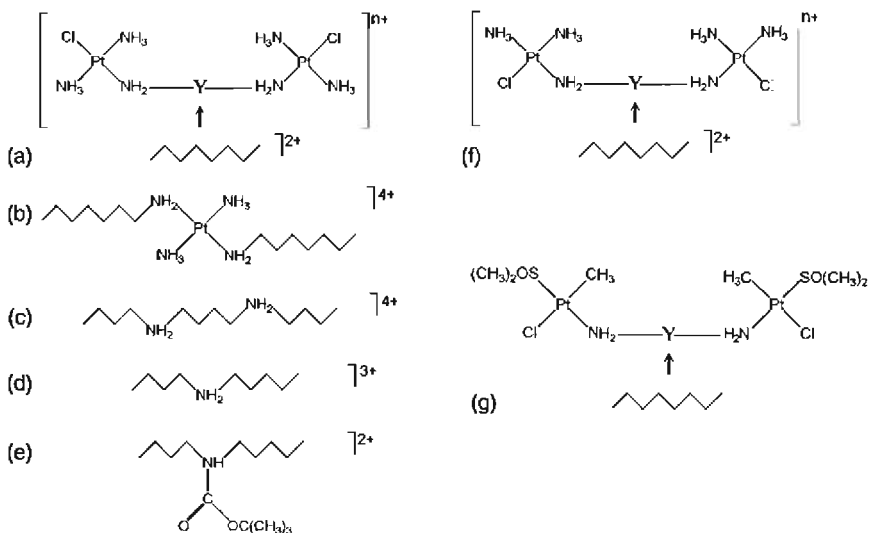


Fig. 8.10. Structures of dinuclear (a, c–g) and trinuclear (b) platinum(II) compounds. (a) 1,1/*t,t*, (b) 1,0,1/*t,t,t* (BBR3464), (c) 1,1/*t,t*-spermine, (d) 1,1/*t,t*-spermidine, (e) 1,1/*t,t* BOC-spermidine, (f) 1,1/*c,c*, and (g) ORGANOBisPt.

compound that differ from those of cisplatin or other antitumor mononuclear counterparts are the 4+ charge and the bifunctional binding character, where the binding sites are separated by large distance. Initial clinical trials have revealed activity in pancreatic, lung, and melanoma cancers, suggesting the complementary clinical anticancer activity of BBR3464 in comparison to cisplatin. Preclinical studies have shown cytotoxicity of BBR3464 at 10-fold lower concentration than cisplatin and collateral sensitivity in cisplatin-resistant cell lines [166–169]. Importantly, BBR3464 also displays consistently high antitumor activity in human tumor xenografts characterized as mutant p53. This important feature suggests that the new agent may find utility in the over 60% of cancer cases where mutant p53 status has been indicated. DNA damage by chemotherapeutic agents is in many cases mediated through the p53 pathway. Consistently, cytotoxicity displayed in mutant cell lines would suggest an ability to bypass this pathway.

BBR3464 has been designed on the basis of systematic studies on various dinuclear compounds. The history of the development of this class of platinum drugs has been recently thoroughly reviewed [170, 171] so that here only the major features of DNA-binding modes of dinuclear and trinuclear compounds capable of bifunctional binding will be described. DNA interactions in cell-free media of the compounds, which are the agents most closely related to trinuclear BBR3464, will be summarized first. Then the focus will be on studies of DNA modifications by BBR3464.

8.5.1

Dinuclear Compounds

Exploring new structural classes of platinum antitumor drugs resulted in the discovery of dinuclear bis(platinum) complexes with equivalent coordination spheres with the single chloride leaving group on each platinum linked by a variable-length alkanediamine chain, represented by the general formula $[\{\text{PtCl}(\text{NH}_3)_2\}_2 \cdot (\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}_2)]^{2+}$ ($n = 2-6$). The leaving chloride ligands are either *cis* (1,1/*c,c*) or *trans* (1,1/*t,t*) (Fig. 8.10a,f). These dinuclear platinum complexes exhibit antitumor activity *in vitro* and *in vivo* comparable with that of cisplatin, but importantly they retain activity in acquired cisplatin-resistant cell lines [171]. However, the 1,1/*c,c* complexes have been shown to be less efficient in overcoming cisplatin resistance than their 1,1/*t,t* counterparts. This situation represents a fundamental difference between mononuclear and dinuclear platinum chemistry and biology – in the mononuclear case cisplatin is active, while its direct isomer transplatin is antitumor-inactive. These differences in biological activity between dinuclear and mononuclear platinum complexes on the one hand and the differences between the dinuclear compounds themselves on the other have provided the impetus for the studies of molecular mechanisms underlying these differences.

Like cisplatin or carboplatin, the dinuclear platinum complexes bind to DNA and inhibit DNA replication and transcription, which indicates that DNA modification by dinuclear platinum complexes plays an important role in the mechanism of their biological action. Initial studies have already shown some significant differ-

ences in DNA modification in cell-free media by individual dinuclear platinum complexes and cisplatin. 1,1/*t,t* isomers bind to DNA more readily than 1,1/*c,c* complexes. Both isomers unwind globally modified DNA by 10–12° (i.e. similar to cisplatin). In contrast to cisplatin, both dinuclear isomers induce the *B*–*Z* transition in poly(dG–dC).poly(dG–dC) [172] and preferentially form interstrand crosslinks in DNA, the 1,1/*c,c* isomer being more efficient [173, 174]. 1,1/*t,t* complexes have also been shown to form minor 1,2-GG intrastrand crosslinks, producing a flexible, non-directional bend in DNA [175]. Intrastrand crosslinks have not been observed in double-helical DNA if it is modified by 1,1/*c,c*. A more recent study [176] has investigated the reasons underlying the observed inability of 1,1/*c,c* complexes to form a 1,2-GG intrastrand crosslink with double-helical DNA. ¹H NMR spectroscopy of samples of very short single-stranded di- or tetranucleotides containing the GG sequence modified by 1,1/*c,c* has provided evidence for restricted rotation around the 3' G in single-stranded 1,2-GG intrastrand crosslinks of 1,1/*c,c*. This steric hindrance, not present in 1,2-GG intrastrand crosslinks of 1,1/*t,t* complexes, has been suggested to be responsible for the inability of 1,1/*c,c* complexes to form 1,2-GG intrastrand crosslinks with sterically more demanding double-helical DNA.

Interesting results have been obtained when modifications of natural DNA by the dinuclear platinum(II) organometallic complex [$\{\text{PtCH}_3\text{Cl}((\text{CH}_3)_2\text{SO})\}_2(\mu\text{-N-N})$] (N–N = $\text{H}_2\text{N}(\text{CH}_2)_6\text{NH}_2$) (ORGANObisPt, Fig. 8.10g) were investigated [177]. The complex binds irreversibly to DNA, but its DNA-binding mode is different from that of the formally equivalent 1,1/*c,c* (Fig. 8.10f). Interestingly, ORGANObisPt binds to DNA considerably faster than 1,1/*c,c* and cisplatin. In addition, when ORGANObisPt binds to DNA it exhibits a strong base sequence specificity to guanine residues. ORGANObisPt forms on double-helical DNA mainly monofunctional adducts and also a small amount of DNA interstrand crosslinks (~2%), i.e. a radically smaller amount in comparison with the complex 1,1/*c,c*. Importantly, these interstrand crosslinks of ORGANObisPt are capable of terminating RNA synthesis *in vitro* while its major monofunctional adducts are not. In addition, the adducts of ORGANObisPt affect conformation of DNA, but in a different way than its dinuclear analog 1,1/*c,c* or cisplatin. Thus, these results demonstrate that some structural features of ORGANObisPt, such as the charge or nature of the *trans* and *cis* activating groups relative to the labile chloride, are responsible for the altered DNA-binding mode and biological activity in comparison with the 1,1/*c,c* compound.

More recent studies have been mainly focused on structural details of major interstrand crosslinks formed by 1,1/*c,c* and 1,1/*t,t* in DNA [174, 178]. The interstrand crosslinks of 1,1/*c,c* are preferentially formed between guanine residues. Besides 1,2 interstrand crosslinks (between guanine residues in neighboring base pairs), 1,3 or 1,4 interstrand crosslinks are also possible. In the latter two long-range adducts, the sites involved in the crosslinks are separated by one or two base pairs. 1,2, 1,3, and 1,4 interstrand crosslinks are formed at a similar rate and are preferentially oriented in the 5' → 5' direction. In addition, the DNA adducts of these complexes inhibit DNA transcription *in vitro*.

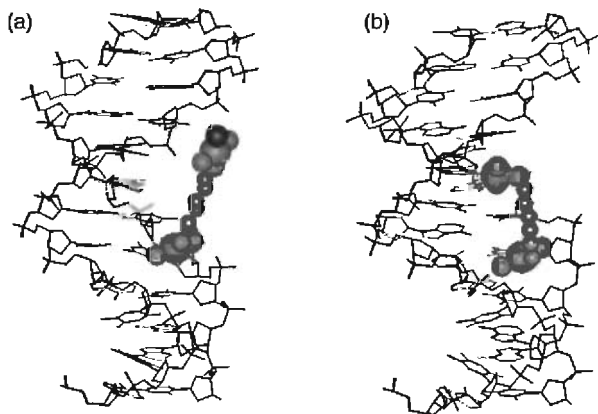


Fig. 8.11. NMR structures of DNA duplexes containing a monofunctional (a) or 1,4-GG interstrand adduct (b) of dinuclear 1,1/*t,t*. Images of the self-complementary duplexes 5'-d(ATATG*TACATAT)/5'-d(ATATGTACATAT) (a) and 5'-d(ATATG*TACATAT)/5'-

d(ATATG*TACATAT) (b), where the asterisk represents the sites involved in the adducts of 1,1/*t,t* crosslink [179]. Colors: black, thymine; orange, adenine; green, cytosine; red, guanine; yellow, platinum atom; blue, nitrogen atom; gray, carbon atom; green, chlorine atom.

The properties of the interstrand crosslinks formed in DNA by 1,1/*t,t* complexes have been investigated more thoroughly [178]. Preferential DNA-binding sites in these lesions are G residues in the base pairs separated by at least one other base pair. The crosslinks between G residues in neighboring base pairs are formed with a pronouncedly slower rate than long-range 1,3 or 1,4 crosslinks. Importantly, the length of the diamine bridge linking the two platinum units does not appear to be a substantial factor affecting DNA interstrand crosslinking by the bifunctional dinuclear platinum compounds. As in the case of the interstrand crosslinks of 1,1/*c,c* [174], these lesions of 1,1/*t,t* complexes are also preferentially formed in the 5' → 5' direction so that this feature of the interstrand crosslinking might be common for this class of dinuclear platinum compound. The reasons for this preference in the orientation of DNA interstrand crosslinks of dinuclear compounds are unknown.

The conformational distortions induced in DNA by the major 1,3- or 1,4-interstrand crosslinks of 1,1/*t,t* have been further evaluated more in detail (Fig. 8.11) [178, 179]. Interestingly, this lesion results in only a very small directional bending of helix axis (~10°), duplex unwinding (9°), and the conformational flexibility of the crosslink, a feature expected to contribute to the distinct differences in biological profile between dinuclear and mononuclear platinum antitumor drugs.

HMG-domain proteins play a role in sensitizing cells to cisplatin (see Sections 8.2.4.1 and 8.2.5). An important structural motif recognized by HMG-domain proteins on DNA modified by cisplatin is a stable, directional bend of the helix axis. The major interstrand crosslinks and even minor 1,2-d(GpG) intrastrand crosslinks of 1,1/*t,t* compounds bend the helix axis much less efficiently than the crosslinks of cisplatin [175, 178]. Therefore, it is not surprising that very weak or no recogni-

tion of DNA adducts of 1,1/*t,t* complexes by HMG1 protein has been observed [178]; this is consistent with the assumption that an important structural motif recognized by HMG-domain proteins is bent or kinked duplex axis. The affinity of HMG-domain proteins to the duplex containing the 1,2-GG intrastrand crosslink of cisplatin is sequence-dependent and is reduced with increasing destabilization of the duplex due to the crosslink. Thus, a lack of an affinity of the minor 1,2-GG intrastrand crosslink of 1,1/*t,t* to HMG-domain proteins is also consistent with the observation that this lesion reduces the thermal and thermodynamical stability of DNA markedly more than the same lesion of cisplatin [180]. Thus, it is clear that the major DNA adducts of antitumor 1,1/*t,t* compounds may present a block to DNA or RNA polymerase [173, 181] but are not a substrate for recognition by HMG-domain proteins. From these considerations it can be concluded that the mechanism of antitumor activity of bifunctional dinuclear platinum complexes does not involve recognition by HMG-domain proteins as a crucial step, in contrast to the proposals for cisplatin and its direct analogs (see Section 8.2.5).

One possible role for binding of HMG-domain proteins to DNA modified by cisplatin is that these proteins shield damaged DNA from intracellular NER (see Section 8.2.5). No recognition of the 1,2 intrastrand crosslink of 1,1/*t,t* by HMG1 proteins has been noticed [178], but effective removal of these adducts from DNA by NER has been observed [66]. These results suggest that the processing of the intrastrand crosslinks of 1,1/*t,t* in tumor cells sensitive to this drug may not be relevant to its antitumor effect. Major adducts formed in DNA by 1,1/*t,t* compounds are, however, interstrand crosslinks, which are, in general, believed to be repaired much less easily than intrastrand adducts (see also Section 8.2.3 and reference [20]). Thus, 1,3- or 1,4-interstrand crosslinks of 1,1/*t,t* are not removed in the *in vitro* assay using mammalian and rodent cell-free extracts capable of removing the 1,2-intrastrand crosslinks of cisplatin (J. Kasparkova, N. Farrell, V. Brabec, unpublished results). Hence, the major DNA adducts of bifunctional dinuclear platinum compounds do not have to be shielded by damaged DNA-recognition proteins, such as those containing HMG domains, to prevent their repair.

As mentioned above, addition of the central tetraamine(platinum) unit as in BBR3464 (Fig. 8.10b) greatly enhances the cytotoxicity and antitumor activity in comparison to the prototype dinuclear compound linked by a simple diamine as in 1,1/*t,t* or 1,1/*c,c*. In examining structure–activity relationships within this class of compounds a logical step was a substitution of the central tetraamine platinum unit by other H-bonding groups that would retain antitumor activity. Designed linear polyamine-linked dinuclear platinum complexes display the same biological profile as BBR3464 and represent a further subclass of dinuclear compounds with promising preclinical activity [170].

The DNA-binding profiles of three bifunctional dinuclear platinum(II) polyamine-linked compounds, $[[trans-PtCl(NH_3)_2]_2\{\mu\text{-spermine-}N^1,N^{12}\}]^{4+}$, $[[trans-PtCl(NH_3)_2]_2\{\mu\text{-spermidine-}N^1,N^8\}]^{3+}$, and $[[trans-PtCl(NH_3)_2]_2\{\mu\text{-BOC-spermidine}\}]^{2+}$ (1,1/*t,t*-spermine, 1,1/*t,t*-spermidine, and 1,1/*t,t* BOC-spermidine, respectively in Fig. 8.10c–e), have been evaluated [182]. All of the compounds bind preferentially in a bifunctional manner. The kinetics of binding of these compounds corresponds to their relative high charge (2+ to 4+). The preference for

the formation of interstrand crosslinks, however, does not follow a charge-based pattern. The preferred sites of binding of the dinuclear polyamine-linked compounds are similar to their trinuclear BBR3464 counterpart (see Section 8.5.2), and charge differences do not contribute solely to the variances between the compounds.

8.5.2

Trinuclear Compound

The identification of bifunctional trinuclear BBR3464 (Fig. 8.10b) as the first polynuclear platinum compound used in the clinic was also the impetus for its DNA-binding studies [183]. The high charge on BBR3464 facilitates rapid binding to DNA with a $t_{1/2}$ of ~ 40 min, significantly faster than the neutral cisplatin. The melting temperature of DNA adducted by BBR3464 increases at low ionic strength but decreases in high salt for the same level of the modification. This unusual behavior is in contrast to that of cisplatin. BBR3464 produces an unwinding angle of 14° in negatively supercoiled pSP73 plasmid DNA, indicative of bifunctional DNA binding. Quantitation of interstrand DNA crosslinking in linearized plasmid DNA has indicated approximately 20% of the DNA to be interstrand crosslinked. While this is significantly higher than the value for cisplatin, it is, interestingly, lower than that for dinuclear platinum compounds such as 1,1/*t,t* or 1,1/*c,c* (Tab. 8.2). Either the presence of charge in the linker backbone or the increased distance between platinating moieties may contribute to this relatively decreased ability of BBR3464 to induce DNA interstrand crosslinking. Moreover, BBR3464 rapidly forms long-range delocalized lesions on DNA with sequence selectivity and strong sequence preference for single dG or d(GG) sites. By choosing an appropriate sequence on the basis of sequence-specificity studies, molecular modeling on 1,4-GG interstrand and 1,5-GG intrastrand crosslinks has further confirmed the similarity in energy between the two forms of crosslink [183].

Finally, immunochemical analysis, which has shown that antibodies raised to cisplatin-adducted DNA do not recognize DNA modified by BBR3464, has confirmed the unique nature of the DNA adducts formed by BBR3464. In contrast, DNA modified by BBR3464 inhibits the binding of antibodies raised to transplatin-adducted DNA. Thus, the bifunctional binding of BBR3464 contains few similarities to that of cisplatin, but may have a subset of adducts recognized as similar to transplatin.

We examined how the structures of the various types of the crosslinks of BBR3464 affect conformational properties of DNA and how these adducts are recognized by HMGB1 protein and removed from DNA during *in vitro* NER reactions. The first analyses [184] focused on the 1,2-GG and other long-range intrastrand crosslinks between guanine residues and revealed that these lesions create a local conformational distortion, but that none of these crosslinks results in a stable curvature. In addition, we have observed no recognition of these crosslinks by HMGB1 proteins, but we have observed effective removal of these adducts from DNA by NER. Thus, as in the case of the intrastrand crosslinks of dinuclear platinum compounds (see Section 8.5.1), the processing of the intrastrand crosslinks of

Tab. 8.2. Summary of the structural properties and DNA-binding characteristics of dinuclear platinum compounds and trinuclear BBR3464 (see Figs 8.1 and 8.10 for structures).

Properties	Cisplatin	1,1/t,t (n = 6)	1,1/t,t BOC- spermidine	1,1/t,t- spermidine	1,1/t,t- spermine	1,0,1/t,t,t (n = 6,6)
Reactive Pt–Pt distance (Å)	N/A	12.4	14.9	14.9	21.1	25.3
Linker charge	N/A	0	0	1+	2+	2+
H bonding in linker	N/A	No	No	Yes	Yes	Yes
Total charge	0	2+	2+	3+	4+	4+
DNA binding (t _{1/2} , min)	~240 ^b	200–300 ^h	9.2 ^j	8.2 ^j	2.8 ^j	40 ^c
Unwinding angle/adduct (degrees)	13 ^c	10–14 ^c	13 ^j	12 ^j	15 ^j	14 ^c
Decrease of EtBr fluorescence ^a	Medium ^{d,e}	Strong ^c	Strong ^j	Strong ^j	Strong ^j	Strong ^c
% interstrand crosslink/adduct at $\eta_b = 2 \times 10^{-4}$	6 ^f	70–90 ^e	74 ^j	40 ^j	57 ^j	20 ^c
Affinity of the major adducts to HMG proteins	Strong ^g	Weak ⁱ	ND	ND	ND	No ^k

^a Ethidium bromide (EtBr) is used to characterize perturbations induced in DNA by bifunctional adducts of platinum compounds [183, 189, 190]. Delocalization of these distortions due to long-range crosslinks reduces EtBr intercalation into DNA and consequently its fluorescence more than monofunctional adducts or crosslinks between neighboring base pairs.

^b Bancroft *et al.*, 1990 [11].

^c Keck and Lippard, 1992 [30].

^d Kasparkova *et al.*, 1999 [174].

^e Brabec *et al.*, 1999 [183].

^f Brabec and Leng, 1993 [41].

^g Ohndorf *et al.*, 1999 [56].

^h Zaludova *et al.*, 1997 [173].

ⁱ Kasparkova *et al.*, 2000 [178].

^j McGregor *et al.*, 2002 [182].

^k Kasparkova *et al.*, 2002. unpublished results.

BBR3464 in tumor cells sensitive to this new drug may not be relevant to its anti-tumor effect.

The analysis of long-range interstrand crosslinks formed by BBR3464 in DNA (J. Zehnulova, J. Kasparkova, N. Farrell, V. Brabec, unpublished results) has demonstrated that these lesions distort DNA only weakly and that these lesions are not recognized by DNA-damaged binding proteins, including those containing HMG domains. On the other hand, the interstrand crosslinks are not removed from DNA in *in vitro* NER reactions and are not recognized by XPA and RPA proteins, i.e. the major damage-recognition proteins involved in the early stage of NER (see Section 8.2.4.2). Thus, as in the case of the dinuclear complex 1,1/t,t, long-range interstrand crosslinks rather than intrastrand adducts appear to be more likely

candidates for the genotoxic lesion relevant to the antitumor effects of BBR3464. An intriguing feature of interstrand crosslinking efficiency of BBR3464 is its ability to form crosslinks in the 3' → 3' direction (J. Zehnulova, J. Kasparkova, N. Farrell, V. Brabec, unpublished results), confirming an ability of this agent to form more types of adducts than the dinuclear counterparts. Thus, these results are consistent with the view that the presence of charge and hydrogen-bonding capacity within the central linker of BBR3464 in the form of a tetraamineplatinum(II) moiety could result in significant pre-association prior to covalent binding, which may affect the extent and direction of interstrand crosslinks.

An important feature of the antitumor efficacy of BBR3464 is also the hypersensitivity of human tumors with mutant p53 to this drug. It has been suggested that apoptosis induced in tumor cells by BBR3464 is not mediated by p53 and that “bypassing” of the p53 pathway may have its origin in the novel DNA-binding mode of this trinuclear platinum agent [170]. In connection with this suggestion the results of our recent studies on the effect of DNA adducts of BBR3464 on the binding of active p53 protein to DNA (J. Kasparkova, S. Pospisilova, N. Farrell, V. Brabec, unpublished results) appear particularly interesting. The adducts of BBR3464 inhibit binding of p53 to the consensus sequence with an efficiency similar to that exhibited by DNA adducts of cisplatin (see Section 8.2.4.2). On the other hand, no adduct of BBR3464 increases the binding affinity of p53 to DNA lacking the consensus sequence. This is in fundamental contrast to cisplatin, whose major 1,2-intrastrand crosslinks increase binding affinity of p53 to DNA lacking consensus sequence [82]. On average, cells with mutant p53 are more resistant to the effects of cisplatin [78], whereas BBR3464 maintains activity in several tumors with mutant p53 with both acquired and inherent resistance to cisplatin [185]. As the DNA-binding activity of p53 protein is crucial to its tumor suppressor function, the plausible explanation for different sensitivities of human tumors with mutant p53 to BBR3464 and cisplatin may be the different efficiency of the adducts of these drugs to increase the binding affinity of DNA lacking the consensus sequence to p53. Confirmation of this hypothesis remains an exciting and potentially significant area of research aimed at further understanding mechanisms underlying the unique antitumor properties of polynuclear platinum agents.

In summary, BBR3464 and antitumor dinuclear bifunctional platinum agents form on DNA adducts which are clearly not those found for cisplatin (Tab. 8.2). Hence, these compounds exhibit a unique profile of DNA binding, strengthening the original hypothesis that modification of DNA binding in manners distinct from that of cisplatin will also lead to a distinct and unique profile of antitumor activity.

8.6

Concluding Remarks

Platinum antitumor drugs represent important agents for the treatment of several different tumors. It appears that changing the chemical structure of platinum compounds may substantially modulate their DNA-binding mode, subsequent

processing of DNA damage, and consequently the mechanism of biological efficacy of these compounds. These structural modifications may also affect not only the spectrum of biological activity of the platinum agents and the development of drug resistance, but also their toxicity profile. Hence, information summarized in this article has implications for the future development of platinum-based clinical agents that new, clinically relevant agents not based on cisplatin analogs can also be found. A further understanding of how new platinum compounds modify DNA and how these modifications are further processed in cells may lead to further insight of “downstream” effects initiated through differential protein recognition and repair that may produce unique antitumor effects. On the other hand, there is still a gap between these molecular events involving DNA interactions and understanding why platinum anticancer drugs are more poisonous to cancer cells than to normal cells. The studies so far performed in this area have implicated multiple systems, including several classes of DNA repair, replication, transcription, cell cycle, and cell death responses involved in the processes associated with cellular sensitivity to the platinum drugs. It is also likely that many other determinants remain to be identified.

Complete knowledge of how modifications of DNA by antitumor platinum compounds and other metal-based drugs affect the components of these pathways should provide a basis for understanding the mechanism of action of platinum antitumor drugs and, thereby, a rational basis for the design of new anticancer agents and chemotherapeutic strategies.

Acknowledgments

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9

Electrochemical Detection of DNA with Small Molecules

Shigeori Takenaka

9.1

Introduction

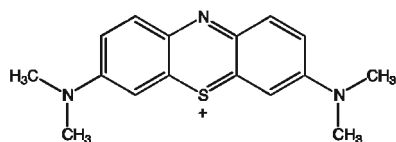
Small molecules that can bind to nucleic acids (DNA and RNA) often show a characteristic change in their absorption spectra upon complexation with nucleic acids [1, 2]. Studies on the interaction of small molecules with DNA have been based on these phenomena. Since most of these DNA-binding small molecules are also electrochemically active (reducible or oxidative, i.e. redox active), and their interaction with DNA can be studied electrochemically. Some examples of electrochemically active DNA-binding molecules are shown in Fig. 9.1. They are classified into the following groups: (1) intercalating molecules that can insert adjacent base pairs, and (2) groove binders such as organic dyes and metal complexes, which can bind to the groove of DNA. Nucleic bases also undergo redox reaction under certain “extreme” conditions and therefore an electrochemical response is obtained by proper selection of electrodes and pH of the electrolyte.

Here, the electrochemistry of nucleic acids is reviewed first and an electrochemical change accompanying the binding of an electrochemical ligand with DNA under conditions in which the DNA is inactive electrochemically will then be described. The ligands exhibiting a preference for double-stranded DNA can be used as hybridization indicators in DNA detection and can be applied to the electrochemical detection of target genes coupled with a DNA probe (a DNA fragment which can form a duplex with the desired DNA sequence) immobilized on the electrode. This system is called a DNA sensor or genosensor. Integration of a multiple DNA probe-immobilized electrode on a small surface is also discussed from the viewpoint of DNA microarray and chip technology, together with their applications.

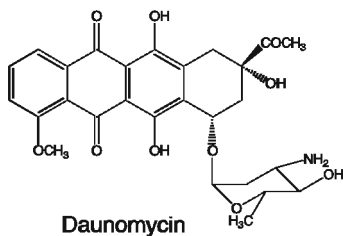
9.2

Electrochemistry of Nucleic Acids

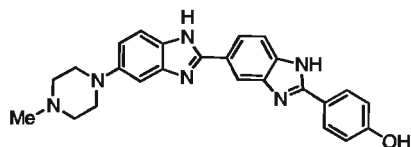
Since electrochemical analysis of DNA and RNA was reported for the first time by Berg in 1957 [3], many papers have been reported, mainly by Berg's and Palecek's



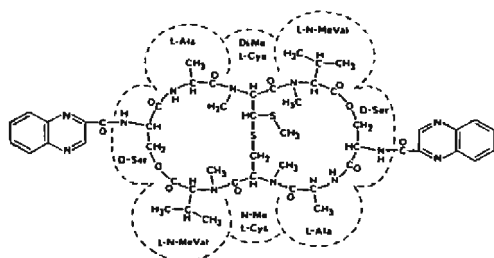
Methylene blue (MB)



Daunomycin



Hoechst 33258



Echinomycin

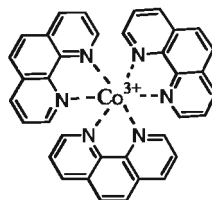
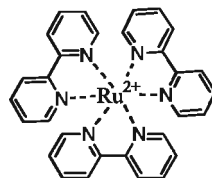
Co(phen)₃³⁺Ru(bpy)₃²⁺

Fig. 9.1. Chemical structures of six electrochemically active DNA-binding ligands: methylene blue (MB), daunomycin, Hoechst 33258, echinomycin, Co(phen)₃³⁺, and Ru(bpy)₃²⁺.

groups [4–6]. DNA and RNA have electrochemically reactive parts on nucleic bases, some of which are illustrated in Fig. 9.2. The imino parts at N1–C6 of adenine, at N7–C8 of guanine, and at N3–C4 of cytosine are reducible by mercury electrodes, as these electrodes have a hydrogen excess potential and hence have a wider potential window in the reducing region. The N1–C6 of adenine and N3–C4 of cytosine are protected from redox reaction by engaging in hydrogen bonding of DNA duplex and therefore protonation at N1 of adenine and N3 of cytosine is necessary for their reduction. On the other hand, C8 of guanine and C2 of adenine are oxidized by graphite electrodes [8].

Differential pulse (d.p.) voltammetry of denatured DNA at pH 7.0 gives rise to two anodic peaks at +0.89 and +1.17 V resulting from guanine and adenine bases of DNA adsorbed on the surface of the electrode, respectively [9, 10]. In 1981, Brabec observed distinct electrochemical oxidation peaks for intact (double-

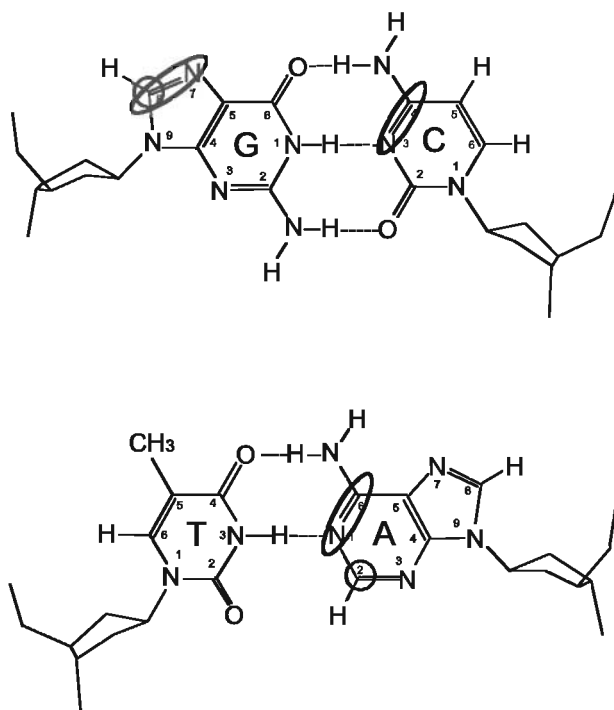


Fig. 9.2 Watson-Crick base pairs and their electroactive sites. Upper and lower sites of base pairs are located in major and minor groove, respectively. Reduction site: N7-C8 of guanine, N3-C4 of cytosine, and N1-C6 of adenine. Oxidation site: C8 of guanine and C2 of adenine.

stranded, ds) and denatured (single-stranded, ss) DNA [11]. The oxidation peak currents of native DNA were markedly smaller than those of denatured one. Since the guanine and adenine bases undergoing oxidation reaction are not involved in the hydrogen bonding of DNA duplex, these differences were ascribed to a difference in the mode of adsorption of nucleic bases of ssDNA and dsDNA on the surface of the graphite electrode; more flexible ssDNA should be adsorbed more and was susceptible to the oxidation reaction. Pang *et al.* reported the electrochemical oxidation of calf thymus DNA on a gold microelectrode using cyclic voltammetry [12]. An oxidation reaction of intact calf thymus DNA took place at +1.09 V, whereas that for denatured DNA took place at +1.16 V, having shifted toward the more anodic potential. The oxidation active bases do not take part in the hydrogen bonding of DNA duplex but they are masked deep in the groove of DNA duplex, making them inaccessible to oxidation reaction on the electrode surface. In all of the cases, a well-defined peak was observed only in the first scan because the oxidation product of the nucleic bases seems to remain adsorbed on the electrode surface, presumably making the electrode unresponsive.

Electrochemical reduction of DNA has been studied at neutral pH by direct cur-

rent (d.c.) polarography [4, 6, 7]. In this case, ssDNA gave an electrochemical signal, whereas dsDNA was electrochemically inactive. This difference appears to be associated with different accessibility of bases in the two types of DNA, since the reduction sites are involved in hydrogen bonding with each other and away from the electrode. These results can be used for the discrimination of ssDNA from dsDNA by d.c. polarography [4, 5, 7]. Palecek and co-workers showed that the reduction peak of dsDNA is observed at potentials different from that of ssDNA [13]. The current intensity for ssDNA was only 1% that for dsDNA. The detection limit of this technique was 20 mg mL^{-1} of ssDNA. Since DNA can be adsorbed on the surface of mercury electrodes, DNA can be detected electrochemically after its concentration onto the surface. This technique is called adsorptive stripping (AdS) analysis. The combination of AdS with a.c. or cyclic voltammetry improved the sensitivity of detection to 1 ppm with calf thymus DNA [14]. Palecek then developed adsorptive transfer stripping voltammetry (AdtSV), in which the electrode is immersed in a solution of nucleic acid (5–10 mL) for a short period of time, washed, and transferred to an electrolyte solution without DNA [15]. A cathodic scan of this electrode gave reduction signals due to adenine and guanine.

9.3

DNA Labeling Through a Covalent Bond

Redox reactions of nucleic acids take place only at “extreme” potentials and are often accompanied by irreversible reactions. Palecek and co-workers found for the first time that osmium tetroxide-pyridine can bind covalently to the thymine base of ssDNA as depicted in Fig. 9.3 [5, 13]. This complex undergoes a reversible redox reaction at -0.2 V or -0.7 V on carbon and mercury electrodes, respectively. This was the first electrochemical marker ever known for DNA. Since then, electrochemical studies of nucleic bases modified by small molecules such as benzo[a]pyrene–DNA adducts have been reported [16].

9.4

Electrochemistry of Metal Complexes Bound to DNA

Since DNA is a polyanion of phosphate esters, it can interact with various metal ions. Until now, the interaction of several metal ions with DNA has been reported

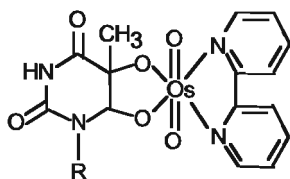


Fig. 9.3. Chemical structure of thymine modified by OsO_4 and bipyridyl.

together with their biological response [17]. For example, copper ion interacts with the phosphate anions or nucleic bases at low or high metal concentrations, respectively [18, 19]. Cis-diaminodichloroplatinum(II), an anticancer drug, is known as a crosslinking reagent for neighboring guanine bases [20, 21]. Metal complexes such as tris(1,10-phenanthroline)ruthenium chelate, $\text{Ru}(\text{phen})_3^{2+}$, are known to bind to DNA reversibly. Especially, the optical isomers, Δ and Λ , of $\text{Ru}(\text{phen})_3^{2+}$ can discriminate left- and right-handed DNA helices [22] and this discrimination was achieved electrochemically [23]. When bound to DNA, absorption and fluorescence spectra of $\text{Ru}(\text{phen})_3^{2+}$ also changed significantly. However, monitoring of the interaction of tris(2,2'-bipyridyl)cobalt(III) chelate, $\text{Co}(\text{bpy})_3^{3+}$, with DNA is difficult because of a small absorption change. In cases like this where spectrophotometric monitoring is difficult, an electrochemical method is particularly useful to study the DNA interaction with small molecules.

The metal complexes that can bind to DNA reported to date are summarized in Tab. 9.1. Bard and co-workers studied electrochemically the interaction of metal complexes with DNA in detail [24, 25]. Here, tris(1,10-phenanthroline)cobalt(III) chelate, $\text{Co}(\text{phen})_3^{3+}$, is taken as an example. A cyclic voltammogram of $\text{Co}(\text{phen})_3^{3+}$ in the absence and presence of calf thymus DNA with a glassy carbon electrode is shown in Fig. 9.4 [25]. A cathodic peak (E_{pc}) at 0.107 V and anodic peak (E_{pa}) at 0.173 V due to the reduction and oxidation reaction, respectively, are observed. The difference between both peak potentials is $\Delta E_p = 60$ mV, close enough to the theoretical value of 59 mV, indicating a diffusion-controlled reversible one electron transfer is taking place. Both peak potentials shifted toward the more anodic side of 0.120 and 0.182 V, concomitant with a decrease in peak currents in the presence of an excess of calf thymus DNA. The latter phenomenon can be explained by an increase in the apparent size of the molecule of $\text{Co}(\text{phen})_3^{3+}$ bound to DNA duplex to result in a smaller diffusion rate. The peak currents in the ab-

Tab. 9.1. Electrochemically active metal complexes, which can bind to DNA.

Compound	References	Compound	References
$\text{Co}(\text{phen})_3^{3+}$	24–28	$\text{Os}(\text{phen})_2(\text{dppz})^{2+}$	37, 41
$\text{Fe}(\text{phen})_3^{2+}$	25, 29	$\text{Os}(\text{phen})_3^{2+}$	41–44
$\text{Co}(\text{bpy})_3^{3+}$	25, 30–34, 41	$(\text{tpy})(\text{bpy})\text{ROH}_2^{2+}$	41
$\text{Fe}(\text{bpy})_3^{2+}$	25, 29	$(\text{tpy})(\text{phen})\text{RuOH}_2^{2+}$	41
$\text{Ru}(\text{phen})_3^{2+}$	23, 28, 35, 36	$\text{Mn}(\text{TMPyP})^{3+}$	45
$\text{Ru}(\text{bpy})_3^{2+}$	37, 38	$\text{Fe}(\text{TMPyP})^{3+}$	45
$\text{Cu}(\text{phen})_2^{2+}$	29	TMAP	46
$\text{Ru}(\text{NH}_3)_6^{3+}$	33, 39	Hemine	47
$\text{Os}(\text{bpy})_3^{2+}$	27, 40, 41	$\text{Ir}(\text{bpy})(\text{phen})(\text{phi})^{3+}$	39

phen, 1,10-phenanthroline; bpy, 2,2'-bipyridine; dppz, dipyrrophenazine; tpy, 2,2',2''-terpyridine; TMPyP, 5,10,15,20-tetra-(*N*-methyl-4-pyridyl)-porphyrin; TMAP, meso-tetra(*p*-*N*-trimethylanilinium)porphyrin; phi, 9,10-phenanthrenequinone diimine.

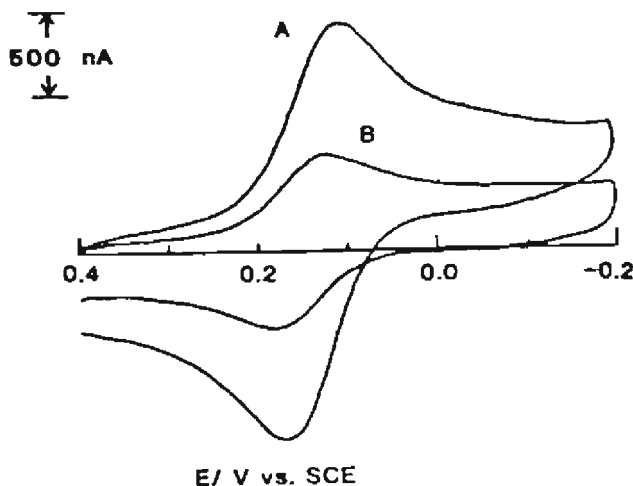


Fig. 9.4. Cyclic voltammogram of 1.0×10^{-4} M Co(phen)_3^{3+} in the absence (A) and presence (B) of 5.0 mM calf thymus DNA in 5 mM Tris-HCl, 50 mM NaCl, pH 7.1. Sweep rate 100 mV s^{-1} .

sence and presence of DNA duplex were proportional to the square root of scan rate, $\nu^{1/2}$, indicating that a diffusion-controlled electrochemical reaction is taking place. From these plots diffusion coefficients $D_f = 5.0 (\pm 0.6) \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and $D_b = 3.2 (\pm 1.2) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ were obtained for Co(phen)_3^{3+} alone and that bound to DNA, respectively.

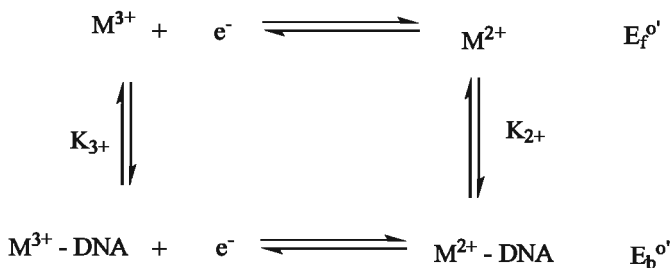
It is manifest that the diffusion rate of Co(phen)_3^{3+} decreased to about 1/20th upon binding to the DNA duplex. Bard and co-workers verified that the current decrease is not due to a decrease in the surface area of the electrode upon binding of the complex by showing that the CV of a mixture of Co(phen)_3^{3+} and octacyanomolybdate(IV), Mo(CN)_8^{4-} , was the same, irrespective of the absence and presence of a DNA duplex [25]. The peak potential and current of Mo(CN)_8^{4-} do not change in the presence of DNA duplex, whereas those of Co(phen)_3^{3+} change in the presence of DNA duplex.

On the other hand, the current shift in the presence of DNA reflects the strength of the interaction of reduced or oxidized species of Co(phen)_3^{3+} with DNA. The thermodynamic cycle in the DNA binding process of these species is shown in Scheme 9.1.

This one electron redox process is expressed by the following modified Nernst equation:

$$E_b^{o'} - E_f^{o'} = 0.059 \log(K_{2+}/K_{3+}) \quad (1)$$

where K_{2+} and K_{3+} represent the binding constants for the reduced (M^{2+}) and oxidized (M^{3+}) species of Co(phen)_3^{3+} , respectively. In other words, the difference



Scheme 9.1

between the redox potentials of the bound and free species represents the binding ratio of the oxidized to reduced species. In the case of $\text{Co}(\text{phen})_3^{3+}$, the current shift in the presence of DNA was +17 mV and $K_{2+}/K_{3+} = 1.74$, implying that the binding of $\text{Co}(\text{phen})_3^{2+}$ is nearly twice greater than that of $\text{Co}(\text{phen})_3^{3+}$. Bard and co-workers concluded that the hydrophobic interaction contributes more than the electrostatic one in the binding of $\text{Co}(\text{phen})_3^{3+}$ with DNA.

Next, the determination of binding constants by electrochemical treatments will be described. As DNA is added, the current decreases to a constant value, which represents that for the bound form. Since the concentration of the ligand bound to DNA, C_b , can be estimated from this value, one can obtain the binding constant, K , from the following Scatchard-type equation:

$$\begin{aligned}
 C_b &= b - \{b^2 - (2K^2 C_l [\text{DNA}])/s\}^{1/2}/2K \\
 b &= 1 + KC_l + K[\text{DNA}]/2s
 \end{aligned} \quad (2)$$

where s is the site size in base pairs excluded by the bound ligand complex and C_l is the total concentration of ligand. If it is assumed that the electron transfer occurs in a reversible, diffusion-controlled manner, two limiting cases may be described for the current in CV from Eq. (2). When the bound and free forms of the ligand cannot interconvert to each other on the time scale of electrochemical measurements (static, S), the square root of the diffusion coefficient ($D^{1/2}$) is simply the sum of the free and bound forms of the ligand weighed by the individual mole fractions and the equation is rearranged into

$$i_{pc} = B(D_f^{1/2} C_f + D_b^{1/2} C_b) \quad (3)$$

If the complexes interconvert rapidly on the experimental time scale, $D^{1/2}$ is the mole-fraction-weighted time average of free and bound values (mobile, M) and the equation is rearranged into

$$i_{pc} = BC_l(D_f C_f/C_l + D_b C_b/C_l)^{1/2} \quad (4)$$

where C_f is the concentration of the free ligand. For Nernstian reaction in CV at 25°C, $B = 2.69 \times 10^5 n^{3/2} A v^{1/2}$, where n is the number of electrons transferred

per metal complex and A is the surface area of the electrode. Using this procedure, the binding constant of Co(phen)_3^{3+} with calf thymus DNA was determined as $K = 1.6 (\pm 0.2) \times 10^4 \text{ M}^{-1}$ (S , $s = 6 \text{ bp}$) or $2.6 (\pm 0.4) \times 10^4 \text{ M}^{-1}$ (M , $s = 5 \text{ bp}$) [25].

9.5

Electrochemistry of DNA-binding Small Molecules

Most of the DNA-binding small molecules reported to date (Tab. 9.2) are more or less electrochemically active, exhibiting redox activity at cathodic potentials. Natural products carrying an anthraquinone skeleton were the first to be studied as this kind of molecules and daunomycin is the ligand used most often for electro-

Tab. 9.2 Electrochemically active small molecules, which can bind to DNA.

<i>Compound</i>	<i>References</i>	<i>Compound</i>	<i>References</i>
Daunorubicin	48, 49	Ethidium homodimer	51
Duborimycin	48	Ethidium monoazide	51
RP33921	48	Chlortetracycline	51
RP21080	48	Tetracycline	51
Daunomycin	39, 50–57	Deoxycycline	51
Adriamycin	50, 57, 58	Minocycline	51
4'-Deoxyadriamycin	57	Hoechst 33258	51, 63, 79
Iremycin	50	Hoechst 33342	51
Carminomycin	50	7-Aminoactinomycin D	51
<i>N,N'</i> -Dibenzyl daunomycin	50	Chromomycin A	51
Steffimycin	50	Olivomycin	51
3'-Hydroxydaunomycin	50	Vinblastine	51
Musetlamycin	50	Propidium iodide	51
Violamycin BI	50	Quinacrine mustard	51
component		DAPI	51
MA144-S1	50	Rifampicin	51
Nogalamycin	50, 57	Stains-all	51
Aclacinomycin	50	Phenothiazine	54
Cinerubin B	50	Chlorpromazine	64
Maecellomycin	50	Promethazine	64
Neutral red	50	Mitoxantrone	65
Crystal violet	59	Khellin	66
Methylene blue (MB)	39, 59–61	Benzyl viologene	67
9-Aminoacridine	28, 51	Echinomycin	4, 68
Acridine orange	51	Chloroquine	28
Aclarubicin	51	Epirubicin	69
Doxorubicin	28, 51	Bis-acridine carrying viologen unit(s)	70–73
Pirarubicin	51	FND-1	74, 75
Ethidium bromide	46, 51, 62	FND-2	76

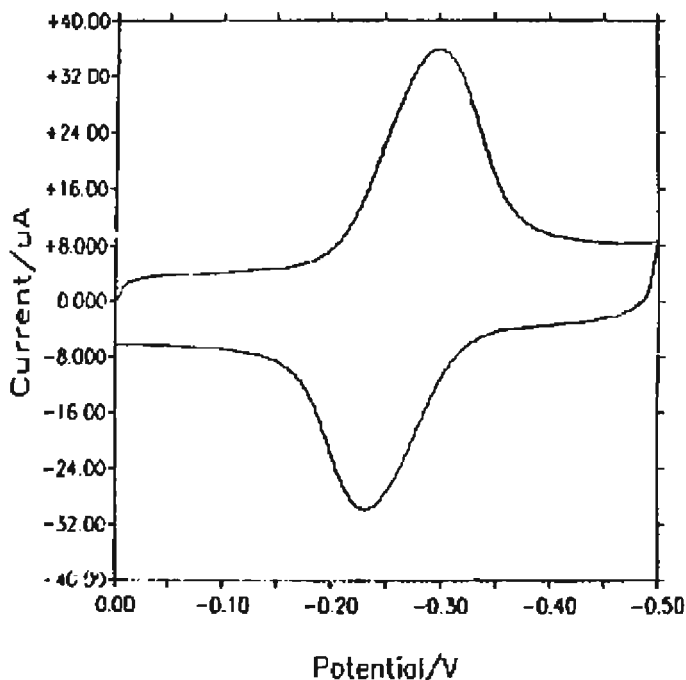


Fig. 9.5. Cyclic voltammetry of 1.0 μM methylene blue (MB) in 50 mM phosphate (pH 7) on a DNA-modified electrode (area = 0.7 cm^2). Scan rate 100 mV s^{-1} .

chemical studies. The molecules carrying an acridine or ethidium skeleton also are electrochemically active, but they often undergo irreversible reactions. Methylene blue (MB) is known to undergo a reversible redox reaction and was studied in detail by Barton and co-workers [60, 88]. Figure 9.5 shows a cyclic voltammogram of MB on a DNA immobilized gold electrode in 50 mM phosphate (pH 7). Peak potentials due to the redox reaction of MB were observed at -0.25 V and -0.30 V (versus SCE) with $\Delta E_p = 0.05\text{ V}$. The reduced form of MB is known as leuco-MB. The binding constant and its difference between the reduced and oxidized forms of such a kind of molecules can be estimated according to the treatment analogous to that for DNA-binding metal complexes. The redox reaction occurs on the same site of these molecules as that of their binding (interacting site).

On the other hand, Takenaka and co-workers synthesized a bis-acridine derivative carrying a viologen linker as a reversible electrochemically active moiety at two acridine parts [70] (Fig. 9.6). Because of the bis-intercalating character, this molecule was expected to exhibit higher affinity for the DNA duplex. Electrochemical studies of the molecule revealed that the reduced form of the molecule possesses higher affinity than the oxidized one, suggesting that the electrostatic interaction is the major contributor in the DNA binding. It was also suggested that its viologen parts take part actively in the DNA interaction process. This notion that the viol-

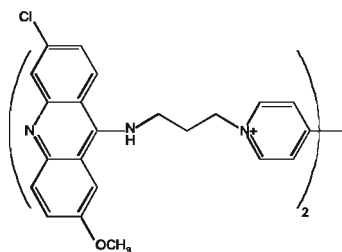
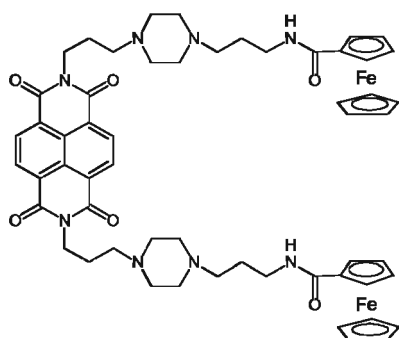
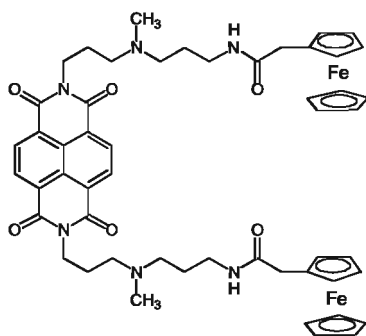
**Bis-acridine****FND-1****FND-2**

Fig. 9.6. Chemical structures of bis-acridine and threading intercalators FND-1 and FND-2 carrying electrochemical signal parts.

ogen interacts with the DNA groove was supported by recent electrochemical studies of dibenzylviologen [67].

Recently, Palecek and co-workers reported an electrochemical study of echinomycin as bis-intercalator in a hope of improving affinity for the DNA duplex (see below) [4, 68]. The quinoxaline parts of echinomycin are electrochemically active. Takenaka and co-workers also succeeded in synthesizing mixed ligands carrying multiple viologen units [72, 73] and the acridinyl viologen connected through a different length of alkyl chain [71]. These bis-intercalators underwent a reversible redox reaction and showed slight preference for AT-rich DNA duplexes.

9.6

DNA Sensor Based on an Electrochemically Active DNA-binding Molecule as a Hybridization Indicator

As described above, the diffusion of small molecules is retarded upon binding to DNA in solution to result in a decrease in the redox current. In addition, the peak

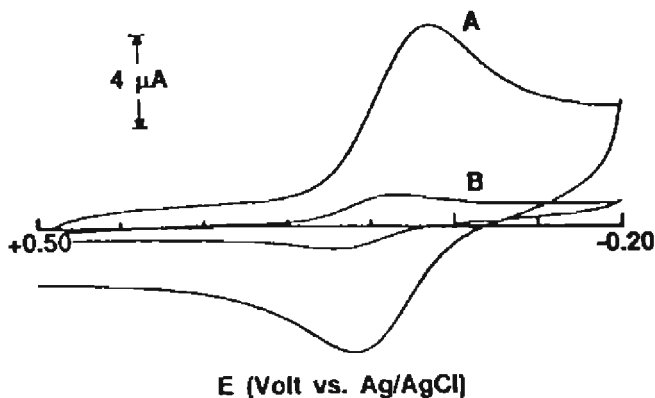


Fig. 9.7. Cyclic voltammogram of $1.6 \times 10^4 \text{ M}^{-1} \text{ Co(phen)}_3^{3+}$ at a glassy carbon electrode alone or modified with $[\text{poly(dG-dC)}]_2$.

potential also shifted upon binding to DNA, suggesting that the microenvironment around the molecule has changed. In contrast, where DNA is immobilized on the electrode, the molecule is concentrated on the surface of the electrode and the current intensity increases accordingly. Such an example is shown in Fig. 9.7 in a cyclic voltammogram for a glassy carbon electrode carrying $[\text{poly(dG-dC)}]_2$ in an electrolyte containing Co(phen)_3^{3+} [31]. The target DNA can be detected by using such molecules coupled with a DNA-immobilized electrode. The DNA sensor or genosensor shown in Fig. 9.8 is based on this principle [77, 78].

First, a DNA probe, which is a denatured DNA fragment capable of forming a duplex with the complementary gene sequence, is immobilized on the surface of an electrode. A double-strand formation (hybridization) reaction of sample DNA with the DNA probe is conducted on the surface of the electrode. Second, the electrode is dipped in an electrolyte containing a hybridization indicator and then electrochemical measurements are made. The amount of molecule bound on the electrode and the amount of DNA duplex formed are estimated from the signal intensity. In this sense, the molecule is regarded as a transducer to convert the DNA duplex formation to an electrochemical signal and can be called a hybridization indicator. In this technology, the selectivity of the indicator for dsDNA is very important to determine the sensitivity of a DNA sensor: if the indicator binds to ssDNA, the DNA probe-immobilized electrode gives rise to an electrochemical signal even in the absence of target DNA, which results in a background noise.

So far, several groups have developed an electrochemical genosensor. Ishimori and co-worker used Hoechst 33258 (Fig. 9.1) as a hybridization indicator [63]. Since this ligand binds to the groove of the DNA duplex, it was expected to have a low affinity for ssDNA. Wang and co-workers achieved highly sensitive detection of target DNA using Co(phen)_3^{3+} coupled with a DNA probe-immobilized electrode by a potentiometric stripping analysis (PSA) technique [78]. Marrazza and co-workers detected PCR products containing a mismatched sequence using dau-

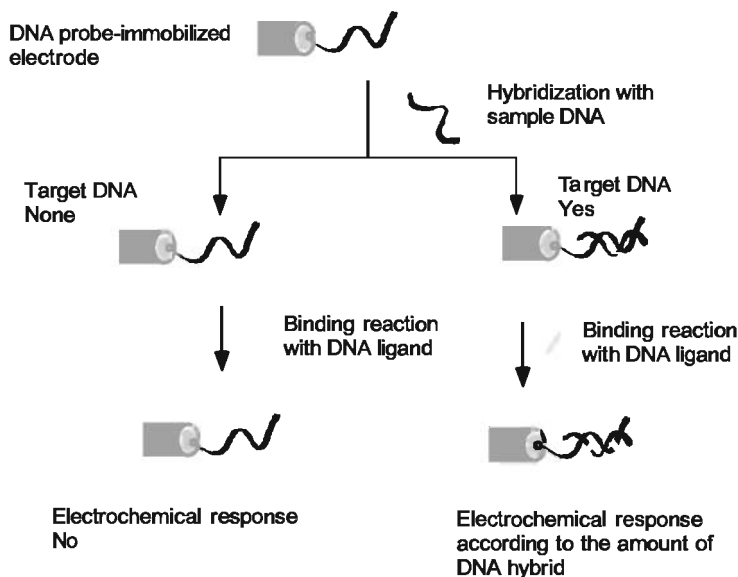


Fig. 9.8. Principle of a DNA sensor or genosensor using a ligand specific for double-stranded DNA coupled with a DNA probe immobilized on the electrode.

nomycin by the PSA technique [56]. However, small molecules such as metal complexes, intercalators, and groove binders can bind to ssDNA as well due to electrostatic and hydrophobic or stacking interactions.

Bis-acridine carrying a viologen unit was synthesized to improve the affinity for dsDNA [70]. Echinomycin is an example; its cyclic voltammogram with a mercury electrode carrying single or double-stranded DNA in an electrolyte is shown in Fig. 9.9 [4, 68]. A peak current due to the quinoxaline rings of echinomycin was observed for the dsDNA-immobilized mercury electrode at a cathodic potential. Both

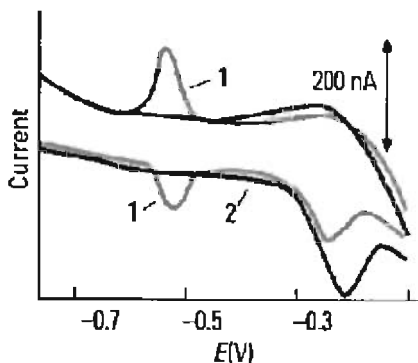


Fig. 9.9. Cyclic voltammograms of 10 μM echinomycin before immersion of native (2) and denatured (1) DNA at a hanging drop mercury electrode.

bis-intercalators undergo redox reactions at cathodic potentials, where the reduction of dissolved oxygen overlaps, hampering sensitive detection of target DNA.

Takenaka and co-workers synthesized ferrocenyl naphthalene diimide [74–77] FND-1 and -2 (Fig. 9.6) as a hybridization indicator with a strong preference for dsDNA and reversible redox response at an anodic potential. When naphthalene diimide binds to the DNA duplex, both of its substituents project out in the major and minor grooves simultaneously. These substituents serve as an anchor to prevent the complex from dissociation, and as a result a very stable complex of naphthalene diimide with DNA duplex is formed. In contrast, the complex of naphthalene diimide with ssDNA is expected to be less stable, as no such a stabilizing factor is present. When the binding process of naphthalene diimide to a DNA duplex is envisaged further, it is easily seen that one of the substituents should penetrate adjacent base pairs. These types of ligand are thus called threading intercalators.

On the basis of these considerations, threading intercalators were expected to be highly selective indicators for dsDNA. FND-1 was designed in such a way that ferrocene moieties are connected to the terminus of a substituent on naphthalene diimide. FND-1 should intercalate between adjacent base pairs with the ferrocene moieties projecting in the major and minor grooves of DNA duplex, with the naphthalene diimide and ferrocene parts acting as a threading intercalator and reversible redox reaction parts at anodic potentials, respectively. Although the discrimination of double-stranded from single-stranded DNA by FND-1 is not complete, because it still binds to ssDNA through electrostatic and stacking interactions, the selectivity for the two types of DNA has been improved significantly.

Thiolated oligonucleotide dT₂₀ was immobilized on a gold electrode through a thiol–gold linkage as a DNA probe and the resulting electrode was allowed to hybridize with complementary dA₂₀ or non-complementary dT₂₀ [75]. After dipping in an electrolyte containing FND-1, the electrode was transferred to another electrolyte without FND-1 and a cyclic voltammogram was measured. As shown in Fig. 9.10, a peak current was obtained only for the complementary oligonucleotide with the bare electrode, whereas the signal intensity for the non-complementary DNA was barely above background. Differential pulse voltammetry was applied to this measurement to suppress background due to the capacitance component and Takenaka and co-workers succeeded in detecting plasmid DNA carrying a target gene at the femtomole level [75]. They also developed FND-2 undergoing a redox reaction at a more negative potential [76]. It was also attempted from an environmental viewpoint, using DNA-immobilized electrodes to detect DNA-binding molecules present in nature [80].

9.7

Mismatched DNA Detection by Hybridization Indicator

There is a diversity by one nucleobase in individual genes. This phenomenon, called single nucleotide polymorphism (SNP), is the cause of the susceptibility of

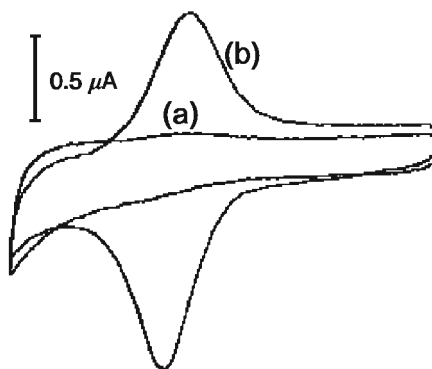


Fig. 9.10. Cyclic voltammograms of a dT₂₀-immobilized gold electrode after hybridization with complementary dA₂₀ (b) and non-complementary dT₂₀ (a) in an electrolyte containing FND-1.

individuals to drugs and diseases. It is thus important to know about SNPs in advance in order to diagnose and prevent various syndromes. When a DNA probe carrying a certain SNP site is allowed to hybridize with sample DNA, a fully matched or mismatched DNA duplex is formed. Takenaka and co-workers developed a mismatched base detection system based on FND coupled with a DNA probe-immobilized gold electrode, the principle of which is illustrated in Fig. 9.11 [82, 83]. The DNA probe is immobilized on the electrode by the sulfur–gold linkage. This method of immobilization is often used because of the ease of controlling the amount of DNA probe immobilized on the electrode.

Electrochemical measurements are made before and after hybridization with denatured sample DNA. First, the d.p. voltammetry of the DNA probe-immobilized electrode is measured in an electrolyte containing FND, and the peak current obtained (i_0) is used to estimate the amount of DNA immobilized on the electrode. Since the binding of FND with ssDNA is proportional to the number of phosphate anions, the peak current should also be proportional to the amount of DNA probe on the surface of the electrode (the diffusion current of FND-1 is also included in this current). After hybridization with sample DNA, d.p. voltammetry is measured and the peak current i is obtained. The current shift, $\Delta i = (i/i_0 - 1) \times 100$ is used for the evaluation of the amount of dsDNA per ssDNA immobilized on the electrode. Under conditions where fully matched and mismatched sample DNA can form a double strand, the Δi value for the mismatched DNA duplex is smaller than that for the fully matched DNA duplex due to the fact that the binding of FND around the mismatched base area is impaired. It was found from other experiments that one mismatched base results in loss of two to three FND molecules otherwise bound there.

One of the advantages of this method lies in the fact that it is not necessary to set the hybridization temperature rigorously, in contrast to the conventional hybrid-

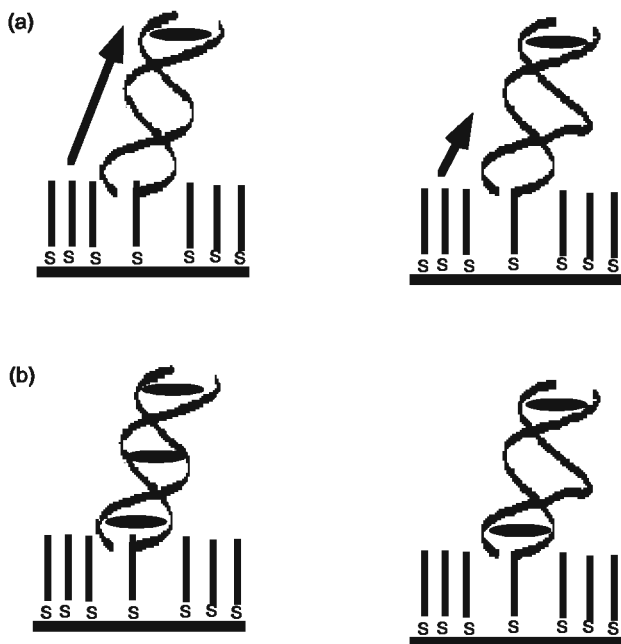


Fig. 9.11. Electrochemical detection of a single base mismatch based on charge transduction through DNA (a) and by reducing the amount of FND-1 bound to mismatched DNA duplex (b).

ization methods where a proper setting of the temperature is essential. Barton and co-workers established a mismatched DNA detection technique using an electron transfer reaction through a π -stack of nucleic bases of DNA duplex [85] (Fig. 9.11). They prepared a gold electrode carrying DNA duplex to high-density (DNA film) [61, 86]. Methylene blue (MB) binds to only the surface of this film and therefore electron transfer reaction occurs between MB and the electrode. Where a mismatched base(s) exists, the efficiency of electron transfer is reduced and hence the existence of a mismatched base on the DNA duplex can be estimated from a change of the amount of their electric charge.

Thus far, discrimination of mismatched bases has been based on the difference in the stability of mismatched and fully matched DNA duplex and hence stringent control of temperatures for hybridization and subsequent washing has been critical. However, it is often difficult to find a common optimum condition for fully matched and mismatched DNA duplexes to discriminate the two. In this sense, these two techniques described above are especially useful in mismatched base detection, as they do not rely on the stability of the DNA duplex.

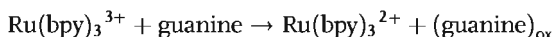
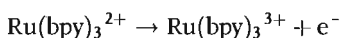
Another mismatched base detection was achieved by Wang's group using peptide nucleic acid (PNA), in which a "duplex" of PNA with DNA is formed more easily than the DNA/DNA duplex [87].

9.8

DNA-detecting System using Hybridization Indicator as a Mediator

The redox reaction of ferricyanide–ferrocyanide, an anionic marker, is blocked on a DNA film prepared by Barton's group because of electrostatic repulsion between the anionic marker and DNA. When MB binds to the surface of the DNA film, the anionic marker can now gain access to MB bound on the DNA film and electron transfer between MB and the electrode can be enhanced by the redox reaction of anionic marker. Barton's group developed a highly sensitive detecting system of mismatched DNA duplex using this electrocatalytic reduction system [85, 88]. The complex of FND with DNA is regarded as a pseudo-polyferrocene coating the DNA duplex because of the intercalator's binding mode (nearest exclusion model), and hence an electron transfer through this array was envisaged. In fact, such an electron transfer was observed between the electrode and the reduced glucose oxidase generated from the reaction of glucose oxidase and glucose [89]. This result suggested that the electrochemical signal is enhanced further upon DNA duplex formation in this system. The electron transfer occurs through the ferrocene array covering the groove of DNA duplex in Takenaka's system, whereas it occurs through the π -stacked base pairs of DNA duplex in Barton's system.

Tris(2,2'-bipyridine)ruthenium chelate, $\text{Ru}(\text{bpy})_3^{3+}$, described above, can mediate the oxidation reaction of guanine bases:



Thorp and co-workers studied the mediation reaction of unpaired guanine bases on DNA duplex through $\text{Ru}(\text{bpy})_3^{2+}$ using an indium tin oxide (ITO) electrode [38, 90]. Figure 9.12 shows an example of the mediation current of $\text{Ru}(\text{bpy})_3^{2+}$ for calf thymus DNA. They proved that the mediation of $\text{Ru}(\text{bpy})_3^{2+}$ derives from the guanine exposed to the solvent on the basis of their observation that this mediation current depended upon the amount of unpaired guanine bases. They developed a DNA-sensing system using a DNA probe-immobilized ITO electrode, on which the mediated current of $\text{Ru}(\text{bpy})_3^{2+}$ increased with an increase in the amount of target DNA. The magnitude of the mediation current was relatively constant with several DNA samples because of the uniformity of the GC content of natural DNA used.

9.9

Application to DNA Microarray

Recently, DNA microarrays are receiving attention as a means of high-throughput analysis of DNA. This technique, developed by Brown of Stanford University, consists of a range of procedures [91]. Many different probe DNAs are spotted for immobilization on a glass plate with a microarrayer (preparation of a DNA microarray). A labeled DNA sample is allowed to hybridize on the microarray plate. After

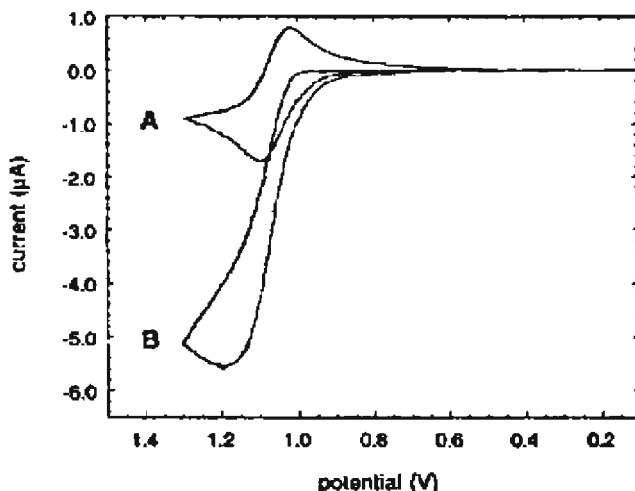


Fig. 9.12. Cyclic voltammograms of $\text{Ru}(\text{bpy})_3^{2+}$ in the absence (A) and presence (B) of calf thymus DNA at 25 mV s^{-1} in $700 \text{ mM NaCl}/50 \text{ mM}$ sodium phosphate buffer, pH 6.8.

washing the plate, the sample DNA bound on the plate is detected by the label's signal. Affymetrix Inc. (USA) developed a highly condensed DNA array called the DNA chip by oligonucleotide synthesis on the silicon surface by photolithography using amidate reagents carrying a photoreactive protecting group [91]. An electrochemical integrated multi-electrode system has been developed using the application of the DNA sensor technique. Xanthox Inc. (USA) constructed a multi-ITO electrode on the well of titer plates and applied it to the mediated DNA-detecting system using $\text{Ru}(\text{bpy})_3^{2+}$ [92]. TUM Inc. (Japan) developed a multi-gold electrode called electrochemical array (ECA), in which many DNA probes were immobilized on a gold electrode and the hybridization events were detected by FND-1 as a hybridization indicator [81]. Takenaka and co-workers tried to visualize a DNA microarray by scanning electrochemical microscopy (SECM) [93]. When a DNA microarray was dipped in an electrolyte containing FND-1, the ligand was concentrated in the DNA duplex region on the glass plate. The reason for the enhanced SECM signal is that the DNA duplex regions of the spots serve as a conductive polymer. Since this system can be used as an ordinary DNA microarray and a fluorescent dye is unnecessary to immobilize and detect the DNA duplex region, it is expected to emerge as the DNA microarray technology of the future.

9.10

Conclusion

As described above, DNA, which is electrochemically inactive under ordinary conditions, can be analyzed electrochemically by using electrochemically active DNA-

binding ligands. This method is a sort of a DNA sensor or genosensor based on the use of DNA-binding ligands as a hybridization indicator. This novel genosensor enables not only general DNA analysis but also mismatch detection including analysis of single nucleotide polymorphisms (SNPs). It is expected that this novel methodology will be seen as a breakthrough in the development of highly sensitive, quick, inexpensive, and compact analytical devices for target DNA. In light of an increased interest in DNA chip technology, research on electrochemical chips based on integrated multi-electrodes is being carried out intensively. The fruits of this research, once achieved, will exert a strong impact or even revolutionize diagnosis in medicine in the future.

9.11

Summary

Electrochemical methods have proved to be an excellent technique for the study of interactions of small molecules with DNA and RNA, especially where the molecules are spectrophotometrically silent. Electrochemical molecules called hybridization indicators can be used in DNA sensors or genosensors coupled with a DNA immobilized electrode. In this regard, hybridization indicators with high discrimination ability for double-stranded DNA (hybrid with target DNA) over single-stranded DNA (unreacted DNA probe) are required. Bis-intercalators and threading intercalators seem to be promising candidates for this. Mismatched DNA detection using the electron transfer reaction through a π -stack of DNA duplex or relying on an decrease in the amount of the bound intercalator per mismatched DNA duplex has provided a new technology for single nucleotide polymorphism (SNP) analysis. Electrochemical array (ECA) and its homolog DNA chip have also been developed for electrochemical DNA sensing. Visualization of DNA microarrays on an ordinary glass plate was realized by ferrocenyl naphthalene diimide as threading intercalator coupled with scanning electrochemical microscopy (SECM).

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10

Design and Studies of Abasic Site Targeting Drugs: New Strategies for Cancer Chemotherapy

Jean-François Constant and Martine Demeunynck

10.1

Introduction

10.1.1

Importance of Abasic Sites in Cells

The emergence of resistance is one of the factors that limits the efficacy of anti-tumor drugs. Some DNA-repair processes have been shown to be implicated in resistance to DNA-damaging agents [1] and an increasing number of studies are being carried out to design DNA repair inhibitors, with the ultimate goal of improving the antitumour effects of DNA-damaging drugs [2, 3]. Among the numerous lesions produced by these drugs on DNA, modified bases are processed by a specific process: the base excision repair (BER) pathway, which can be summarized as consisting of three major steps (Fig. 10.1):

1. Recognition and excision of the damaged base by a specific glycosylase, leaving a 2'-deoxyribose residue, the abasic site (apurinic/apyrimidinic or AP-site).
2. DNA breakage at the abasic site by a specific AP endonuclease (usually HAP1 enzyme in humans).
3. DNA resynthesis by two possible mechanisms named the short patch and long patch repair pathways.

The short patch repair pathway replaces only one or two nucleotides and involves DNA polymerase β (pol β), XRCC1 (X-ray cross-complementation protein 1), and a ligase. In the long patch repair pathway up to 10 nucleotides are replaced, involving other enzymes such as FEN1 (flap endonuclease 1), PCNA (proliferating cell nuclear antigen), a polymerase (β , δ , or ϵ) and a ligase [1].

Base modifications that result from the action of alkylating agents are handled by the BER pathway. Thus a deficiency of this repair pathway could result in increased cellular sensitivity to alkylating agents [4]. BER has not been fully investigated as a potential actor in resistance to antitumor drugs and very few reports have addressed the potential for targeting abasic site processing.

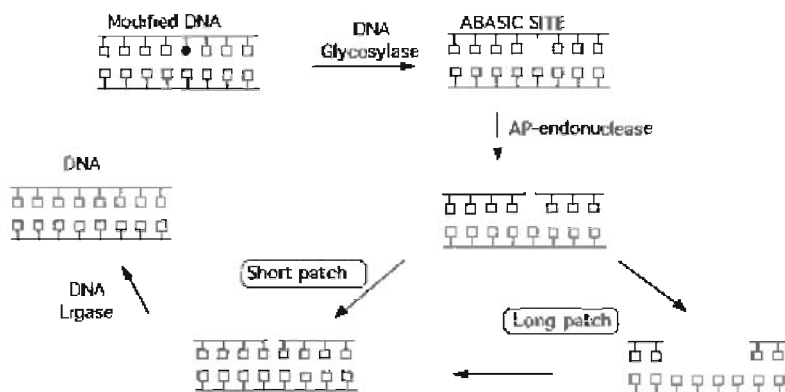


Fig. 10.1. Base excision repair (BER) pathway.

As mentioned above, AP-sites are key intermediates in the BER pathway. They are formed by enzymatic cleavage of the C1'-N glycosidic bond of a damaged or inappropriate nucleic base [1]. Many BER glycosylases have been isolated and characterized [5, 6].

Recent work has shown that abasic sites may interfere with other vital enzymes in cells such as ligases [7] and topoisomerases [8]. Apurinic sites appear to stimulate DNA breakage up to 20-fold when located within the four-base overhang produced by topoisomerase II-mediated cleavage [9–11]. This lesion appears to be 1000 times more potent than specific topoisomerase II inhibitors [12]. Topoisomerase I activity is also affected by the presence of abasic sites. Depending on its position, a single abasic site is able to induce new topoisomerase I cleavage sites or to trap the topoisomerase I-cleavable complex [13]. Abasic sites and other DNA damage may therefore be considered to be site-specific topoisomerase poisons [14].

All these studies demonstrate the central role played by abasic sites as key intermediates in BER process and as potential poisons for other DNA-modifying enzymes. In our group we came up with the idea that targeting this lesion by specific drugs might give rise to new pharmacological activities (Fig. 10.2):

- Drugs that bind strongly at abasic site might mask the lesion to the hydrolytic activity of AP endonucleases, the enzymes that initiate the AP-site repair by incising DNA at the adjacent phosphodiester bond.
- A similar effect might be expected from drugs that react with AP-sites in their open aldehydic form, leading to covalent adducts, the repair of which would be less efficient.
- Drugs able to catalyze single-strand cleavage at abasic sites might trigger other enzymatic activities such as polyADP ribose polymerase (PARP). PARP production is induced by high levels of single-strand breaks in DNA [15]. This enzyme interacts at the cleavage site and catalyzes the synthesis of NAD oligomers. The cell depletion of this essential cofactor might participate in the numerous processes leading to γ -irradiated cell death.

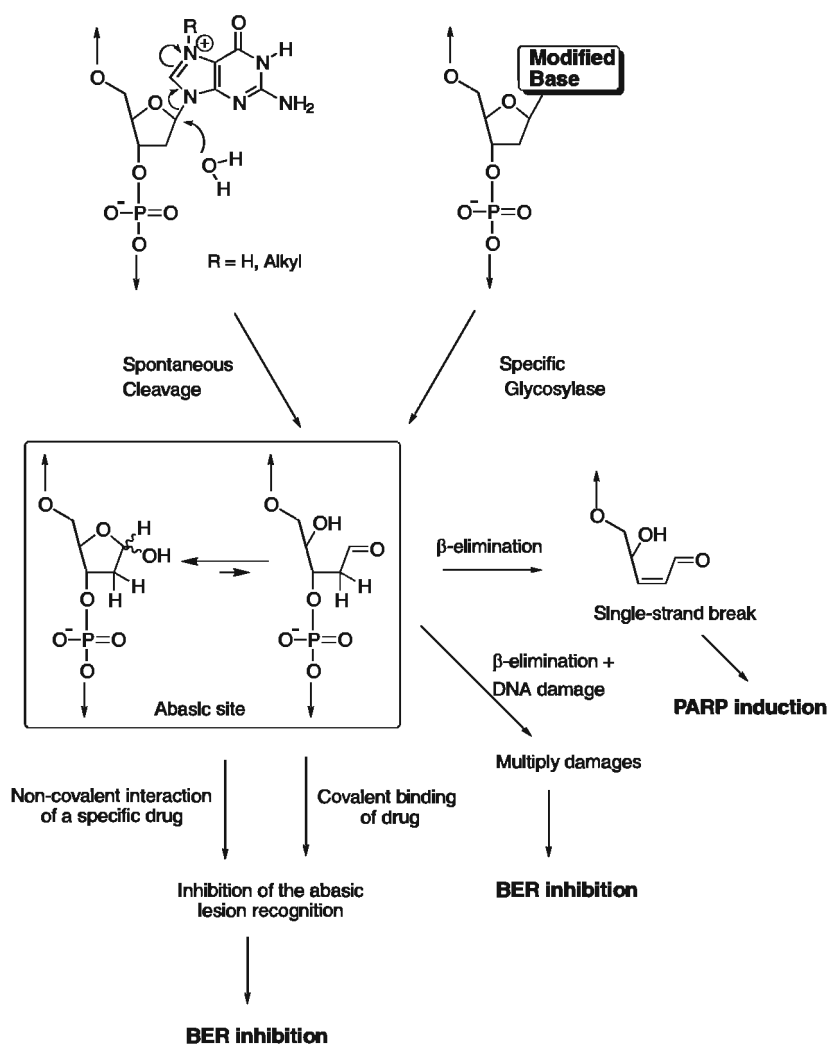


Fig. 10.2. Importance of abasic sites as a target for specific drugs.

- Another class of drugs is derived from the single-strand breakers mentioned above. These compounds also possess a DNA-damaging motif. When binding at abasic sites, such compounds might induce multiply-damaged sites (single-strand breaks + other damage) known to be more difficult to repair.

10.1.2

Structure of Abasic DNA

The chemical structure of abasic sites was studied by NMR spectroscopy analysis of oligodeoxynucleotides containing ¹⁷O- and ¹³C-labeled abasic sites [16–18].

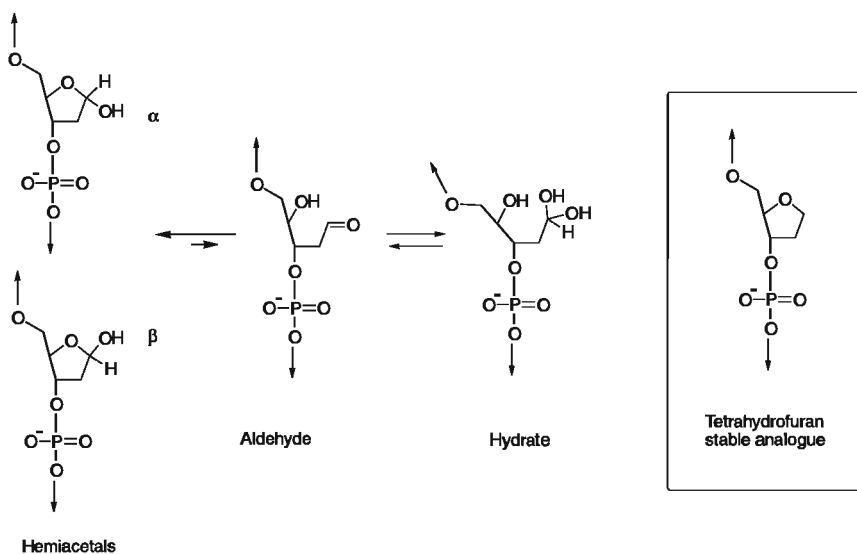


Fig. 10.3. Chemical structure of the abasic lesion.

Abasic sites can exist in three different forms in equilibrium: open-chain aldehyde, α and β hemiacetals, or hydrate. NMR studies of this equilibrium in solution indicated that the mixture of α/β cyclic hemiacetals is the predominant form in the duplex and that the aldehyde represents only 1% of the total (Fig. 10.3).

The use of synthetic oligonucleotides containing stable analogs of the abasic lesion, such as the tetrahydrofuran (Fig. 10.3) [19–23] allowed studies of the conformational influence of an abasic site [24–27]. Different types of abasic duplexes were examined by high-field NMR spectroscopy, calorimetry, and restrained molecular dynamics. The influence of the nature of the base opposite the abasic site, a purine or a pyrimidine (i.e. respectively an apyrimidinic or an apurinic site), and the nature of the flanking bases was studied. The main results that emerged were as follows: (1) the backbones of the abasic duplex DNAs are regular with right-handed B-form helices, (2) the conformational changes due to the presence of the abasic site depend on the nature of the flanking and opposite bases. In the duplexes containing unpaired purines (apyrimidinic sites) the purine remains stacked within the helix in an intrahelical conformation [22, 23, 25, 27–29]. The situation is more complex for apurinic sites, the pyrimidine opposite the abasic site may be either stacked inside the helix or expelled outside, or in equilibrium between the two situations [20, 26, 29]. However, some differences are observed between the “true” abasic site (also called the “aldehydic” abasic site) and the stable tetrahydrofuran analog. In the case of the “true” abasic site, which exists predominantly as a mixture of α - and β -anomers, the deoxyribose residue is within the helix for the β -anomer, while the sugar in the α -configuration is out of the helix [25].

Another feature that distinguishes the structure of an abasic duplex from that of the parent unmodified oligonucleotide, is formation of a kink at the site of the lesion. The bending value into the major groove was estimated to be approximately 10° in an apyrimidinic undecamer [28] or approximately 30° for an apurinic dodecamer [30]. Furthermore the abasic lesion considerably increases the flexibility of duplex DNA [28].

10.1.3

Abasic Site Reactivity

In its opened aldehydic form, deoxyribose may undergo two types of reaction (Fig. 10.4): (1) strand cleavage in alkaline conditions and (2) condensation with amino nucleophiles. In mild alkaline conditions, abasic sites undergo a β -elimination reaction with formation of an α,β -unsaturated aldehyde [31, 32]. In more drastic alkaline medium, a second elimination reaction (δ -elimination) may occur. Similar reactivity was observed in the presence of polyamines [32, 33]. These β - and δ -elimination reactions are implicated in the cleavage of AP-sites by class I AP endonucleases or AP lyases (see Section 10.1.4).

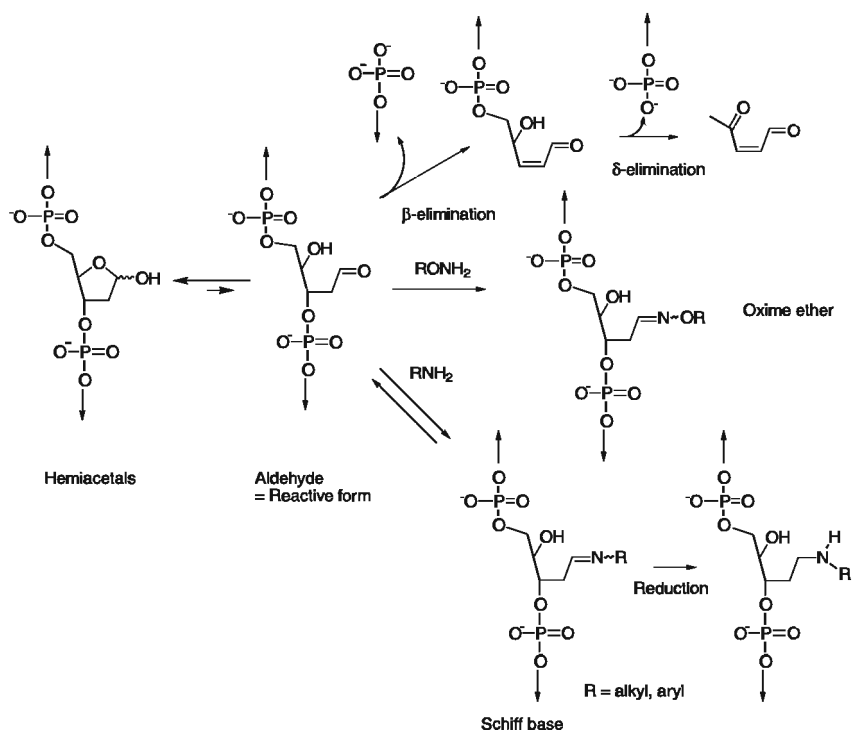


Fig. 10.4. Chemical reactivity of the 2'-deoxyribose residue.

The β -elimination process occurs on the open aldehydic form of the deoxyribose residue. NMR spectroscopy experiments confirmed the stereospecific abstraction of the 2'-pro-S hydrogen atom [34–36].

The reactivity of the abasic site with amino nucleophiles has been used by several groups to derivatize oligonucleotides either by formation of a Schiff base between the electrophilic aldehydic abasic site and amino group containing ligands, followed by reduction (reductive amination) or by reaction with alkoxyamines leading to stable oxime ethers. This reaction has been largely used for detection and titration of abasic sites.

10.1.4

Enzymology of the Abasic Site

As shown in Fig. 10.1 the very first step in the abasic site-repair process is an incision of DNA at one of the phosphodiester bonds adjacent to the lesion. AP-sites can be processed following two enzymatic pathways (Fig. 10.5). Class II enzymes named AP endonucleases, such as exonuclease III from *Escherichia coli* [37] and APE-I, its human homolog [38], cleave the DNA backbone at the 5' side of the AP-site via hydrolytic process [39], thus generating a 3'-OH group and a 5'-deoxyribose 5'-phosphate residue. Class I AP endonucleases, also termed AP lyases [40], are associated with glycosylase activities and proceed via a β -elimination mechanism leaving a 3'- α,β unsaturated aldehyde residue and a 5'-phosphate group. The formation of a Schiff base between an amino residue of the enzyme and the aldehyde group of the abasic site has also been postulated [41]. δ -Elimination has also been proposed to follow β -elimination cleavage of AP-sites during enzymatic repair of damaged DNA [42].

If not repaired, the abasic site was shown to be mutagenic [43–46] or lethal [47]. If replication proceeds through the abasic lesion, *in vitro* experiments found that that DNA polymerases from various organisms preferentially (but not exclusively) incorporate dAMP opposite abasic lesions [48–51]. This is known as the “A rule.” Precise kinetics studies were made with synthetic gapped matrix containing a site-specific abasic analog (tetrahydrofuran). The bypass synthesis by DNA polymerases was studied by varying the position of the 3' and 5' terminus of the primer relative to the abasic site [52, 53]. In all cases the “A rule” was obeyed. It was also shown that the persistence of the non-coding AP-site in the cell may lead to the blockage of DNA replication [54].

10.2

Drug Design

10.2.1

Introduction

Various compounds have been shown to interact or react with DNA at abasic sites. As already mentioned in Section 10.1.3, alkoxyamine derivatives bind covalently to

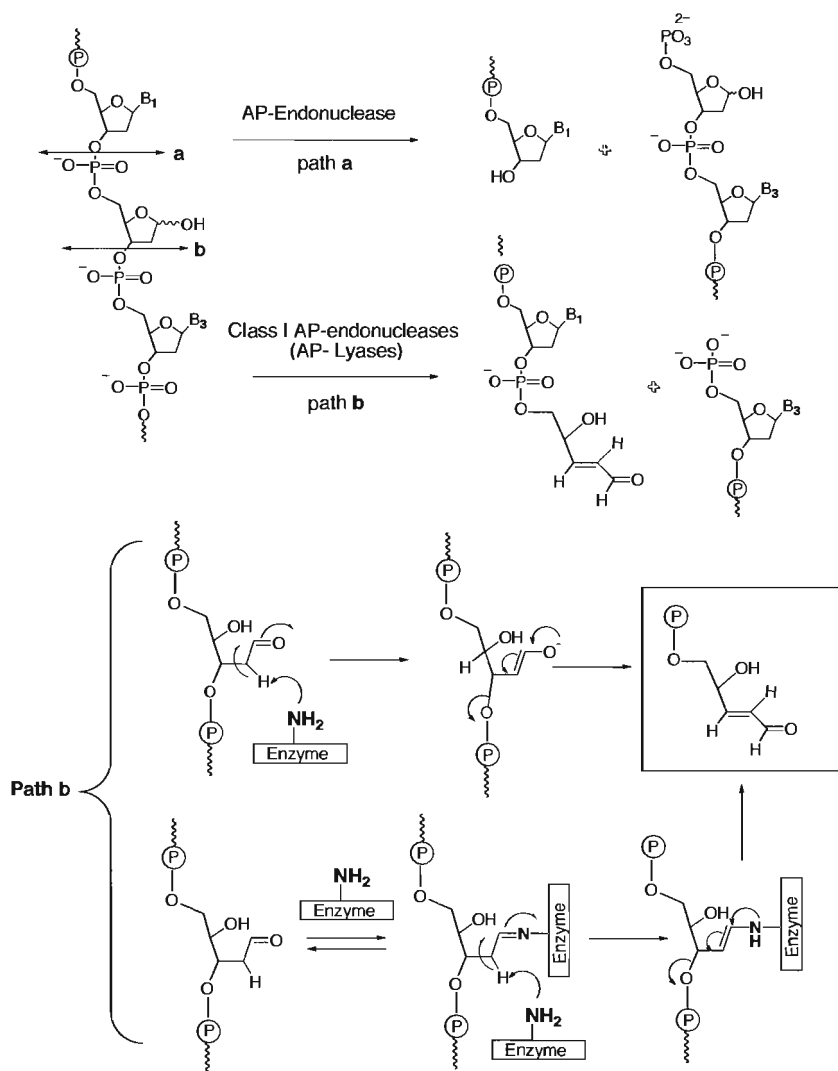


Fig. 10.5. Enzymatic cleavage of the abasic lesion.

the abasic site. Several derivatives were designed for the detection and quantification of the lesion. The simplest one, ^{14}C -labeled methoxyamine, allows quantification of AP-sites by measuring radioactivity of labeled DNA resulting from the formation of ^{14}C -labeled oxime methylether [55, 56]. Other probes have also been prepared (Fig. 10.6), such as ARP, a biotin derivative used in an ELISA-like assay [57–60], and the lissamine probe for fluorescence detection [61, 62].

Two intercalating drugs, 9 amino-ellipticine (9-AE) and 3-aminocarbazole, have been shown to interact with AP-sites and induce DNA cleavage at these sites at low

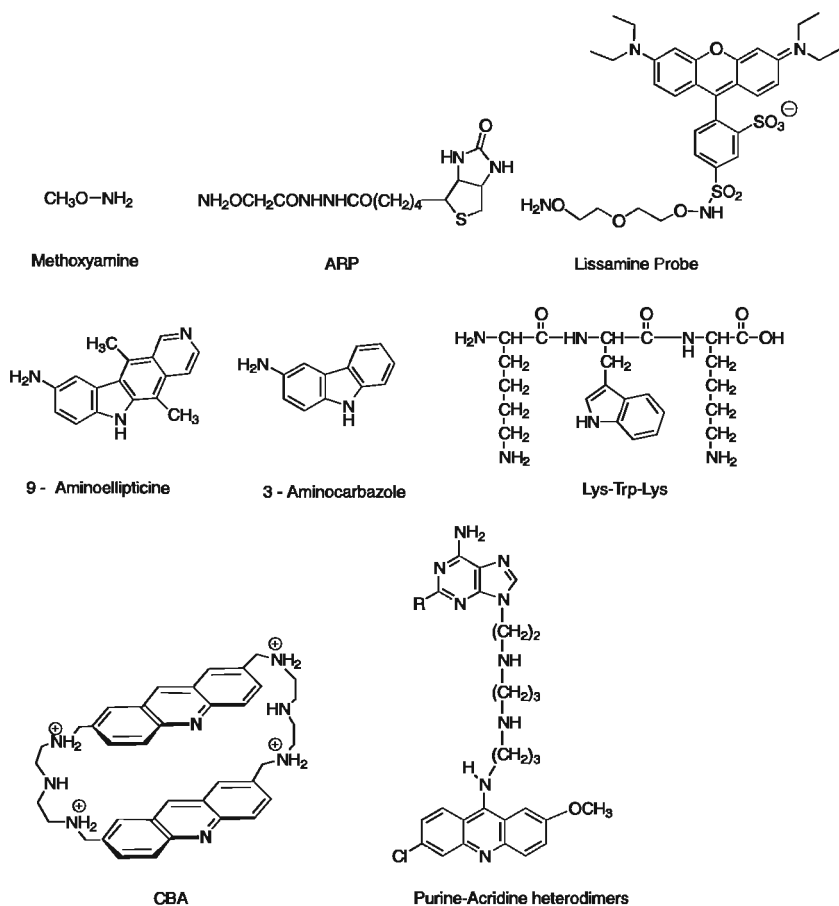


Fig. 10.6. Molecules showing specificity for abasic lesion.

doses [63–65]. The mechanism of cleavage by 9-AE has been thoroughly examined; it is proposed to involve formation of a Schiff base by reaction of the amino group of the drug with the aldehyde function of the AP-site, followed by β -elimination reaction. This results in the formation of a 2',3'-unsaturated deoxyribose and cleavage of the DNA strand at the 3'-side of the site. In presence of a reducing agent, the Schiff base is converted into a stable covalent adduct, and the fluorescence of 9-amino-ellipticine can be exploited to detect and quantify the abasic lesions [66].

Similar reactivities have been observed with oligopeptides containing aromatic and basic amino acids. The interaction of the tripeptide Lys-Trp-Lys with abasic sites containing DNA has been studied in detail. The tripeptide catalyzes the cleavage of the phosphodiester bond adjacent to the AP-site via a β -elimination reaction [67–70]. Specific binding of the tripeptide at the AP-site places the amino

group of a lysine residue in close proximity to the aldehydic function of the ring-opened deoxyribose residue. Proton abstraction at the C'-2 carbon, occurring either on the free aldehyde and/or on the imine formed with an amino group of the Lys-Trp-Lys results in β -elimination of the 3'-phosphate [35].

More recently the macrocyclic bisacridine CBA, which belongs to the cyclobisintercaland family [71], was reported to interact at abasic sites in a very specific mode known as threading intercalation. Combined high-field NMR spectroscopy and restrained molecular dynamics calculations suggest the formation of a major complex in which CBA penetrates the double helix at the abasic site, sandwiching the base pair 3' to the lesion between the two acridine rings, and leaving one protonated linking chain in each groove [72]. Furthermore, CBA induced selective photocleavage in the vicinity of the abasic lesion on both strands of a 23-mer duplex containing tetrahydrofuran analog [73]. For more on the cyclobisintercaland family, see Chapter 11.

With the ultimate goal of developing molecules capable of interfering with the repair processes in the cell, we designed a series of heterodimers for specific recognition and binding to abasic sites in DNA [74, 75]. These tailor-made molecules include: (1) a recognition unit, i.e. a nucleic base that inserts into the abasic cavity and gives hydrogen bonds with the complementary base in the opposite strand; (2) an intercalator for strong but non-sequence-specific binding to DNA; and (3) a linking chain endowed with binding and/or cleavage activities. These molecules may act as "artificial nucleases" and/or DNA-repair inhibitors (Fig. 10.7).

Following this hypothesis, a large series of heterodimers was prepared [74–79]. The most representative compounds are depicted in Figs 10.8–10.10.

Compounds 1–9 that contain aliphatic amines in the linker behaved as "artificial nucleases" (see Section 10.2.4). They are able to cleave the abasic strand very efficiently via a β -elimination catalysis. A second DNA-modifying motif was also introduced on the intercalating moiety as exemplified by molecules 10–16 (Fig. 10.9). Their nuclease properties are described in Section 10.2.3.

In a third group of compounds, the linker containing amide and/or guanidi-

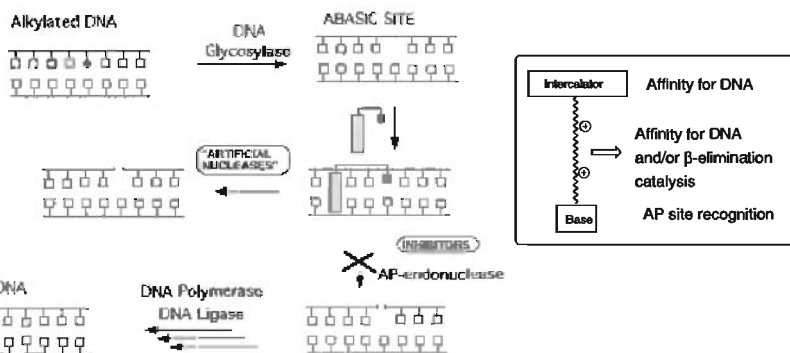


Fig. 10.7. Design of a family of base-acridine heterodimers.

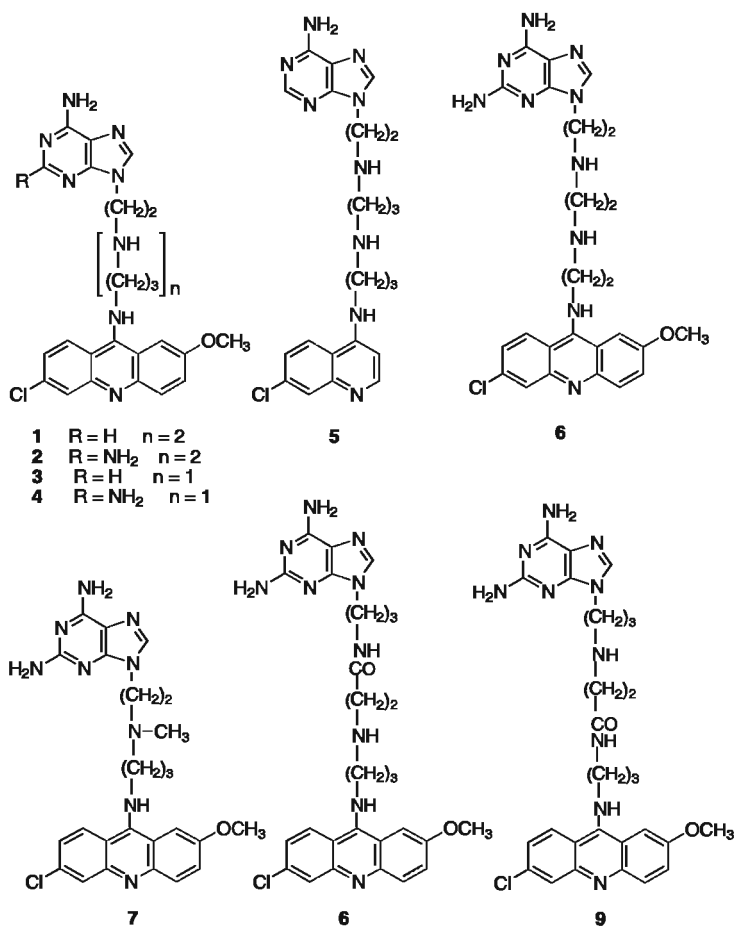


Fig. 10.8. "Artificial nucleases."

nium functions is totally devoid of cleavage properties (compounds 17–28, Fig. 10.10). The biological properties of these compounds are discussed in Sections 10.2.6 and 10.3.

10.2.2

Synthesis of the Heterodimers

As shown in Schemes 10.1 and 10.2, two main strategies were designed to prepare the different series of heterodimers. Molecules containing polyamines as linkers were synthesized in three steps (Scheme 10.1): (1) *N*-9 alkylation of the nucleic base, adenine or 2,6-diaminopurine, by 1,2-dibromoethane; (2) chain elongation by reaction with the polyamine; and (3) introduction of the intercalating nucleus, ac-

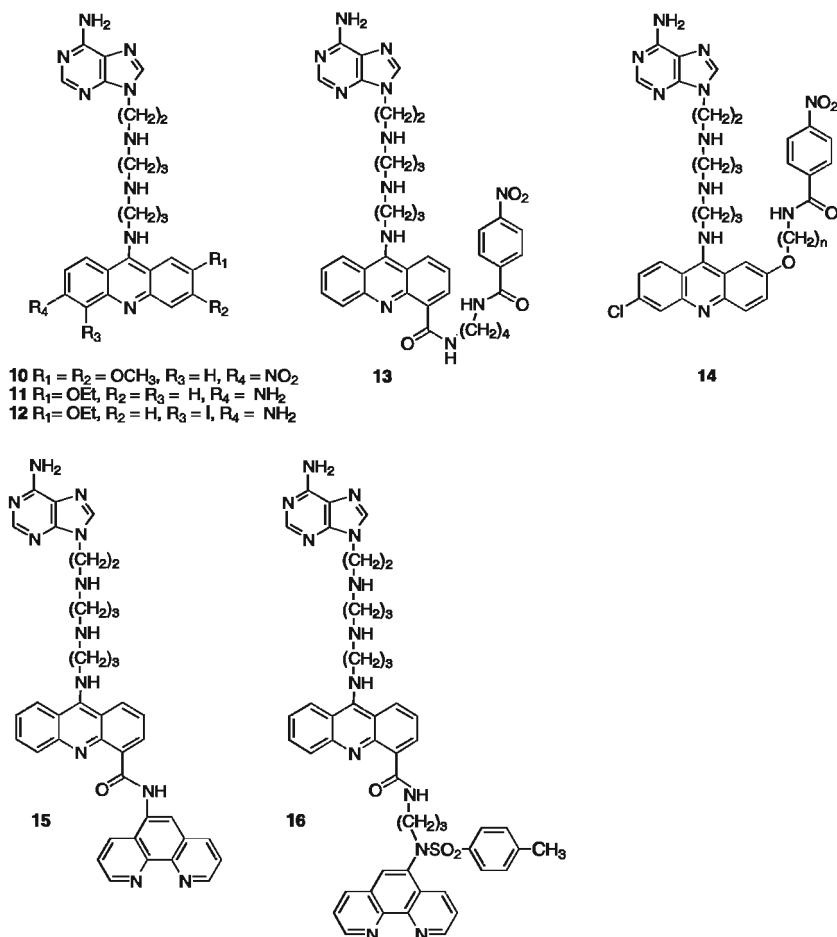


Fig. 10.9. Bifunctional “nucleases.”

ridine or quinoline, by reaction of the terminal primary amine of the linker with the phenoxy or chloro heterocycle [75]. Post-functionalization of the secondary amines of compounds 4 and 1, afforded the tertiary amino-substituted compound 7 [80] and the bis-guanidino derivative 18 [79], respectively.

For the preparation of the bifunctional nucleases 10–16, two strategies were used: (1) the acridine nucleus itself was converted into a photocleavage agent by introduction of various substituents (R_2 , $R_3 = \text{NO}_2$, NH_2 , or iodine) on the heterocycle, (2) a modifying agent, i.e. nitrobenzamide or phenanthroline, was tethered to the acridine nucleus. In both strategies, the purine linker synthon is introduced on the functionalized 9-chloro or 9-phenoxy acridines in the last step.

Compounds 20–28, in which the two chromophores are tethered via N,N' -dialkylguanidine, were prepared by the step-by-step construction of the guanidine

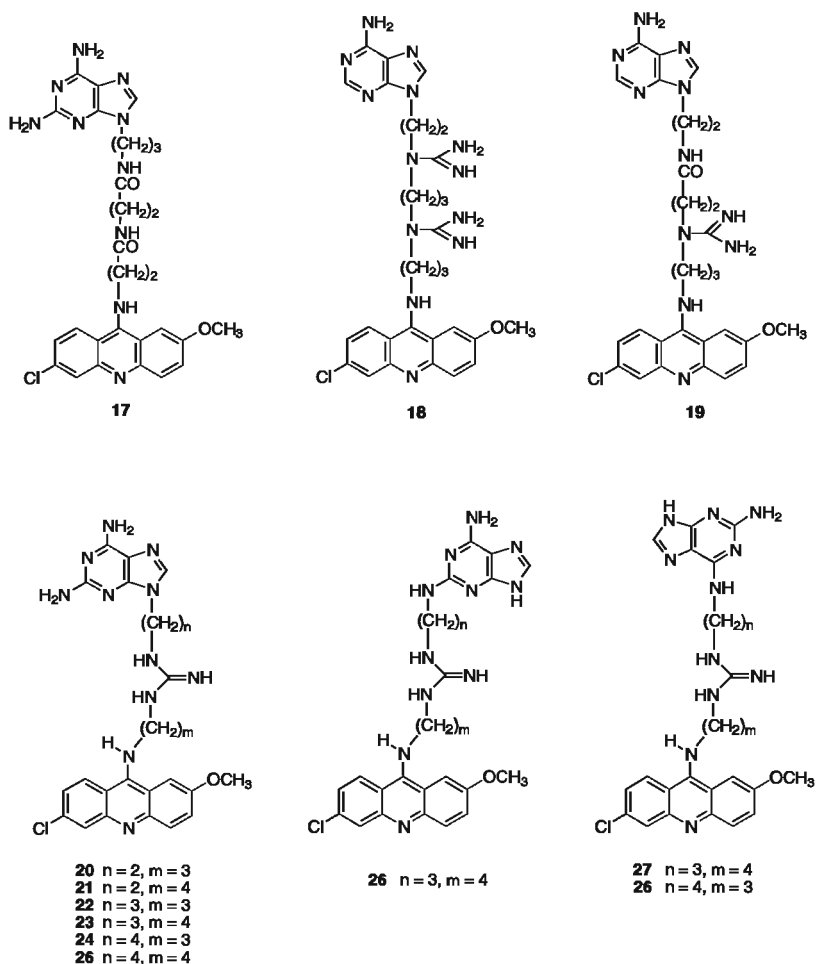
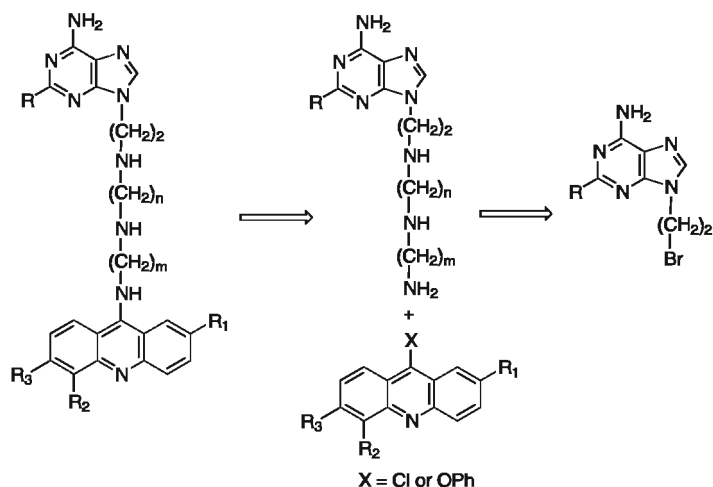


Fig. 10.10. DNA repair inhibitors.

linker (Scheme 10.2) [76, 77]. The key step involves coupling of an ω -alkylamino-substituted purine with a protected amino thiourea.

The linker was introduced on various positions of the 2,6-diaminopurine and different pathways were adopted to prepare the ω -alkylamino-substituted purine key intermediates (Scheme 10.3) [77].

A slightly modified route was used to prepare molecules possessing amido or mixed amino-amido linkers [74, 78]. The key step is the synthesis of the poly-functionalized chains $\text{Cl}-(\text{CH}_2)_n-\text{X}-(\text{CH}_2)_m-\text{Y}-(\text{CH}_2)_p-\text{NH}_2$, bearing a halogen atom at one end for purine alkylation, a protected primary amine at the other end for reaction with the activated acridine, and including amido and protected secondary amino groups (X, Y). A typical synthesis is shown in Scheme 10.4.



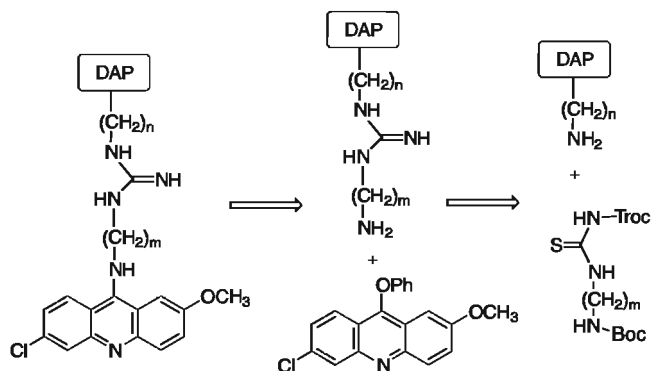
Scheme 10.1. General strategy for the synthesis of the “artificial nucleases.”

10.2.3

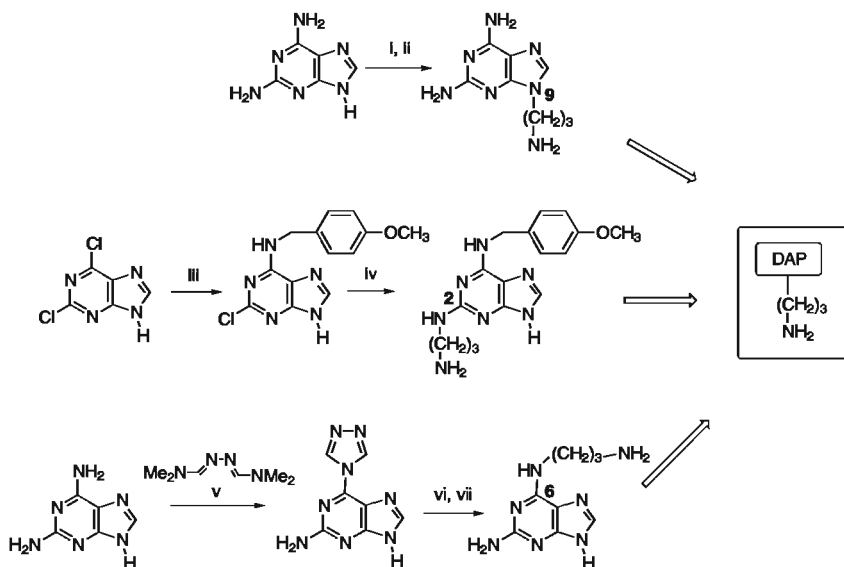
Nuclease Properties

In a first approach to optimize the selectivity of the heterodimers for abasic sites, we used their ability to cleave the DNA strand at AP-sites. The cleavage efficiency was evaluated by agarose gel electrophoresis using supercoiled pBR322 plasmids containing an average of two abasic sites per DNA molecule [75]. Single-strand cleavage at the abasic site converts the supercoiled form into a relaxed circular form, which migrates more slowly. To illustrate the methodology, the results obtained with Lys-Trp-Lys and compound **2** are shown in Fig. 10.11.

We also measured the association constant of each compound with calf thymus DNA. The data are collected in Tab. 10.1. All molecules bind strongly to DNA. The three parts of the molecules are necessary for cleavage, the “half molecules” **29**



Scheme 10.2. Synthesis of guanidinium containing heterodimers.



i) 3-Bromopropylphthalimide, DMF, NaH, rt, 6 days; ii) 12N HCl/H₂O/AcOH 1/1/1 mixture, 100°C, 24h; iii) *para*-methoxybenzylamine, NEt₃, DMF, 80°C, 2h; iv) 1,3-diaminopropane, 140°C, 2h; v) DMF, 180°C, 18h; vi) BocNH-(CH₂)₃-NH₂, 140°C, 1.5 days; vii) 1N HCl in AcOH, rt, 1h.

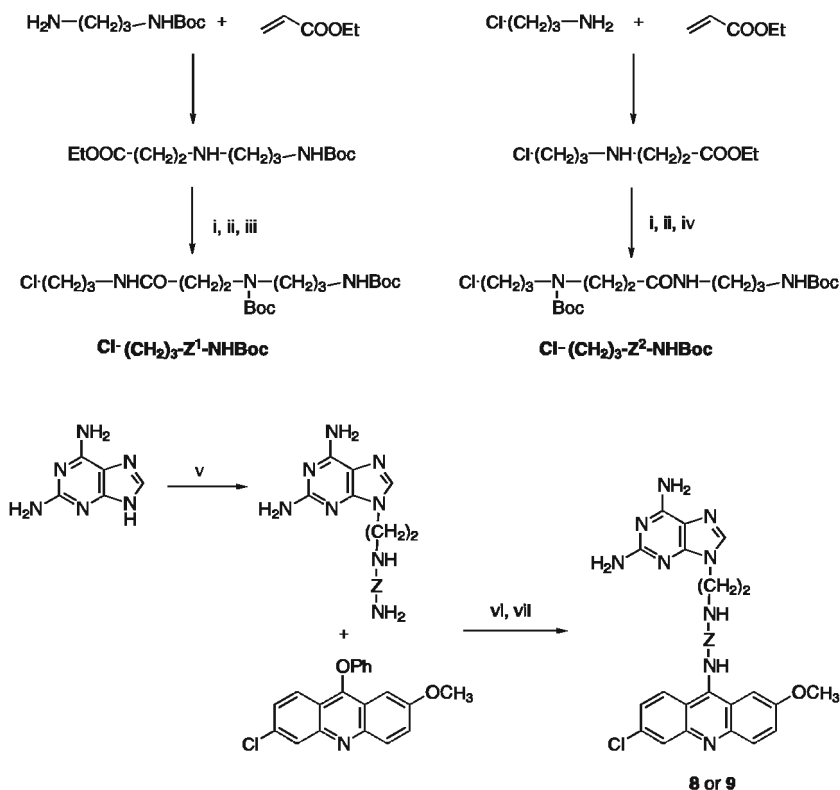
Scheme 10.3. Regioselective functionalization at position 2, 6, or 9 of the 2,6-diaminopurine [76, 77]. (i) 3-Bromopropylphthalimide, DMF, NaH, rt, 6 days. (ii) 12N HCl/H₂O/AcOH 1/1/1 mixture, 100°C, 24 h. (iii) *para*-

Methoxybenzylamine, NEt₃, DMF, 80°C, 2 h. (iv) 1,3-diaminopropane, 140°C, 2 h. (v) DMF, 180°C, 18 h. (vi) BocNH-(CH₂)₃-NH₂, 140°C, 1.5 days. (vii) 1N HCl in AcOH, rt, 1 h.

and **30** (Fig. 10.12) being almost totally inactive in the conditions of the experiments, and for molecules possessing identical linker a relationship is observed between binding and cleavage. The presence of at least one secondary aliphatic amine in the linking chain is necessary for cleavage activity.

The most efficient molecule, **2**, possesses 2,6-diaminopurine as nucleic base, 9-aminoacridine nucleus as intercalator, and two secondary amines in the linker. This molecule is able to cleave plasmid DNA at nanomolar concentrations, and can therefore be considered as the most potent abasic selective artificial nuclease. All data are in agreement with formation of a specific complex to which all three modules participate, prior to cleavage, as was confirmed by high-field NMR experiments (see Section 10.2.5) [30].

It is interesting to note the critical effect of the protonation state of the amines present in the linker. For the most active molecules, **1** and **2**, which possess the same linking chain, one of the amino groups of the linker that is essentially protonated at neutrality (pK_a 8.5), participates in the formation of the specific complex by ionic interaction with the DNA phosphates, while the second amino group of



i) NaOH, EtOH, rt, 24h; ii) Boc_2O , dioxane/ H_2O ; iii) Chloropropylamine hydrochloride, isobutylchloroformate, NEt_3 , THF, 0°C ; iv) *t*-butoxycarbonylamine-propylamine, isobutylchloroformate, NEt_3 , THF, 0°C ; v) $\text{Cl}-(\text{CH}_2)_3-\text{Z}-\text{NHBoc}$, K_2CO_3 , DMF, 80°C , 48h; vi) 1N HCl in AcOH, rt; vii) PhOH, 80°C

Scheme 10.4. Synthesis of compounds 8 and 9 containing amine/amide linkers [74]. (i) NaOH, EtOH, rt, 24 h. (ii) Boc_2O , dioxane/ H_2O . (iii) Chloropropylamine hydrochloride, isobutylchloroformate, NEt_3 , THF, 0°C .

(iv) *t*-Butoxycarbonylamine-propylamine, isobutylchloroformate, NEt_3 , THF, 0°C . (v) $\text{Cl}-(\text{CH}_2)_3-\text{Z}-\text{NHBoc}$, K_2CO_3 , DMF, 80°C , 48 h. (vi) 1N HCl in AcOH, rt. (vii) PhOH, 80°C .

the linker, being partially unprotonated (pK_a 6.7), catalyzes the cleavage. Changing the number of methylenes in the bridge as in molecule 6 dramatically lowers the pK_a values of the aliphatic amines (pK_a 4.7 and 7.8), and the resulting molecule exhibits a very different pH/activity profile, with the highest cleaving activity observed at pH 4 [80].

Molecules such as 18 that contain highly basic guanidine groups ($\text{pK}_a > 11$) display the highest affinity for DNA but do not induce any cleavage as they are fully protonated at physiological pH. The most important features that emerge from this study are summarized in Fig. 10.13.

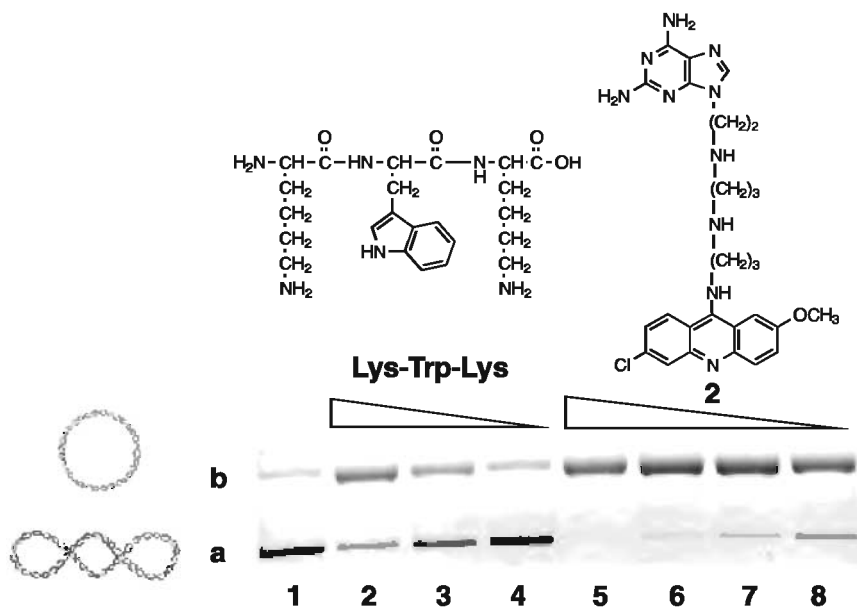


Fig. 10.11. Agarose gel analysis of the nuclease activity of Lys-Trp-Lys and compound 2. (a) pBR322 supercoiled plasmid containing two AP sites/plasmid $2 \cdot 10^{-9}$ M. (b) Relaxed circular form. Lane 1: Reference; lanes 2–4: Lys-Trp-Lys 10^{-3} , 10^{-4} , and 10^{-5} M; lanes 5–8: Compound 2 10^{-5} , 10^{-6} , 10^{-7} , and 10^{-8} M.

10.2.4

Molecules Inducing Multiple DNA Damage

Clustered lesions, or locally multiply damaged sites (LMDS), in tracks of a few base pairs appear to be very toxic as they present a challenging repair problem for the cell [81, 82]. Hence the production of new lesions in the vicinity of the abasic site seems an attractive strategy to increase the biological activity. This can be done by introducing a second DNA-damaging or cleavage group on the artificial AP nuclease 1 (Fig. 10.14).

The DNA-modifying activity of these bifunctional compounds (Fig. 10.9) was tested on a 23-oligomer containing the tetrahydrofuran analog (Fig. 10.15).

In compounds 10–12 the acridine nucleus bears amino-, nitro-, or iodo- substituents. The modification of the acridine does not alter the specificity of the drugs for the abasic site and their cleavage efficiency. The three molecules show photodamaging activity that occurs mainly in close proximity to the lesion [83].

In compounds 13–16, a reporter group capable of inducing DNA damage or cleavage is tethered to the acridine 2- or 4-positions [84]. No DNA-damaging activity was observed with 13, but nitrobenzamide-acridine conjugates 14 ($n = 4$ or 6) appeared to be AP-site-specific photodamaging agents [84]. As illustrated by molecular modeling calculations, the molecules interact with DNA in a very simi-

Tab. 10.1. Binding constants (K_a) for native calf thymus DNA, measured by ethidium bromide displacement and cleavage activity on pBR322 DNA plasmid containing approximately two apurinic sites.

Molecules	Affinity $K_a \times 10^{-5} (M^{-1})$	Cleavage activity	
		a (%)	b
1 Ade-C ₂ -NH-C ₃ -NH-C ₃ -NHAc	2	90	5×10^{-9}
2 DAP-C ₂ -NH-C ₃ -NH-C ₃ -NHAc	11	100	1×10^{-9}
5 Ade-C ₂ -NH-C ₃ -NH-C ₃ -NHClQ	0.1	90	8×10^{-8}
6 DAP-C ₂ -NH-C ₂ -NH-C ₂ -NHAc	4	80	7×10^{-7}
3 Ade-C ₂ -NH-C ₃ -NHAc	0.7	23	$>10^{-5}$
4 DAP-C ₂ -NH-C ₃ -NHAc	2	26	$>10^{-5}$
5 DAP-C ₂ -N(CH ₃)-C ₃ -NHAc	0.4	10	$>10^{-5}$
17 DAP-C ₂ -NHCO-C ₂ -NHCO-C ₂ -NHAc	1.2	0	
8 DAP-C ₂ -NHCO-C ₂ -NH-C ₃ -NHAc	5	23	$>10^{-5}$
9 DAP-C ₂ -NH-C ₂ -CONH-C ₃ -NHAc	3.5	23	$>10^{-5}$
18 Ade-C ₂ -Gua-C ₃ -Gua-C ₃ -NHAc	20	0	
29 Ade-C ₂ -NH-C ₃ -NH-C ₃ -NH ₂		0	
30 AcrNH-C ₃ -NH-C ₃ -NH ₂		0	

Plasmid concentration 2×10^{-9} M, incubation time 20 min. pH = 7.4.

$T = 37^\circ\text{C}$.

(a) Observed cleavage rate percentage for 1×10^{-5} M drug concentration.

(b) Drug concentration (M) inducing 50% cleavage.

Abbreviations: C₂, (CH₂)₂; C₃, (CH₂)₃; Acr, 6-chloro-2-methoxyacridine; ClQ, 7-chloroquinoline; Gua, guanidine.

lar way to that of the original artificial nucleases **1** or **2** [85]. Both side chains of the intercalated acridine are positioned in the minor groove.

The acridine–phenanthroline conjugate **16** is very promising as it cleaves efficiently and selectively the DNA strand at the base opposite the abasic site (Fig. 10.16). This molecule probably interacts with DNA by threading intercalation, i.e.

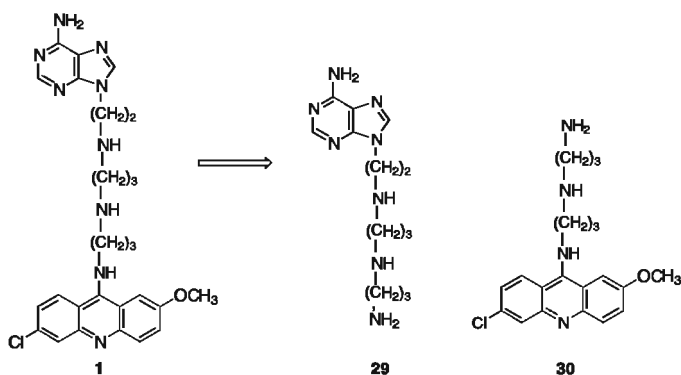


Fig. 10.12. “Half-molecules” **29** and **30**.

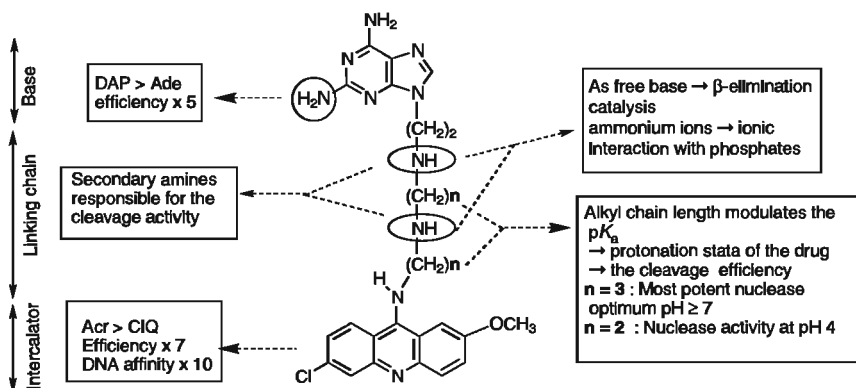


Fig. 10.13. Optimization of the AP site cleavage activity.

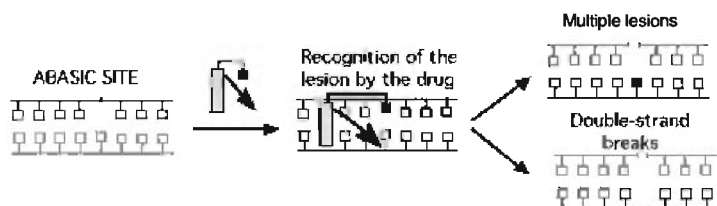


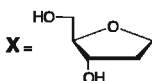
Fig. 10.14. Induction of multiply-damaged sites.

by positioning a side chain in each groove of the DNA [86, 87]. The presence of the chain joining the phenanthroline to the acridine 4-position confers to the conjugate the degree of freedom required for adequate positioning of the DNA-cleavage agent inside one groove. This compound thus seems to be a good candidate for the generation of double-stranded DNA cleavage (via its intrinsic β -elimination cleaving activity on the abasic strand and radical cleavage on the opposite strand).

10.2.5

Drug–DNA Interaction

The mode of recognition of the abasic sites by drugs and the structure of the drug/DNA complexes were investigated by studying the interaction of the drugs with a



Strand 1: $5' \text{C}_1 \text{G}_2 \text{C}_3 \text{G}_4 \text{T}_5 \text{A}_6 \text{C}_7 \text{G}_8 \text{C}_9 \text{A}_{10} \text{C}_{11} \text{X}_{12} \text{C}_{13} \text{A}_{14} \text{C}_{15} \text{G}_{16} \text{C}_{17} \text{A}_{18} \text{T}_{19} \text{G}_{20} \text{C}_{21} \text{G}_{22} \text{C}_{23} 3'$
 Strand 2: $3' \text{G}_{46} \text{C}_{45} \text{G}_{44} \text{C}_{43} \text{A}_{42} \text{T}_{41} \text{G}_{40} \text{C}_{39} \text{G}_{38} \text{T}_{37} \text{G}_{36} \text{T}_{35} \text{G}_{34} \text{T}_{33} \text{G}_{32} \text{C}_{31} \text{G}_{30} \text{T}_{29} \text{A}_{28} \text{C}_{27} \text{G}_{26} \text{C}_{25} \text{G}_{24} 5'$

Fig. 10.15. A 23-oligomer containing the tetrahydrofuran analog.

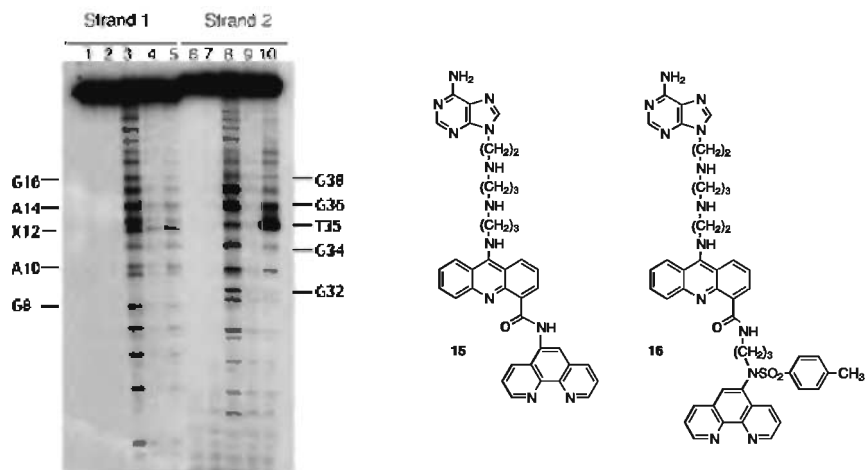


Fig. 10.16. DNA cleavage induced by phenanthroline-acridines **15** and **16**. Lanes 1 and 6: DNA alone; lanes 2 and 7: Cu; lanes 3 and 8: phenanthroline-Cu; lanes 4 and 9: **15** and Cu; lanes 5 and 10: **16** and Cu. (Mercaptopropionic acid, MPA, was used as reducing agent.)

short (11-mer) synthetic DNA duplex containing the stable tetrahydrofuran analog of the abasic site. The sequences are shown in Fig. 10.17.

Different methods such as thermal denaturation studies, ¹H NMR, and electron paramagnetic resonance (EPR) were used. UV Spectrophotometry is a simple method used to study the thermal denaturation of DNA duplexes. We determined the *T_m* values (melting temperatures) of the abasic site-containing duplex (TX) and of the conventional TA duplex in the absence and presence of interacting drugs [88]. The loss of a base leads to a significant decrease of *T_m* value (37°C for the TX duplex versus 55°C for the TA duplex in 10 mM phosphate buffer at pH 7, containing 20 mM NaCl). We investigated the effects of drugs such as **1**, **2**, **30**, and ethidium bromide (BET) on TA and TX duplexes. All four molecules stabilized the TA duplex quite efficiently due to non-specific binding of the intercalating moiety and ionic interaction contribution of the amino chain. The stabilizing effect of these drugs on the abasic duplex was considerably increased. At low drug-to-AP-site ratios (less than 1), the specific contribution of the recognition of the abasic pocket by the nucleobase of the drug could be evaluated (the *T_m* increase was more significant for **1** and **2** than for **30** and BET). At higher drug concentrations, the

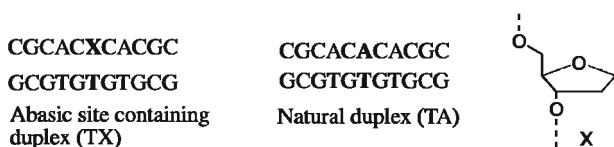


Fig. 10.17. Sequence of the synthetic oligonucleotide used for interaction studies.

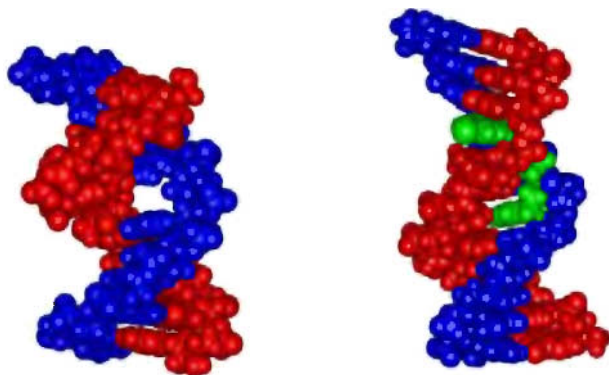


Fig. 10.18. CPK representations of the abasic oligomer (left) and of its complex with **1**. The strand containing the abasic lesion is shown in red, the complementary strand in blue, and the interacting drug **1** in green [30].

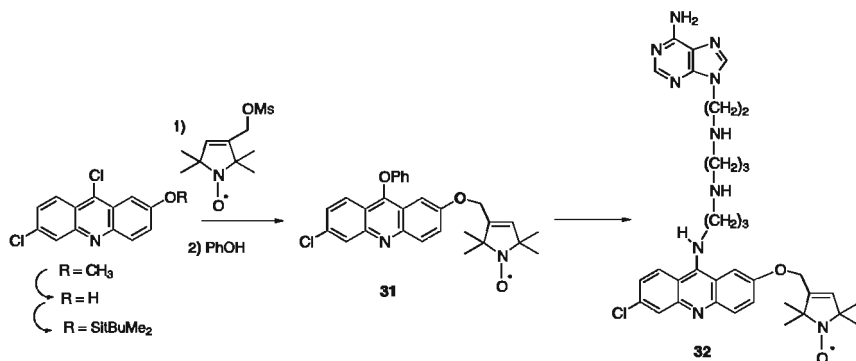
specific contribution of the recognition moiety was overcome by the ionic interaction contribution of the protonated amino groups of the aliphatic chain (the T_m increases for **2** and **30** became similar). Other compounds, e.g. guanidinium-containing molecules **20–28**, were also tested, and similar T_m increases were measured [76].

High-field NMR spectroscopy coupled to molecular modeling revealed a similar mode of binding for molecules **1** and **2** [30]. Two complexes are formed in which the purine base of the drug inserts into the abasic pocket and pairs with thymine in the opposite strand, most probably in the Hoogsteen mode. The acridine intercalates at a two-base-pair distance 5' to the abasic site, and the polyamino chain lies in the minor groove (Fig. 10.18). The two complexes only differ by a 180° rotation of the acridine ring around the C9–N bond.

Compound **18**, the analog of **1** that possesses two guanidinium functions in the linker [79], also forms specific 1:1 complexes with the stable model abasic duplex (TX), as indicated by thermal denaturation experiments and high-field NMR spectroscopy. Two types of specific complexes were identified by ^1H NMR. In both complexes the adenine moiety of the drug is inserted into the abasic cavity. Watson–Crick base-pairing of this adenine with thymine in the opposite strand was definitely established by measurement of characteristic NOEs between protons of the complementary bases (T17–H3 and Ade–H2 of the drug).

EPR studies of the interaction were done with compound **32**, a spin-labeled analog of **1** in which a nitroxide probe was grafted on the acridine 2-position (Scheme 10.5). Its binding to abasic oligonucleotide provided additional data on the kinetics of complex formation [89, 90].

The observed correlation time is significantly increased by the interaction with the abasic duplex ($\tau_c = 1.3 \times 10^{-10}$ s for the free probe in solution and $\tau_c = 6 \times 10^{-9}$ s in the presence of the abasic duplex). More interestingly, it was shown



Scheme 10.5. Synthesis of the nitroxide labeled compound **32** [89].

by this technique that the affinity of **32** is much higher for the abasic duplex than for the natural duplex ($K_{\text{aff}} = 1.5 \times 10^6 \text{ M}^{-1}$ and $4 \times 10^4 \text{ M}^{-1}$ respectively).

10.2.6

Enzyme Inhibition

As discussed in the introduction, abasic sites are formed as intermediates during the repair of modified bases produced by anticancer alkylating agents through the BER mechanism. Molecules that bind specifically and strongly to abasic sites may inhibit this repair process and thus sensitize tumor cells by potentiating the activity of alkylating drugs.

The BER pathway has only been recently suggested as a target for such a potentiation and very few BER inhibitors have been reported. The first inhibitor mentioned in the literature was methoxyamine, a molecule that reacts with abasic sites and inhibits AP endonucleases *in vitro*, but at very high doses (50 mM) [91]. Malvy *et al.* reported that 9-aminoellipticine (9-AE) and the structurally related isopropyl-oxazolopyridocarbazole potentiate the cytotoxic effect of alkylating drugs such as dimethyl sulfate in *E. coli* through a mechanism involving apurinic sites [92]. They later showed that, *in vitro*, 9-AE inhibits 65% of the endonuclease activity of *E. coli* exonuclease III [93]. It was suggested that inhibition might result from irreversible adduct formation at the 3'-side of the apurinic site following cleavage [26, 63, 94].

In our laboratory we have been developing for the last decade a new concept of inhibition in relation to the AP-site. This strategy is based on specific recognition of the abasic lesion by the synthetic heterodimeric compounds described above. By masking the AP-site to AP endonucleases these compounds may inhibit their repair activity. Even the heterodimers acting as artificial endonucleases might increase the toxicity of DNA-damaging agents whose adducts are repaired by the BER pathway, since this chemical cleavage activity occurs irrespective of the co-regulation proteins involved in the BER pathway. The BER inhibition was inves-

tigated [95] using the 3-D assay, a chemiluminescence microplate assay summarized by the following four steps:

1. A plasmid DNA is adsorbed in the wells of sensitized microplates.
2. DNA is treated with methyl methane sulfonate (MMS).
3. The compounds to be tested are incubated with a mixture containing all the constituents necessary for the *in vitro* DNA repair reaction [96]. During this reaction, damaged DNA fragments are excised and replaced by neo-synthesized DNA fragments incorporating digoxigenylated dUTPs.
4. The DNA repair activity is quantified with an anti-digoxigenin antibody conjugated with alkaline phosphatase. The conjugated enzyme dephosphorylates a substrate (lumigen) which becomes fluorescent.

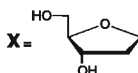
The results indicated that **2** and **1** were potent inhibitors of BER with IC_{50} values of 70 and 62 μM respectively. These compounds were clearly more efficient than methoxyamine (480 μM), a BER inhibitor used as a reference [97].

Since the BER pathway involves key steps such as abasic site incision by specific AP endonucleases, some of the heterodimeric compounds described above were tested for their ability to inhibit the AP-endonucleolytic activity of exonuclease III *in vitro*. Exonuclease III is a class II AP endonuclease, which accounts for more than 80% of the cellular AP endonuclease activity in *E. coli* [98]. The substrates of exonuclease III were synthetic oligonucleotides (23-mers) containing the abasic analog X (Fig. 10.19).

The sequences were chosen in order to form duplexes containing the four different abasic sites (A, C, G, or T facing the lesion).

We tested the effect of **1** and **2** on the activity of exonuclease III. For comparison, we also investigated molecules devoid of recognition motif: **30** and BET, a classical intercalator. In our conditions, the four drugs decreased very slightly the cleavage activity of exonuclease III at apyrimidic sites (A or G opposite X). In contrast, a significant inhibitory effect was observed with **1**, **2** and even **30** at the apurinic site where a thymine is facing the lesion. At a drug-to-AP-site ratio of 2, the percentage of cleavage by the enzyme decreased from 30% to 15% after an incubation of 30 min (unpublished results). All drugs were ineffective on the other apurinic site where a cytosine faces the abasic lesion.

The specific effect of **1** and **2** was interpreted in terms of a specific interaction in which the nucleobase of the drug (adenine or diaminopurine) pairs with the complementary single thymine of the baseless duplex. The surprising inhibitory effect



Strand 1: 5' C₁ G₂ C₃ G₄ T₅ A₆ C₇ G₈ C₉ A₁₀ C₁₁ X₁₂ C₁₃ A₁₄ C₁₅ G₁₆ C₁₇ A₁₈ T₁₉ G₂₀ C₂₁ G₂₂ C₂₃ 3'

Strand 2: 3' G₄₆ C₄₅ G₄₄ C₄₃ A₄₂ T₄₁ G₄₀ C₃₉ G₃₈ T₃₇ G₃₆ N₃₅ G₃₄ T₃₃ G₃₂ C₃₁ G₃₀ T₂₉ A₂₈ C₂₇ G₂₆ C₂₅ G₂₄ 5'

N = A, G, C, T

Fig. 10.19. 23-mer duplexes used for enzyme inhibition experiments.

(a decrease of the cleavage activity from 30% to 20% in the presence of two drugs per lesion) of **30** (devoid of abasic site recognition unit) at both apurinic sites was attributed to a sequence context effect. In our duplex, the local environment at apurinic sites (GpyrG facing CXC) might be more flexible than at apyrimidic sites (GpurG facing CXC). This flexibility might allow the acridine insertion in the apurinic pocket with a significant contribution of the protonated amino chain to the binding. Similar observations were done when investigating the specific recognition of apurinic sites by these same compounds using melting temperature (T_m) measurements (see Section 10.2.3).

In a similar approach, **19** was tested for exonuclease III inhibition on the natural abasic site (2'-deoxyribose). Compound **19** (Fig. 10.10) shows no cleavage activity since the amino groups of the linker in **1** were replaced by one amido and one guanidine functions (fully protonated at pH 7). At a concentration of one molecule of **19** for six base pairs, a 50% inhibition of the AP endonucleolytic activity of exonuclease III was observed on a depurinated plasmid DNA (pBR322) containing two apurinic sites per DNA molecule [78]. The overall effects of these drugs on mammalian cells have been also investigated (see Section 10.3).

In addition, more recently, it has been reported that genomic damage, such as abasic sites, may influence the activities of topoisomerases [12, 13]. This represents an additional impulse for designing abasic site-specific drugs.

10.3

Pharmacological Data

Compounds **1** and **2** tested against L1210 cultured cells are moderately cytotoxic (IC_{50} 1.5 and 2 μ M respectively) but more interestingly potentiate the DNA-methylating agent methyl methane sulfonate (MMS) as the IC_{50} drops from 350 μ M with MMS alone to 10 μ M with MMS combined with either **1** (0.7 μ M) or **2** (1 μ M) [95]. In the same study, sensitization to bis-chloroethylnitrosourea (BCNU), a clinically useful anticancer drug was also observed. In the presence of BCNU the IC_{50} value shifts from 5.6 μ M with BCNU alone to 0.43 or 0.85 μ M with a combination of BCNU and either **1** (0.7 μ M) or **2** (1 μ M). A synergistic effect was also observed with thiotepa used in combination with **2**, but none with alkylating agents such as mitomycin C and streptozocin [95].

Following this study, the heterodimers **18–28** specifically designed as DNA-repair inhibitors (devoid of DNA-cleavage activity) were also investigated and compared with the artificial nucleases **1** and **2**. Intrinsic cytostatic activity was estimated by measuring the dose-dependent growth inhibition of L1210 murine leukemia cells. Cytostatic/cytotoxic properties as well as synergistic potency with BCNU were determined by measuring the clonogenicity of A549 human pulmonary carcinoma cells. Results are given in Tab. 10.2 [76, 77, 79].

Compared with molecules **1** and **2**, the cytostatic activity of **18–28** alone on L1210 cells appeared to be in the same range and rather weak, and cytotoxicity on A549 was moderate.

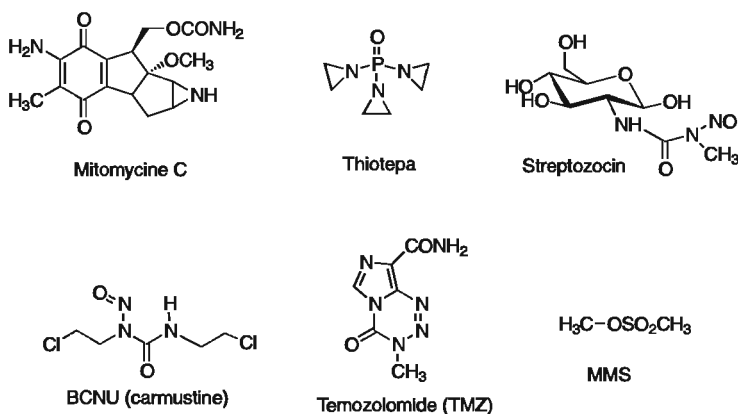


Fig. 10.20. Alkylating agents used for potentiation studies.

Tab. 10.2. Binding constants (K_a) and cytostatic/cytotoxic properties (IC_{50} alone and in association with BCNU) of the drugs tested as DNA-repair inhibitors.

Compound	IC_{50} L1210 (μM) ^a (Growth inhibition)	IC_{50} A549 (μM) ^a (Clonogenic inhibition)	Synergy ^b	K_a ($\times 10^{-5} \text{ M}^{-1}$)
1	$>10^{\text{d}}$	0.1	+ ^c	2
2	$>10^{\text{d}}$	0.05	—	11
18	33	10	+94%	20
19	>100	4	—	2
20	74	3	—	6
21	26	6	—	8
22	>100	28	+74%	0.6
23	60	7	+16%	3.7
24	>100	31	—	2.1
25	>100	43	+49%	4
26	36	5	—	2
27	>100	5	—	3
28	70	1	—	2.2

^aTested compounds were added at various concentrations and incubated for 24 h.

^bApparent synergy was measured by simultaneous exposition of A549 cells to 10 μM of the tested compound and 10 μM BCNU and expressed as the % of toxicity increase with regard to the addition of the toxic effects of both drugs alone (theoretical combined toxicity) taken as 100%.

^c+25% synergy observed after exposition to 0.05 μM of 1 and 10 μM BCNU. Cytotoxicity was too strong at higher doses of 1.

^d IC_{50} of respectively 1.6 and 2.2 μM have been previously observed after 48 h incubation with L1210 cells [95].

Tab. 10.3. Activity of compound **18** alone or in association with BCNU on the P388 murine leukemia.

Treatment	Average survival (days $\pm$ s.d.)	T/C (Survival of treated animals/Survival of controls) $\times$ 100	Number of surviving animals at 60 days
Controls	14.9 $\pm$ 8	–	0/7
18 (2 mg kg ⁻¹)	16.2 $\pm$ 3	109	0/6
BCNU (6 mg kg ⁻¹)	46 $\pm$ 17	309	3/6
18 (2 mg kg ⁻¹) + BCNU (6 mg kg ⁻¹)	–	–	6/6

Compounds **18**, **22**, **23**, and **25** displayed a significant synergy with BCNU. Interestingly, **22** and **25** are among the less toxic compounds on both cell lines. The most active compound, **18**, was also tested *in vivo* on the murine leukemia P388 (Tab. 10.3). Compound **18** showed no antitumor property by itself but definitely potentiated the action of BCNU (treatment with BCNU alone led to a 60-day survival rate of 50%, while association with **18** resulted in 100% survival). However this compound elicited curare-like acute toxicity, possibly due to the presence of two guanidinium cations in the linker, that severely limited further *in vivo* experiments [79].

Recently, Taverna *et al.* reported sensitization of resistant cell lines to chemotherapeutic alkylating agents by methoxyamine (MX) [99]. Methoxyamine increases significantly the cytotoxicity of temozolomide (TMZ) against HCT116 colon cancer cells and SW480 cell line (Tab. 10.4). HCT116 cells are deficient in DNA mismatch repair (MMR) and are therefore resistant to methylating agents such as TMZ and nitrosourea.

Due to the extreme complexity of the system, these biological data cannot be directly interpreted in terms of mechanism of action. Nevertheless, most of the compounds that were designed to bind specifically and strongly to the abasic site present the most interesting activities *in vitro* and even *in vivo*, most particularly in association with alkylating and/or anticancer drugs that create multiple abasic lesions in the cell. It thus seems that targeting the abasic site by specific drugs may be a pertinent and useful strategy to improve the clinical efficiency of currently available DNA-damaging agents.

Tab. 10.4. Synergistic activity of methoxyamine.

Cell line	TMZ IC₅₀ (μM)	MX IC₅₀ (mM)	MX + TMZ IC₅₀ (μM)
SW480	395	50	172
HCT116	950	28	306

Cells are exposed to 0–1500 μ M TMZ for 2 h or 6 mM MX and TMZ for 2 h.

Dedication

We dedicate this chapter to Professor Jean Lhomme on the occasion of his retirement. Together with the many colleagues who participated in the long and stimulating story of abasic site recognition, we wish Professor Jean Lhomme an enjoyable retirement. He helped us to progress in the fascinating world of bioorganic chemistry, and many young scientists benefited from his teaching and advice.

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11

Interactions of Macrocyclic Compounds with Nucleic Acids

Marie-Paule Teulade-Fichou and Jean-Pierre Vigneron

11.1

Introduction

The selective recognition of single- and double-stranded nucleic acids, as well as their many secondary and tertiary structures, constitutes a challenge to chemists and biologists. Among the many DNA ligands discussed in the literature, the macrocyclic compounds are the most intriguing ones. Their very structure implies that it would be very difficult for them to bind to double-stranded (ds) DNA, at least in the intercalative mode. However, natural antibiotics of the triostin family, for instance, which are composed of a cyclic peptide with two intercalator quinoline rings, are able to associate with double-stranded DNA by inducing a strong local distortion of the double helix. This means that the double-stranded DNA matrix can accommodate large complex synthetic molecules. Moreover, increasing knowledge on nucleic acids structure, particularly on the conformational flexibility of double helices, makes it possible to envisage new binding modes for synthetic molecules.

In the past decade, a number of macrocyclic synthetic ligands have been designed and synthesized, inspired by both the cyclic structure of many natural compounds and the numerous studies centered on bisintercalators. Indeed, in addition to their high affinity for dsDNA, the latter, such as bisanthracyclines and ditercalinium, elicited significant antitumor activity which strongly stimulated research in this field. More or less simultaneously, several groups synthesized topologically constrained macrocyclic bisintercalators in order to test the ability of DNA to accommodate such structures and possibly to point up new binding modes or unprecedented structural binding selectivities.

In many cases, synthetic strategies and the design of the cyclic structures are rooted in studies on the complexation of nucleotides by synthetic receptors that were carried out in the past two decades. Indeed, work on the molecular recognition of nucleosides and nucleotides by well-defined synthetic models can shed light on the non-covalent interactions between nucleic acids and various important biological compounds. Moreover, the nucleotides not only participate in the funda-

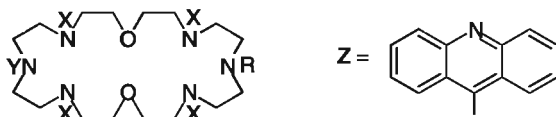
mental mechanisms of storage, replication and transcription of genetic information as building blocks of nucleic acids, but also play a role in a variety of biological processes as chemical energy sources. This research topic led to the rapid development of various molecular architectures essentially based on acyclic dimeric aromatics (molecular tweezers) and macrocyclic polyamines. Noticeable synthetic difficulties came from the need for compounds with large aromatic moieties that were water soluble at a physiological pH. It is important to briefly summarize these studies before reviewing the macrocycle–nucleic acids interactions themselves.

11.2 Nucleotide Complexation

The selective complexation of nucleotides is the result of an interplay between many parameters; of these, electrostatic forces, hydrogen bonding, stacking interactions, and hydrophobic effects play the most important roles. Many multifunctional receptors, such as macrocyclic polyamines, azoniacyclophanes, and cyclo-intercaland compounds, have been designed so as to optimize the participation of each of these different contributions. So far, most of them make use of electrostatic and hydrophobic forces only, hydrogen bonding being highly disfavored in water.

11.2.1 Macrocyclic Polyamines

Like biological polyamines (i.e. putrescine, spermidine, and spermine), macrocyclic polyamines, when protonated, bind to nucleotides strongly and selectively by electrostatic interactions between the cationic ammonium groups of the receptor and the negatively charged phosphate groups [1]. The measured affinities are comparable to those found in enzyme–substrate complexes; for instance, when it is fully protonated, the [24]-N₆O₂ macrocycle **1** binds AMP^{2−}, ADP^{3−}, and ATP^{4−} with log *K_s* values of 6.95, 8.30, and 11.00, respectively [2, 3]. But, in order to improve the selectivity of the nucleotide recognition, macrocycles also need to contain other binding sites capable of interactions with the other parts of the molecule, the sugar moiety, and/or the nucleic base. Nucleic base recognition may be achieved either by stacking interactions or by hydrogen bonding; in the former case the distinction between different nucleic bases rests on differences in stacking energy, while in the latter it results from the presence in the receptor of sites capable of forming complementary hydrogen-bonding patterns. A combination of both is necessary to obtain strong recognition and good selectivity.



Compound **2a** was the first synthetic multifunctional structure able to bind nucleotides by an interplay of electrostatic and stacking interactions [1, 4]. It combines a macrocyclic polyammonium moiety as anion-binding site and an acridine side arm for stacking interactions. Due to this double recognition, protonated **2a** forms stronger complexes with the nucleotides than the parent macrocycle **1**, which does not possess the acridine side arm. However, protonated **2a** is unable to discriminate between the different nucleic bases of the nucleotides. To perform molecular recognition of nucleic bases more specific information features must be introduced in the structure. Of note, **2a** binds strongly to the supercoiled circular dsDNA plasmid pBR322, probably via double interaction involving both intercalation of the acridine subunit within the base pairs and electrostatic attraction between the ammonium groups of the receptor and the phosphate groups of DNA.

The bis-functional analog **2b** possessing two acridine subunits was synthesized subsequently [5]. Unexpectedly, this structural modification did not provide the macrocycle with higher affinities either for nucleotides or for dsDNA as compared with **2a**.

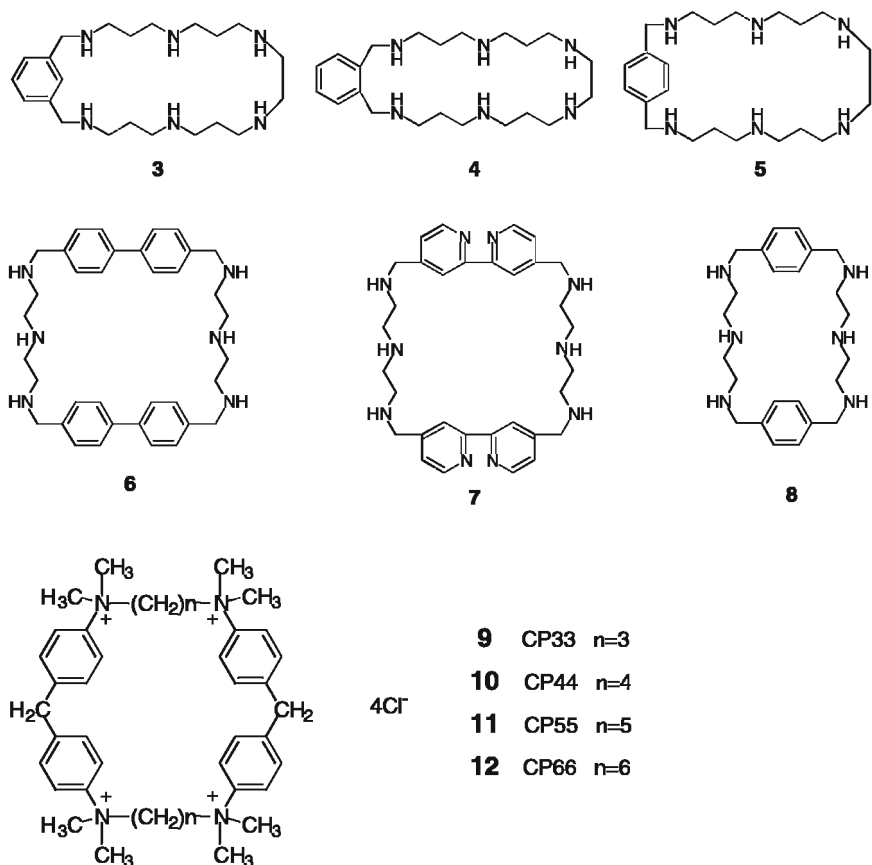
11.2.2

Azoniacyclophanes

Adding stacking interactions to coulombic forces was also the purpose of the design of azoniacyclophanes **3–8**, but the binding of AMP^{2-} , ADP^{3-} , and ATP^{4-} by these ligands results essentially from electrostatic interactions, although NMR studies provided unambiguous evidence for participation of π -stacking to the stabilization of the various complexes [6–9].

Compared with the synthetic receptors studied so far, the **CPnn** azoniacyclophanes **9–12** delineate more structured cavities. These lipophilic compounds are polyfunctional macrocycles composed of two apolar diphenylmethane subunits connected by two polar bridges having variable length and bearing positively charged nitrogen atoms. The geometrical features of the diphenylmethane group make it a suitable structural element for the design of molecular cavities capable of including substrates, particularly aromatic flat compounds [10]. The number of methylene groups in the linkers, n , varies from three to six, which allows the cavity to be tailored to fit specific guests, the ammonium groups providing the receptor

with water solubility. They were prepared according to Koga by cyclization of bis-tosylated bis(4-aminophenyl)methane with α,ω -dibromoalkanes followed by removal of the tosyl groups and permethylation [11].



Complexation of nucleosides and nucleotides by CP66, in aqueous solution, has been extensively studied [12]. It binds purine derivatives strongly and the complexation-induced NMR shift values, up to -1.7 ppm, are in favor of the inclusion of the adenine moiety into the cavity of CP66 with the sugar ring outside. NMR titration curves showed that the stoichiometry of the complexes is 1:1 for every substrate tested. Among purines, the selectivity for adenine derivatives is remarkable: for example, the association constant is 5 times higher for adenosine than for guanosine; the complexation-induced NMR shifts are also smaller for all the other nucleobases. CP66 gives only loose associations with pyrimidine derivatives. Clearly, on the route towards the search of selective receptors for nucleotides, a great step was accomplished with the design of the CP n azoniacyclophanes.

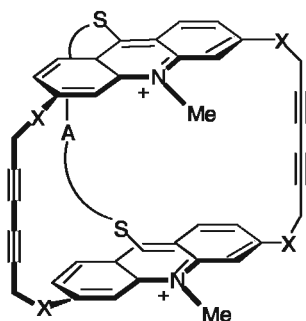
11.2.3

Cyclobisintercalands

The concept of cyclointercalation, developed by Lehn's group, was a breakthrough towards the design of selective synthetic receptors for nucleotides and nucleic acids. In a manner reminiscent of the binding of intercalators between the plateaux of base pairs in double-stranded nucleic acids, the incorporation of flat subunits into macropolycyclic structures gives receptor molecules which may be expected to display molecular recognition of flat substrates. These planar subunits must be of sufficient surface and positioned at a distance suitable for the intercalation of planar substrates such as nucleic bases; moreover the bridges which link them must be sufficiently rigid in order to prevent the collapse of the cavity because of hydrophobic effects in water. Of special interest, as structural groups, are the planar molecules known to interact with nucleic acids by intercalation. In that case, receptors of cyclointercaland type are obtained; they may be expected to form supramolecular complexes with every flat substrates, especially with the nucleic bases of nucleosides and nucleotides, and to present selective properties towards nucleic acids due to their bulky structure. Of note, this generic name refers to structural factors but not to intercalative properties [13, 14].

11.2.3.1 **Acridinium derivatives**

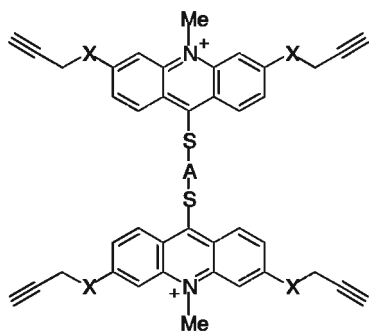
The triply bridged bisintercalands **13** and **14**, based on acridine subunits, were among the first to be prepared and studied in the context of nucleosides and nucleotides binding in water [14–16].



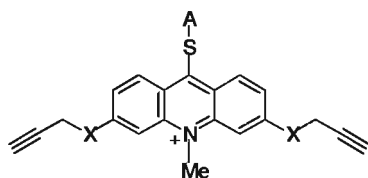
13a²⁺	X=O	A= -(CH ₂) ₆ -
13b²⁺	X= O	A= -CH ₂ CH ₂ OCH ₂ CH ₂ -
13b²⁺	X= O	A= -CH ₂ (CH ₂ OCH ₂) ₂ CH ₂ -
14²⁺	X= NH	A= -CH ₂ CH ₂ OCH ₂ CH ₂ -

The addition of increasing amounts of a series of nucleosides and nucleotides to their aqueous solutions resulted in a decrease of the intensity of the band located around 400 or 470 nm in their electronic absorption spectrum. Stability constants of 10^3 to 10^4 were determined and the stoichiometry of the complexes formed by **13** and **14** was found to be 1:1 for all substrates, indicating that a well-defined species is generated. The marked hypochromism observed in all cases reveals the formation of stacked structures between π -systems. Taken together, the 1:1 stoichiometry and the hypochromism suggest a sandwich-type structure for the complexes, the substrate being located between the two flat acridine units. Several fea-

tures indicate that substrate binding by **13** and **14** is dominated by stacking effects that involve both van der Waals and hydrophobic effects: there is no significant increase of the stability constants with increase of the charge of the substrate, the binding constants for AMP^{2-} , ADP^{3-} , and ATP^{4-} being the same; likewise the stability constants measured for the neutral nucleosides are comparable to those of the doubly charged nucleotides.



- 15a²⁺** X = O, A = $-(\text{CH}_2)_6-$
15b²⁺ X = O, A = $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-$
15c²⁺ X = O, A = $-\text{CH}_2(\text{CH}_2\text{OCH}_2)_2\text{CH}_2-$
16²⁺ X = NH, A = $-\text{CH}_2\text{CH}_2\text{OCH}_2\text{CH}_2-$

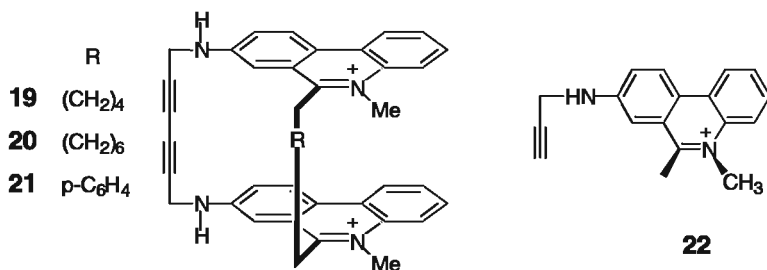


- 17** X = O, A = $-\text{CH}_2\text{CH}_2\text{CH}_3$
18 X = NH, A = $-\text{CH}_2\text{CH}_2\text{CH}_3$

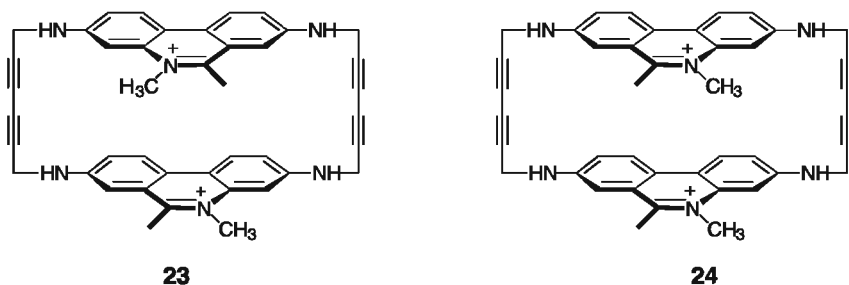
Although the stability of the complexes formed in aqueous solutions are about two orders of magnitude higher than for the monomers reference compounds **17** and **18**, they are however smaller than those measured with the acyclic analogs **15** and **16** by factors of 5–10. This could indicate that the macrobicyclic is not well tailored for the intercalation of planar substrates. Indeed, the crystal structure of **13** indicates that the two acridine walls are too far apart to perfectly accommodate flat compounds, particularly since the rigidity of the diacetylenic bridges prevent any adaptation of the size of the cavity to allow a close contact with the substrate [17].

11.2.3.2 Phenanthridinium derivatives

Diacetylenic bridges have also been used to connect two planar phenanthridinium units in a series of cyclobisintercaland receptors [18–20]. The addition of increasing amounts of nucleotide to aqueous solutions of each of compounds **19–21** resulted in the quenching of their fluorescence emission, which allowed the determination of the stability constants by fluorometric titrations; very high values, ranging from 10^5 to 10^6 , were measured for various nucleotides and the neutral adenosine. These constants were found to be practically charge-independent, which indicates that the stacking interactions are the driving force for the binding. The high stability of the complexes is, thus, remarkable for receptors which bind only the nucleic base of the nucleotide.



The observed 1:1 stoichiometry for the complexes and the fact that the K_s values for the corresponding acyclic monomer **22** are more than one order of magnitude lower than those of macrocyclic compounds **19–21**, are in accordance with an intercalative type of binding.



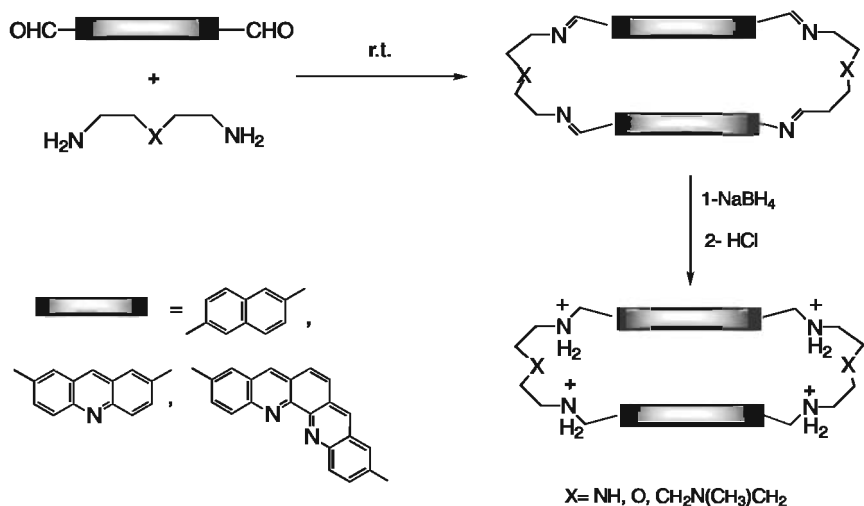
Macrocyclic ligands **23** and **24** are remarkable since they differentiate AMP^{2-} from GMP^{2-} or UMP^{2-} : an increase of the fluorescence is observed upon complexation of the former whereas only a very slight emission change is seen upon the addition of GMP^{2-} and UMP^{2-} [20].

In summary, except for the K_s values, which are higher for the phenanthridinium compounds, acridinium and phenanthridinium cyclobisintercalands behave in the same way. The binding of nucleotides by this first series of cyclointercalands is mainly due to stacking and hydrophobic effects and is virtually not selective. This is why a second generation of compounds, provided with positive sites able to interact with phosphate groups, was designed.

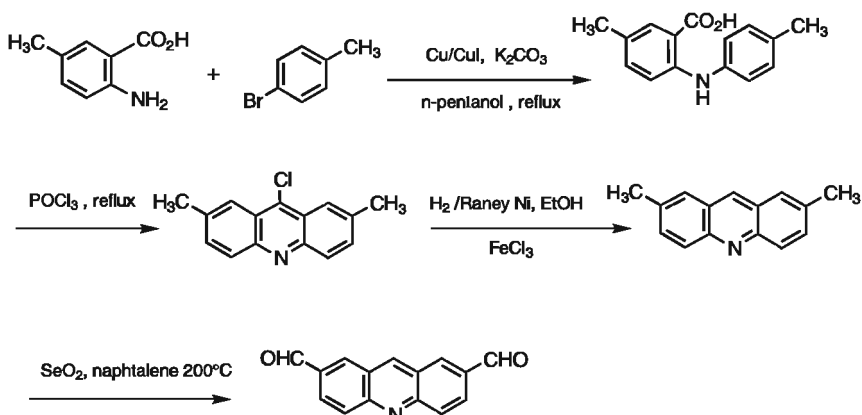
11.2.3.3 Polyamino naphthalenophanes and acridinophanes

According to the results obtained with the rigid acridinium compounds (Section 11.2.3.1), it appeared necessary to render the structure more flexible to help the fit of interactions with nucleotidic substrates and also to introduce binding sites for electrostatic interactions. The macrocycles **BisNP** and **BisA**, in which the two aromatic units are bridged by two flexible diethylene triamine (DIEN) chains, have thus been designed [21, 22]. In these compounds the length of the triamine allows a positioning of the two aromatic units at a distance compatible with the trapping of an aromatic nucleus (~ 3.5 Å) and the four positive charges, globally developed at physiological pH (5–7), greatly help the interaction with phosphate groups;

these charges also prevent the flattening of the cavity in water. **BisNP** and **BisA** were obtained via the very efficient [2 + 2] condensation between DIEN and the corresponding naphthalene or acridine dialdehydic derivative (Scheme 11.1). The naphthalene dialdehyde was prepared in two steps from the dimethylester derivative and the 2,7-acridinedicarboxaldehyde was synthesized through a four-step pathway using usual acridine reactions (Scheme 11.2). Crystals of complexes of **BisA** and **BisNP** with flat aromatic dicarboxylates have been obtained [23, 24]. Structural X-ray analysis showed that the macrocycles adopt a semi-closed conformation with an interchromophoric distance of ~ 7 Å suitable for π -sandwiching of an aromatic nucleus (Fig. 11.1).



Scheme 11.1. General synthetic pathway of cyclobisintercaland macrocycles.



Scheme 11.2. Synthetic scheme of 2,7-acridine dicarboxaldehyde.

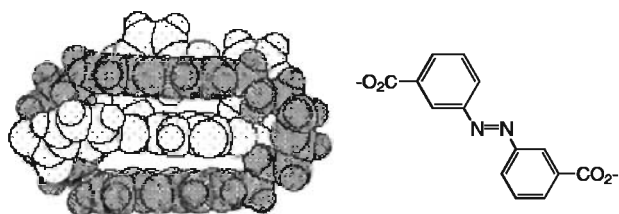
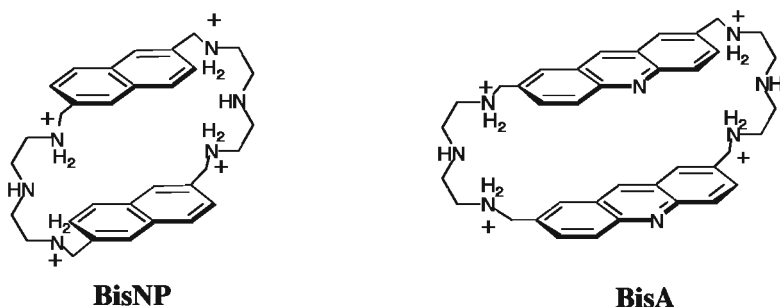


Fig. 11.1. Space-filling representation of the X-ray structure of the complex between **BisA** (gray) and *trans*-3,3'-azobenzene dicarboxylate (white).



The affinity of **BisNP** and **BisA** for various nucleotides has been measured by NMR and fluorimetric titrations. In all cases 1/1 complexes were formed and remarkably high stability constants were determined (Tab. 11.1). In particular **BisA** displays one of the highest affinity constants for ATP^{4-} ever measured in water (K_s for $\text{ATP}^{4-} > 10^8 \text{ M}^{-1}$). For both macrocycles the same trends in selectivity were observed: purinic derivatives are bound more strongly than pyrimidinic ones and the binding constant increases with the anionic charge (one order of magnitude per phosphate group). These features indicate clearly the respective contributions of electrostatic forces and stacking/hydrophobic interactions between the macrocyclic polycationic structure and the nucleotide. A more detailed comparison of the binding properties of the two macrocycles shows that **BisA** is much more efficient than **BisNP**. Indeed, the replacement of the bicyclic naphthalene by the larger tricyclic acridine produces a large increase of the binding constants specially for the purine derivatives (an increase of 2–3 orders of magnitude is observed

Tab. 11.1. Stability constants ($\log K_s$) calculated for the complexes of macrocycles **BisA** and **BisNP** with nucleotides

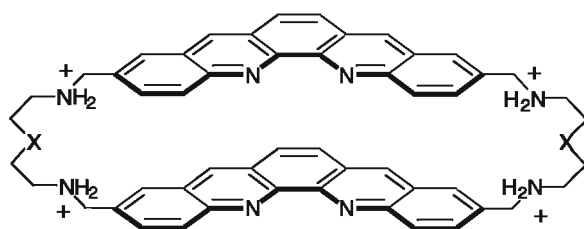
Nucleotide	BisA	BisNP
AMP^{2-}	6.1	4.1
ATP^{4-}	8.4	5.2
GMP^{2-}	4.6	3.6
UMP^{2-}	3.8	3.8

for AMP^{2-} , GMP^{2-} , and ATP^{4-} , see Tab. 11.1). This might simply be due to the larger size of the cavity delineated by the bisacridine derivative but it might also reflect the considerable influence of the van der Waals and solvophobic effects on the complexation since the strength of the stacking interaction is strongly dependent on the area of the aromatic surfaces in contact.

The variations of the fluorescence properties of **BisA** when binding to nucleotides are worth noting. The macrocycle displays a remarkable behavior with two types of response: strong quenching of the fluorescence intensity (70–80%) with the purine derivatives and considerable enhancement of the emission (70–130%) with pyrimidine ones. On the other hand, when **BisNP** associates with nucleotides a strong quenching of the emission of the naphthalene units is observed in any case. The ability of **BisA** to discriminate between the two types of nucleobases is remarkable both qualitatively and quantitatively and will be used further to monitor interactions with DNA hairpins (see Section 11.3.4.2).

In conclusion, thanks to an interplay of electrostatic and stacking/hydrophobic interactions, the naphthalenophane and acridinophane macrocycles are among the most efficient synthetic compounds designed for complexation of nucleotides in physiological conditions.

In an effort to improve the strength and the selectivity of the nucleotides binding again and, also to mimick the base pairing that occurs in biology, larger macrocycles **BisQ**_{1–3} were designed. They consist of crescent-shaped quinacridine subunits linked by the same linkers as in **BisNP** and **BisA** and they were synthesized according the general Scheme 11.1 [25].



BisQ₁ X= NH

BisQ₂ X= O

BisQ₃ X= $-\text{CH}_2\text{N}(\text{CH}_3)\text{CH}_2-$

Absorption and fluorimetric experiments showed that **BisQ**_{1–3} display non-covalent associations with nucleotides and revealed a new recognition pattern for such host–guest systems [26]. As indicated by fluorimetric titrations of **BisQ**₁ by GMP^{2-} and also by mass spectrometry studies, the stoichiometry of the complexes is 1:2 (host/guest) with nucleoside monophosphates. This unusual binding of a nucleoside monophosphate pair involves π -stacking/hydrophobic effects between the nucleobases and the quinacridine subunits of the macrocycles as schematically represented in Fig. 11.2. The formation of hydrogen bonding between the two

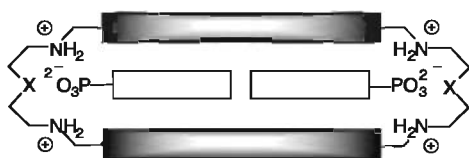


Fig. 11.2. Schematic representation of the 1/2 complex formed between **BisQ** and GMP^{2-} .

nucleobases within the complex could also contribute to the high stability of the ternary association and to the selectivity for GG base pairs.

11.3

Nucleic Acids Complexation

On the way to nucleotide recognition, efforts have mainly been devoted to the design of ligands able to selectively bind isolated planar substrates, including the nucleic bases. In the case of nucleic acids the situation is totally different since the monomer nucleotides are engaged in much more complex architectures with a variety of secondary and tertiary structures. However, in such structures, the efficient ligands for nucleotide recognition should be able to bind isolated nucleic bases when present. Thus, some azoniacyclophanes and cyclointercaland compounds, exhibiting interesting binding properties for nucleotides, have been tested on nucleic acids.

11.3.1

Azoniacyclophanes

The tetracationic azoniacyclophanes **CPnn** (Section 11.2.2) also interact strongly with nucleic acids [27, 28]. Competitive displacements of ethidium bromide intercalated into calf thymus DNA were used to estimate the interactions of the various macrocycles with DNA (calf thymus). The effect of the four CP compounds, **9–12**, was up to 15 times higher than that of the corresponding open-chain analogs. Moreover, the stabilization against thermal denaturation brought by **CP44** is higher than with the other cyclophanes: $\Delta T_m = +5.1^\circ\text{C}$ instead of $+2.2$, $+1.4$, $+3.0$ for **CP33**, **CP55**, and **CP66** respectively. NMR and viscosity measurements ruled out the participation of intercalation and, thus, electrostatic interactions remain the main factor to explain the strong binding of azoniacyclophanes. Molecular modeling showed that **CP44** fits particularly well in the large groove of the DNA double helix, thereby allowing very efficient coulombic interactions between the positively charged ammonium groups of the macrocycle and the negatively charged phosphate groups of the DNA. Because of their size, other azoniacyclophanes do not fit so easily in the major groove and cannot develop the same electrostatic interactions as **CP44**.

More interestingly, the interactions of all these azoniacyclophanes with DNA and

RNA duplexes of the same sequence have also been investigated, by thermal denaturation and viscosity measurements as well as by CD and NMR studies [27]. A surprising behavior was observed: all the compounds stabilize DNA but, depending on the size of the macrocycles and on the experimental conditions, they can either stabilize or destabilize RNA duplexes. With DNA duplexes, as was already seen with DNA from calf thymus, **CP44** forms the most stable complex and **CP66** the least stable one. It is very different with RNA duplexes: whereas a strong stabilization is observed with **CP33**, the RNA ΔT_m values measured with **CP44**, **CP55**, and **CP66** are smaller than the corresponding variations of melting temperature on DNA. With **CP66**, ΔT_m even becomes negative, indicating that RNA is actually destabilized in the conditions of the experiment. Thermal melting curves studies, viscosity, and CD results showed that there are two types of complexes for **CP66** depending on the ratio of macrocycle to RNA. At lower ratios, the major complex involves mainly electrostatic interactions with little deformation of the double helix, whereas at higher ratios, CD studies suggested base-pair opening with inclusion of one or more bases into the cavity of the macrocycle; in addition to the ionic interactions this process also involves van der Waals and hydrophobic forces. Further information provided by NMR experiments at different temperatures confirmed this mechanism and showed that only adenine bases are inserted in the cavity of **CP66**. Two effects can explain the different behaviors of **CP66** with DNA and RNA: stabilization with the former, destabilization with the second. First, the grooves in DNA and RNA are quite different and so **CP66** may fit well in the major groove of DNA but not in that of RNA. Second, NMR experiments clearly demonstrated a stronger interaction of **CP66** with an adenine base in an RNA strand than the same in a DNA strand and only the cavity of **CP66** is large enough to accommodate a purine base.

The interactions of compounds **6–8** and of some analogs with nucleic acids have also been examined [29]. It is worth noting that **6**, which was shown to be large enough to allow insertion of a purine base into the macrocycle cavity, displays the same behavior as **CP66**. The RNA-melting temperature increases at low macrocycle/RNA ratio and then decreases as more **6** is added. These results reveal an initial electrostatic stabilization of the double-stranded RNA followed by base-pair opening and ultimately by denaturation of the double helix.

These surprising observations are reminiscent of biological mechanisms such as base flipping by DNA enzymes, which is a common event in biology, but the fact that a small organic molecule can do the same selectively with RNA is amazing.

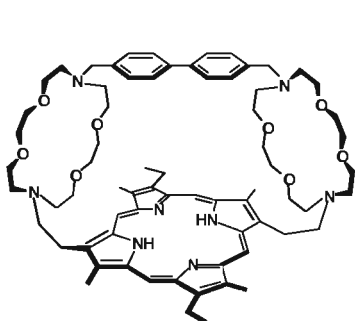
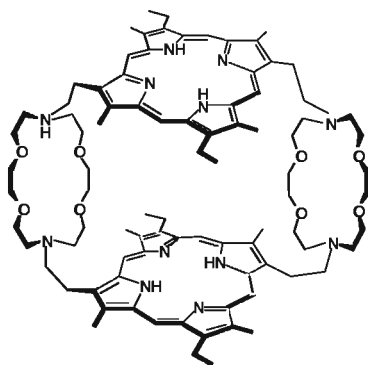
11.3.2

Porphyrim Derivatives

The first cyclointercaland compounds were described as early as 1984 [30]. They are macrotetracyclic and macropentacyclic compounds which combine one or two porphyrin rings and [18]-N₂O₄ aza-oxamacrocycles. In the first derivative, **25**, one porphyrin subunit is linked by two macrocycles to a biphenyl group; the amine groups being protonated around pH 7 in water, it may be expected to interact with

DNA via coulombic interactions. Moreover its bulky structure may allow discrimination between single-stranded and double-stranded nucleic acids, the former being much more flexible. Actually, studies using a set of photophysical methods indicate that it is the case [31].

The binding of **25** to polynucleotides occurs in two steps: the first one is a highly non-specific cooperative binding of the macrocycle along the polymer, whereas the second one is the binding to isolated sites. Using absorption and fluorescence spectroscopy, binding and competition studies on the affinity of **25** for isolated sites on single-stranded and double-stranded polymers indicated that it binds more strongly to single-stranded polynucleotides than to double-stranded ones.

**25****26**

Thermal denaturation measurements showed that it binds efficiently to denaturated DNA whereas there is no significant stabilization of the double helix. This selectivity is pH-dependent, the highest affinity being observed at pH 6.6 for poly(dA) and the ss/ds selectivity is larger at pH 4.6 than at pH 6.6. Moreover, the binding constants of **25** for poly[d(A-T)]₂ and poly(dA)-poly(dT) obtained from fluorescence measurements are comparable, while known intercalators bind more tightly to the former [32]. The similarity between the binding constants of **25** for these two polynucleotides and the absence of stabilization of the double helix upon binding of **25** show that it does not intercalate into double helices like planar aromatic cations. In addition, fluorescence anisotropy results and the fluorescence quenching by the bromine atom of poly[d(A-⁵BrU)] suggested that it binds double helices into the major groove. The binding selectivity and the binding location may be attributed to the macrocyclic cryptand cage structure into which the porphyrin subunit is incorporated. The rigidity of the cage walls causes a steric hindrance which prevents the intercalative insertion of the porphyrin ring between the base pairs and the overall bulky structure of the molecule cannot accommodate the narrow minor groove of the double helix.

The porphyrin derivatives, like most of cyclobisintercalands based on dye molecules, present another interesting feature. Since the porphyrin is a photoactive moiety, it may induce DNA photocleavage when irradiated in visible light. This has

been shown using the macropentacyclic compound **26** which is fitted with two porphyrin rings (Section 11.3.4.2).

11.3.3

Phenanthridinium Derivatives

The affinities of phenanthridinium derivatives **23** and **24** (Section 11.2.3.2) for single- and double-stranded polynucleotides of DNA and RNA have been studied by fluorescence, viscometric, and thermal denaturation measurements and the results have been compared to those obtained with the corresponding monomer **22** [20]. With regard to the structural features of these molecules, particularly the position of the connecting bridges, which are unfavorable for insertion in double-stranded nucleic acids, preferred binding to single-stranded nucleic acids may be expected.

Actually, the two macrocyclic ligands bind single-stranded polynucleotides more strongly than double-stranded ones; for instance, their affinity for poly(dG) is 25 times higher than that for poly(dG)-poly(dC). The K_s values for **23** and **24** with ss-poly(dA) and poly(dG) are two orders of magnitude higher than those of monomer **22**. A similar result was already found with bis- and monointercalators; this is in favor of a participation of both phenanthridinium subunits, with a single base inserted between them and validates the concept that has led to the design of cyclo-bisintercaland compounds. However, other studies would be necessary to assert that true bisintercalation is the mode of binding. In contrast, complementary studies indicated nonintercalative binding of **23** and **24** with double-stranded polynucleotides and were in favor of groove binding driven by hydrophobic and electrostatic interactions.

11.3.4

Acridinium Derivatives

Two bisacridine compounds, **SDM** and **BisA** (Section 11.2.3.3), have been extensively studied by the groups of Zimmerman and Lehn respectively [22, 33, 34]. They differ by the nature and the position of the bridges that connect the two acridine subunits. Depending on the position of attachment of the linkers on the acridine rings, different modes of intercalation into the double helix of DNA can be envisaged (Fig. 11.3):

1. When the two linkers are closely attached on the same side of the ring, bisintercalation can occur in a normal way with both chains in a single groove; this situation is schematized in Fig. 11.3a.
2. But when these linking chains are positioned on opposing sides of the major axis of the acridine ring three complexes are theoretically possible. In Fig. 11.3b, the two acridine rings are partially inserted between the base pairs with the linkers on the same groove as in Fig. 11.3a and with the long axes of the acridines almost perpendicular to those of the base pairs; this complex can be supposed to be weak because of the small overlap with the base pairs.

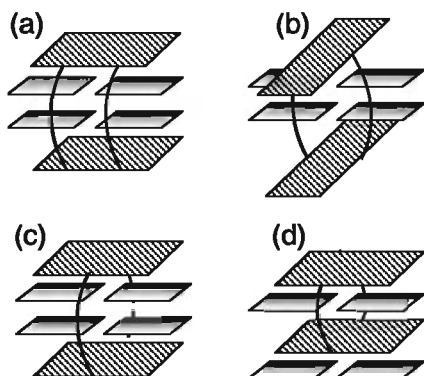
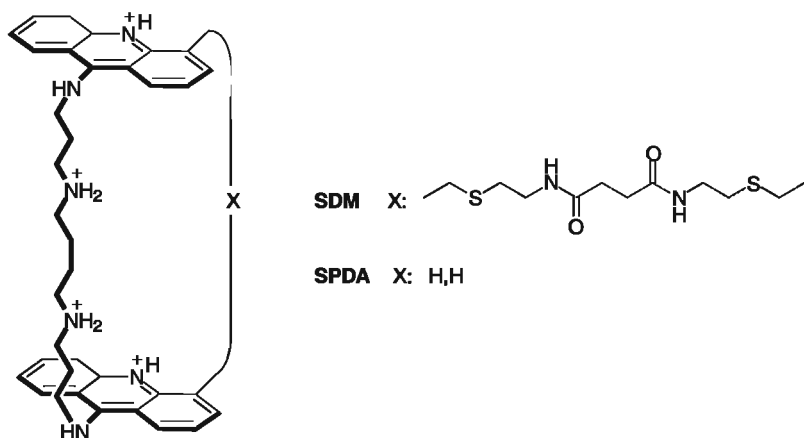


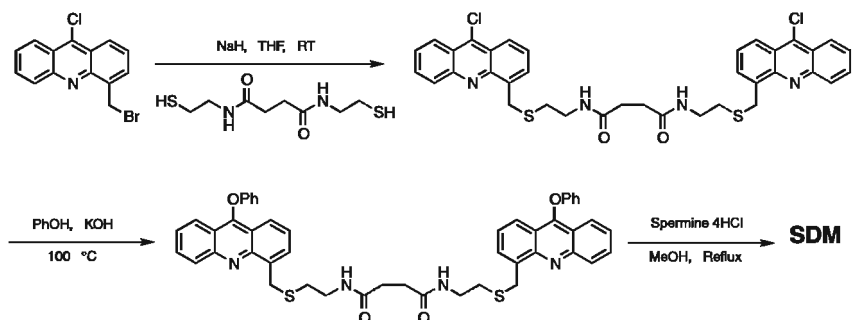
Fig. 11.3. Various binding models of cyclobisintercalation.

3. In Fig. 11.3c, a catenated complex is formed, with the long axis of the intercalator and that of the base pairs approximately parallel and with the linkers in the opposite grooves, which requires a transient disruption of the Watson–Crick base pairs. This case is reminiscent of the threading intercalators and polyintercalators which bear two substituents at the extremities of the diagonal line of an aromatic ring [35].
4. In these three cases, the bisintercalation is supposed to obey the neighbor exclusion principle [36], although violation of this principle, as in Fig. 11.3d, with less favorable bisintercalation at adjacent sites, cannot be excluded. It is also possible that the cyclobisintercaland compound does not bind as an intercalator at all or that it behaves as a monointercalator with one single ring intercalating between the base pairs.



11.3.4.1 SDM Macrocycle

The compound studied by Zimmerman's group is composed of two acridine rings connected by two different linking chains: a spermine unit linking the 9-positions and a 2-aminoethanethiol succinamide motif connecting the 4-positions of the ac-



Scheme 11.3. Synthesis of **SDM** macrocycle.

ridine parts [33]. It was prepared, in three steps, by reaction of 4-(bromomethyl)-9-chloroacridine with *N,N'*-bis(2-mercapto-ethyl)succinamide, in presence of base, followed by conversion of the resulting dichloride into bis(9-phenoxyacridine), which reacted with spermine tetrahydrochloride under high dilution conditions (Scheme 11.3). In **SDM** structure, the 14 atoms linking chains provide water solubility and are long enough (about 16 Å in their fully extended conformation) to allow bisintercalation according to the neighbor exclusion principle. Binding studies performed with various nucleic acids indicated a high affinity. Thermal denaturation measurements, spectroscopic data, viscometric analysis, ability to unwind a closed circular supercoiled DNA and comparison with the known bis-intercalator spermine bisacridine **SPDA**, all were in favor of a bifunctional intercalator behavior [33]. However further experiments were necessary to try to discriminate, if possible, between the binding models discussed above. In addition to visible spectroscopy and stopped-flow kinetics, NMR studies confirmed that **SDM** binds DNA according to an intercalation mechanism with the long axes of the acridine rings parallel to the long axes of the base pairs. All the data were analyzed for consistency with the different binding models. The attachment of the linkers in the 4- and 9-positions of the acridine ring rules out the participation of a binding mechanism of type in Fig. 11.3a and all the accumulated data, included modeling studies, suggest that the model in Fig. 11.3b as highly unlikely. In contrast, all the results fit with the models in Fig. 11.3c and d, in which side chains are in opposite grooves. At this stage it is difficult to distinguish between these two models although NOESY NMR spectra of the **SDM** complex with $d(\text{CGCG})_2$ seem more consistent with Fig. 11.3d, that is to say bisintercalation with violation of the neighbor exclusion principle [36].

In fact, the interactions of **SDM** with dsDNA might involve an even more complicated binding pattern as shown by a recent study in which X-ray diffraction analysis of the complex between **SDM** and CGTACG was performed [37]. The refined crystal structure, at 1.1 Å resolution, presents several unexpected features. The terminal base pairs, C1.G12 and G6.C7, are unravelled, thus leaving only four central base pairs in the duplex. Only one acridine ring of the cyclobis-intercaland is intercalated between the C5pG6 step while the other acridine ring and the linkers are completely disordered. A number of additional interactions

generate a very complex structure in which two acridine rings, belonging to different **SDM** molecules, are intercalated between two sets of GC base pairs. Moreover, NMR studies of the binding of **SDM** to the AACGATCGTT sequence showed that more likely it binds to two different duplexes by intercalating each acridine to a CpG site from different duplexes. A crosslinking would result from such a mechanism which is consistent with the precipitation that occurs during the titration. Of course, this precipitation is also facilitated by the charge neutralization between the bisacridine and the DNA.

In conclusion, these studies show that non-conventional intercalation structures are possible and provide new insights into the design of new intercalating compounds.

11.3.4.2 BisA Macrocycle

The macrocycle **BisA** has been the most extensively studied compound amongst the macrocyclic series of DNA ligands. Its interaction with various DNA conformations has been characterized qualitatively and quantitatively in terms of binding affinities, selectivities and also at the structural level by NMR studies.

Hairpin recognition and duplex destabilization Hairpins, one of the simplest DNA and RNA secondary structures, result from an intramolecular folding of a partially complementary sequence. They are thus composed by a double helix, the stem, linked to a single-stranded loop. Such structures are found at various regulatory sites such as gene transcription regions and in origins of DNA replication; they are therefore possibly involved in biological processes.

The binding of **BisA** with short oligonucleotides (9–11-mer, referred to as sA_3 , sA_5 , sT_5 , see Fig. 11.4) has been investigated using melting temperature experiments, fluorescence measurements, and gel-filtration experiments [38]. Throughout these studies a monochromophoric compound **MonoA** was used as a reference for comparison with the binding behavior of the dimeric macrocyclic structure.

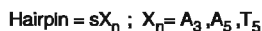
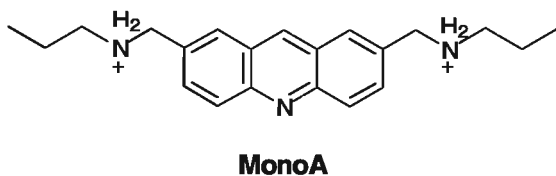


Fig. 11.4. Intramolecular transition from a hairpin to a random coil.

Tab. 11.2. T_m values of the hairpin-to-coil transition of sA_3 in the presence of **BisA**.

BisA/sA₃	T_m (°C)	ΔT_m ($\pm 2^\circ\text{C}$)
free sA_3	47 ± 1	
1/4	47 ± 1	
1/2	49 ± 1	2
1/1	57 ± 2	10
2/1	67 ± 2	20
4/1	70 ± 2	23
5/1	75 ± 2	28

BisA strongly stabilizes the hairpin structure which is indicated by a strong increase of the melting temperature of the hairpin to random coil intramolecular transition (Tab. 11.2). For instance a high affinity constant, $K_s = 4.5 \times 10^7 \text{ M}^{-1}$, was calculated for the 1/1 complex of **BisA** with sA_3 . In contrast, the melting temperature of a short duplex, $d(\text{GCGCGC})_2$, mimicking the stem of the hairpin, is not affected by the presence of the macrocycle. This demonstrates that **BisA** exhibits a low affinity for short duplexes and that the binding should arise in the loop part of the hairpin. Compound **MonoA** does not significantly alter the melting of the hairpin, showing that the macrocyclic structure is responsible for the selective binding into the loop. The affinity of **BisA** for the loops appeared dependent on both their sequence and size, small loops sA_3 and sA_5 being preferred. The fluorescence signature of the macrocycle that distinguishes the heterocyclic base in interaction with the acridine ring (see Section 11.2.3.3) and the comparison of the fluorescence relative yields with various oligo- and polynucleotides allowed the assignment of the binding site to the loop. Gel-filtration experiments performed on the complexes of **BisA** with the sA_3 oligonucleotide confirmed the 1/1 stoichiometry but led also to the detection of a 1/2 species that is much less soluble and thereby was not detected by UV-vis spectroscopy. Comparison of the binding constants of **BisA** for a hairpin loop and a single-stranded dA_3 ($K_s = 6 \times 10^5 \text{ M}^{-1}$) demonstrated that the hairpin loop structure was preferred to single strands.

In conclusion, the whole experimental work demonstrated the selectivity of **BisA** for hairpins compared with both double helices and single-stranded oligomers. It seems that 1/1 associations are formed predominantly and that the binding could occur in the vicinity of the loop. However more structural data would be required to gain information on the interaction at the molecular level.

Complementary work based on electrophoresis gel-shift experiments [39] using two DNA hairpins forming a fully paired duplex showed that **BisA** is able to shift the equilibrium towards the hairpin conformation (Fig. 11.5), which confirms the higher affinity of the macrocycle for DNA hairpins compared with double-stranded oligonucleotides. In addition, the premelting of $\text{poly}[d(\text{A-T})]_2$ induced in the presence of the macrocycle indicates its preference for single- versus double-stranded oligonucleotides. These observations suggested that the selective binding of **BisA** could be potentially applied for destabilizing DNA double helices. It appeared interesting to further investigate this property since DNA opening processes play a

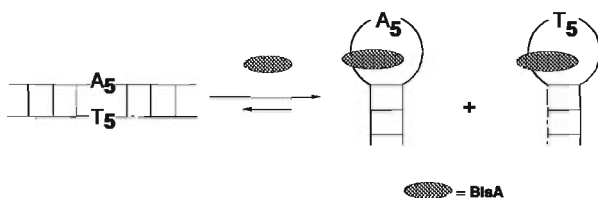


Fig. 11.5. Equilibrium between the complementary hairpins sA_5 , sT_5 and the fully paired duplex.

crucial role in the initiation of key step events such as replication, transcription, and recombination. The ability of **BisA** to destabilize duplexes has been examined using hybrid systems constituted of a primer (17-mer) hybridized to its complementary sequence on the circular ss DNA of phage M13 (Fig. 11.6).

These DNA constructions were initially designed to evaluate the DNA helix-destabilizing ability of single-strand-binding proteins SSB [40]. By analogy with the behavior of these proteins, this simple assay consisted in measuring the ability of the macrocycle to displace the short ^{32}P -labeled primer [41]. The analysis is carried out by gel electrophoresis, which allows an easy quantification of the free/bound oligonucleotide ratio due to the large difference in mobility of the two species (Fig. 11.7). When this assay is carried out at increasing temperature with samples taken at regular intervals, denaturation curves are obtained that allow the measurement of a melting temperature with a satisfying accuracy. In these conditions, a strong destabilizing effect of **BisA** ($\Delta T_m = -20^\circ\text{C}$) could be detected at low **BisA**/DNA ratio and at high salt concentration (100 mM NaCl). The very low ionic strength dependence of the effect suggests a specific and strong interaction of the macrocycle with DNA.

This led to further investigation of the ability of the macrocycle to compete with SSB proteins for binding to DNA. Competitive binding experiments were monitored by nitrocellulose binding assays using the SSB protein from *E. coli* (*Eco* SSB), which forms 1/1 complexes with a single-stranded 36-mer. The protein was immobilized on a nitrocellulose membrane and incubated with the labeled 36-mer oligonucleotide in the presence of **BisA** and control compounds. The activity of the compounds were evaluated by quantification of the radioactivity retained on the membrane after washing (Fig. 11.8). In the concentration range examined, the formation of the complex SSB/36mer was inhibited by the macrocycle, whereas spermine and the reference monomer **MonoA** elicited no effect. This clearly demonstrated the inhibitory effect of **BisA** likely through a competitive binding to the

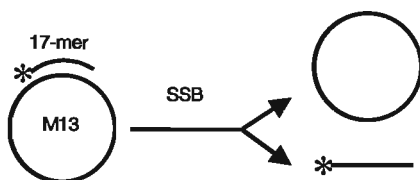


Fig. 11.6. Schematic representation of the hybrid M13:17-mer and of its dissociation by an SSB protein.

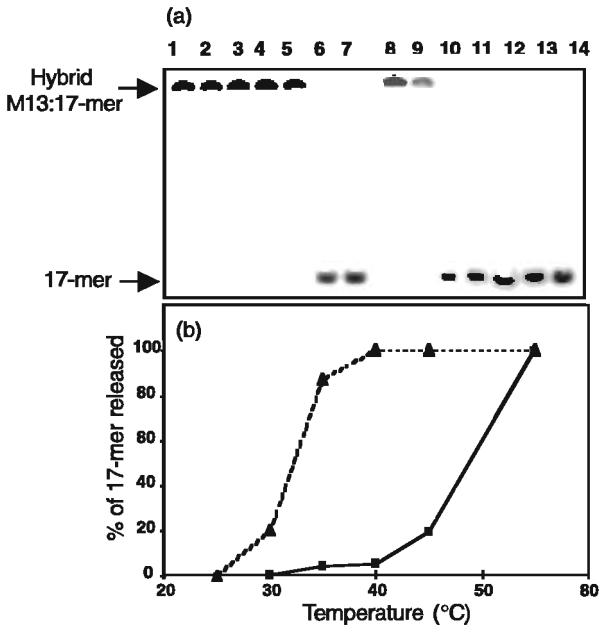


Fig. 11.7. (a) Gel electrophoresis pattern after thermal denaturation of M13:17-mer hybrid alone (lanes 1–7) and in presence of **BisA** (lanes 8–14). Temperature varies from 25°C up to 55°C in 5°C steps. [DNA] in phosphate unit = [BisA] = 1 μ M. (b) Thermal denaturation curves of M13:17-mer hybrid alone (squares) and in presence of **BisA** (triangles), Tris–HCl buffer (pH 7), 10 mM, NaCl 100 mM.

oligonucleotide. This method also provides a rough evaluation of the affinity of **BisA** for binding to the 36-mer. Comparison with the inhibitory effect of unlabeled oligonucleotides (15-mer and 36-mer) shows that the 15-mer and **BisA** have similar efficiencies. This is clearly indicative of a high affinity of **BisA** for the target oligonucleotide since the apparent binding constant of 15-mer to SSB is $>10^7$ M $^{-1}$.

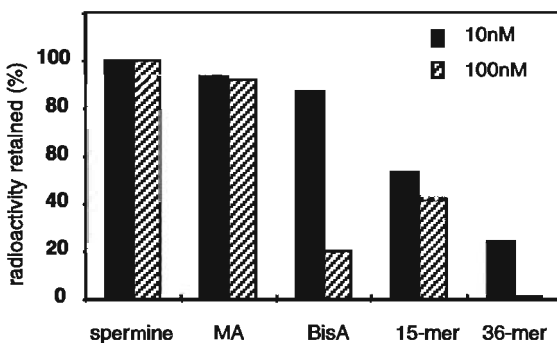


Fig. 11.8. Nitrocellulose binding assay. Inhibition of the formation of complex 36-mer/SSB by various competitors at two different concentrations (10 and 100 nM).

The capacity of **BisA** to discriminate between single-stranded and double-stranded oligonucleotides is likely responsible for the helix-destabilizing effect through equilibrium displacement. This also leads to disruption of protein/oligonucleotides complexes. Whether this property could be applied to a cellular context is not known and is under current investigation.

Selective photocleavage As previously mentioned, acridine and porphyrins are photochemically active groups, a property widely applied for inducing redox damages to nucleic acids, suitable for detecting the binding site of compounds and of potential interest for photodynamic therapy. Moreover, photoactive ligands, able to act as charge injectors via direct electron transfer from guanine bases, have raised an extensive curiosity from many groups through the past 5 years (see Chapter ■). The ability of **BisA** and compound **26** to induce photocleavage of DNA was examined to confirm the selectivity for single-stranded nucleic acids regions shown by the two cyclobisintercalands. To this end, a simple assay has been set up which consists of comparing the ability of the macrocycles to cleave circular dsDNA (plasmids) and circular ssDNA (DNA of phage M13) [42]. The two forms of DNA are commercially available and their cleavage is easily detected on agarose gel. Photocleavage experiments were conducted on circular supercoiled ds plasmids pBR322 and pUC18 and on circular ssDNA M13mp19 and M13mp18. A single cut of either type of DNA results in the formation of new species with very different electrophoretic migration properties. The double-stranded supercoiled DNA yields a relaxed circular form which may be followed by a linear double-stranded one after multiple cleavage events, whereas single-stranded circular DNA gives a linear single-stranded species after a single cut (Fig. 11.9).

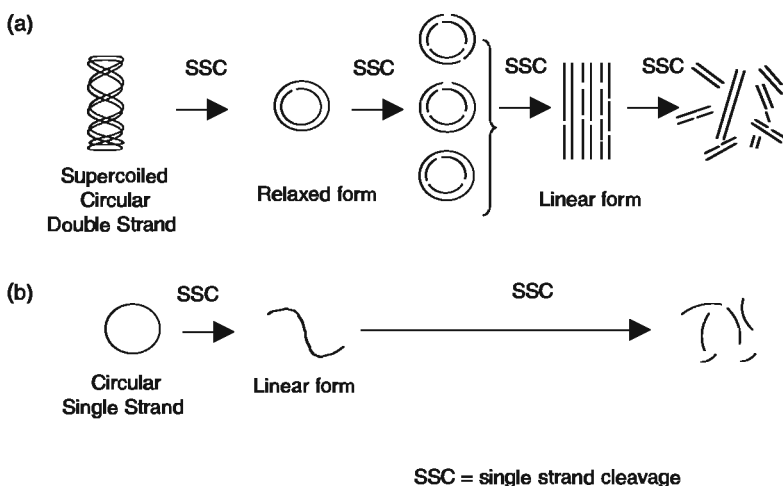


Fig. 11.9. Schematic representation of the species generated by single-strand cleavage reactions of (a) supercoiled circular dsDNA and (b) circular ssDNA.

Tab. 11.3. Photocleavage of equimolar mixtures of supercoiled ds pBr322 and circular ss M13mp19 by cyclobisintercaland **26** (DNA concentration = 0.06 mM in phosphate units).

Concentration of 26 (μM)	0.8	1.6	2	3	6.25	12.5	25	50
ds PBR322 remaining (%)	100	100	100	75	80	60	40	0
ss M13mp19 remaining (%)	90	50	12	0	0	0	0	0

Both compounds induced efficient and selective cleavage of the ssDNA at a low compound/DNA ratio (see Tab. 11.3 and Fig. 11.10). This preferential degradation was observed when irradiation was conducted on each form separately or on mixtures of single- and double-stranded DNA. In both cases significant cleavage of the double-stranded species requires much higher concentration in macrocycles and/or longer irradiation times. Again the reference acridine **MonoA** induces equal degradation of both single- and double-stranded species, thereby confirming that the selectivity of the cyclobisintercalands is provided by the macrocyclic structure. Irradiation experiments were also carried out with **26** on a transfer RNA (tRNA^{asp}) which presents both double- and single-stranded domains. After analysis by electrophoresis, the cleavage patterns have been related to the X-ray crystallographic data of tRNA^{asp} and this revealed that the preferred binding sites are almost exclusively at the ssRNA domains.

According to these experiments **BisA** and **26** display similar level of photocleavage activity which could reflect their similar affinities for single-stranded conformations. However, it is difficult to completely parallel the effects since the efficiency of the cleavage is strongly dependent on both the photophysical properties of each compound (quantum yield) and on the cleavage mechanism. The undisputed advantage offered by **BisA** is that it is active in physiological conditions whereas **26** requires a slightly acidic pH (pH ~4.5) for efficient cleavage.

Altogether these results point to the potential of cyclobisintercaland compounds to function as structural probes for single-stranded domains such as loops, bulges, hairpins, or for local pairing defects in complex nucleic acids.

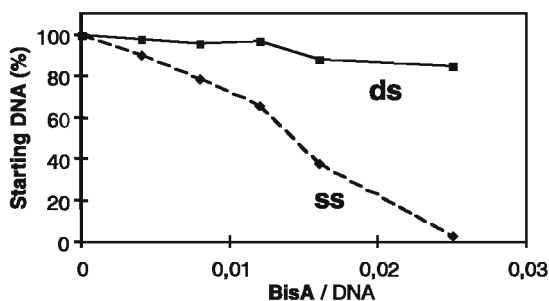


Fig. 11.10. Photocleavage of mixtures of pUC18 and M13mp18 by **BisA**: variation of ds and ssDNA as a function of the concentration of **BisA**. [dsDNA] = [ssDNA] = 0.3 mM in phosphate unit.

Abasic site recognition The loss of a nucleobase is one of the most frequent lesions in DNA and is highly cytotoxic and mutagenic. Abasic sites are produced *in vivo* through the base excision repair pathway (BER) and might also result from the action of ionizing radiations and of alkylating agents, in particular anticancer drugs [43]. It is thus of interest to design molecules capable of interacting specifically with abasic sites either as probes for the detection of damage or as drugs to interfere with the repair process. This fundamental and challenging topic is reviewed in Chapter 1. The structure of an abasic site depends on its flanking sequences and on the nature of the missing base (apurinic and apyrimidinic); flipped out and flipped in positioning of the unpaired base have therefore been described. Abasic sites can be regarded as particular cases of monobase bulges. Consequently, with regard to these structural features and to the selectivity of BisA for unpaired bases in DNA, this compound appeared as a potential new candidate ligand for abasic lesion.

The binding of the drug was investigated with short DNA duplexes (11-mer and 23-mer) oligonucleotides containing a THF cycle facing a thymine in the middle of the sequence [44]. This synthetic modification is commonly used as a stable analog of apurinic (AP)-site (see Fig. 11.11, upper part). A whole set of physico-chemical methods including thermal denaturation, photocleavage experiments, displacement of an abasic site-specific RPE probe, and NMR have been used to demon-

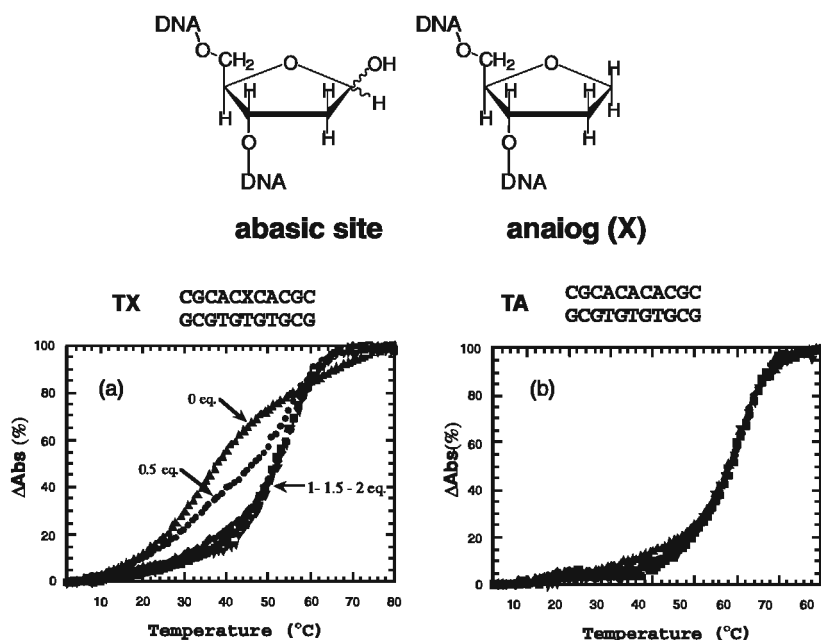


Fig. 11.11. (Upper) Representation of an abasic site and of the THF analog X. (Lower) Melting temperature curves (a) of the undecamer TX and (b) of the regular duplex TA in the presence of BisA (0–2 eq.).

strate the specificity and the mode of binding of **BisA** [44]. Thermal denaturation experiments were carried out with duplex TX (11-mer) in the presence of **BisA**. A strong stabilization ($\Delta T_m = +14^\circ\text{C}$) was induced whereas no modification of the melting temperature could be detected for the parent unmodified duplex (Fig. 11.11, lower part). This effect levels off at a 1/1 drug/DNA ratio, indicating the existence of only one binding site. In addition, irradiation of a mixture of ^{32}P -labeled oligonucleotide (23-mer) containing the model abasic site X and **BisA** (drug/DNA ratio = 4/1) leads to selective photocleavage in the vicinity of the abasic site on both strands of the duplex (Fig. 11.12). Finally, EPR measurements involving dis-

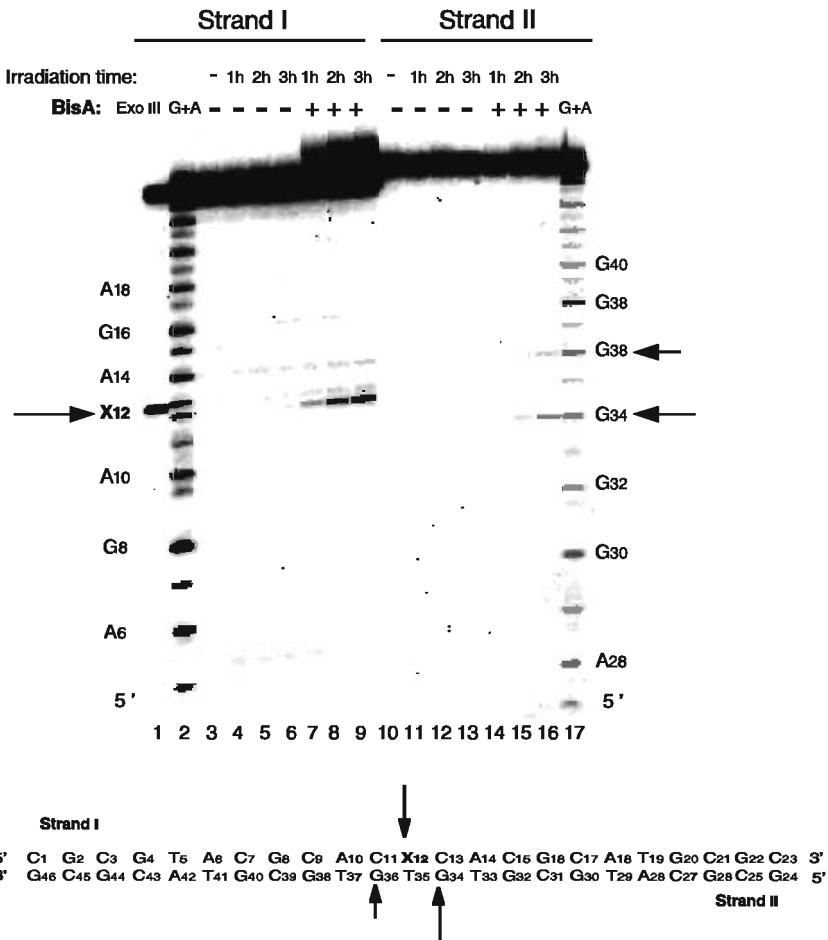
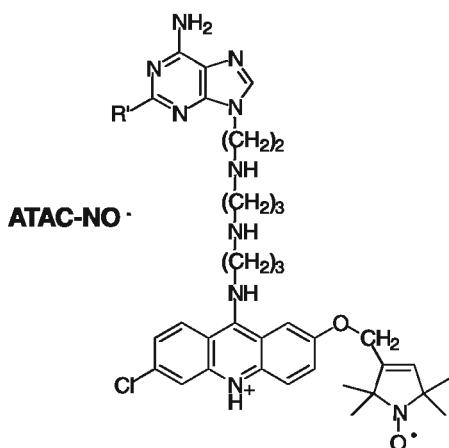


Fig. 11.12. Cleavage pattern of a 23-mer duplex containing an apurinic site X. Lane 1: duplex incubated with exonuclease III to localize the abasic lesion; lanes 2 and 17: G + A for strand I and II; lanes 3 and 10:

non-irradiated strands I and II in the absence of **BisA**; lanes 4–6 and 11–13: duplex irradiated in the absence of **BisA** for 1, 2, and 3 h; lanes 7–9 and 14–16: duplex irradiated in the presence of **BisA** (4 μM) for 1, 2, and 3 h.

placement by BisA of an abasic site probe labeled by a nitroxide (ATAC-NO $\cdot$) confirmed the specificity of the binding.



These results obtained by three different methods demonstrate unambiguously that BisA binds specifically and cleaves AP-sites.

Study of the interaction between the drug and the undecamer TX by ^1H NMR confirmed the binding site [45]. The greatest DNA proton chemical shifts were detected for the two GC base pairs flanking the unpaired thymine and also for the neighboring AT base pair. Data revealed that BisA forms two different intercalation complexes with the abasic site containing undecamer. These complexes are present in a 80/20 ratio. The NMR results for the major complex showed clear evidence that one of the two acridine residues intercalates between the C7-G16 and A8-T15 base pairs while the other acridine moiety inserts between the C7-G16 and C5-G18 base pairs (Fig. 11.13a).

Further evidence of BisA intercalation was given by the differences in chemical shifts of the aromatic protons in the free and bound molecules since on complexation most of the acridine protons resonances are shifted upfield up to 1.6 ppm. Intermolecular nuclear Overhauser effects (NOEs) were detected between one of the linkers and minor groove markers (A8-H1', H4', and C7-H1') indicating that the two linker chains lie in opposite grooves. Intermolecular distance restraints determined from the NOESY spectra were used to perform a molecular modeling study in order to obtain a model of BisA-binding mode. The structure of the major complex proposed after calculations is represented in Fig. 11.13b. The macrocycle is inserted into the abasic pocket with one acridine unit replacing the missing base and the other one intercalated between the two adjacent base pairs. Dynamics calculations showed that the two acridine rings have quite different positions relative to their flanking base pairs. The long axis of the acridine moiety intercalated between C7-G16 and A8-T15 is oriented parallel to the long axis of the A8-T15 residues. This chromophore stacks partially over its adjacent C7 nucleotide while no stacking is observed with the G16 residue. By contrast, the major axis of the second

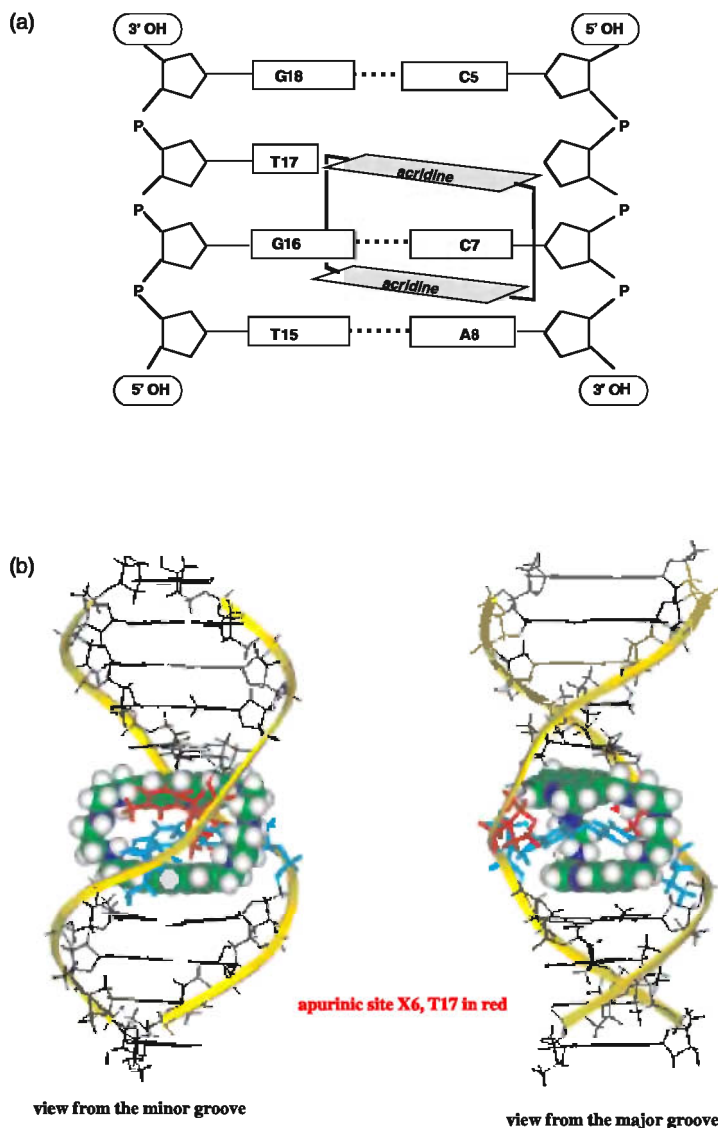


Fig. 11.13. (a) Schematic representation of the major **BisA**-DNA complex. The chromophores thread through the helix with the linker chains lying in either groove. (b) Molecular modeling-based structure of the major **BisA**-DNA complex. The drug is drawn in CPK. Left: view from the major groove. Right: the same representation after 90° angle rotation.

acridine moiety located in the abasic pocket forms approximately a 90° angle with the major axis of the C5-G18 base pair.

Actually, this chromophore stacks with the bases located on one strand (i.e. C5 and C7) and not with their complementary counterpart (G16 and G18). Similar

partial intercalation overlap involving stacking of the drug with the two flanking bases on the same strand has only been described previously for threading intercalators such as nogalamycin. The two acridine moieties are oriented with the ring nitrogens pointing toward the major groove and the linking chains positioned in the middle of each groove. The abasic sugar is pushed out away from the minor groove, while the T17 residue is slightly shifted toward the major groove, stacking partially with G16 but the glycoside torsion angle remains roughly anti. The C7-G16 base pair that is sandwiched between the two acridine ring exhibits substantial buckling up to 36°C.

The existence of hydrogen bonds between the NH_2^+ groups of the linkers and hetero-atoms of the bases in both grooves are suggested from examination of the refined structure. In addition, other electrostatic interactions between the positively charged side chains and the negatively charged functional group of the nucleotides in both groove can contribute to the stabilization of the complex. The structure of minor complex was more difficult to determine. It seems that one acridine unit is inserted in the abasic pocket but the position of the second acridine moiety could not be defined. Formation of the minor complex could result from intercalation of the first acridine ring in the abasic pocket, the second acridine ring remaining outside the duplex. Opening of the C7-G16 would allow insertion of the second acridine ring between the two base pairs giving the major complex. This seems a reasonable hypothesis since the opening of a single base pair was previously demonstrated to be a rapid event. The presence of competing complexes and the few intermolecular NOEs available prevented complete quantitative analysis. Finally no complex formation was observed with the parent unmodified duplex TA in the same conditions.

For the first time, this study provides structural data on the interaction of BisA with oligonucleotides. It confirms the specific binding of the macrocycle to the abasic site and demonstrates clearly the lack of affinity for duplex DNA. Moreover a new binding mode was evidenced that could be defined as threading bisintercalation, which involves the opening of a base pair. The cost in energy of this event should be compensated by the high stability of the resulting complex formed with the macrocycle. Also it further opens the possibility of investigating the potential of BisA and other related macrocycles to act as reagents for the stabilization of short-lived single-stranded regions and for the detection of locally altered structures in DNA.

Quadruplex recognition More recently, the interaction of BisA with DNA quadruplexes has been investigated [46]. From a structural point of view, intramolecular quadruplex structures formed from intramolecular folding of G-rich sequences exhibit hairpin loops that could constitute recognition motifs for cyclobisintercalands (Fig. 11.14a,b, left). From a biological point of view, the formation of quadruplexes in regulatory regions containing repetitive stretches of guanine is increasingly likely. Therefore their role in the regulation of DNA function, as well as their use as artificial tools, is the focus of intense research (see Chapter ■).

Currently, one of the most concrete applications of G-quadruplexes is their

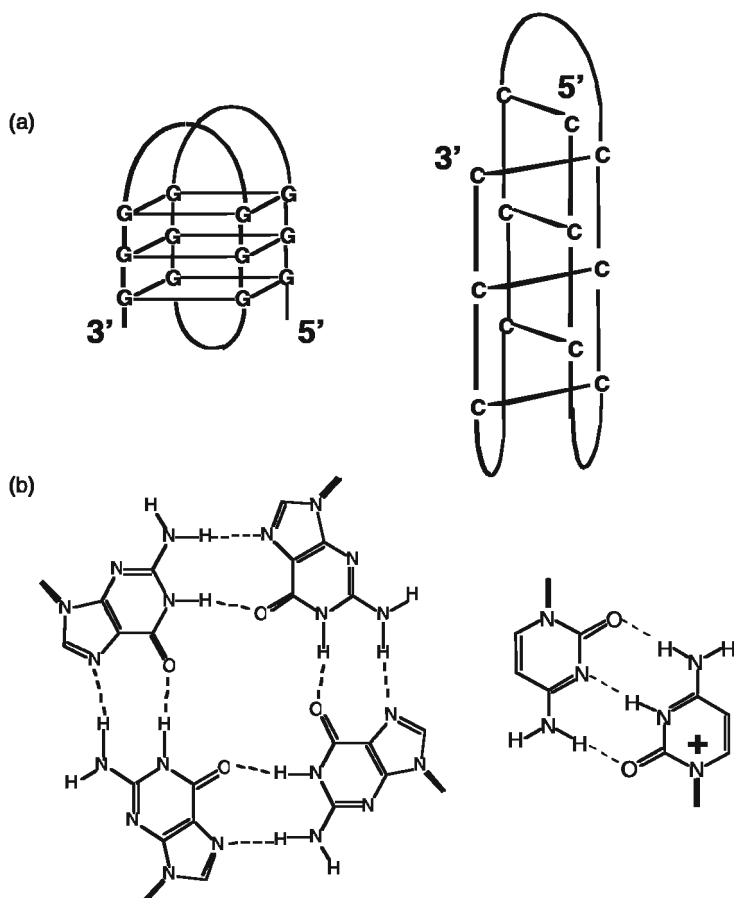


Fig. 11.14. (a) Possible folded structure of the G-rich (left) or C-rich (right) strand of human telomere. (b) Building blocks for quadruplex formation: the G-quartet (left) and the C.C⁺ hemiprotonated base pair (right).

capacity to inhibit telomerase. The single-stranded form of the telomere end is required for optimal telomerase activity and its folding into a G-quadruplex conformation has been shown to directly inhibit telomerase elongation. This enzyme, which is reactivated in most cancers, is an exciting target for designing new anti-cancer agents [47]. Therefore, a number of small ligands have been prepared to inhibit the function of telomerase by stabilizing G4-DNA structures. Moreover, telomeres are essential for DNA replication and for protecting chromosomes ends against double-strand breaks; they also participate in various aspects of the functional organization of the nucleus. All these functions might be altered by G-quadruplex-specific ligands independently of telomerase inhibition.

Thermal denaturation measurements have been carried out by fluorescence using doubly labeled oligonucleotides according to the FRET method developed by

Mergny *et al.* [48]. The oligonucleotide F21GT (5'-fluo-(GGGTTA)₃GGG-tamra 3'; fluo = fluorescein, tamra = tetramethylrhodamine) was used; this models the human telomere repeats and forms an intramolecular quadruplex in the presence of KCl. Fluorescence melting curves showed that **BisA** is a good stabilizer of the G4-stranded structure ($\Delta T_m = +15^\circ\text{C}$) whereas **MonoA** has little effect on the melting behavior of F21GT (Fig. 11.15a). A similar experiment was performed with the oligonucleotide F23CT, which corresponds to the complementary C-rich strand (5' fluo-TA(CCCTAA)3CCC-tamra 3') and forms a particular quadruplex called an i-motif (Fig. 11.14a,b, right). The melting profile revealed a strong stabilization by the macrocycle ($\Delta T_m = +33^\circ\text{C}$) (Fig. 11.15b). The difference in the stabilizing activity of **BisA** towards the two quadruplexes should be interpreted in terms of kinetic effect of the folded forms rather than as a neat preference for the i-motif. However, **BisA** is one of the first i-DNA ligand to be discovered. A T_m experiment performed with the duplex formed from annealing F21GT and its complementary strand 21C revealed that **BisA** is able to prevent duplex formation (Fig. 11.16). This is attributed to the high affinity of **BisA** towards G- and C-tetraplexes that locks the oligonucleotides into a conformation that is no longer suitable for complementary hybridization. It is tempting to attribute the location of the macrocycle to the three loops which result from the folding of the C- and G-rich strands into quadruplexes. These results are fully in line with the helix-destabilizing properties of **BisA** described above.

The ability of **BisA** and **MonoA** to inhibit telomerase was determined using the TRAP assay (TRAP = telomerase repeat amplification protocol). **MonoA** was found to be completely inactive ($\text{IC}_{50} \gg 10 \mu\text{M}$, IC_{50} being the concentration of ligand required for a 50% decrease of the enzymatic activity). In contrast **BisA** is a potent inhibitor of telomerase ($\text{IC}_{50} = 0.75 \mu\text{M}$). This result confirms the correlation between the G-stabilizing effect and the inhibition of the activity of the telomerase, already observed for many G-quadruplex ligands [49].

The selectivity of **BisA** for various DNA conformations has been studied using a large panel of physico-chemical and biochemical methods. The results obtained on hairpins, helix destabilization, abasic sites, and quadruplexes all fit in with the general trend: **BisA** cannot bind DNA duplexes in a specific manner. Therefore, **BisA** preferentially recognizes any secondary structure with more accessible sites: single strands, hairpin loops, loop-containing quadruplexes, and abasic pockets. Quantitative data would be very informative in order to compare the affinities of **BisA** for these various structures as well as structural studies in order to characterize the interactions at the molecular level.

11.3.5

Miscellaneous

11.3.5.1 Naphthalene diimide derivatives

The macrocyclic bisintercaland **27**, composed of two naphthalenediimide subunits connected by two permethylated ammonium linkers, has been reported recently

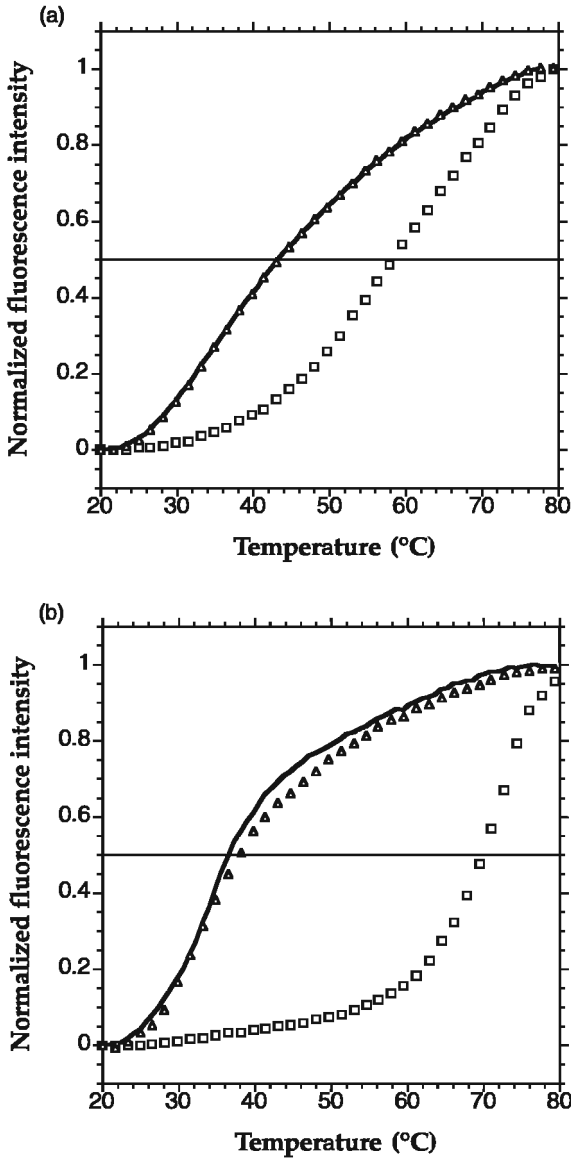


Fig. 11.15. FRET studies: fluorescence emission was monitored at 520 nm using a 470 nm excitation wavelength. (a) G-rich strand F21GT was chosen as the fluorescent probe for quadruplex formation. Solid line: oligonucleotide alone; triangles: +1 μM MonoA; squares: +1 μM BisA. Buffer conditions: 10 mM

sodium cacodylate, 100 mM LiCl, pH 7.2. (b) C-rich strand F23CT was chosen as a fluorescent probe for i-DNA formation. Solid line: oligonucleotide alone; triangles: +1 μM MonoA; squares: +1 μM BisA. Buffer conditions: 10 mM cacodylate, pH 6.8 (heating curves).

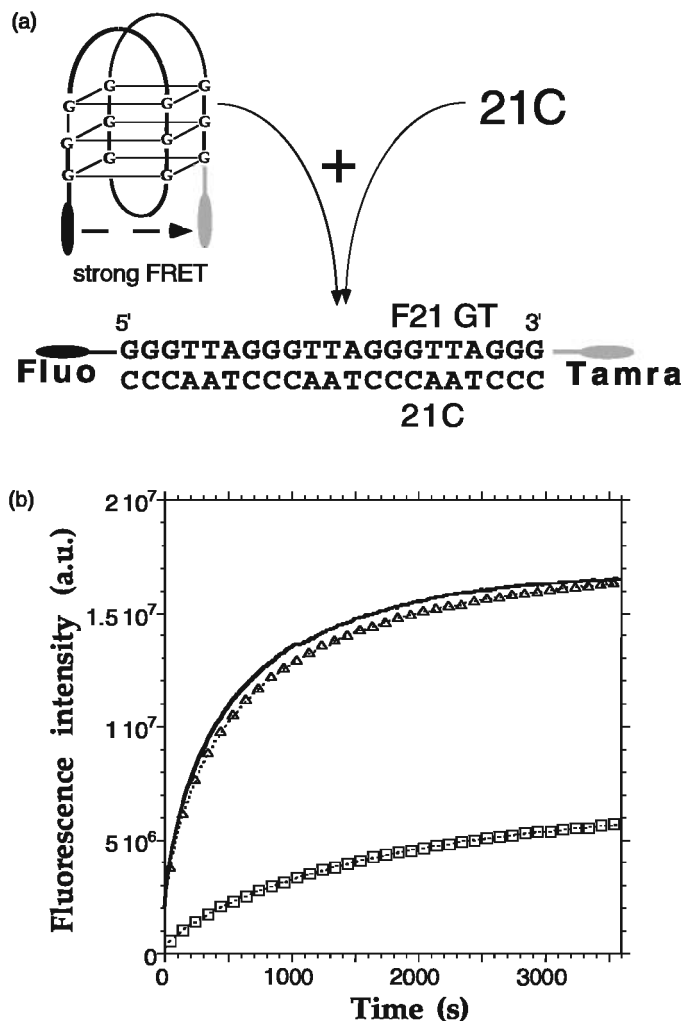
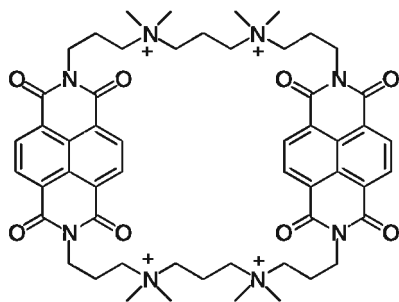


Fig. 11.16. (a) Design of the experiment: the G-rich strand is labeled with fluorescein (Fluo) and a rhodamine derivative (Tamra) at its 5' and 3' ends respectively. (b) Kinetic analysis of duplex formation: 21C (0.25 μM strand concentration) is added at 37°C to a preformed F21GT quadruplex alone (0.2 μM strand concentration, solid line) or in the presence of 0.5 μM **MonoA** (triangles) or **BisA** (squares), 100 mM KCl, 10 mM cacodylate buffer (pH 7.2).

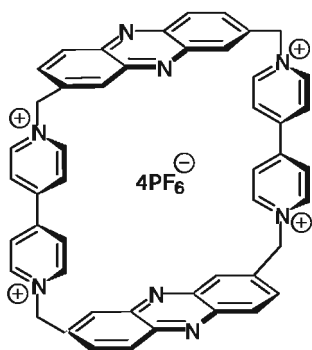
[50]. Based on UV-vis measurements, a catenated structure was suggested for its complex with CpG. Likewise, it forms a strong complex with calf thymus DNA which dissociates 10^3 -times more slowly than the corresponding monointercalator; this result also supports a catenated structure for the complex of 27 with dsDNA. However more experimental results would be necessary to assess the reality of this threading intercalation mode of binding.



27

11.3.5.2 Phenazine derivatives

A tetracationic cyclointercalator made of two photosensitizing intercalating phenazine subunits linked by two viologen moieties, **28**, has been prepared [51]. Upon irradiation this compound appears to strongly cleave supercoiled pBR322 plasmid, generating mainly single-strand breaks.



28

11.3.5.3 Aminocalixarenes and aminocyclodextrins

Since calixarenes and cyclodextrins can be considered to be special macrocycles, the interactions of some of their amino derivatives with nucleotides and nucleic acids are worth mentioning. It has been reported that cyclodextrins bearing amino groups at their 6-positions bind nucleotides strongly and can discriminate between isomeric 3'- and 5'-phosphates as well as between 2'-oxy and 2'-deoxy sugar phosphates [52]. Some permethylated aminocalixarenes, for their part, exhibit a remarkable preference for DNA in comparison to RNA [53]. In any case, these interactions are dominated by coulombic forces.

11.4

Conclusion and Perspectives

The synthetic macrocycles conceived by analogy with natural bisintercalators and on the basis of supramolecular concepts such as geometrical and functional complementary, show amazing and interesting binding behaviors towards DNA. The results reported throughout this chapter demonstrate that most macrocycles studied so far cannot form classical intercalation complexes with double-stranded DNA. More interestingly they display binding preferences for single-stranded secondary structures and some of them even exhibit the ability to destabilize DNA or RNA duplexes. Obviously, their particular behavior is determined by the steric clash expected from their cyclic framework, but the exhibited selectivities are also related to the chemical properties and to the topology of their constitutive subunits. As reported above, the combination of flat intercalative units and flexible polyammonium linkers led to the most selective and efficient binders (**BisA** and **SDM**). The flexibility of the latter could be the dominant structural feature contributing to fit the drug into its DNA target by allowing the optimization of specific polar and hydrophobic interactions.

Besides, the binding of macrocycle to DNA can result in strong distortion from normal double helix geometry. For instance, the 'intercalation platform' caused by **SDM** binding to duplexes DNA is a particularly intriguing phenomenon. It would be of great interest to know if these crosslinked species are biologically relevant and if they can be controlled for constructing well-defined multistranded DNA assemblies.

Similarly, the insertion of **BisA** into the abasic lesion through a threading bisintercalation in violation of the neighbor-exclusion paradigm was difficult to predict. This behavior, which is unprecedented either for synthetic or natural ligands, demonstrates that cyclic ligands are compatible with dsDNA if a local pairing defect is present, allowing insertion. Moreover, this leads to remarkably stable associations resulting from interlocking of the two partners. This study offers the possibility of finding novel unusual binding modes which in turn should stimulate the conception of new multifunctional chemical architectures.

The above examples demonstrate that it is essential to develop structural analysis of the molecular interactions to shed light on the ability of DNA to accommodate complex compounds, thus providing new insight on the plasticity of this biopolymer. Furthermore, there is a growing need for real-time measurements of complexation events (i.e. quantitative access to kinetic parameters (K_{on}/K_{off})), especially when competitive binding of small molecules with proteins are contemplated. However, the use of NMR or of optical biosensors technology to monitor DNA/macrocycle interactions is systematically hampered by serious precipitation due to charge neutralization and/or crosslinking. Indeed, all the groups working with cationic macrocyclic compounds have reported that aggregation of DNA is frequently induced by ligand binding. This phenomenon also makes it difficult to use gel-mobility shift assays due to the inhibition of the migration of the complexes. The condensation of DNA with polyamines is a complicated process, which

is not currently well understood. However, this phenomenon is related to fundamental biological problems such as gene vectorization and chromatin organization, and one can speculate that macrocycles might be potential candidates for studying or inducing condensation of DNA [54].

In most cases the molecular recognition of DNA by macrocycles has been investigated using model oligonucleotides or polynucleotides. The next step will be to examine the behavior of macrocycles with biologically relevant DNA targets, as has been done with **BisA** and G-quadruplexes. This could lead, for instance, to the use of macrocycles for the identification of structural-specific DNA elements either *in vitro* or *in vivo*; the detection could be achieved by using macrocycles containing fluorescent moieties or by grafting a fluorescent marker on the molecule. In addition, the trapping of nucleic bases by π -sandwiching between photo- or chemically active subunits could improve the redox-damaging activity. Such improvement could be expected from a longer residence time on the DNA or from a more efficient chemical reaction.

Another specific feature of macrocyclic structures possessing polyamino and heterocyclic moieties is their ability to form multinuclear coordination complexes with metallic cations (Cu^{2+} , Rh^{2+} , etc.). It would be of interest to take advantage of this property to induce redox damages at specific sites. The same rationale is valid for photocleavage, and this is currently under investigation with **BisQ** cyclobis-intercalands which possess quinacridine subunits able to damage DNA either when coordinated to Cu^{2+} [55] or via photoinduced electron transfer [56].

Finally, investigating more deeply the recognition of RNA by macrocyclic ligands could also be very fruitful. RNAs exhibit a large array of secondary and tertiary structures; among these a variety of bulge loops, hairpins, and pseudoknots present single-stranded regions which offer attractive specific binding sites for macrocyclic derivatives. Moreover the diversity of tertiary folding exhibited by RNAs can delineate clefts and pockets to accommodate macrocyclic compounds that could permit discrimination between DNA and RNA, which is still a challenge.

Altogether, the above observations may be seen as potent arguments for retaining consideration of macrocyclic structures as selective DNA ligands either in the drug discovery process or for use as biochemical tools.

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12

Triplex- versus Quadruplex-specific Ligands and Telomerase Inhibition

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12.1

Introduction

A number of alternative DNA structures have been described to date. As stressed previously, DNA comes in many forms [1]. It can exist in a variety of double-, triple-, and quadruple-stranded forms. The list of alternative conformations that can be adopted by this molecule is still growing with the recent addition of DNA pentaplexes [2, 3]. Multistranded structures rely on the formation of specific base pairs, triplets, or quartets (Fig. 12.1).

G-quadruplexes are a family of secondary DNA structures formed in the presence of monovalent cations that consist of four-stranded structures stabilized by G-quartets (Fig. 12.1a) [4]. There is a renewed interest in quadruplex structures due to their putative biological regulatory function. Optimal telomerase activity requires the non-folded single-stranded form of the primer, and G-quartet formation has been shown to directly inhibit telomerase elongation *in vitro* [5]. Therefore, ligands that selectively bind to G-quadruplex structures may modulate telomerase activity [6, 7].

Other multistranded nucleic acid structures may also be observed. Cytosine-rich oligomers may also form a quadruplex called i-DNA [8–10], based on the formation of hemiprotonated base pairs (Fig. 12.1b). Triplexes are obtained when a third strand binds to the major groove of double-stranded nucleic acids [11, 12]. Triple helices were first observed in 1957 formed by polyribonucleotides [13]. At least three structural classes of triple helix exist that differ in the base composition of the third strand, the relative orientation of the phosphate-deoxyribose backbone and the thermodynamical parameters [14]. In the first class, the pyrimidine motif (TC motif), the third strand binds parallel to the purine strand of the duplex by Hoogsteen hydrogen bonds, forming T.A**T* and C.G**C*+ triplets (Fig. 12.1c, left). This class of triple helix is stabilized by an acidic pH. In the second class, the GA motif, oligonucleotides containing guanines and adenines bind in antiparallel ori-

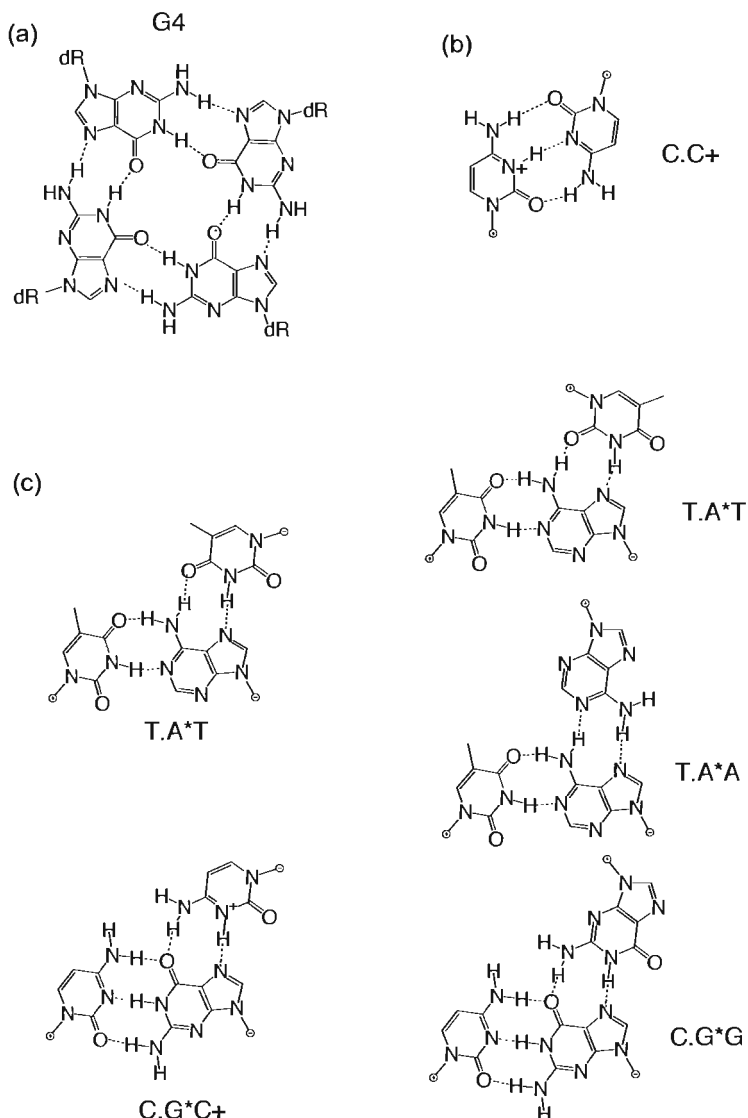


Fig. 12.1. Base pairing schemes. (a) A G-quartet involving four guanines. (b) A C.C⁺ base pair found in i-DNA. (c) Base triplets. Left: Hoogsteen pairing. Right: reverse-Hoogsteen. TC triplexes are composed of T.A^{*}T and C.G^{*}C⁺ base triplets; GA and GT triplexes are composed of T.A^{*}A and C.G^{*}G or T.A^{*}T and C.G^{*}G base triplets, respectively. Watson–Crick

base pair is indicated by a dot, Hoogsteen or reverse-Hoogsteen pairing by *. The base belonging to the third strand is indicated last. Strand orientation is indicated by – and +, except for the G-quartet, where it may vary. Note that the formation of the C.C⁺ base pair and of the C.G^{*}C⁺ base triplet requires protonation of one cytosine at its N3 position.

entation to the purine strand of the duplex by reverse Hoogsteen hydrogen bonds, forming C.G*G and T.A*A base triplets (Fig. 12.1c, right). In the third class, the GT motif, oligonucleotides containing thymines and guanines bind either parallel or antiparallel to the purine strand of the duplex depending on the base sequence [15].

Significant progress has been made over the past few years in studies of drug–DNA interactions. Alternative DNA structures offer significant differences in terms of electrostatics, shape, and rigidity compared with single- or double-stranded DNA. Therefore, specific recognition of unusual DNA structures by small molecules should be possible. This assumption was shown first to be correct for triplexes [16], then for quadruplexes [6]. A drug that stabilizes quadruplexes could interfere with telomerase and telomere replication [17, 18]. A range of molecules containing tricyclic, tetracyclic, and pentacyclic aromatic chromophores has been shown to inhibit telomerase [6, 19–24]. Nevertheless, recent reports have shown that some molecules thought to be quadruplex-specific are rather triplex-specific [25], arguing for a systematic binding evaluation to a variety of nucleic acids structures. The sequence and structural selectivity of various different DNA-binding agents has been previously explored using a thermodynamically rigorous competition dialysis procedure [25–27]. In this competition dialysis method, different nucleic acid structures are dialyzed against a common ligand solution. More ligand accumulates in the dialysis tube containing the structural form with the highest ligand-binding affinity. We have decided to modify the experimental format chosen by Chaires and colleagues in order to accommodate several new triplex structures, as well as a few other DNA conformations. We will first present the nucleic acids structures chosen for this assay before analyzing the selectivity of some new ligands towards these conformations.

12.2

Nucleic Acids Samples

The low specificity of some compounds towards telomerase inhibition prompted us to evaluate the specificity of these compounds for quadruplexes with a competitive dialysis experiment. In such an experiment, different nucleic acids structures are dialysed against a common ligand solution [25]. More ligand accumulates in the dialysis tube containing the structural form with the highest ligand binding affinity. Because our laboratory is interested in the design of triplex- as well as quadruplex-specific ligands, we decided to select a slightly different set of structures (8 different, 10 identical) than previously described [26]. Formation of some of these structural motifs requires a slightly acidic pH and/or magnesium. As a consequence, the buffer chosen for all experiments is somewhat different (pH 6.5, with 10 mM MgCl₂), keeping the same relatively high monocation concentration (0.185 M NaCl) in order to limit electrostatic interactions between positively charged dyes and nucleic acids.

Tab. 12.1. Nucleic acid structures.

Number	Name	Type ^a (length)	Structure	T _m (°C) ^d
1	TC triplex	oligos (30 + 13)	Triplex	38
2	GA triplex	oligos (30 + 13)	Triplex	53
3	GT triplex	oligos (30 + 13)	Triplex	53
4	poly(dA)-2poly(dT)	poly	Triplex	71
5	GA duplex	oligo (24)	“Duplex” ^b	37
6	ps duplex	oligos (24 + 24)	“Duplex” ^b	39
7	24CTG	oligo (24)	“Duplex” ^b	64
8	poly(dA-T)	poly	Duplex	66
9	poly(dG-C)	poly	Duplex	>90
10	CT DNA	poly	Duplex	86
11	ds26	oligo (26)	Duplex	75
12	poly(dC)	poly	i-DNA	51 ^c
13	22CT	oligo (22)	ss/i-DNA ^c	13 ^c
14	22AG	oligo (22)	G4	62
15	24G20	oligo (24)	G4	>90
16	poly(dT)	poly	single-str	– ^f
17	poly(dA)	poly	single-str	– ^f
18	poly(rU)	poly	single-str	– ^f

All polynucleotides were ordered from Amersham-Pharmacia. Oligonucleotides were synthesized by Eurogentec, Belgium on the 1 μmol scale, checked by denaturing gel electrophoresis and used without further purification. Eighteen different nucleic acids structures are used (samples labeled 1–18, left column). The TC, GA and GT triplexes result from the association of two strands of different lengths (13 and 30 nucleotides) [14]: 5'-GAAAGAGAGGAGG and 5'-CCTCCTCTCTTTCCCTTCTTCTCTCTCTCC (TC triplex, sample 1); 5'-CCTCCTCTCTTTC and 5'-GAAAGAGAGGAGGCCTTGGAGGAGAGAAAG (GA triplex, sample 2); 5'-CCTCCTCTCTTTC and 5'-GAAAGAGAGGAGGCCTTGGTGCTGTGTTTG (GT triplex, sample 3). The GA “duplex” (sample 5) results from the self-association of the 24 GA oligonucleotide (5'-GAGAGAGAGAGAGAGAGAGAGA) which probably leads to the formation of a parallel-stranded duplex [28, 29]. The parallel-stranded duplex (sample 6) results from the association of two AT strands [30]: 5'-AAAAAAAAATAATTTAAATATT and 5'-TTTTTTTTTTATATAAATTTATAA. The 24CTG (sample 7) mimics eight repeats of the trinucleotide unit: 5' CTGCTGCTGCTGCTGCTGCTGCTG. ds26 (sample 11) is a 26-base-long duplex formed with the self-complementary oligonucleotide 5'-CAATCGGATCGAATTCGATCCGATTG. 22CT (sample 13) is an oligonucleotide that mimics the cytosine-rich strand of human telomeres: 5'-CCCTAACCTAACCTAACCT [31], whereas 22AG (sample 14) is an oligonucleotide that mimics the guanine-rich strand of human telomeres: 5'-AGGGTTAGGGTTAGGGTTAGGG [32]. 24G20 (T₂G₂₀T₂, sample 15) may form an intermolecular quadruplex 5'-(TTGGGGGGGGGGGGGGGGGGGTT)₄ [26]. Due to the

The 18 samples chosen for analysis and the sequence of oligonucleotides are listed in Tab. 12.1. Structural forms included in the assay range from single strands (poly(dA), poly(dT), and poly(rU)), through a variety of duplexes, to four different triplexes (TC, GA, and GT as well as poly(dA)-2poly(dT)), as well as tetraplex forms. Among these samples, two correspond to G-quartet-containing motifs: 24G20 ($T_2G_{20}T_2$), which forms a parallel-stranded intermolecular quadruplex and 22AG which forms a folded antiparallel quartet structure. 22CT and poly(dC) are prone to i-DNA formation.

The stability of the various structures may be analysed by thermal denaturation experiments. Absorbance versus temperature is recorded for each sample at two different wavelengths (260 and 295 nm) and the results are shown in Figs 12.2 and 12.3. Absorbance at 260 nm was chosen as it corresponds to the absorbance maximum of nucleic acids of mixed purine–pyrimidine content. Absorbance at 295 nm has been previously shown to be useful as the melting of some DNA structures is easily evidenced at this wavelength [35]. Data for poly(dT), poly(dA), poly(rU) (which fail to form any structure between 0 and 90°C), poly(dA-T), poly(dG-C), and calf thymus DNA (which melt at 66, >90 and 86°C, respectively) is omitted for clarity. All the other 12 melting profiles are shown. The two i-DNA-forming oligonucleotides exhibit a non-reversible melting behavior: one cannot superimpose the heating and cooling profiles (Fig. 12.3c,d). At this pH, the 22CT structure is relatively unstable and this oligonucleotide is mostly single stranded at 20°C (Fig. 12.3d). All other structures are formed at room temperature as their T_m is $\geq 37^\circ\text{C}$. The melting of the pyrimidine triplex is biphasic at pH 6.5: the triplex dissociates at a lower temperature than the duplex (Fig. 12.2a). Only the melting of the pyrimidine triplex gives a clear transition at 295 nm (Fig. 12.2a) as a result of cytosine deprotonation upon triplex unfolding [36–39]. Melting of the GA and GT triplexes is monophasic (Fig. 12.2b,c) and the transition corresponds to a triplex-to-single-strand phenomenon [14]. As expected, the stability of the poly(dA)-2poly(dT) triplex is much higher.

Three unusual duplexes were then studied. The GA duplex (Fig. 12.2e), resulting from the self-association of a (GA)-repeat and the parallel-stranded DNA duplex (Fig. 12.2f) have relatively similar stabilities with T_m values of 37 and 39°C, re-

presence of magnesium in the incubation buffer which favors ribonucleotide degradation, only one RNA sample is present (poly rU) among the 18 samples. This oligomer was aliquoted in the absence of MgCl_2 .

^apoly, polynucleotide; oligo, oligonucleotide; oligos, structure formed by the association of two different oligonucleotides. The length of the oligomer(s) is shown within parenthesis. Polynucleotides are >100 bases long.

^bThese three duplexes are rather unusual as they involve the formation of non-classical base pairs.

^c22CT may form an i-DNA structure but is mainly single stranded at room temperature.

^dObtained in a buffer identical to the equilibrium dialysis protocol.

^eHysteresis. T_m obtained while heating.

^fNo transition.

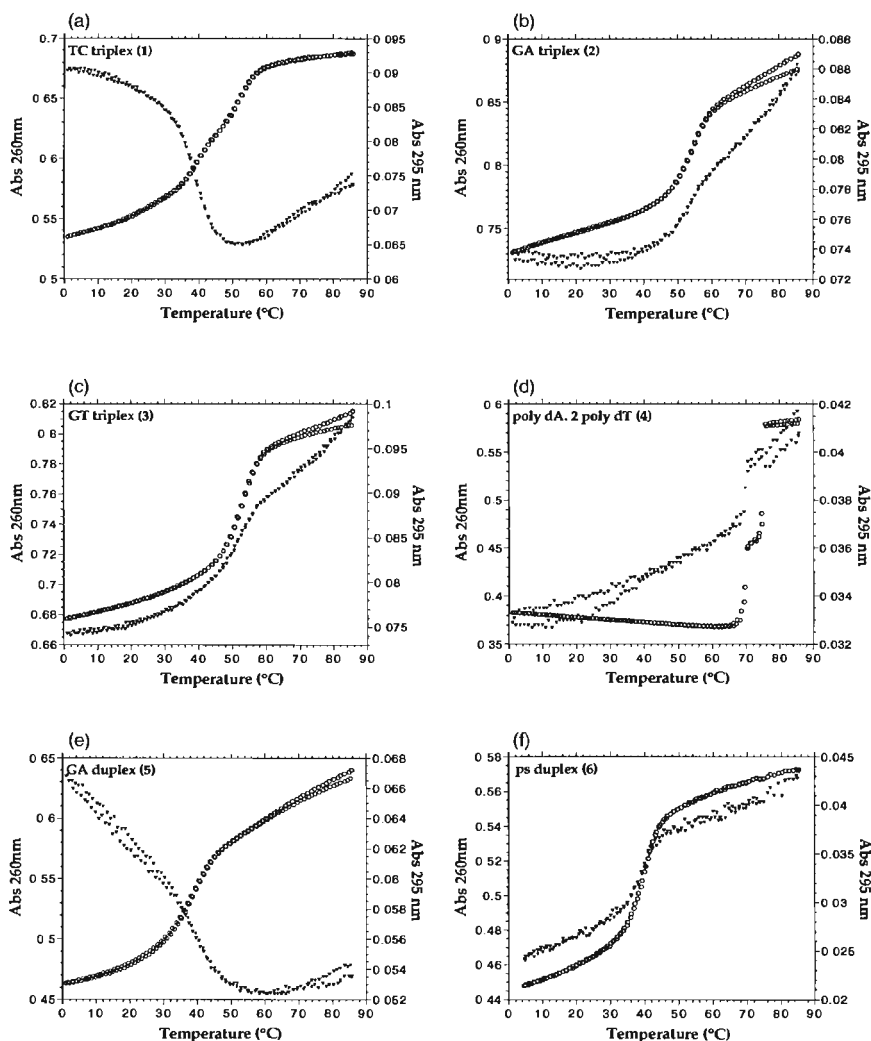


Fig. 12.2. Stability of some DNA structures: T_m analysis (1). The experimental conditions are: 0.185 M NaCl, 15 mM Na cacodylate pH 6.5, $MgCl_2$ 10 mM. All T_m values were recorded starting from high temperature (90°C) cooling at $0.2^\circ\text{C min}^{-1}$ to 0°C followed by a heating cycle at $0.2^\circ\text{C min}^{-1}$ [14]. T_m values were obtained at 2–5 μM strand concentration. The profiles were recorded at

260 nm (open circles) and 295 nm (filled triangles). Note the separate y-axis for these two wavelengths. All melting profiles are kinetically reversible, as shown by the superimposition of the heating and cooling curves. (a) TC triplex (1); (b) GA triplex (2); (c) GT triplex (3); (d) poly(dA)-2poly(dT) triplex (4); (e) 24 GA duplex (5); (f) parallel-stranded AT duplex (6).

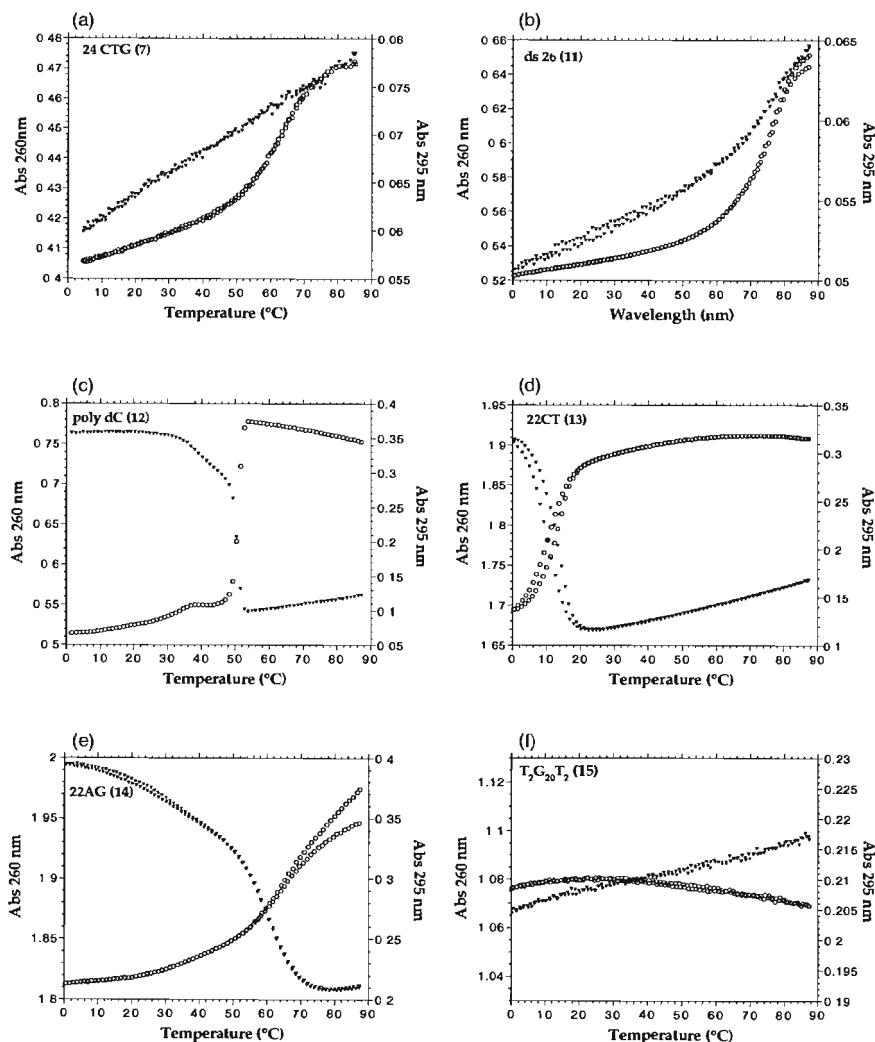


Fig. 12.3. Stability of some DNA structures under dialysis conditions: T_m analysis (2). Same conditions as in Fig. 12.2. All melting profiles were recorded at 260 nm (open circles) and 295 nm (filled triangles). Note the separate Y-axis for these two wavelengths. All melting profiles except c and d are kinetically reversible, as shown by the superimposition of the heating and cooling curves. i-DNA formation at near neutral pH is slow, leading to an hysteresis phenomenon. As a result the

cooling profile of 22CT is shifted towards lower temperatures. For poly(dC), the effect is even more pronounced, and i-DNA formation is extremely slow. Only the denaturation profile is shown. (a) 24 CTG trinucleotide repeat (7); (b) short 26-bp duplex (11); (c) poly(dC) (12); (d) i-DNA forming oligonucleotide 22CT (13); (e) intramolecular G-quadruplex 22AG (14); (f) parallel G-quadruplex 24G20 (15) ($T_m > 90^\circ\text{C}$, no fusion observed).

spectively. The 24-base-long oligonucleotide that mimics a trinucleotide repeat, (CTG)₈, forms a highly stable structure with a T_m of 64°C (Fig. 12.3a). This T_m value is close to the melting temperature (75°C) of a perfectly matched 26-base-long Watson–Crick autocomplementary oligonucleotide ds26 (Fig. 12.3b). Among the two i-DNA-forming samples only poly(dC) gives a relatively high T_m (51°C) at pH 6.5 (Fig. 12.3c,d). Finally, an oligonucleotide mimicking the G-rich strand of human telomeres has a T_m of 62°C under these conditions (Fig. 12.3e), whereas the parallel-stranded quadruplex involving 20 contiguous guanines cannot be unfolded even at 90°C (Fig. 12.3f).

The nature of the folded form may also be analyzed by recording absorbance spectra below and above the melting temperature. The resulting differential absorbance spectrum shown in Fig. 12.4 may be seen as specific signatures for each sample. For example, the relatively broad peak between 245 and 270 nm (Fig. 12.4a) and the small negative peak at 295 nm is specific for a pyrimidine triplex requiring the protonation of the cytosines in the third strand in order to form C.G⁺C⁺ triplets. On the other hand, it is difficult to distinguish between the melting of a GT triplex (Fig. 12.4b) or a GA triplex (not shown) and the melting of a classical Watson–Crick duplex [14]. Both unusual duplexes have specific, differential absorbance signatures. The shoulder shown in Fig. 12.4c between 285 and 300 nm is absolutely specific of a parallel duplex, whereas the trinucleotide repeats (Fig. 12.4d) gives a maximum differential at an unusually high wavelength (277 nm).

Concerning quadruplexes, both types of structures give highly specific differential absorbances. Melting of an i-DNA structure (Fig. 12.4e) is associated with a positive peak at very short wavelengths (238 nm) and a large negative peak at 295 nm resulting from cytosine deprotonation [37]. On the other hand, melting of a G-quadruplex gives three reproducible peaks at 242, 255, and 271 nm and a negative peak at 295 nm (Fig. 12.4f) [35]. This figure illustrates the practical use of recording absorbances at several wavelengths to study nucleic acids structures.

12.3

Dialysis Results

We have recently identified several families of quadruplex ligands [23, 24, 34, 40], and we decided to analyse their binding specificity in the dialysis assay. The formulas of some of these molecules are shown in Fig. 12.5. Results are shown in Figs 12.6–12.7 for eight different compounds. Figure 12.6a shows the dialysis profile for ethidium bromide, a well-known intercalator. Its profile has previously been determined by Ren *et al.*, and this compound was therefore chosen as the reference. This dye interacts preferentially with regular duplexes such as poly(dA-T), poly(dG-C), and calf thymus DNA. Surprisingly, its preferred substrate is the unusual CTG repeat duplex. A detailed analysis demonstrates that this dye weakly interacts with triplexes [41], with a slight preference for poly(dA)-2poly(dT) [42].

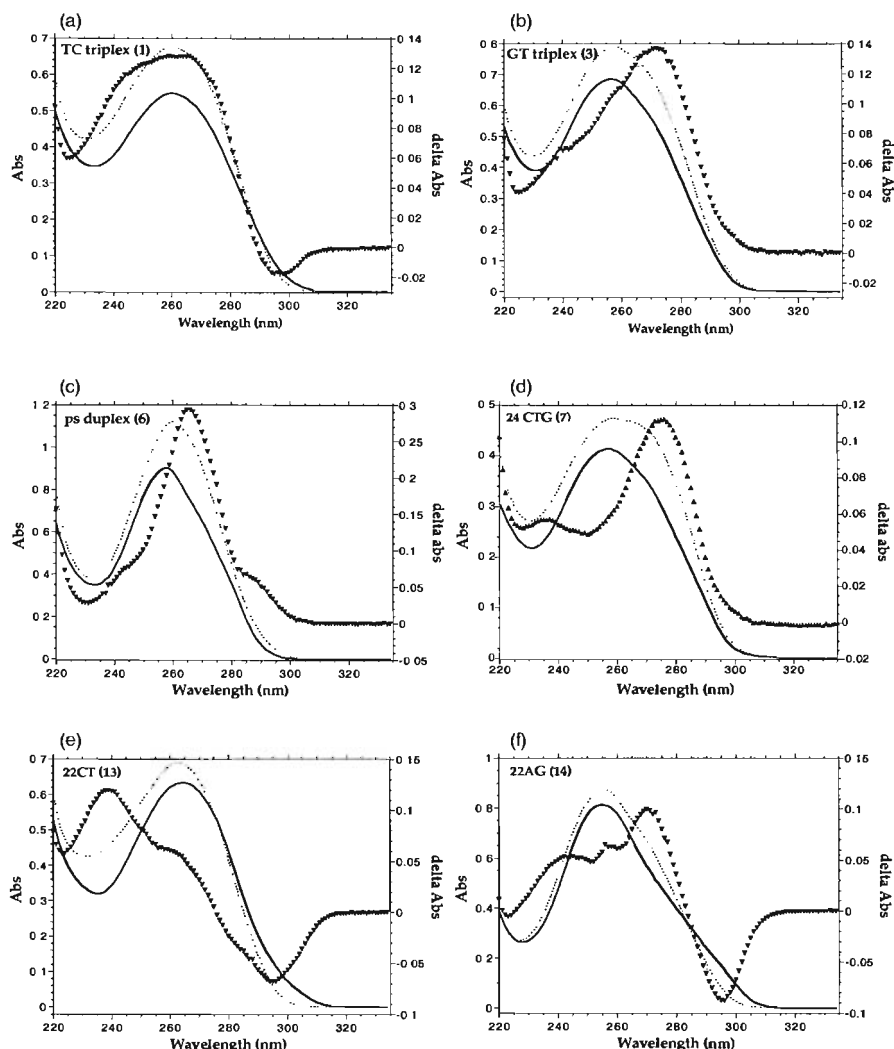


Fig. 12.4. Absorbance of some DNA structures under dialysis conditions at low and high temperature. Experimental conditions: 0.185 M NaCl, 15 mM Na cacodylate pH 6.5, MgCl_2 10 mM. The UV-scan was recorded at 0° (full line) and 85° (dotted line). The absorbance values are shown on the left y-axis. The difference between high and low

temperature absorbance is shown by black triangles (right scale). A negative ΔAbs means that the absorbance at 85°C is lower than the absorbance at 0°C. (a) TC triplex (1); (b) GT triplex (3); (c) parallel-stranded AT duplex (6); (d) 24 CTG trinucleotide repeat (7); (e) i-DNA forming oligonucleotide 22CT (13); (f) intramolecular G-quadruplex 22AG (14).

Binding to quadruplexes is confirmed [43], although the amount of dye bound to G-quadruplexes is low [23]. On the other hand, 9944, an ethidium derivative that efficiently inhibits telomerase, shows a marked preference for G-quadruplexes and antiparallel triplexes (Fig. 12.6b).

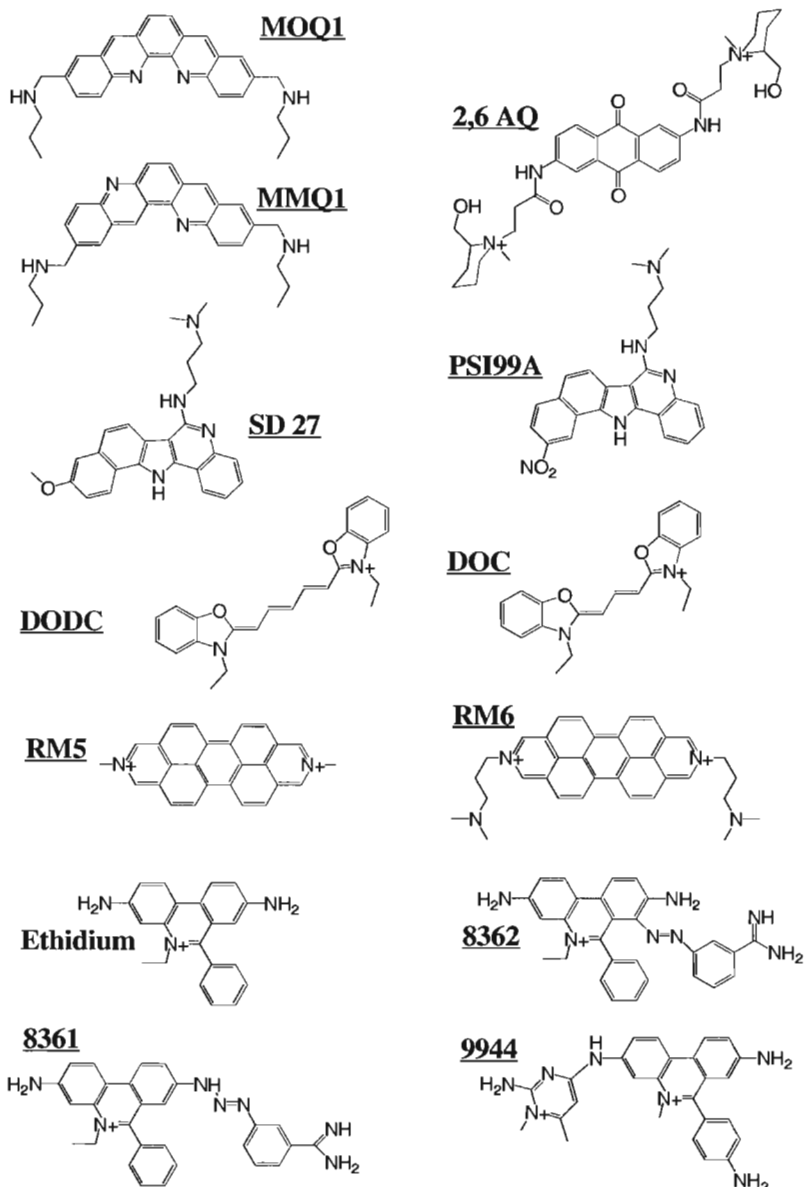


Fig. 12.5. Formulas of the compounds. **2,6 AQ** stands for 2,6-disubstituted amidoanthraquinone (or BSU 1051). **DODC** is the abbreviation of 3,3'-diethyloxadibenzocyanine. **DOC** is the abbreviation of 3,3'-diethyloxadibenzocyanine. The synthesis of ethidium derivatives [23],

dibenzophenanthrolines [24], **RM5** and **RM6** [33] and benzoindoloquinolines [34] has been described previously. **DODC** and **DOC** were obtained from Sigma-Aldrich. **2,6 AQ** (or BSU 1051) was synthesized and purified according to a published protocol [6].

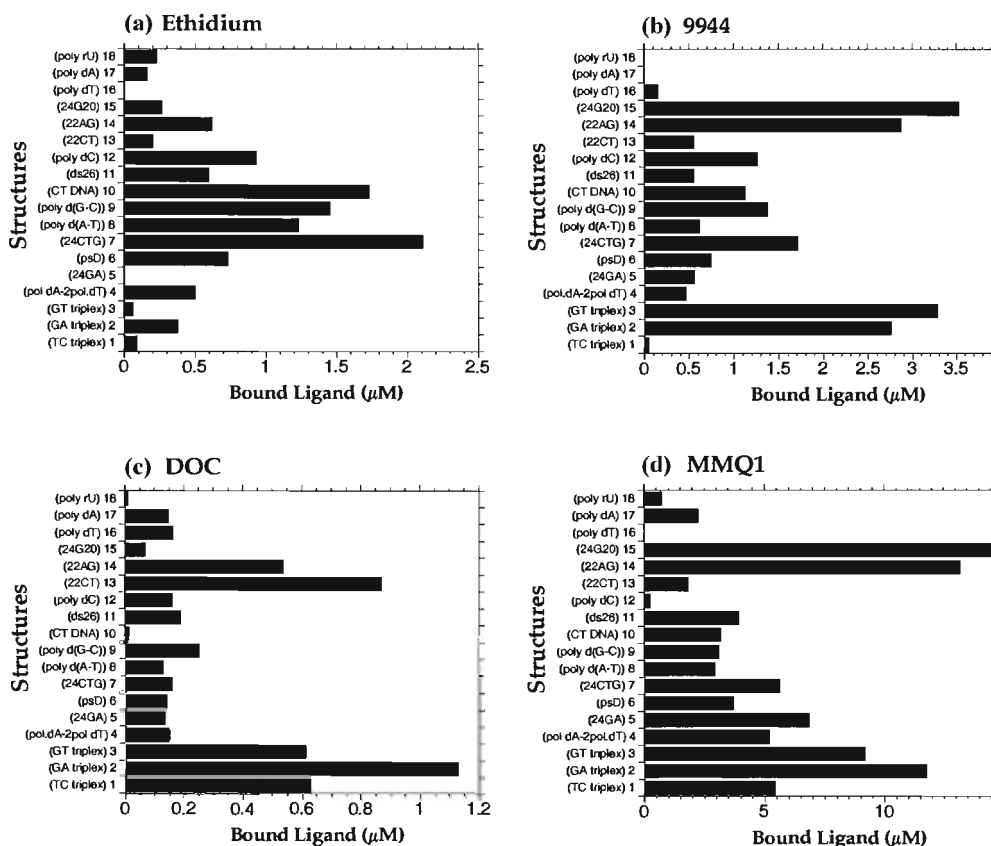


Fig. 12.6. Dialysis results (part 1). All measurements were performed using a methodology adapted from Chaires and colleagues [25–27]. Different nucleic acids were tested against a 1 μM dye solution in 500 mL of dialysis buffer (0.185 M NaCl, 15 mM Na cacodylate pH 6.5, 10 mM MgCl_2). A volume of 200 μL (at 75 μM monomeric unit: nucleotide, base pair, base triplet, or quartet) of each of the DNA samples listed in Table 12.1 was pipetted into a separate dialyzer unit (Pierce). All 18 dialysis units were then placed in the beaker containing the dialysate solution. The beaker was covered with Parafilm and

wrapped in foil, and its contents were allowed to equilibrate with continuous stirring at room temperature (20–22°C) overnight. At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes, and treated as described previously [26]. The ligand concentration in each sample was determined by absorbance or fluorescence. $\text{T}_2\text{G}_{20}\text{T}_2$ and 22AG form G-quadruplexes. Note the differences in x-axis limits between various compounds, which reflect large differences in binding affinities. (a) **Ethidium**; (b) **9944**; (c) **DOC**; (d) **MMQ1**.

This compound illustrates the structural difference between the different classes of triplexes: binding to the pyrimidine triplex is approximately 30 times weaker than to the GA and GT parallel triplexes. Such differences could be the result of the positive charges present on each $\text{C.G}^*\text{C}^+$ triplet in the TC triplex [41] or of differences in strand orientation and stacking interactions. On the other hand, there

is little difference between the classes of quadruplexes (antiparallel, no. 14 and parallel no. 15). Binding to single strands is very low, whereas binding to duplexes is intermediate. Within the duplex set, a slight preference for the CTG repeat is obtained.

Other classes of DNA ligands were then analysed. Carbocyanines (**DODC**) have been reported to interact with triplexes [25] and quadruplexes [44, 45]. We chose to analyse **DOC**, a close relative of **DODC**. As shown in Fig. 12.6c, **DOC** binds preferentially (but weakly, note x-axis values) to triplexes and some quadruplexes. The marked **DODC** preference for triplexes is lowered for **DOC**, showing that the net distance between the cyanine groups could fine-tune the structural selectivity. Surprisingly, binding to the 22CT oligonucleotide is much higher than to the other i-DNA prone sample (poly(dC)). **DOC** is also the compound for which the relative difference between antiparallel (no. 14) and parallel (no. 15) quadruplexes is the largest. Binding to all other structures is very low ($<0.2 \mu\text{M}$), especially when considering that the uncertainty of the measurement is $\sim 0.1 \mu\text{M}$ for that compound.

MMQ1 is a G-quadruplex ligand that efficiently inhibits telomerase [24]. Figure 12.6d confirms that this molecule interacts preferentially with G-quadruplexes, but does not distinguish between antiparallel and parallel structures. This molecule also strongly interacts with triplexes, especially the GA and, to a lesser extent, GT parallel triplexes. On the other hand, binding to single strands is almost negligible. Binding to duplexes is lower than to multistranded structures. Within the duplex class, very little difference is found between the different classical Watson–Crick duplexes (samples 8–11). The affinity for the three unusual duplexes (samples 5–7) is at least as high as for the regular duplexes.

13*H*-benzo-[6,7]-indolo[3,2-*c*]quinolines (latter referred to as benzoindoloquinolines) are pentacyclic aromatic molecules that carry a positively charged chain [46] and recognize quadruplexes [34]. Two benzoindoloquinolines (**PSI99A** and **SD27**) have been tested. As shown in Fig. 12.7a, **PSI99A** preferentially interacts with quadruplex-forming DNA samples. It also binds strongly to the GA triplex. Binding to duplexes is moderately strong, whereas binding to single strands is very low. On the other hand, **SD27** exhibits a limited structural specificity (Fig. 12.7b).

Another ligand, PIPER, was shown to interact with quadruplexes [47, 48]. Its shape is similar to dimethyldiazapyrenium dications, which interact with nucleic acids [33, 49]. We therefore tested the affinity of two derivatives, **RM5** and **RM6**, for various DNA structures. As shown in Fig. 12.7c,d, both molecules interact with most types of nucleic acids. This observation allows us to conclude that most planar polyaromatic cationic molecules interact with quadruplexes, but not necessarily in a specific manner. Compounds with a bend shape might be more selective for quadruplexes and/or triplexes.

All these profiles confirm that equilibrium dialysis is a useful method to test the specificity of G4-based telomerase inhibitors [50]. Some, but not all, of the compounds presented here exhibit a significant preference for quadruplexes as compared to duplexes or single strands. However, it has been relatively difficult to dissociate triplex and quadruplex affinity. Most of the quadruplex ligands exhibited similar and sometimes higher affinity for antiparallel triplexes. Another potential

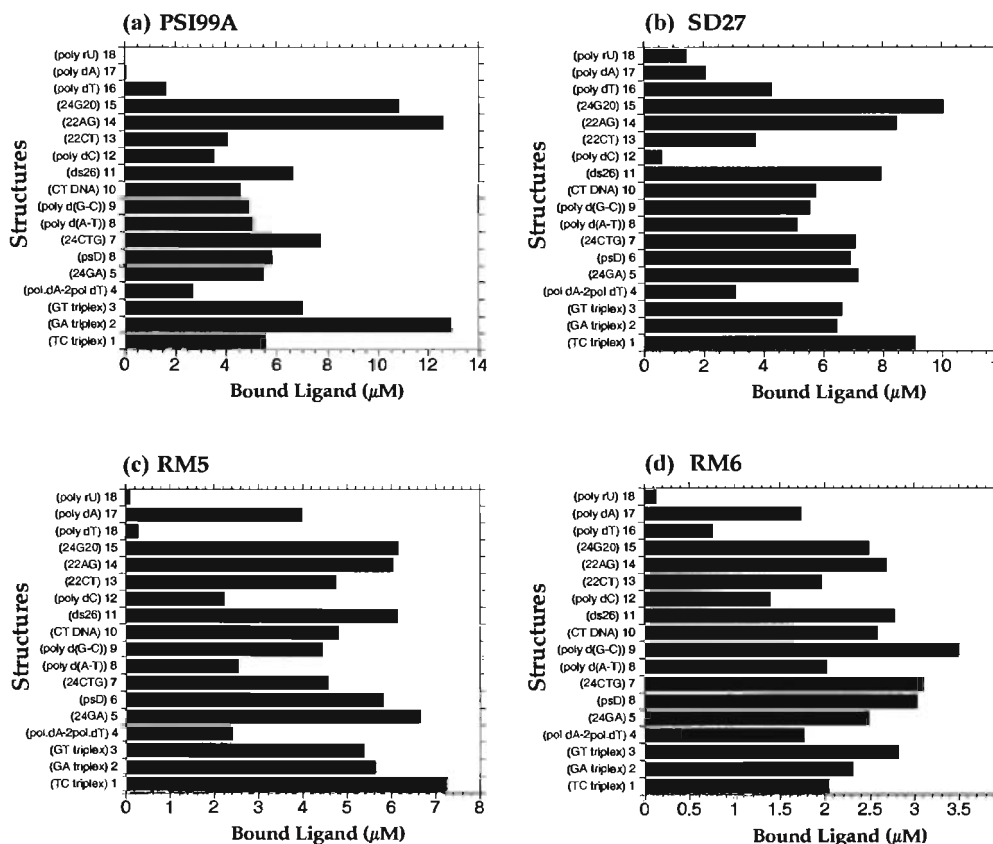


Fig. 12.7. Dialysis results (part 2). Same conditions as in Fig. 12.6. (a) PSI 99A; (b) SD27; (c) RM5; (d) RM6.

problem, when *in vivo* applications are contemplated, comes from the fact that very few molecules, with the possible exception of **DOC**, distinguish between the various classes of G-quadruplexes (intra- or intermolecular, parallel or antiparallel). In order to achieve specific telomerase inhibition, it might be interesting to identify compounds that would only bind to the antiparallel telomeric quadruplex (sample 14). As none of the ligands fulfill this condition, non-specific and undesired cellular effects are expected to occur [50].

12.4

Induction of Quadruplex Structures

In all previous experiments, binding of ligands to preformed DNA structures (and G-quadruplexes in particular) was measured. Another point would be to demonstrate that these molecules may induce the formation of the multistranded struc-

tures starting from single strands. The formation of some bimolecular quadruplex structures may be a very slow process. Small ligands such as perylene diimide [48] or ethidium derivatives [23] may behave as drivers for the assembly of nucleic acid secondary structures. It has been demonstrated by a gel-shift experiment that PIPER can dramatically accelerate the association of a DNA oligomer (Tr2) containing two tandem repeats of the human telomeric sequence (TTAGGG) into di- and tetrameric G-quadruplexes [48]. By analogy, eight different quadruplex ligands were tested for their ability to promote two different quadruplex-folded oligonucleotides, and the results are shown in Figs 12.8 and 12.9.

As expected, ethidium is a poor G-quadruplex ligand, it fails to induce significant amount of quadruplex (Fig. 12.8a). On the other hand, **8361**, **8362**, and **9944** efficiently promote quadruplex formation in a dose-dependent manner. Among these ethidium derivatives, only **8361** (Fig. 12.8b) seems to distinguish between the two quadruplexes: Tr2 (human telomeric quadruplex, crosses) is induced at a lower concentration than Ox-1T (*Oxytricha* telomeric repeat, circles). One should remember that this accelerated assembly is measured at a relatively high dye concentration and that other factors such as dye aggregation, binding stoichiometry, or dimerization could also play a role. The four other G-quadruplex ligands also promote quadruplex formation, but with a slightly lower efficiency (Fig. 12.9). **SD27** and **2,6 AQ** seem to prefer the *Oxytricha* over the human telomeric motif (Fig. 12.9a,b).

The situation is more complex for the dibenzophenanthrolines **MMQ1** and **MOQ1**. At low compound concentrations, Ox-1T is partially converted to a quadruplex species, whereas Tr2 remains single-stranded. However, with 20 μ M **MMQ1** or **MOQ1**, G-quadruplex formation is favored with the Tr2 sequence. The following compounds were also tested: **DODC** is a very poor quadruplex inducer (<5% quadruplex at 20 μ M dye concentration) whereas **RM5** induces a significant conversion to a multistranded species (data not shown).

12.5

Triplex versus Quadruplex Stabilization

Our laboratory has been involved in the search of triplex ligands for a decade. Various methods have been implemented to evidence triplex stabilization, such as UV-melting [16, 51], footprinting, and gel retardation experiments [52]. Of particular interest is the observation that some parallel triplexes may be formed only in the presence of these ligands [53] (Takasugi, unpublished results). We then reasoned that if a triplex stabilizer exhibits some affinity for other nucleic acid structures, adding increasing amounts of such competing structures should trap the triplex ligand and destabilize the triplex. This experiment was implemented using non-denaturing gel electrophoresis. Formation of a 16-base-long triplex is evidenced by a retarded band when the target duplex (40RY) is radiolabeled. Under these experimental conditions, no triplex is obtained in the absence of **SD27**,

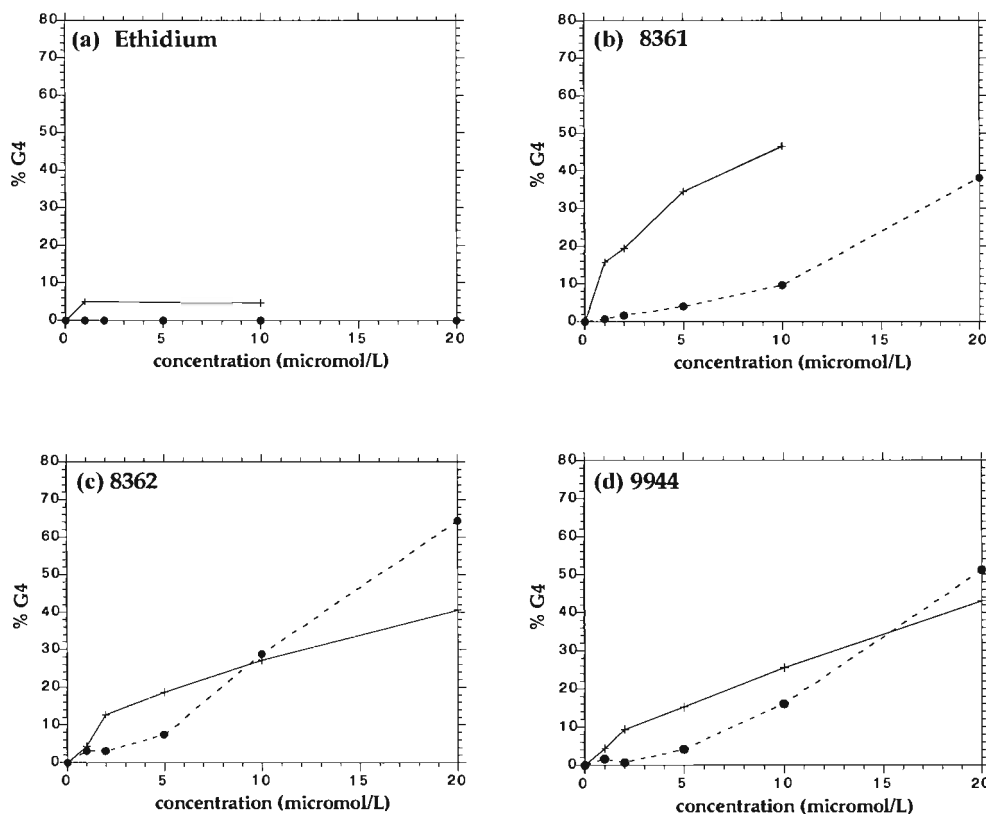


Fig. 12.8. G4 induction by ethidium derivatives. The purified Tr2 (5' TACAGATAGTTAGGGTTAGGGTTA) and Ox-1T (5' ACTCTCGTACTTGATACGGGTTTTGGGGAATGTGA) oligodeoxynucleotides were 5'-end labeled and used at a final strand concentration of 8 μM . The solution was heated to 95°C for 5 min in a TE buffer containing 0.1 M KCl and slowly cooled to room temperature. Ligands were added (0–20 μM), and reaction mixtures were then incubated for 1 h at room temperature, then loaded on a native 12% acrylamide vertical gel in a 0.5 $\times$ TBE buffer supplemented with 20 mM KCl. The gel was run at 4°C for 6 h, dried and analyzed with a phosphorimager. Quadruplex formation is efficiently promoted by some derivatives as shown by the appearance of a new band of retarded mobility. The dye concentration is indicated in micromolars. %G4 represents the relative amount of radioactivity found in the band corresponding to a quadruplex species. Crosses: Tr2 oligonucleotide; filled circles: Ox-1T. (a) **Ethidium**; (b) **8361**; (c) **8362**; (d) **9944**.

whereas a complete conversion to the triplex form is observed in the presence of 5 μM **SD27** (Fig. 12.10a, lane 1 and 2). Adding increasing amounts of a non-radiolabeled quadruplex leads to the disappearance of the triplex band (Fig. 12.10, lane 3–5). Adding an excess of the ligand (50 μM) restores the triplex band, showing that the trapping phenomenon is saturable and therefore does not result

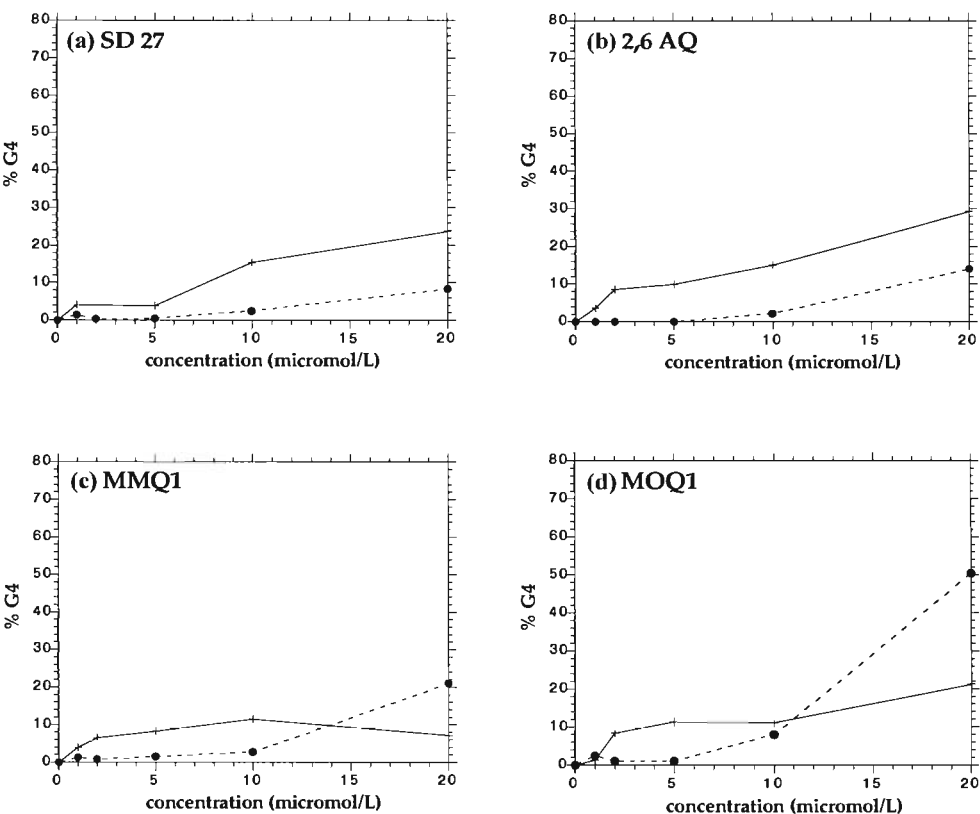


Fig. 12.9. G4 induction by other compounds. Conditions as in Fig. 12.8. %G4 represents the relative amount of radioactivity found in the band corresponding to a quadruplex species. Crosses: Tr2 oligonucleotide; filled circles: Ox-1T. (a) **SD27**; (b) **2,6 AQ**; (c) **MMQ1**; (d) **MOQ1**.

from an interaction between the competitor nucleic acid and the third strand of the duplex (16GT). Identical results are obtained in the presence of some, but not all, nucleic acid species. For example, single-stranded poly(dA) is an inefficient competitor, whereas an unrelated triplex leads to the rapid disappearance of the retarded band (Fig. 12.10a, lanes 7–9, 11–13).

The results of this method are quantified on Fig. 12.10b. The structures that inhibit triplex formation at low concentrations are expected to strongly bind **SD27**. This ranking is in agreement with the equilibrium dialysis result for most of the competitors structures: **SD27** has the best affinity for triplexes and G-quadruplexes structures. Nevertheless, some discrepancies are found: poly(dA)-2poly(dT) which is the best competitor according to this method has a relatively low affinity for **SD27** in the equilibrium dialysis experiment.

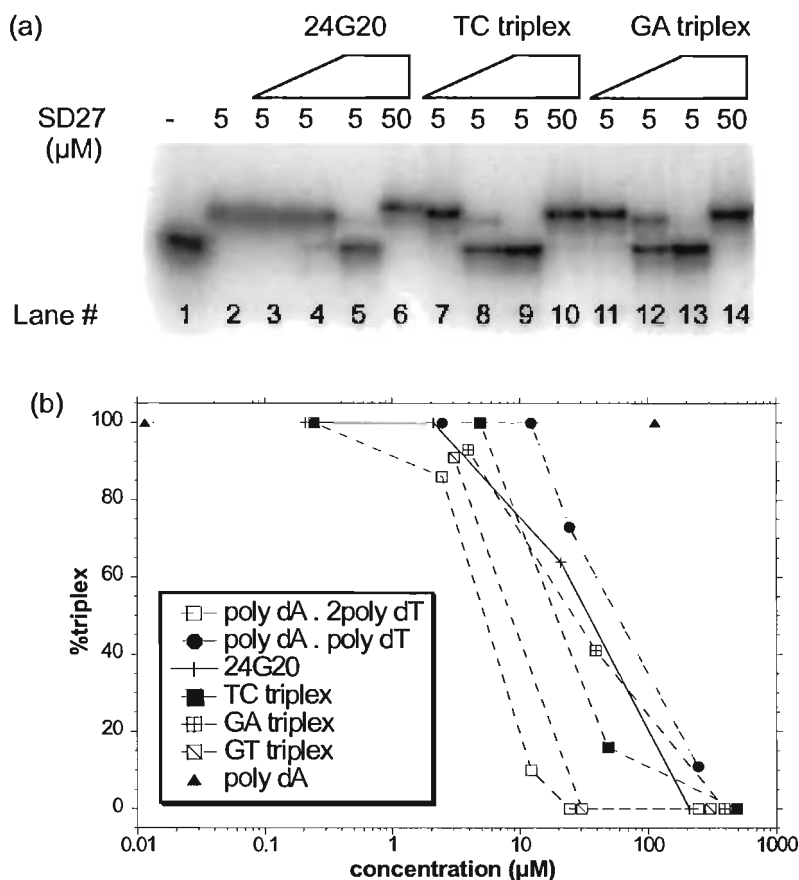


Fig. 12.10. Competition for triplex stabilization: a gel-shift assay. A triplex can be formed by the interaction of a 40-mer duplex (40R + 40Y) and a GT third strand (16GT) in the presence of ligand (**SD27**, see Fig. 12.5).

40R 5'-CATTCTGACGTTAGGAGGAAAAGGAGGATGCAAGTAATAC-3'

40Y 5'-GTATTACTTGATCCTCCTTTTCTCCTAACGTACGAATG-3'

16GT 5'-TGGTGGTTTTGGTGGT-3'

The triplex radiolabeled on the 40Y strand mixture was incubated in the absence or in the presence of **SD27** (5 or 50 μM) in a 50 mM HEPES (pH 7.2) buffer containing 140 mM KCl. This triplex mixture was also incubated in the presence of several non-radiolabeled nucleic acids prone to form different structures (single strands, duplexes, triplexes, and quadruplexes). For each competitor, the incubation at the higher competitor concentration was also performed with a 10-fold excess of **SD27** (50 μM). Duplex (40R + 40Y) and triplex (40R + 40Y + 16GT) species were separated by non-denaturing gel electrophoresis (12% acrylamide (acrylamide:bisacrylamide 19:1), HEPES 50 mM, pH 7.2) at 4°C. The gels were then dried and analyzed with a Phosphorimager SP instrument (Molecular Dynamics). Incubation 3 days at 4°C (HEPES 50 mM pH 7.2, KCl 140 mM, sucrose 10%, duplex 40R + 40Y* 10 nM (* = 5' ³²P labeled), 16GT 10 μM). (a) Gel shift: Lane 1: nothing added; lanes 2–5, 7–9, 11–13: **SD27** 5 μM; lanes 6, 10, and 14: **SD27** 50 μM. Competitor added (concentrations are expressed in base triplets or quadruplets according to the competing structure): lanes 3–6 24G20 (2.1, 21, 210, 210 μM); lanes 7–10 TC triplex (4.9, 48.9, 489, 489 μM); lanes 11–14 GA triplex (3.9, 39.3, 393, 393 μM). Electrophoresis at 4°C in a 12% non-denaturing polyacrylamide gel (50 mM HEPES pH 7.2). (b) Quantification of the triplex signal detected in the gel-shift assay for various competitors.

12.6

Conclusion and Further Developments

Equilibrium dialysis is a useful method for the analysis of the structural selectivity of nucleic acids ligands. The data presented here, as well as previous observations, confirm that a significant overlap exists between triplex and quadruplex recognition [6, 19, 55, 56]. Within a given family of molecules, the detailed positioning of key substituents modulates triplex versus quadruplex preference. However, it is relatively difficult to completely prevent binding to one form without losing affinity for the other multistranded structure. A number of small ligands have been discovered to inhibit the function of telomerase by stabilizing G4-DNA structures. Evidence for the recognition of G4-DNA may also be provided by absorbance and fluorescence spectroscopy [23, 24, 34]. These ligands lock a telomeric G-overhang into a folded conformation which cannot be extended by telomerase. Once G4 stabilization and induction had been established, it is interesting to test whether these molecules inhibit telomerase as measured by a modified TRAP assay [23, 54]. Most G-quadruplex ligands described in this chapter inhibit telomerase with a sub-micromolar IC_{50} and may therefore be considered as potent inhibitors [23, 24, 34, 40].

Even more important is the compared affinity with duplexes [50]. Although some evidence has been presented to demonstrate that some drugs prefer to bind tetraplexes rather than duplexes, an exquisite specificity would be required to target this particular structure in the presence of a massive excess of double-stranded DNA [57]. Such preference is difficult to obtain as only a 2- to 10-fold preference for a tetraplex over a duplex has been demonstrated [58]. One trisubstituted acridine has a better specificity (40-fold) [59]. Most quadruplex ligands are polyaromatic molecules bearing one or more positive charge (see Fig. 12.5). A notable exception to that rule is NMM [60], an anionic porphyrin, which binds rather exclusively to quadruplexes. Equilibrium dialysis assay leads to the conclusion that this derivative, perhaps thanks to its negative charge and despite a relatively low affinity, is the most selective quadruplex ligand studied so far [26]. The race for a high-affinity highly specific G-quadruplex ligand has not ended yet!

12.7

Summary

Specific recognition of a given DNA conformation by small molecules may be demonstrated by equilibrium dialysis using a specific set of nucleic acid structures. We have designed a set of 18 different nucleic acid structures, ranging from single strands to G- or C-quadruplexes, and analyzed the binding of several ligands. Some of the molecules that bind to preformed G-quadruplexes were also shown to induce G-quadruplexes in a gel-shift assay. These families of derivatives were shown to inhibit telomerase *in vitro*.

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13

Design and Analysis of G4 Recognition Compounds

Shozeb Haider, Gary N. Parkinson, Martin A. Read, and Stephen Neidle

13.1

Introduction

Guanine is uniquely rich in its pattern of hydrogen-bonding donors and acceptors, on both the Watson–Crick and Hoogsteen faces (Fig. 13.1). This enables guanine to readily self-associate to form stable structures – it is a common experience that guanine-rich oligonucleotides all too readily form gels and aqueous-insoluble material as a result of such interactions. Guanine-rich sequences in general can form a variety of multistranded structures, termed guanine- or G-quadruplexes. Four guanines can associate together via Hoogsteen hydrogen bonding in a coplanar cyclic array to form a G-quartet (also sometimes termed a G-tetrad). Eight hydrogen bonds stabilize a quartet (Fig. 13.2). G-quartets can stack on top of another to make four-stranded G-quadruplex (G4) DNA structures. These can be formed by the folding of a single contiguous guanine-rich sequence (intramolecular) or by the association of two or four separate strands (intermolecular). All G4 structures comprise two distinct categories of feature, namely a set of base G-quartets stacked together, and unpaired bases. All quadruplexes formed from two or fewer strands have these unpaired, thymine-rich strands in the form of loops. If two connecting strands are antiparallel, then the loop between them is positioned above the plane of stacked G-quartets. On the other hand, if the two strands are parallel, then the connecting loop is necessarily positioned within the groove formed between two sugar–phosphate backbones of the guanines.

The overall topologies available to G4 structures were until recently considered to be restricted to two categories: all-parallel for intermolecular G4s with all four strands from separate molecules, and combinations of antiparallel/parallel for hairpin dimers and intramolecular G4s. Some of these possibilities are shown in Fig. 13.3. The determination of crystal structures for two quadruplexes formed from repeats of the human telomeric sequence d(TTAGGG) has revealed an extra layer of complexity, which is described below in detail. In brief, both structures, one of an intermolecular dimer formed from two repeats, and the other of an intramolecular quadruplex from four repeats, show all-parallel arrangements of

Hoogsteen face

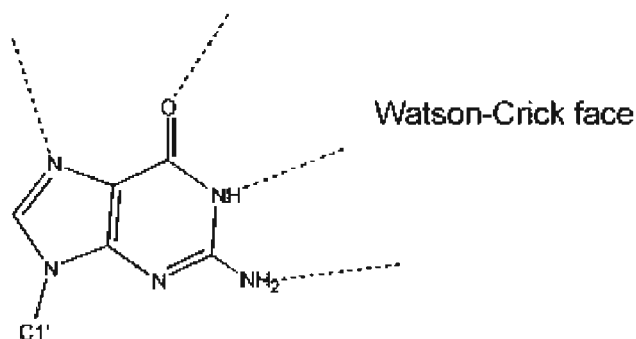


Fig. 13.1. The two hydrogen-bonding faces of a guanine base.

strand directions. There is insufficient structural data at present to be able to define the rules governing quadruplex folding in general, but the limited current data set of crystal and NMR structures suggests that the major factors in defining strand polarity and loop formation are likely to be:

- the nature of the sequence(s) intervening between the guanine repeats themselves,

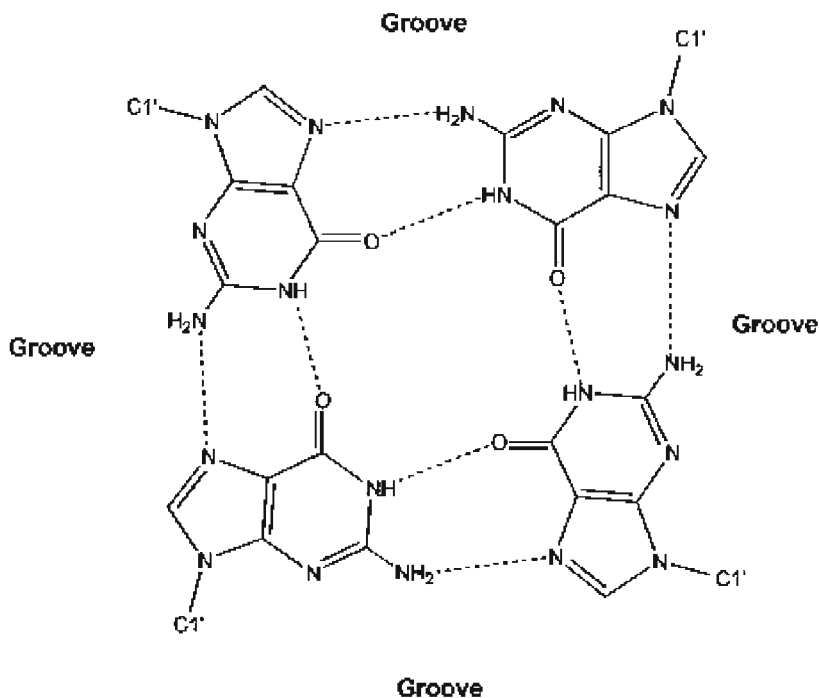


Fig. 13.2. The hydrogen-bonding arrangement in a G-quartet.

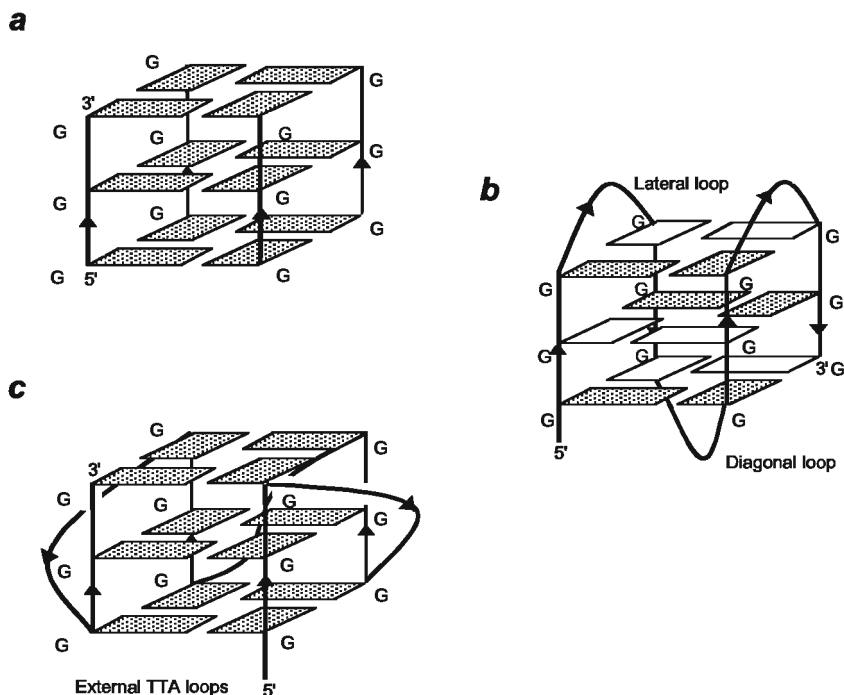


Fig. 13.3. Schematics of various quadruplex connectivities. Shaded guanine bases are in the *anti* conformation, and unshaded ones are in the *syn* conformation. (a) An intermolecular quadruplex formed from four separate strands. (b) The human intramolecular quadruplex, as

determined by NMR methods [63], with alternation of *syn* and *anti* conformations and strands being parallel/antiparallel. (c) The parallel strand orientations in the crystal structure of the human 12-mer dimer quadruplex [55].

- the nature of the cation involved,
- the length of the guanine repeat itself, and
- the guanosine glycosidic angle.

The G-quadruplex family of structural motifs are of considerable current interest because of their potential occurrence throughout the genomes of many organisms (guanine-rich DNA sequences can be found dispersed throughout their genomes). A number of biological functions have been attributed to them. They can potentially form, not only at the telomeric sequences of eukaryotic chromosomes [1, 2], but in the regulatory regions of a number of genes including the switch recombination that immunoglobulin heavy chains undergo during differentiation of B lymphocytes [3], at the promoter regions of oncogenes such as *c-myc* [4, 5], in retinoblastoma susceptibility genes [6], in the regulation of the insulin gene [7] and have also been implicated in the dimerization [8] and inhibition of the human immunodeficiency virus (HIV) RNA genome [9]. The functional relevance of

quadruplex formation at these sites has been supported by the isolation of proteins that bind and promote the formation of quadruplex structures [10–15], such as transcription factors [16], nucleases [17–20], topoisomerase I [21], resolvases [22], and helicases [23, 24]. Furthermore, the fact that appropriate sequences can inter-convert to intermolecular G-quadruplex DNA at about 100–150 mM cation concentrations, suggests a plausible way for their formation inside cells [25, 26]. However, only very recently has unambiguous evidence for their existence *in vivo* been demonstrated, using monoclonal antibodies [27].

13.2
Telomeric DNA

In eukaryotic systems guanine-rich sequences tend to be concentrated at the ends of chromosomes, and are termed telomeric sequences. Their function is to maintain chromosome integrity, avoiding end-to-end fusions and unwanted recombination events, protecting the ends from chromosomal degradations and from being recognized as damaged DNA. Telomeric DNA comprises tandem repeats of simple guanine-rich motifs, such as (TTTGGGG)_n in lower eukaryotics such as the ciliates *Oxytricha* and *Euplotes*, (TTGGGG)_n in *Tetrahymena*, and (TTAGGG)_n in vertebrates (Tab. 13.1). The value of *n* can be large, with the telomeres of human

Tab. 13.1. Telomeric DNA sequences from various species.

Organism	Sequence
Protozoa	
Holotrichous ciliates	
<i>Tetrahymena</i> , <i>Paramecium</i>	T ₂ G ₄
Hypotrichous ciliates	
<i>Oxytricha</i> , <i>Euplotes</i>	T ₄ G ₄
Sporozoites	
<i>Plasmodium</i>	TTAGGG(G/C)
Flagellates	
<i>Trypanosoma</i>	T ₂ AG ₃
Yeast	
<i>Saccharomyces</i>	(TG) _{1–3} TG _{2–3}
<i>Schizosaccharomyces</i>	T _{2–2A} CA _{0–1} C _{0–1} G _{1–6}
Slime molds	
<i>Dictyostelium</i>	AG _{1–8}
<i>Didymium</i>	T ₂ AG ₃
Plants	
<i>Arabidopsis</i> , <i>Triticum</i>	T ₃ AG ₃
Animals	
Insects	
<i>Bombyx</i> , <i>locusta</i>	T ₂ AG ₂
Vertebrates	
<i>Ictalurus</i> , <i>homo sapiens</i>	T ₂ AG ₃

somatic cells being typically 6–10 kb in length. The G-rich strand has a 5' to 3' orientation and the 3' end of this G-rich strand protrudes beyond the C-rich strand in a single-strand overhang, in contrast to the remainder of telomeric DNA, which is double stranded [28–30]. The length of this overhang is species-dependent, and is especially long in humans, being typically 100–200 bases. Recent electron microscopic studies have demonstrated that the TRF2 telomeric binding protein can cause telomeric DNA to fold back, forming a large duplex loop (the t-loop), at least *in vitro*. The loop model has the extreme single-strand overhang inserted into a duplex region via a junction type of arrangement [31].

The cellular replicative machinery cannot copy the extreme ends of chromosomes due to the basic asymmetry of semiconservative DNA replication, the so-called “end replication problem.” Successive rounds of cell divisions in normal somatic cells thus lead to the progressive erosion of telomeric repeats, leading to critically short telomere lengths, when cells enter the senescent state of irreversible growth arrest, and finally succumb to cellular apoptosis. The maintenance of telomere length and the single-strand overhang is thus essential for chromosomal integrity and cellular survival. Loss of this results in end-to-end fusion events and erosion that causes genomic instability and cellular senescence [32]. Hence telomeres have a central role in the protection of chromosome ends [33]. They have also been implicated in the formation of nuclear domains that may be important for transcriptional regulation, in sister chromatid pairing at mitosis and in homologous meiotic synapses [34]. DNA damage-surveillance proteins sense DNA breaks and invoke nucleases that then degrade DNA or are involved in end-to-end fusion. Thus telomere shortening can be thought of as a molecular clock that signals entry into cellular senescence, when cells cease to divide and may be directed to cell death [35, 36]. This mechanism has been related to aging and cellular immortality, since the telomeres of immortalized cells do not shorten upon division.

Different cells have evolved different mechanisms to counteract the progressive loss of telomeres, including recombination [37–39] and retrotransposition [40, 41]. However in higher eukaryotes, telomere length is maintained by the action of a specialized RNA-dependent DNA polymerase complex, sometimes termed telomere terminal transferase or more commonly telomerase [42–45] which also plays a key role in tumorigenesis [46]. It is present in 80–85% of tumor cells, and essentially absent in normal somatic cells. These observations have generated much interest in the potential of the telomere and telomerase as anticancer targets and more generally on the role of telomeres and their protein complexes and the maintenance of chromosome integrity.

An important link between telomeres and possible higher order structure was shown by the finding that the enzymatic activity of telomerase is directly inhibited by the formation of G-quadruplex structures stabilized by K^+ ions [47]. This has also been developed into the hypothesis that stabilization of such higher ordered telomeric structures by small molecules can be used to inhibit the enzymatic activity of telomerase [48], and hence activate the pathway to telomere shortening and senescence in tumor cells.

There are a number of approaches available for inhibition of telomere main-

tenance in tumors, and consequently, activation of the telomere-shortening pathway. Inhibiting telomerase, including targeting the active site and the template of its RNA domain, has been the subject of a large number of studies. In therapeutic terms these are all broadly equivalent and will only be effective in telomerase-positive cells and may suffer from acquired telomerase resistance. By contrast the approach of targeting the telomere substrate is more general and is likely to affect telomerase-negative cells where telomere maintenance is achieved by the ALT recombination pathway. The focus of this chapter is telomere targeting, and specifically the induction or stabilization of G-quadruplex structures at the 3' end of telomeres, using small molecules to achieve this. Such molecules can be discovered either by serendipidity, by screening of combinatorial libraries, or by rational structure-aided molecular design. The last is the approach that is described in detail here. We first discuss the database for design, the small number of G-quadruplex structures solved by X-ray crystallography. NMR methods have also been used to study quadruplex structure. In general these are to significantly lower effective resolution, necessitated by the relatively small number of NOE signals available in a nucleic acid backbone.

13.3

Crystal Structures of G-quadruplexes

13.3.1

The d(TG₄T) Quadruplex

The crystal structure of d(TG₄T) was solved at 1.2 Å resolution [49] and refined to the exceptionally high resolution of 0.95 Å [50]. This is a parallel-stranded quadruplex (Fig. 13.4), formed in the presence of Na⁺ ions. There are two pairs of quadruplexes in the asymmetric unit, which are co-axially stacked in a 5' to 5' orientation. The terminal 5' G-tetrad of one quadruplex is stacked head to head over the terminal 5' G-tetrad of the other, thus generating a ladder of eight stacked tetrads. The thymine bases contribute only to the stabilization of the crystal lattice and not to the conformation of the four-stranded structure.

Seven Na⁺ ions stabilize the structure and can be seen along the central cavity formed by the quartets. The sodium ions exhibit bipyrimidal coordination geometry, each forming eight equidistant bonds with the carbonyl oxygen atom of guanine in the quartets. Nine Ca²⁺ ions are also present and help to stabilize the contacts between the adjacent quadruplexes. The Ca²⁺ ions adopt hepta-coordinate hydration shells in the structure.

A pattern of hydration is clearly visible in the grooves and around the phosphate groups. The average strand separation of phosphates is similar to that of B-form DNA. However no water molecules bridge adjacent phosphate groups, as observed in A- and Z-form DNA [51]. The 4-fold symmetry of each quadruplex gives rise to four relatively narrow grooves that are equivalent with respect to the hydration structure. The preferred hydrated atoms in the grooves are the exposed N2 amino

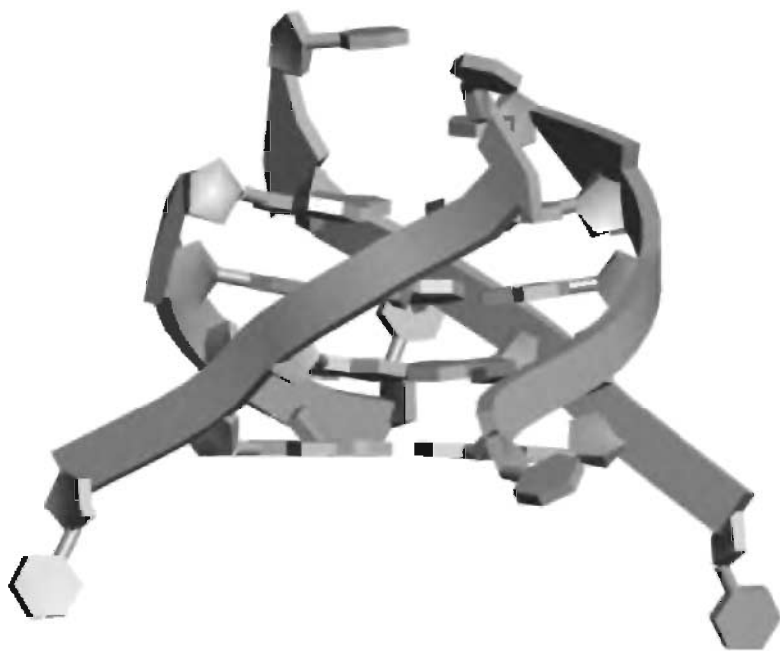


Fig. 13.4. View of one quadruplex in the crystal structure of the four-stranded quadruplex formed from d(TGGGGT) [50].

group and the ring N3 atom. This is similar to the pattern of hydration observed for the guanines in Z-form DNA, in which the water molecules have a preference for N2 over N3 relative to A- and B-form DNA, where N3 is preferred over N2 [52].

The structure exhibits a relatively underwound helical trajectory. It has 12 bases per turn as compared to 11 bases per turn in A-form DNA and 10.5 bases per turn in B-form DNA. This twist is thought to help in guanine base stacking. The guanine bases in the quadruplex are all in the *anti* conformation.

13.3.2

The Na⁺ form of d(G₄T₄G₄) *Oxytricha nova* telomeric DNA

This crystal structure was solved inadvertently during the course of a study of how the single-stranded DNA d(G₄T₄G₄) binds to the *Oxytricha nova* telomere end-binding protein. On increasing the resolution they were able to solve the crystal structure of the Na⁺ form of d(G₄T₄G₄) at 1.86 Å resolution [53], bound to the protein, at a site that is distinct from the single-stranded DNA-binding site.

The quadruplex is formed by the association of two strands of sequence d(G₄T₄G₄) containing four sets of guanine quartets stacked on top of one another. The d(TTTT) sequence diagonally crosses over the quartet at either ends creating two loops. The diagonal-loop topology results in a combination of parallel and antiparallel strands. As a result of this, the grooves vary in width, one being wide,

two medium, and one narrow. Both wide and narrow grooves have antiparallel strands, while the medium grooves have parallel strands. The mixed strand polarities and the diagonal topology also results in alternating *syn/anti* conformation about the *N*-glycosyl bond of the guanines. The d(TTTT) loop is present at both ends of the quadruplex. Thymine 5, 6, and 8 stack together on guanine 4 while thymine 7 flips out, stacking on top of the first guanine of the second strand. It is the loops that are involved at the protein–DNA interface.

Four sodium ions were clearly seen along the central channel of the system. The sodium ions have been shown to occupy three types of sites: (a) sandwiched between the two G-quartets, (b) coplanar with the G-quartet, and (c) intermediate, less symmetric positions. The sodium ions are thought to be less constrained by steric clashes and thus can occupy multiple positions that reduce electrostatic repulsions between adjacent ions. The central ions are coplanar with the quartets and exhibit distorted octahedral coordination geometry while the outer ones are positioned above and below the planes of the quartets.

There is a distinct order in hydration pattern in the grooves with preference for involvement of N2 atoms. These form stable water bridges with the O4' and phosphate oxygen atoms through multiple contacts. The methyl groups of the thymine bases are also involved in water-mediated contact with the protein.

13.3.3

The K⁺ form of d(C₄T₄C₄) *Oxytricha nova* Telomeric DNA

The K⁺ form of the d(C₄T₄C₄) quadruplex has been solved in two space groups, one at 1.6 Å resolution [54]. The structures adopt an almost identical fold (Fig. 13.5) in both space groups, with the two strands forming an antiparallel/parallel diagonal arrangement. Moreover, both quadruplexes are closely similar to that in the crystal structure grown in the presence of Na⁺ ions and *Oxytricha* protein (see above). These observations lead to the conclusion that the nature of the counterion does not affect the overall fold of this quadruplex. The structure forms a bimolecular quadruplex with the thymine tracts contributing to the diagonal loops. This diagonal topology results in the formation of one wide, two medium, and one narrow groove. The glycosidic torsions for the guanine residues are alternating *syn-anti* along each strand and *syn-syn-anti-anti* within each quartet.

The thymine tracts form the diagonal loops of the structures. Thymine 4, 5, and 8 show base stacking while thymine 7 flips and stacks on the guanine from the other strand. Distinct thymine–thymine interactions are present between the loops of quadruplexes in the orthorhombic crystal form. This pattern of interaction is identical to that occurring in the crystal lattice between symmetry-related molecules and the loops at either ends of the quadruplex structures, but is distinct from that observed in the Na⁺ form.

Three K⁺ ions lie between the planes of the G-quartets while one each is present in the thymine loops at either end. The ions in the loops play an important role in the stabilization of the loop conformation. The coordination around the K⁺ ions exhibits square antiprismatic geometry. It has been suggested that the primary



Fig. 13.5. View of the tetragonal form of the K^+ -containing quadruplex formed by two strands of $d(GGGGTTTTGGGG)$ [54], as found in the crystal structure.

function of the highly electronegative potassium channel might be to ensure the rapid influx of K^+ ions and ejection of Na^+ ions, thereby ensuring maximum structural stability.

All the grooves are hydrated and there is no set pattern of hydration observed in any groove of either of the quadruplexes. Water molecules tend to cluster around phosphate residues joining one group to another. There is some preference for hydration around the N2 exocyclic amino groups and the O4' sugar atoms.

13.3.4

The Crystal Structure of the Human Telomere G-quadruplex

The human telomeric sequence $d(TAG_3T_2AG_3T)$ comprises two telomeric repeats. Its crystal has been solved to 2.4 Å resolution [55], and shows two DNA strands forming a dimeric intermolecular G-quadruplex in which all four strands are parallel to each other, thereby generating a topology in which the $d(TTA)$ loops protrude from the sides of the G-quadruplex. Strikingly, the crystal structure of the sequence $d[AGGG(T_2AG_3)_3]$, ie almost four repeats, shows an intramolecular quadruplex with the same parallel polarity of the strands. The loops are diagonal and located externally to the G-quartets. These loops each link the top to the bottom face of the G-quartets. The intermolecular 12-mer has two loops opposite to each other whereas the intramolecular 22-mer generates three loops, thus giving



Fig. 13.6. View of the intramolecular quadruplex formed in the presence of K^+ by the sequence $d[AGGG(TTAGGG)_3]$, as found in the crystal structure [55].

the structure a propeller-like appearance (Fig. 13.6). The adenine in each loop swings back such that it intercalates between the two thymines, generating a loop with the apparent sequence $d(TAT)$. Thus the loop generates three different surfaces: (a) a highly charged backbone (b) an aromatic planar surface, and (c) a TAT hydrogen-bonding interface. The TAT loop interface also resembles that of A-form RNA of sequence $r(AUA)$. The parallel strandedness of the structure results in an arrangement in which the glycosidic angles of guanines all adopt the *anti* conformation. The guanines are stacked into three tetrads with 12 guanine bases per turn. The overall structure is 41 Å wide and 6.3 Å high.

Monovalent K^+ ions are positioned between the planes of the quartets and exhibit a bipyrimidal antiprismatic arrangement, as observed in the K^+ form of the *Oxytricha nova* quadruplex crystal structure. There is a single spine of hydration,

present in all four grooves. The preferred atoms for interaction with solvent are O3', N2, and N3. However, they are not observed to bridge adjacent phosphate residues. The clustered networking of solvent atoms is much more complex than the simpler spine of hydration observed in the quadruplex structures from protozoan ciliates.

13.3.5

The r(UG₄U) RNA Quadruplex

Roles for G-quadruplexes have been identified in RNA, such as in the dimerization of retroviral RNA [8], in translational repression, and in mRNA turnover [20]. They have also been observed in *E. coli* 5S RNA, in the presence of K⁺ ions [56]. The crystal structure of the RNA quadruplex r(UG₄U) was solved at 0.61 Å resolution, which is one of the highest for any nucleic acid [57]. There are four RNA strands in the asymmetric unit, each forming a right-handed parallel structure. There are 12.5 bases per turn. The quadruplex stacks in opposite polarity on top of one another (5' to 5'), forming a pseudo-continuous column. All the nucleosides are in the *anti* glycosidic conformation and exhibit mixed sugar puckering. Sr²⁺, Na⁺, Ca²⁺ ions, and spermines have been observed in the structure. Sr²⁺ and Na⁺ are placed within the centre of the quadruplex. The bipyrimidal-distorted-antiprism geometry exhibited by divalent Sr²⁺ is analogous to that observed for monovalent cations in DNA quadruplexes. Each ion is located between every alternating quartet plane and is coordinated to the O6 of the guanines within the central channel. Thus each guanine O6 is coordinated to a single Sr²⁺ ion. This association of divalent cations sandwiched between every other plane of G-quartets is a unique feature of this RNA quadruplex.

The most striking features of the structure are the octads formed by the combination of uridines and guanines residues. The 5'-terminal uridine rotates around the backbone of 5'-terminal guanine to hang in the groove of the neighboring quadruplex. This hydrogen bonds to the second guanine and to phosphate groups. This arrangement leads to two octads sandwiching two G-quartets. The 3' uridines are stacked inward to form the uridine tetrads. The stacking arrangement of the guanines is similar to that in the structure of the parallel-stranded G-quadruplex d(TG₄T).

13.4

NMR Studies of Quadruplexes

This section is confined to those studies that relate to the crystallographic studies outlined above; others have been reviewed elsewhere.

The analysis of the four-stranded G-quadruplex d(TG₄T) in solution [58, 59] has found a parallel-stranded arrangement that is closely similar to that in the crystal structure. The *Oxytricha* sequence d(GGGGTTTTGGGG) has excited much controversy since the NMR studies [60, 61] in the presence of either Na⁺ or K⁺ ions



Fig. 13.7. View of the intramolecular quadruplex formed in the presence of Na^+ by the sequence $\text{d[AGGG(TTAGGG)}_3\text{]}$, as found in the NMR structure [63].

have indicated that the topology is distinct from that reported in an X-ray study in the presence of K^+ ions [62]. It was initially suggested that this discrepancy represented real differences between the folding in the solid versus solution states. However the more recent crystallographic analysis described above [54] has shown that the earlier analysis was incorrect and that there is no discrepancy between the X-ray and NMR analyses in terms of the fold topology. Some differences are apparent between them for the T_4 loop geometry, indicating its flexible nature.

The intramolecular sequence $\text{d[AGGG(TTAGGG)}_3\text{]}$, on the other hand, forms a quite distinct antiparallel/parallel fold structure (Fig. 13.7) in solution in the presence of Na^+ ions [63], compared with the parallel quadruplex observed (Fig. 13.6) in the K^+ crystal structure [55]. This sequence is present at the single-stranded extreme 3' end of human telomeres, so the definition of a particular structure is of considerable importance when examining ligand interactions with this sequence.

13.5

Quadruplex-binding Ligands

An early study of ethidium showing binding to guanine-rich oligonucleotides [64] suggested that intercalative-type small molecules possessing planar aromatic chromophores could interact with quadruplex structures, although no detailed analysis was made at this time. A subsequent finding that suitable disubstituted amino-

alkylamido anthraquinones (AQs) could preferably interact with higher order DNA structures such as triple-helical ones, led to the extension of this concept to quadruplex DNAs, when it was shown that 2,6-disubstituted AQs could bind to them, and also inhibit the ability of telomerase to extend a telomere primer sequence [48]. This was the first demonstration of telomerase inhibition by a non-nucleoside agent. The proposal was made that the ligand stabilizes this sequence into a quadruplex complex, which inhibits the primer from interacting as a single-stranded species with the RNA template of telomerase. This remains the prevailing hypothesis for AQs and other ligands that have been subsequently designed or discovered.

A wide range of such ligands have been reported, and a number of structure–activity relationships derived. The majority of these ligands possess the common feature of extended electron-deficient planar aromatic chromophores, together with side chains having terminal cationic groups. They include:

- disubstituted aminoalkylaminoamido anthraquinone derivatives [65, 66], fluoronones [67], and acridines [68]
- *N*-methyl tetrapyrrolyl porphyrin derivatives [69–71]
- disubstituted aminoalkyl perylene derivatives [72, 73]
- bisamidinium-substituted ethidium derivatives [74]
- disubstituted aminoalkyl dibenzophenanthroline derivatives [75]
- an acridine dimer [76]
- derivatives of several tetracyclic chromophores such as benzo[*b*]naphtha[2,3-*d*]furan, 10H-indolo[3,2-*b*]quinoline and benzo[6,7]indo[3,2-*c*]quinolines [77]
- simple derivatives of the pentacyclic chromophore 8H-quino[4,3,2-*k*]acridinium [78], which carries a permanent positive charge on the chromophore
- the natural product telomestatin, comprising a cyclic arrangement of eight heteroaromatic five-membered rings [79, 80]
- substituted fluoroquinophenoxazine derivatives [81]
- carbocyanine dyes [82]
- substituted triazine derivatives [83].

Telomerase inhibitory activity has been reported to be between <10 nM and 50 μ M (quoted as the concentration for 50% inhibition of telomerase activity), although it is not possible to directly compare different series of compounds since experimental conditions for the TRAP assay vary from one laboratory to another. Similarly estimates of quadruplex affinity cannot readily be compared, with there being many differences in experimental conditions (notably potassium ion concentration and the precise nature of the quadruplex sequence and type).

13.6

NMR and Modeling Studies of Quadruplex–Ligand Complexes

The initial study of a 2,6-disubstituted AQ binding to the 7-mer parallel quadruplex formed by d(TTAGGGT) showed upfield shifts of guanine imino protons, sugges-

tive of intercalative-type binding [48]. A key finding in this study was that a minimum of four telomeric DNA repeats is synthesized by telomerase even when there is total subsequent inhibition by an AQ compound, strongly suggestive of the presence of a stable intermediate (i.e. the four-repeat intramolecular quadruplex). A subsequent molecular-modeling study on a series of AQ regioisomers [66] utilized the NMR structure (PDB ID 143D) for the Na^+ -containing human 22-mer quadruplex (of sequence d[AGGG(TTAGGG)₃] as a starting point, and defined a plausible binding site to be between the diagonal T₂A loop and the G-quartet stack. This assumption of a single high-affinity binding site was consistent with the 1:1 stoichiometry found by isothermal titration calorimetry, and strongly suggested that binding of AQ and other similar ligands occurs at the end of the G-quartet stack in a quadruplex. Intercalation between individual G-quartets is thermodynamically and kinetically disfavored.

The model has also been successfully used in the definition of structure–activity relationships for series of disubstituted AQs and acridines [84]. These ligands are predicted to bind so that the planar electron-deficient chromophore surface lies across the G-quartet surface, with π - π interactions playing a significant role in the overall stabilization. The substituents each lie in a groove of the quadruplex, with the terminal cationic charges involved in electrostatic interactions with phosphate backbones.

An analogous model of quasi-external stacking was suggested for the porphyrin TMPy4 [71], and has been supported by a more recent detailed gel mobility and molecular dynamics study [69], the latter also focusing on the 22-mer parallel/anti-parallel NMR quadruplex structure. NMR 1D and 2D spectra of ligand–quadruplex complexes have not always been amenable to analysis, in part because of persistent line-broadening effects. An NMR study of the perylene-based compound PIPER [72] with the sequences d(TTAGGG)₄ and d(TAGGGTTA)₄, has shown that this telomerase inhibitor also binds at the ends of the G-stacks, threading between them and the hydrogen-bonded AT base pairs. The sequence d(TTAGGG)₄ forms a 2:1 complex with a PIPER molecule, which is sandwiched between the two intermolecular quadruplexes formed by it. This type of model for PIPER, with external binding to the G-quartets, is also supported by chemical cleavage experiments with a perylene–ETDA– Fe^{2+} complex [85], and by an NMR study (although this was hindered by the common problem of line-broadening, precluding a detailed analysis).

Greater detail has been obtained in an NMR study of a complex between the pentacyclic dibenzoacridine derivative RHSP4 with the intermolecular quadruplex formed by four strands of d(TTAGGGT)₄. Again, this shows chromophore stacking onto the end of the stack of three G-quartets in this quadruplex [86].

The general quadruplex-binding model that emerges from these studies emphasizes the planar electron-deficient chromophores common to all the ligands, which impart much of the overall binding energy, by virtue of their π - π interactions with the two categories of surface, the G-quartet, and the base/base pairs of the T/A regions. A substituent would lie in one of the four grooves, each bounded by sugar–phosphate backbones.

The Na^+ NMR structure of the human intramolecular quadruplex has also been used in a structure-based design study [87] to successfully develop second-generation acridine derivatives that have a third substituent (at the 9-position of the acridine ring). It was predicted that this substituent, an anilino group, would be capable of binding in a third groove of the quadruplex, and thereby have enhanced quadruplex affinity, selectivity, and telomerase potency. These predicted properties have been borne out by experiment, and the lead compounds have also shown antitumor activity in animal models [88].

The finding that the K^+ quadruplexes formed by the d(TTAGGG) repeats of the human telomere have all strands parallel with diagonal loops has significant implications for ligand interactions. The NMR studies outlined above are all consistent with this type of structure, and indeed it is notable that modeling studies that have attempted to fit the larger ligands into the relatively restricted site in the Na^+ intramolecular quadruplex produce considerable site distortions. Although no X-ray or detailed NMR analyses are yet available for the parallel intramolecular quadruplex with bound ligand, a plausible model is one in which the ligand molecule is sandwiched between two 22-mer quadruplexes. This model emphasizes, even more than that with the Na^+ quadruplex, stacking interactions with a ligand chromophore held between two G-quartet stacks. The K^+ intramolecular quadruplex has a complex pattern of grooves, since the TTA loops are held within them. These factors present significant future opportunities for the design of quadruplex-binding ligands with better affinity and specificity than existing ones.

Appendix. Methodology for Ligand Quadruplex Modeling

Quantitative molecular modeling can be performed in order to visualize and describe in energetic terms how small molecules interact with and stabilize G-quadruplex structures. The appropriate target structure may be taken from a database (either from a crystal or a NMR structure analysis) and provides initial coordinates for the simulations. An appropriate binding site for the ligand can be chosen depending on the model being used in the study, for example within a loop in the human intramolecular quadruplex NMR structure (Figs 13.9 and 13.10a) or externally binding in the human intramolecular quadruplex crystal structure (Figs 13.8 and 13.10b).

A typical procedure involves initial starting coordinates being first subjected to molecular mechanics energy minimization (1000 steps steepest descent with line searching and 3000 steps Polak-Ribiere conjugate gradient minimization with a derivative convergence of $0.05 \text{ kJ } \text{\AA}^{-1} \text{ mol}^{-1}$), followed by molecular dynamics (1.5 fs time-step, 40 ps at 300K equilibrium). A subsequent final molecular mechanics minimization of the time-averaged dynamics structure is employed to obtain a low-energy final model.

Since the solution and the crystal structure of the intramolecular G-quadruplex formed by four repeats of the human sequence are different, there are at least two plausible ways by which a ligand can bind to these systems. If there is no loop

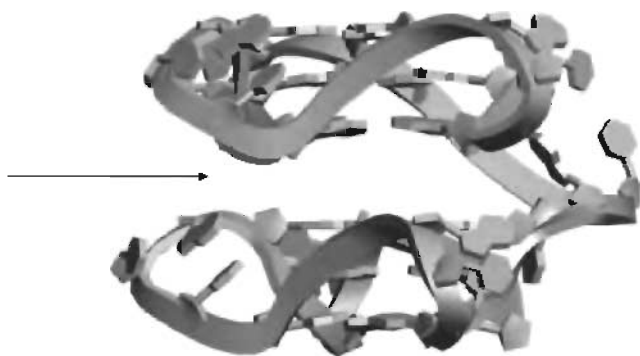


Fig. 13.8. Quadruplex dimer derived from two quadruplex of the human 22-mer crystal structure, indicating the plausible site for ligand binding between them.

present or if the loops are positioned laterally, then the molecule can sit externally on the quartets, as in the case of the human telomere crystal structures (Fig. 13.8). The other alternative is that the ligand intercalates between the diagonal loop and the quartets, which is the most plausible model for ligand binding to the Na^+ solution structure of the human 22-mer intramolecular quadruplex, or to any quadruplex with a loop positioned above the G-stack (Fig. 13.9).

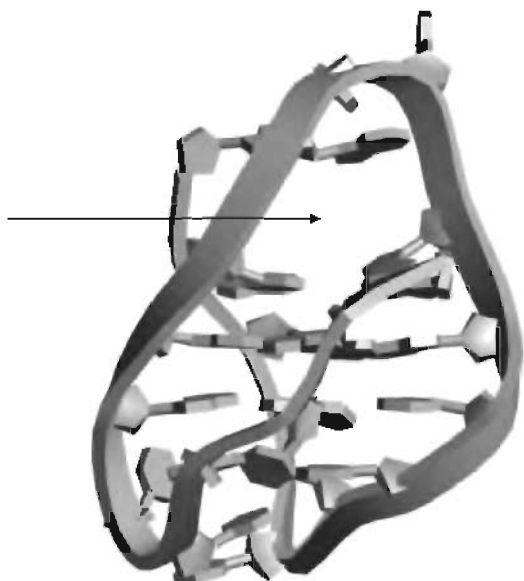


Fig. 13.9. Quadruplex derived from the human 22-mer Na^+ NMR structure, indicating a plausible site for ligand binding, between a G-quartet and a TTA loop.

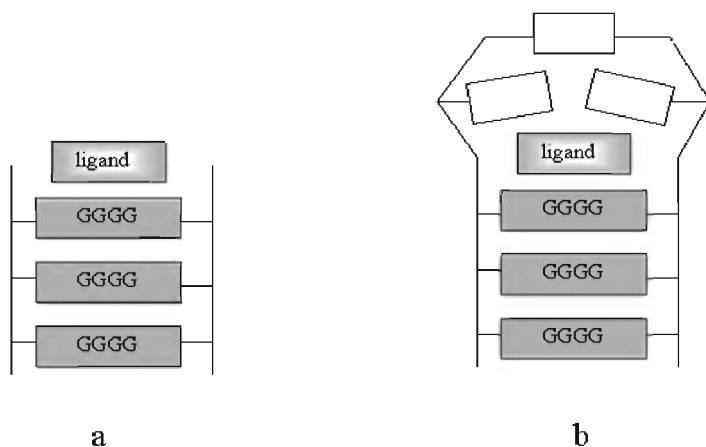


Fig. 13.10. Schematic diagrams illustrating ligand binding (a) at the end of a stack of G-quartets, and (b) between the G-quartet end and an antiparallel loop.

If the initial starting model has the latter type of diagonal loop-over structure, then an intercalation site for the ligand needs to be constructed. In the case of the human Na^+ solution structure, the optimal site is between the diagonal T_2A loop and the G-quartet segment of the structure. It can be formed computationally by breaking the relevant sugar–phosphate backbones and increasing the appropriate base separation from 3.4 Å to 6.8 Å. The sugar–phosphate backbones are then re-connected and molecular mechanics energy minimization is employed to relieve any steric clashes while positionally restraining the G-quartets and loop motifs.

Molecular models of the ligands themselves may be constructed, minimized, and partial charges calculated semi-empirically using the MOPAC package. The ligands can then be docked into the intercalation/binding site, for example with the AFFINITY and DOCKING modules within the InsightII suite of programs. A particular feature of these modules is that they explore molecular orientations while interactively monitoring changes in electrostatic and van der Waals ligand–DNA interactions. The CFF force field within the package is adequate for these molecular mechanics minimizations, typically using 1000 steps. Many different positions in three-dimensional space are evaluated in this procedure and a selection of low-energy ones is made on the basis of the maximization of π -electron overlap between the G-quartet, chromophore and the nucleobases that sit over the ligand. A typical chromophore may be positioned directly over the center of the adjacent G-quartet while its substituent side chains protrude out through to the sugar–phosphate backbone.

The complete molecular system can then be transferred to the AMBER suite for further simulations. The force-field parameters for the ligand can be extrapolated from the existing values for analogous groups in the AMBER and CFF force fields; the latest version of AMBER (version 7) does have a general force field that is applicable to the majority of organic molecules.

Meaningful molecular dynamics simulations are best performed in simulated solvent. The DNA–ligand complex structure at this point can be “solvated” in a periodic TIP3 water box that extends at least 10 Å from any solute atom. A box of dimensions $\sim 50 \times 50 \times 50$ Å is suitable for a typical quadruplex complex. High-resolution X-ray studies have revealed a vertical alignment of consecutive cationic charged ions along the helical axis. These monovalent ions are located between the guanine residues of each G-quartet, forming a central charged channel running through the entire quadruplex. Thus internal cations can be positioned in the central channel of the modelled quadruplex, between quartets and using crystallographically determined distances. Additional positively charged counterions are included in the system to neutralise the charge on the DNA backbone. They are usually Na^+ or K^+ depending on the system. The counterions are automatically positioned by the LEAP program within AMBER, throughout the cell at grid points of negative coulombic potential. The final system is such that the overall net charge is zero.

The resulting complex is then equilibrated with the explicit solvent molecules by 1000 steps of minimization and 25 ps of molecular dynamics at 300K, by keeping the complex itself constrained, while allowing the ions and the solvent molecules to equilibrate. The complex is then subjected to a series of minimization and dynamics in which the constraints are gradually relaxed over six successive steps, till such a time that there are no constraints at all on the system. The final production run is carried out without any restraints on the complex, typically for at least 1000 ps. Coordinates are saved after every 10 ps, in order to allow analysis of their trajectories. These calculations are carried out using the SANDER module of AMBER, with the shake algorithm enabled for hydrogen atoms. A 10 Å non-bonded Lennard-Jones cutoff is normally used and the non-bonded pairs list is updated every 20 steps. The particle-Mesh-Ewald summation term is increasingly used for all simulations, since this includes all the long-range electrostatic interactions in the calculations. The PME charge grid spacing is ~ 1.0 Å, and the charge grid is chosen to be products of the powers two, three, and five to ensure efficiency for the fast Fourier transform calculations.

A similar protocol may be used to calculate the interaction energies of the ligand with the quadruplex. The first step is to solvate the ligand in a waterbox using the protocols described above, with charges neutralized. The resulting neutral system is equilibrated in a manner identical to that for the complex. The equilibration steps consist of six rounds of 1000 steps of minimization and 25 ps of dynamics, keeping the ligand constrained while allowing the ions and solvent to equilibrate with it, followed by 1000 ps of unrestrained production run dynamics. Interaction energies can then be calculated. Direct calculations of the contribution of the van der Waals and electrostatic components of the total interaction energy between each ligand and its environment is made for both bound and unbound states. The relative binding energies can then be calculated by subtracting the total interaction energy of the explicitly solvated ligand and its surrounding in a neutral periodic box from an identical system where the ligand is bound to the quadruplex structure. The resulting differences in interaction energy correspond to the relative

binding energy. This method is adapted from the semiempirical method for calculating free energy of binding developed by Åqvist and co-workers.

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14

Triple Helix-specific Ligands

Keith R. Fox and Richard A. J. Darby

The formation of intermolecular DNA triple helices offers the possibility of designing compounds with extensive sequence recognition properties, which may be useful as antigene agents or tools in molecular biology. In these structures a third strand oligonucleotide binds in the DNA major groove, making specific contacts with substituents on the exposed faces of the base pairs. Although triplexes form with exquisite specificity they are often less stable than their duplex counterparts. One means of promoting the formation of these structures is to employ ligands that selectively bind to triplex (not duplex) DNA and which therefore pull the equilibrium towards triplex formation. This chapter outlines the use of triplex-specific binding ligands for promoting the formation of triple helical DNA.

14.1**Introduction**

Compounds which bind to duplex DNA in a sequence-specific fashion have the potential to inhibit the activity of individual genes, and may be useful for treating a variety of diseases including cancer or viral infections. In order to be selective for a unique DNA sequence within the human genome of 3×10^9 base pairs, such agents need to recognize at least 16–17 consecutive bases. A simple method for achieving this level of specificity is the use of oligonucleotides as sequence specific agents, generating intermolecular DNA triplexes at appropriate target sites.

The formation of DNA and RNA triple helices was first demonstrated in 1957 by mixing poly(rU) and poly(rA) in the ratio of 2:1 [1, 2]. This and other triplexes, formed with synthetic polynucleotides, remained an obscure part of DNA chemistry until 1987 when it was realized that a base triplet code might offer a means for designing agents which can bind to specific targets on duplex DNA [3, 4]. Their properties and uses as antigene agents have been the subject of several reviews [5–9]. In these triple helical complexes the third strand lies in the major groove of the DNA duplex, where it makes specific hydrogen bond contacts with substituents on the exposed faces of the Watson–Crick base pairs (Fig. 14.1). Two triplex motifs

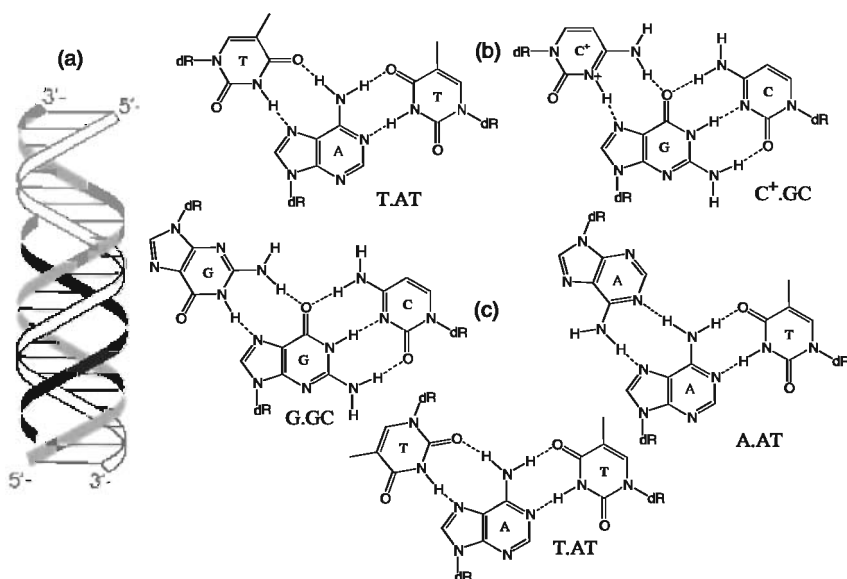


Fig. 14.1. (a) Schematic representation of a DNA triple helix. The third strand is shown in black and lies in the major groove of the DNA duplex for which the strands are in grey and white. (b) Parallel base triplets, T·AT and C⁺·GC. (c) Antiparallel base triplets G·GC, T·AT, and A·AT.

have been characterized which differ in the orientation and base composition of the third strand. Those in which the third strand runs parallel to the duplex purine strand are characterized by T·AT and C⁺·GC triplets (Fig. 14.1b), and are stabilized by the formation of Hoogsteen base pairs [3, 4, 10]. One major restriction of this motif is that the C⁺·GC triplet only forms at low pHs (<6.0), because of the requirement for protonation of the third strand cytosine. In contrast, antiparallel triplexes are characterized by A·AT, T·AT, and G·GC triplets (Fig. 14.1c) and are stabilized by reverse-Hoogsteen base pairs [11–13]. (In the following, the notation X·ZY denotes a triplet in which the third strand base X interacts with a ZY base pair, forming hydrogen bonds to base Z.) Several other triplets have also been described [14], including G·GC [15, 16], G·TA [17], and T·CG [18] for the parallel motif and T·CG [19, 20] for the antiparallel motif, though these are generally less stable.

Although intermolecular triple helix formation offers the possibility of selectively targeting unique DNA sequences, it currently suffers from several limitations. These include the requirement for low pH, necessary for formation of the C⁺·GC triplet; the restriction to homopurine tracts, since the stable triplets contain hydrogen bonds to G and A in the target duplex; and their lower stability than the duplex counterparts. This chapter describes one method for overcoming this limitation, which is caused by the slow rate of triplex formation and the charge repulsion between the three polyanionic DNA strands.

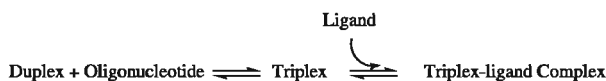


Fig. 14.2. Equilibrium for stabilization of triplexes by triplex-binding ligands.

14.2

Triplex-binding Ligands

One means of stabilizing triple helical nucleic acids is to add compounds which bind selectively to triplex (and not duplex) DNA, thereby drawing the equilibrium in the direction of triplex formation (Fig. 14.2). This can be used to enhance the apparent affinity of a third strand for its target or to promote the formation of unstable complexes. The first ligands to be shown to interact with triplex DNA were well-known duplex intercalators such as ethidium [21]. These are not selective for triplex DNA and in some instances destabilize triple helical structure. These are considered in further detail below (Section 14.4.1). The main part of this chapter concerns compounds which selectively target triplex rather than duplex DNA and the main classes are considered in turn below.

14.2.1

Benzopyridoindole Derivatives

The first triplex-specific ligand to be described was benzo[e]pyridoindole (BePI) (Fig. 14.3a) [22]. It is presumed to bind by an intercalative mechanism since viscometric studies showed that BePI unwinds supercoiled DNA and increases the persistence length of double- and triple-stranded polynucleotides [22–24]. The ligand increases the melting temperature of triplex DNA much more than duplex DNA and it has a greater effect than the addition of polycations such as spermine. On irradiation with UV light BePI induced covalent modification of both strands of the duplex DNA target, which was greatly enhanced by the presence of the third strand oligonucleotide. BePI also strongly enhanced oligonucleotide-directed sequence-specific inhibition of transcription initiation. Each of these observations confirms that BePI binds selectively to triple-helical DNA. Triplex formation is known to be slow and the melting and annealing profiles of intermolecular triplexes often do not overlap, as the system is not at thermodynamic equilibrium. This hysteresis is removed in the presence of BePI, suggesting that the ligand increases the rate of triplex formation, possibly by stabilizing the transient intermediates that contain a few triplets [22].

The selectivity for triplex DNA is thought to arise from the large ring structure, which shows good overlap with the surrounding triplets and which is too large to stack efficiently into duplex DNA. Tricyclic derivatives, lacking the left-most benzene ring, do not stabilize triplex DNA [22]. The ligand is most effective at stabilizing triplexes containing T·AT triplets; its activity decreases with increasing C⁺·GC content and it requires stretches of adjacent T·AT triplets. This selectivity is

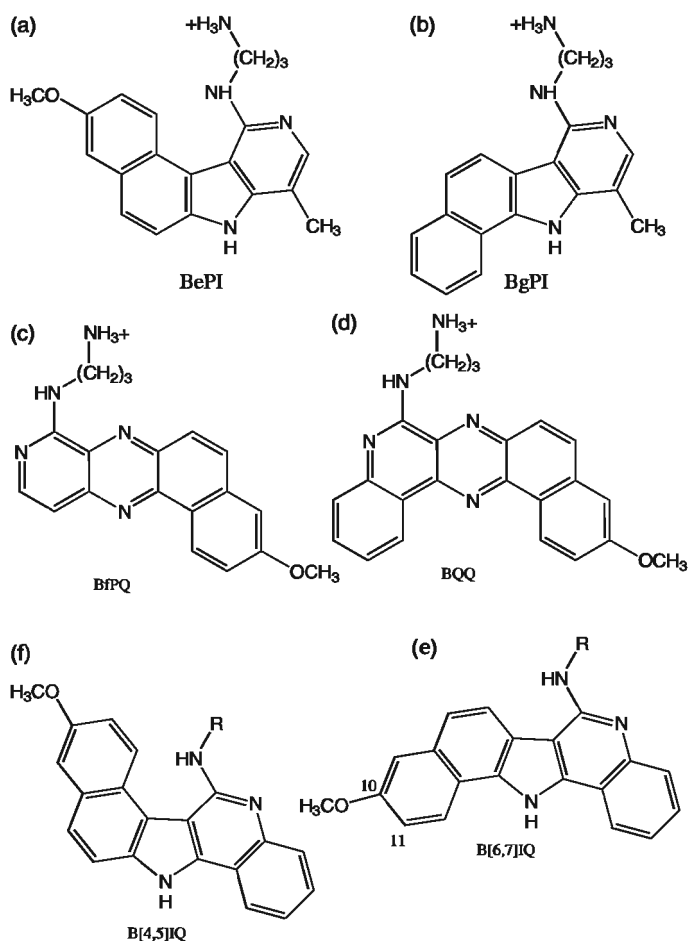


Fig. 14.3. Chemical structures of benzopyridoindole triplex-binding ligands. (a) BePI [22–24]; (b) BgPI [25]; (c) BfPQ [27–29]; (d) BQQ [31]; (e) B[6,7]IQ [32, 33]; (f) B[4,5]IQ [32, 33].

common to many other triplex-binding ligands (see below) and is thought to be due to charge repulsion between the cationic ligand and the positively charged $\text{C}^+\text{-GC}$ triplet. BePI derivatives bearing no alkylamino side chain or no charge in the side chain stabilize $\text{C}^+\text{-GC}$ - and T-AT -containing triplexes to a similar extent. The position of the methoxy group, located at position 3, is important. Derivatives in which this is moved to positions 2 or 4 show much lower triplex stabilization. Replacement of the methoxy with a hydroxyl or hydrogen reduced the ΔT_m to 13°C and 3°C respectively compared to a value of 20°C for the parent compound [22].

Modeling studies suggested that the side chain of BePI lies in the major groove [24], between the duplex pyrimidine strand and the third strand (the Watson–Hoogsteen groove), in an unfavorable position. Several derivatives of BePI have

been evaluated for triplex binding activity by Hélène and co-workers, who have shown similar good activity with benzo[*g*]pyridoindoles (BgPI) (Fig. 14.3b) [25]. Whereas the alkyalmino side chain of BePI makes unfavorable contacts with the DNA backbone, the side chain of BgPI contributes toward the triplex stabilization, especially when it contains a positive charge. This difference is caused by the different locations of the alkyamino groups in BePI and BgPI and is confirmed by cleavage studies in which these ligands are conjugated to EDTA [26] (see below). Modeling studies suggest that, in contrast to BePI, the side chain of BgPI lies in the minor groove between the two strands of the underlying duplex (the Watson–Crick groove) [25]. BgPI possesses a more linear structure than BePI, suggesting that the lower base stacking is compensated by electrostatic interactions. In contrast benzo[*f*]pyridoindole does not stabilize triplexes [26].

The benzo[*f*]pyridoquinoxalines (BfPQ) were synthesized as analogs of BePI in which the five-membered indole ring is replaced by a quinoxaline ring [27–29]. This compound again showed high selectivity for triplex over duplex DNA and modeling studies suggested that the four-ring structure mainly stacked with the Hoogsteen base pairs, showing poor overlap with the pyrimidine strand of the duplex. This poor stacking with the pyrimidine strand was consistent with the observation that this compound (like BePI) could induce the formation of a parallel, Hoogsteen-paired duplex (i.e. a triplex lacking the duplex pyrimidine strand) [28, 30]. On the basis of this model a further compound was prepared (BQQ) (Fig. 14.3d) which contained an additional benzene ring positioned so as to provide better stacking with the duplex pyrimidine strand. This compound again stabilized the triplex to such an extent that in the presence of 10 μ M ligand the triplex–duplex transition for the melting of a 14-mer third strand coincided with the melting of the underlying 26-base-pair duplex. This compound is one of the best triplex-stabilizing ligands developed and shows the highest specificity for triplex over duplex DNA.

Modeling studies predicted that it should be possible to enhance the triplex binding of BePI and BgPI by extending the stacking interactions and adding a benzene ring onto the pyrimidine rings of the parent compounds, producing the pentacyclic derivatives B[4,5]IQ and B[6,7]IQ [32] (Figs 14.3e and 14.3f). Modeling studies with these compounds suggested that both pentacyclic derivatives are positioned with their aminoalkylamine side chains in the minor groove. These compounds do indeed show selective binding to triplex structures. In general the B[4,5]IQ derivatives stabilized the triplex–duplex transition by between 9°C and 19°C less than their B[6,7]IQ counterparts, though both compounds were more potent than BePI and BgPI. Indeed the B[6,7]IQ series are some of the most potent derivatives described to date. More recent studies have examined the effects of different substituents at the 10 and 11 position of B[6,7]IQ derivatives [33]. The different derivatives showed only modest changes in their affinity for duplex DNA as expected, since modeling studies suggest that this region of these molecules interacts with the third strand bases. However, substitution at position 11 increased triplex affinity in order the $\text{NH}_2 < \text{OCH}_3 < \text{NO}_2$; the most electron-withdrawing groups promoting the greatest triplex stabilization [33].

Further related triplex-binding ligands containing a pentacyclic ring structure

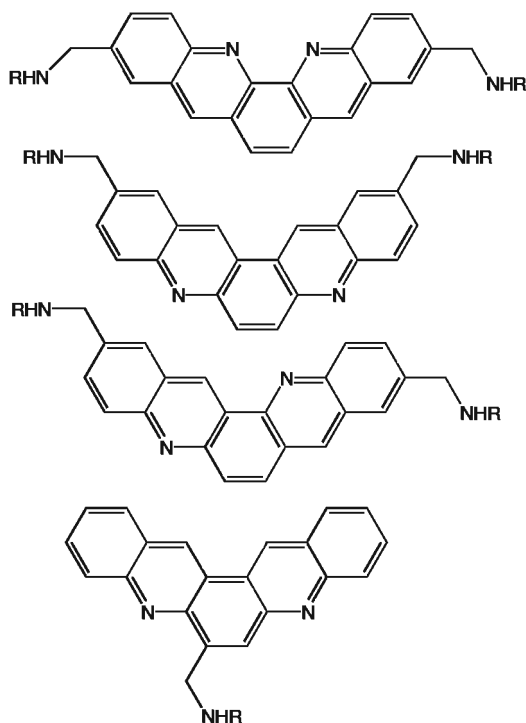


Fig. 14.4. Chemical structures of the dibenzo[*b,j*]phenanthroline series of triplex-binding ligands [34]. The lower, monosubstituted, compound is the best triplex-binding ligand in this series.

have been developed based on the crescent-shaped dibenzo[*b,j*]phenanthroline nucleus (Fig. 14.4) [34]. The upper three compounds in this figure, which are disubstituted and contain nitrogens at different positions in the aromatic rings, showed modest triplex-stabilizing ability with poor discrimination between duplex and triplex DNA, especially for triplexes containing a higher proportion of $C^+ \cdot GC$ triplets. However the fourth monosubstituted structure showed good triplex stabilization with only a moderate effect on duplex DNA. This nucleus may therefore provide a new lead for developing further triplex-binding ligands.

14.2.2

Coralyne

The berberine alkaloid coralyn (Fig. 14.5a) is a natural product containing four fused aromatic rings, which has been shown to stabilize triplexes. Initial reports showed that it bound to triplexes much better than duplexes and claimed that it had little sequence specificity, stabilizing both T·AT and $C^+ \cdot GC$ triplets [35]. However more recent studies have shown that it preferentially binds adjacent to T·AT

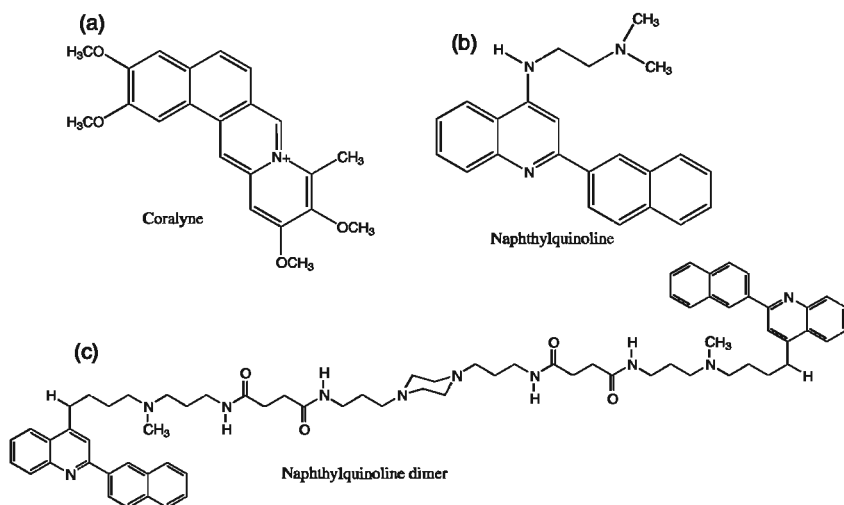


Fig. 14.5. Chemical structures of triplex-binding ligands. (a) Coralyne [35–37]; (b) naphthylquinoline [40–44]; (c) naphthylquinoline dimer [47].

[36]. The ligand promotes the formation of a triplex with T_6C_2 , but not with T_2C_6 . In addition it increased the melting temperature of triplexes of the kind $T_n \cdot A_n T_n$, but not $(CT)_n \cdot (GA)_n (TC)_n$. This preferential binding to T·AT triplets is similar to that of other positively charged ligands and most probably arises from unfavorable interactions with the charged $C^+ \cdot GC$ triplets. Coralyne has also been shown to promote the formation of antiparallel GT-containing triplexes [36]. Several coralyne derivatives have been prepared, all of which have a lower affinity for triplex DNA, again exhibiting a preference for T·AT triplets [37]. Coralyne has been shown to increase the efficiency of triplex-directed DNA alkylation [38, 39], using GA-containing oligonucleotides. Modification of the oligopurine tract in the chemokine receptor gene by a 12-mer oligonucleotide with a nitrogen mustard attached to the 5' end was greatly enhanced by addition of 8 μM coralyne. The efficiency of alkylation was further improved by replacing the G with 8-aza-7-deaza-G.

14.2.3

Naphthylquinolines

The naphthylquinoline triplex-stabilizing ligands (Fig. 14.5b) were also designed to bind to triplex DNA by intercalation [40]. These compounds possess a large aromatic surface area to stack with the base triplets, but since the aromatic portions are not fused, they possess torsional flexibility and can accommodate the propeller twist of the triplets in which the three bases may not be coplanar. They also contain a positively charged side group, which can counteract the negative charges from the phosphodiester backbones. In this series of compounds the 2-naphthyl deriva-

tive (naphthylquinoline) afforded the greatest triplex stabilization. The first studies showed that this ligand increased the melting temperature of poly(dA)-2poly(dT), while having little effect on duplex DNA. Footprinting studies showed that 10 μ M of this ligand enhanced triplex affinity by as much as 100-fold, and that it could also stabilize triplexes which contain a central triplet mismatch [41–43]. Further footprinting studies demonstrated that naphthylquinoline stabilized T-AT triplets to a greater degree than C⁺·GC and that it also stabilized antiparallel triplexes [44]. Although antiparallel triplex formation is normally pH-independent, the ligand induced a greater increase in triplex affinity at low pHs indicating that the active species is protonated [44]. The ligand can also be used to promote the formation of triplexes which contain multiple G·TA and T·CG triplets [45, 46].

A further derivative in this series is a naphthylquinoline dimer [47] (Fig. 14.5c). This was developed in the expectation that a bifunctional triplex intercalator should bind with greater affinity and have increased selectivity for triplex over duplex DNA. Footprinting studies with this ligand demonstrated that it stabilized a 9-mer parallel triplex containing a single C⁺·GC triplet approximately 30-fold more than the monofunctional naphthylquinoline at equivalent concentrations [47]. The increased affinity of these compounds for triplex DNA is consistent with bifunctional intercalation, though this has not been directly demonstrated. In this compound the two naphthylquinoline moieties were attached by a flexible linker through the amino groups on the quinoline rings. Other derivatives containing phenylquinolines linked via the phenyl rings do not bind to either duplex or triplex DNA (unpublished observations).

14.2.4

Bis-amidoanthraquinones

Bis-substituted anthraquinones (Fig. 14.6a) have been shown to stabilize DNA triplexes in a manner dependent upon the position of substitution. 1,4-Disubstituted compounds bind preferentially to duplex DNA, whereas 2,6-disubstituted compounds are selective for triplex DNA [48]. Their stabilization properties are related to the position of substitution, with 2,7-disubstituted amidoanthraquinone displaying the greatest triplex affinity. The order of affinity for parallel (CT-containing) triplexes decreases in the following order: 2,7 > 1,8 = 1,5 > 2,6 [49]. The different properties of the two series of compounds are thought to arise from differences in the stacking of the chromophore with the base triplets, as well as variations in the accessibility of the DNA grooves. This selectivity was confirmed by a thermodynamic study using calorimetric and spectrophotometric techniques which showed that the 1,4 compounds destabilize DNA triplexes, leading to dissociation of the third strand, whereas their 2,6 analogs stabilize triple-stranded structures [50]. Molecular modeling studies showed that all the ligands (except the 1,8 isomer) bind by intercalation, placing one substituent chain in the minor groove and the other in the major groove [49]. The 1,8-disubstituted amidoanthraquinone can also bind by intercalation but does so by inserting both side chains into the same triplex groove [49].

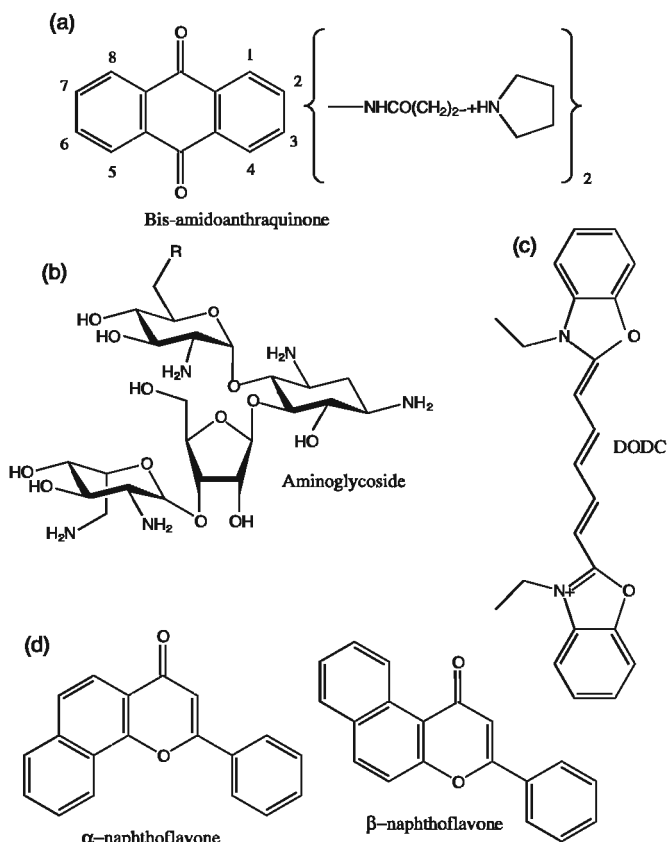


Fig. 14.6. Chemical structures of triplex-binding ligands. (a) bis-substituted amidoanthraquinones [48–51]; (b) aminoglycosides [54–56], neomycin R = NH₂, paromomycin R = OH; (c) DODC [57]; (d) α - and β -naphthoflavone.

The relative affinities of the different compounds are very different for anti-parallel triplexes [51]. Only the 2,7 and 1,8 isomers stabilize TG-containing triplexes, whereas AG-containing triplexes are only stabilized by the 1,5 and 2,6 isomers. These variations must be due to differences in the stacking interactions between the ligands and the DNA bases since the various antiparallel triplets are not isomorphic. Although these compounds bind to triplex DNA much better than to duplex structures it is worth noting that they also stabilize G-rich quadruplex DNA [52].

Anthraquinone sulfonamides have also been shown to stabilize triplex DNA. Again the position of substitution influences triplex stabilization [53], and the 2,7-disubstituted anthraquinone sulfonamide affords the best triplex stabilization. It was further shown that the ligands with greater numbers of positively charged substituents have a greater effect on triplex stability.

14.2.5

Aminoglycosides

Aminoglycoside antibiotics are antibacterial agents comprised of two or more amino sugars joined to a hexose nucleus. Among these compounds neomycin, paromomycin, gentamycin, tobramycin, and sisomycin have been shown to stabilize triplex DNA [54–56]. Neomycin (Fig. 14.6b) is the most potent compound in this series and stabilizes the triplex poly(dA)-2poly(dT), while the duplex DNA poly(dA)-poly(dT) is unaffected [54, 55]. It shows greater discrimination between triplex and duplex DNA than most intercalating agents and 25 μ M ligand increases the triplex–duplex melting temperature by 25°C but only stabilizes the duplex–single strand transition by 1°C. Their structure prohibits intercalation as the mode of action and their binding to triplexes is thought to arise from binding to one of the triplex grooves [54]. The stabilization is sensitive to the placement of the charges on the antibiotic, and related aminoglycosides have a lower affinity for triplex DNA [55]. This group of antibiotics also stabilizes the RNA triplex poly(rA)-2poly(rU), increasing its melting temperature by 49°C. Moreover, neomycin can promote the formation of some hybrid DNA/RNA triplexes which are usually only stable at high ionic strengths. It stabilizes both poly(rA)-2poly(dT) and 2poly(rA)-poly(dT) though it has a greater effect on the latter [55]. The stabilization of RNA triplexes is also very sensitive to the structure of the antibiotic and neomycin is much more potent than paromomycin, though these compounds only differ by replacing an amino group with a hydroxyl, and these compounds bind to duplex RNAs with equal affinity. Although these results demonstrate the unusual triplex-binding capability of these compounds, it should be noted that they also bind to other RNA-containing structures. Molecular modeling studies suggest that neomycin binds in the Watson–Hoogsteen groove, bridging the two pyrimidine strands [56].

14.2.6

Other Ligands

Diethyloxadadicarbocyanine (DODC) (Fig. 14.6c) was first identified as a quadruplex-specific ligand [56] using a database searching procedure. However, competition dialysis experiments showed that, although it does bind to quadruplex DNA, it has greater affinity for triplex structures [57]. DODC selectively stabilizes triplex DNA and has almost no effect on the duplex–single strand thermal transition [57]. Both α - and β -naphthoflavones have been shown to stabilize triplex DNA [58 and J. B. Chaires, personal communication]; the α compound is about four times as potent as the β . These compounds are unusual as they are uncharged, whereas most triplex–ligands are cations. Other intercalating compounds with the ability to stabilize triplexes include ruthenium–phenanthroline complexes, which are thought to intercalate from the minor groove [60] and imidazoethioxanthones [61]. Other non-intercalating triplex–ligands may include anthocyanins [62], polycationic oligopeptides [63], comb-type polycations [64], and hydralazine [65].

14.3

Sequence and Structural Selectivity of Triplex-specific Ligands

14.3.1

Sequence Selectivity

Most triplex-binding ligands contain positively charged heterocycles and this positive charge is thought to prevent binding adjacent to protonated cytosine residues. These ligands therefore stabilize triplexes containing T·AT triplets better than those that are rich in C⁺·GC. Indeed there are very few triplex-binding ligands that do not possess positively charged chromophores: the only exceptions are the bis-substituted amidoanthraquinones, which contain positively charged side groups, and the naphthoflavones, which do not contain any formal charges. Our studies suggest that both these compounds can bind to C⁺·GC and T·AT triplets with similar affinities. We find that the triplex formed with (CCT)_n is stabilized by an uncharged ligand (a disubstituted amidoanthraquinone) but not by naphthylquinoline (charged). In contrast, both ligands stabilize the triplex formed with (CCTT)_n.

A further complication is the observation that triplex stability is itself affected by the triplet composition. Several groups have shown that isolated C⁺·GC triplets impart a greater stability than T·AT, presumably as a result of favorable interactions between the protonated cytosines and the π -electrons of the stacked bases [66–68]. This is related to the observation that sequences containing alternating C⁺·GC and T·AT triplets have the greatest stability [69]. In these structures the intercalation of any ligand, whether charged or uncharged, will disrupt the pattern of alternating charged and uncharged bases. Consistent with this suggestion we find that most ligands are unable to stabilize the triplex formed with (CT)_n. The most stable structures are therefore formed when the stacked rings (bases or ligand) alternate between charged and uncharged species. Triplexes containing alternating C⁺·GC and T·AT triplets are not stabilized by ligands since they would interrupt the alternating pattern of charged and uncharged residues (unpublished observations).

14.3.2

Binding to Different Motifs

Most studies with triplex-binding ligands have examined their interaction with parallel (CT-containing) triplexes in contexts in which all three strands are composed of DNA (rather than RNA). BePI has been shown to stabilize triplexes containing antiparallel G·GC and T·AT triplets, but does not promote the binding of parallel GT-containing oligonucleotides [70]. Coralyne and naphthylquinoline also promote the formation of antiparallel GA- and GT-containing triplexes, though the ligands are less effective on these structures than the parallel CT-containing triplexes [36, 44]. The greatest differences in binding to various motifs are exhibited by the series of bi-substituted amidoanthraquinones [51]. The 2,7 and 1,8 isomers

stabilize TG-, but not GA-containing triplexes, whereas AG-containing triplexes are only stabilized by the 1,5 and 2,6 isomers; these compounds have no effect on the GT-containing sequences. These variations are presumed to arise from differences in the ways in which the ligands stack between different DNA base triplets, which themselves adopt a range of structures.

14.4

Interaction of Duplex-specific Ligands with Triple Helical Nucleic Acids

14.4.1

Intercalators

Ethidium bromide is well known as a duplex DNA intercalating agent and early thermal denaturation studies demonstrated that it destabilized the triplex helix formed by poly(A)-2poly(U) [71]. However, a later study showed that ethidium stabilized poly(dA)-2poly(dT) more than poly(dA)-poly(dT) [21]. This apparent selectivity for triplex DNA is partly due to the poor binding of ethidium to poly(dA)-poly(dT) because of its unusual conformation [72–74]. A separate study further demonstrated that parallel triplexes containing both C⁺·GC and T·AT triplets are destabilized by ethidium [75]. However, intercalation of duplex-specific ligands is especially likely at triplex–duplex junctions, which have been shown to generate a good intercalation site [76].

14.4.2

Minor Groove Binders

In principle, one might expect that ligands which bind in the DNA minor groove will not affect the formation of triple helical nucleic acids since the ligand and the third strand are located in different DNA grooves. However, it is conceivable that minor groove ligands could enhance or inhibit triplex formation by altering the dimensions and/or flexibility of the major groove, with cross-talk between the two grooves. At first sight the literature on this subject is confusing since the precise effect depends on the ligand, the ionic strength, and the type of triple helix.

Early studies showed that netropsin can bind to parallel DNA triplexes containing only T·AT triplets, though the binding constant to triplexes is lower than to duplex DNA. CD spectral analysis suggested that the ligand is bound in a similar conformation and environment in both duplex and triplex structures [77, 78]. However, thermal denaturation studies showed that the ligand destabilizes triplex DNA, whereas it stabilizes duplexes and in the presence of 200 mM sodium, netropsin displaces the third strand of poly(dA)-2poly(dT) [79]. However, on increasing the sodium ion concentration to 1 M the triplex is maintained with netropsin bound to the minor groove. Biophysical studies suggested that netropsin binding displaces more water molecules from triplex than from duplex DNA [79]. Other studies showed that it binds to the central A₄·T₄ site of an intramolecular DNA

triplex containing antiparallel G·GC and T·AT triplets with an affinity of about $1 \times 10^8 \text{ M}^{-1}$ and increases the thermal stability of this structure [80]. In contrast, it binds about an order of magnitude less well to the similar, though less stable, intramolecular structure containing parallel G·GC and T·AT triplets [81].

A number of studies have examined the binding of berenil to triple helical structures [82, 83]. Berenil binds to both poly(dA)·2poly(dT) and poly(rA)·2poly(rU) triplexes in a salt-dependent fashion: at low salt ($<0.08 \text{ M}$ sodium) the triplex to duplex transitions are stabilized, while they are destabilized at higher salt concentrations ($>0.125 \text{ M}$). Both triplexes also reveal that the effect of berenil depends on the ligand:triplet ratio. At low ratios the ligand appears to bind in a non-specific fashion and weakly destabilizes the triplex, while at higher ratios the triplex is stabilized. The ligand shows a higher affinity for the DNA than the RNA triplex.

The ligand DAPI has been shown to bind in the minor groove of AT-rich duplexes, in a manner similar to other minor groove binding ligands. It also binds to GC-containing sequences by a mechanism which probably involves partial intercalation. This ligand can induce the formation of the hybrid triplex poly(rA)·2poly(dT) which possesses an RNA-containing purine strand [84]. This triplex is unstable and is not formed in the absence of the ligand. Several studies have shown that DNA pyrimidine third strands do not bind to DNA/RNA hybrid duplexes in which the purine strand is composed of RNA [85–88] and that the most stable parallel triplexes contain RNA in both pyrimidine strands and DNA in the purine strand. Flow dichroism studies suggest that DAPI stabilizes poly(rA)·2poly(dT) by an intercalative mechanism. Netropsin, berenil, and ethidium also stabilize this structure, though less is known about the details of the interaction [89]. DAPI can also bind to poly(dC)⁺·poly(dG)·poly(dC), possibly by partial intercalation from the major groove [90]. DAPI and berenil can also induce the formation of the antiparallel triplex poly(rA)·poly(rA)·poly(dT), which is also unstable in the absence of added ligand [89].

Distamycin has been shown to bind to intramolecular parallel triplexes containing T·AT triplets at high ionic strength, though spectral analysis suggests that it is bound in a slightly different configuration than to duplex DNA [91]. This only occurs in the 1:1 binding mode and no complex formation is observed in the 2:1 mode. In contrast, distamycin caused dissociation of a similar intermolecular triplex. NMR, IR, CD, and LD studies have also shown that distamycin can bind to poly(dA)·2poly(dT) [92–94]. Neither distamycin nor berenil affected the formation of an antiparallel GT-containing triplex [95], though this may not be surprising since the target did not contain any AT-tracts long enough to accommodate the ligand. In contrast the GC-selective minor groove ligand mithramycin prevented formation of this triplex.

Even though one report suggested that distamycin does not bind in the 2:1 mode to a short intramolecular triplex [91] it has been shown that a polyamide dimer (ImPyPy-Dp) specific for the sequence 5'-AGACA does not affect triplex formation when its binding site overlaps with that for a triplex forming oligonucleotide [96]. No cooperative effect was observed for binding of the polyamide dimer on oligonucleotide association. This is surprising since dimer binding produces a groove width of 6.8 Å compared with 3.4 Å for netropsin binding in the 1:1 mode. How-

ever, in this instance the binding sites only overlapped by one base pair, and there is no information on polyamide binding to a site within a triple helix. In contrast, the attachment of one monomer of a polyamide dimer to the end of a triplex-forming oligonucleotide can be used to induce oligonucleotide dimerization, and so induce triplex formation in a cooperative fashion at adjacent triplex sites which are separated by the preferred sequence for the polyamide [97].

14.5

Tethered DNA-binding Agents

One of the first methods to be attempted for increasing triplex stability was to attach a DNA-binding ligand to one end of the oligonucleotide. In this way sequence selectivity is achieved by the triplex-forming oligonucleotide, while the ligand acts as a non-selective anchor increasing the affinity. Most of these studies have used duplex intercalators as DNA-binding ligands, though some recent examples have employed triplex-binding ligands.

14.5.1

Tethered Triplex-binding Ligands

Benzopyrindoindole and benzopyridoquinoline triplex-binding ligands have been attached to either the 5'-end or to an internucleotide position of triplex-forming oligonucleotides [98, 99]. All these conjugates stabilize triple helical structures more than the addition of free ligand at an equivalent concentration. As expected the attached ligand had a greater effect on sequences containing longer run of contiguous thymines. Benzo[h]quinoxinoxaline was the most effective compound when attached to the 5'-end of the third strand, while BePI was the best ligand for the internal site. These modifications did not alter the stringency of triplex formation, and introduction of a single mismatch caused a large decrease in binding affinity. Naphthylquinoline-oligonucleotide conjugates have also been shown to stabilise triplexes to a greater extent than equivalent concentrations of free ligand [100]. However, it should be noted that tethered ligands only introduce the benefit of one stabilizing event at each triplex, whereas with free ligand several ligand molecules may be able to bind simultaneously at different positions along the triplex.

Inclusion of a duplex-binding agent within a third-strand oligonucleotide has also been used to enhance triplex formation at sites containing pyrimidine interruptions. In these experiments an internal acridine group was placed adjacent to the third strand base, opposite the inverted pyrimidine-purine base pair [101, 102]. This arrangement uses the binding free energy of the intercalator to compensate for the reduction in triplex stability, in a similar manner to that achieved by attaching an intercalator to the third strand. In the absence of a base inversion, inclusion of an internal acridine has little or no effect on triplex stability, possibly because it is a duplex- rather than a triplex-specific intercalator. Inclusion of an internal acridine moiety increases the stability of triplexes at both CG and TA in-

versions, using both natural and synthetic bases opposing the pyrimidine. Recognition of TA is strongest using either acridine or propanediol with an acridine on its 3'-side. In contrast, recognition of CG is greatest with either cytosine with acridine on its 3'-side or guanine with acridine on its 5'-side [102]. Oligonucleotides containing 5'-acridine moieties have also been used to stabilize the triplex formation at target sites containing pyrimidine interruptions [112]. The base mismatches had a greater destabilizing effect when placed close to the intercalator end of the oligonucleotide.

14.5.2

Tethered Intercalators

The covalent attachment of intercalating agents to the end of a triplex-forming oligonucleotide generates a complex in which the intercalating agent acts as a non-specific anchor, while the oligonucleotide provides the sequence selectivity. One of the first intercalating agents to be linked in this way was acridine (usually [2-methoxy, 6-chloro, 9-amino]acridine) producing a significant increase in triplex stability [103–108]. Attaching the acridine to the 5' end has a greater effect than at the 3' end [104], possibly reflecting structural difference between the 5' and 3' triplex–duplex junctions. Most of these intercalator–oligonucleotide conjugates have used parallel, pyrimidine-containing oligonucleotides, though this strategy can also be used to stabilize antiparallel GA- and GT-containing triplexes [109–113] as well as alternate-strand triplexes which contain both parallel and antiparallel motifs [114]. Acridine conjugation generally increases triplex stability by about 100-fold, though the stringency of triplex formation is reduced [107, 114].

Other intercalating agents which have been successfully linked to third-strand oligonucleotides include orthophenanthroline [115], anthraquinone derivatives [116], ellipticine [117], and the UV-sensitive crosslinking agent psoralen [118–120].

14.5.3

Other Tethered DNA-binding Agents

Other DNA-binding agents which have been tethered to triplex-forming oligonucleotides include the AT-selective minor groove-binding ligand Hoechst 33258 [121, 122], the topoisomerase I inhibitor camptothecin [123], duocarmycin [124], the DNA cleavage agent bleomycin [125], a platinum-containing DNA crosslinking agent [126], and a polyamide minor groove binding agent [97].

14.6

Other Uses of Triplex-binding Ligands

To date the main use of triplex-binding ligands has been to enhance the stability of triplex helical structures *in vitro*. However their binding to triplex DNA has several other implications, some of which are described below.

14.6.1

Relaxing the Specificity of Triplex Formation

Triplex-binding ligands can be used for increasing the strength of binding to sites which contain pyrimidine interruptions. By increasing the strength of binding, by up to 1000-fold, complexes can be formed at sequences for which there are no clear triplex coding rules and for which triplex formation is very weak. The naphthylquinoline derivatives have been shown to promote the formation of triplexes at sites containing up to three consecutive base pair inversions using T·CG and G·TA triplets [45]. The least destabilizing triplets are the same in both the presence and absence of BePI, though the identity of the third strand base is less important in the presence of the ligand [127]. However addition of the ligand does provide some discrimination between different inverted base pairs. In particular 3-nitropyrrole discriminates CG from GC, TA and AT pairs in the presence, but not the absence of BePI [127]. Although blocks of alternating T·AT and G·TA triplets are not stable, even in the presence a triplex-binding ligand, these complexes can be stabilized by attaching to a block of consecutive T·AT triplets, which can be further stabilized by ligand binding. In this way it is possible to target alternating AT-tracts with GT-containing oligonucleotides in the presence of the naphthylquinoline triplex-binding ligand [128, 129]. In this way the duplex $A_{11}(AT)_6 \cdot (AT)_6 T_{11}$ can be targeted with the third strand $T_{11}(TG)_6$ [129].

14.6.2

Triplex Cleaving Agents

Addition of an EDTA moiety to a triplex-binding ligand can convert it into a triplex-specific cleavage agent in the presence of Fe^{2+} and a reducing agent. EDTA conjugates, attached to the alkylamino side chain, have been prepared for BePI, BgPI [26] and BQQ [130]. The ligands cleave DNA at sites to which a triplex-forming oligonucleotide is bound, though the background cleavage in non-targeted duplex DNA is greater than that produced by EDTA-tethered oligonucleotides [3]. BgPI and BQQ conjugates produce double-stranded cuts at triplex target sites, cleaving the purine and pyrimidine strands of the duplex with equal efficiency. As a result of their selectivity for triplex DNA these agents can be used to cleave plasmid DNA at a single site to which a triplex-forming oligonucleotide is bound. In contrast BePI-conjugates only cleave the duplex pyrimidine strand while the purine strand is actually protected from background cutting. As a result, this conjugate cannot be used to produce site-specific cleavage of duplex DNA. None of these agents cleave the triplex-forming oligonucleotide itself. The different cleavage properties are explained by the location of the alkylamino side chains to which the EDTA moieties are attached. These are positioned in the minor groove for BQQ and BgPI, close to the likely site of free radical attack at C4', in contrast to BePI for which the side chain is located in the major groove.

Another example of DNA cleavage by a triplex-specific ligand is the photocleavage of DNA by quinacridine derivatives [131]. These compounds display pho-

toactive properties, which can be used to cleave DNA close to their site of binding. These compounds induced site-specific cleavage of plasmid DNA in the vicinity of a pre-formed triplex, though high-resolution mapping showed that the actual site of damage was at guanines located 6–9 bases away from the 5′ end of the oligonucleotide. Both strands of the duplex were cleaved in a reaction that did not require alkaline treatment; the purine-containing strand was modified at a single base, while cleavage occurred at three closely spaced guanines on the pyrimidine-containing strand. Photocleavage was also observed at the same location when the PQ moiety was tethered to the 5′ end of the oligonucleotide. This, together with the observation that this site is still cut when it is in a single stranded form adjacent to the triplex, suggests that the ligand is not actually bound at the cleavage site.

14.6.3

Antigene Activity

Despite the clear demonstration that many ligands can selectively stabilize triplex DNA, there have been comparatively few studies on the use of these compounds, along with triplex-forming oligonucleotides, for inhibiting gene activity. The original work with BePI showed that this ligand enhanced the inhibition of transcription initiation produced by a triplex-forming oligonucleotide in an *in vitro* transcription assay [22]. BePI has also been shown to promote the formation of intramolecular DNA triplexes (H-DNA) leading to inhibition of the expression of an adjacent gene [132]. Coralyne has been used to increase the efficiency of modification of a chemokine receptor gene by a triplex-forming oligonucleotide containing a nitrogen mustard at its 3′ end [38, 39], and amidoanthraquinones have been used to enhance the stability of CT-containing triplexes on a shuttle vector [133].

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15

Polyamide Dimer Stacking in the DNA Minor Groove and Recognition of T-G Mismatched Base Pairs in DNA

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15.1

Introduction: Sequence-specific Recognition of DNA by Synthetic Molecules

15.1.1

DNA Sequence Recognition

The two most successful modes of recognition of specific sequences of DNA with four or more base pairs involve the two grooves of the double helix. These two modes are formation of a triple helix by oligonucleotides or analogs in the major groove and interaction of organic cations in the minor groove. A quite diverse array of DNA sequences, including both Watson–Crick and mismatched base pairs, can be recognized with current compounds that bind in the DNA minor groove and the focus of this chapter will be on agents of this type. Distamycin and netropsin (Fig. 15.1) are classical examples of polyamide compounds that bind in the DNA minor groove [1–6] at AT-rich sequences. Studies of these compounds and their analogs have led to the development of sequence-specific DNA-binding ligands that show excellent possibilities for use as agents for the regulation of gene expression [7]. These new compounds are synthetic oligopeptides, typically distamycin analogs, that contain imidazole (Im)/pyrrole (Py) heterocycles. They have the broadest sequence recognition capability of synthetic compounds prepared to date. Synthetic polyamides generally recognize DNA as stacked dimers. This binding mode is enhanced by covalently linked hairpin dimers that have been studied in detail by Dervan and co-workers [8]. The paradigm for this type of compound originated with the discovery by Wemmer and co-workers that the natural product distamycin (Fig. 15.1) could form antiparallel dimers in the minor groove and could thus read the sequence of DNA from both strands of the duplex [2].

On the basis of an X-ray structure of netropsin bound to an AATT sequence in DNA [9], Lown and Dickerson proposed that the AT base pair recognition of the polyamides could be altered to GC base pairs by replacement of the pyrrole (Py) by imidazole (Im) units [9, 10]. The imidazole rings would be able to form hydrogen bonds with and provide more space than pyrrole heterocycles for the guanine N2 amino group. This group protrudes into the minor groove in GC-containing se-

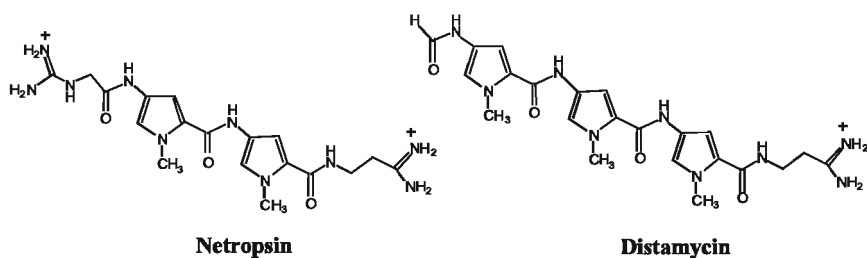


Fig. 15.1. Structures of netropsin and distamycin.

quences and blocks the binding of many AT-specific binding agents. With the Im heterocycles molecules are allowed to penetrate deeper into the minor groove in GC sequences. Such imidazole-containing polyamides were shown experimentally to have enhanced ability to recognize GC-rich sequences of DNA [1, 5, 6, 8, 10–13]. Heterocycles other than imidazole (e.g. furan and thiazole) have also been substituted for the polyamide pyrrole units [14, 15]. NMR and MPE-Fe footprinting studies on furan derivatives as well as on thiazole derivatives with the sulfur atom pointing out of the minor groove revealed that the synthetic imidazole compounds were able to bind to AT-GC mixed sequences. Unfortunately, both the specificity and the affinity for the synthetic compounds were low compared with netropsin under similar conditions [14].

These results indicated that specificity and affinity of minor groove-binding agents is sensitive to the local width of the minor groove. In AT-rich sequences the minor groove is unusually narrow, 0.3–0.4 nm [16]. Therefore, the distamycin and netropsin molecules and modified analogs could fit snugly and make favorable van der Waals contacts with the groove walls. The mixed sequences have a wider minor groove, 0.5–0.6 nm [17], and one molecule of ligand is not thick enough to make contacts with both walls of the groove, considering that the thickness of one ring is about 0.34 nm [4]. Detailed studies by Wemmer and co-workers [18] of distamycin-DNA binding (with the recognition sequence AAATT) revealed that at ratios lower than one compound per DNA duplex the prevalent species was a 1:1 complex, while at ratios above 1, the 2:1 complex formed at the expense of the 1:1 species. It was also observed that for the formation of the 2:1 complex in AT sequences the minor groove had to widen to bind the two distamycin molecules [2, 4, 18]. Since in the wider GC sequences, the formation of a dimeric motif could occur with a lower conformational energy penalty, strategies to design hydrogen bond-accepting polyamides able to form stacked dimers were developed [1, 5, 6, 8, 11, 13, 19–25]. These new agents were designed as analogs of distamycin because netropsin was not observed to form the dimer motif [5]. This modified strategy was quite successful and new agents with high affinity for GC-rich sequences were developed [5, 6, 8, 13, 15, 18–37]. In general these compounds bind to GC sequences in DNA with positive cooperativity and form antiparallel dimers [22–25].

Lown and collaborators reported the first distamycin analog with the central pyrrole of a trimer polyamide replaced with an imidazole heterocycle. This deriva-

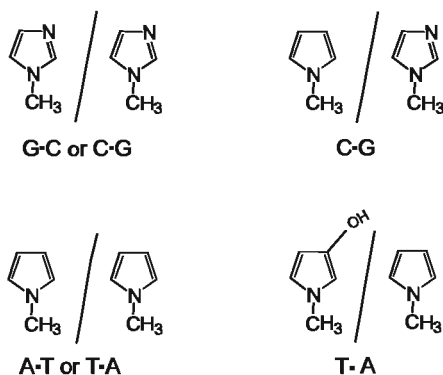


Fig. 15.2. Heterocyclic pairing rules for the interaction of polyamides with DNA base pairs, as defined by Dervan and collaborators [24], are shown.

tive was found by NMR studies to bind with cooperativity to AAGTT in preference over AAATT [5, 23]. The preferred binding to the GC-containing sequences is a result of the formation of hydrogen bonds between the imidazoles and the guanine N2 amino group. In the investigation of both dimeric and linked polyamides in designing gene-specific probes, Dervan and collaborators [5, 6, 8, 20–22, 24, 28–33, 36–38] have developed a set of base pair recognition rules for DNA–polyamide interactions (see Fig. 15.2). Their results revealed that two polyamide heterocyclic rings paired side-by-side were able to recognize certain base pairs depending on the rings that were paired. A pair of stacked methylpyrroles recognize either A·T or T·A while pyrrole paired with hydroxypyrrole (Py/Hp) can distinguish A·T from T·A base pairs. Two paired imidazoles (Im/Im) bind with degeneracy for either G·C or C·G. Pyrrole-paired imidazole (Py/Im) distinguishes C·G from G·C while Im/Py selectively binds G·C [24].

These studies of polyamide–DNA interactions have shown that the affinity of the polyamides for DNA decreases as the number of base pairs that are not a match for the corresponding heterocyclic pair increases and vice versa [36]. Although paired imidazoles (Im/Im) do not bind well to Watson–Crick base paired sequences, they have been found to selectively recognize T·G mismatched base pairs [27, 38]. This finding provides the basis for identification of single nucleotide polymorphisms in DNA that involve T·G mismatched base pairs and will be described in detail below.

15.1.2

Stacking Behavior of Polyamides and DNA Recognition

N-terminal substituents have a very significant effect on the recognition of DNA by polyamides [37, 39, 40]. Dervan and co-workers have shown that the presence of a terminal acetyl group alters the binding orientation of hairpin polyamides from the normal 5′-to-3′ orientation to 3′-to-5′. The substitution also reduces the affinity of the polyamide for DNA [37]. On the other hand, Lown and Dickerson have pro-

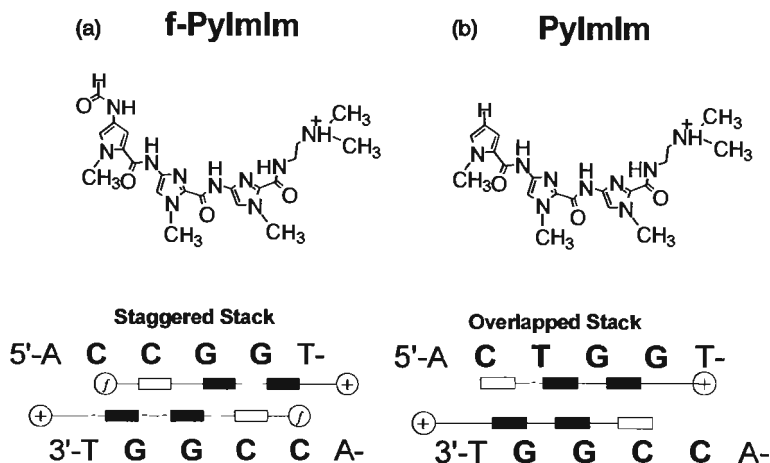


Fig. 15.3. Staggered and overlapped stacking modes of PyImIm (a) and ImPyPy (b) are illustrated with and without a terminal formamido group, respectively. In the two sequences the heterocycles are switched.

Polyamides with a terminal formamido group prefer to stack in a staggered fashion, but the non-formamido analogs stack in an overlapped manner.

posed that the stacking of polyamides in the formation of the dimer complexes with DNA is different when the compounds have a terminal formamido group than when lacking this substituent [40]. There are two different possible low energy ways to form the stacked dimer and these are sensitive to the presence of a terminal formamido group: (i) a fully overlapped stacking mode in which the N-terminal heterocycles of the dimer stack on the amide groups between the two heterocycles at the C-terminal end of the opposite molecule in the dimer and (ii) a staggered stacking mode in which the N-terminal heterocycles are shifted by approximately one unit in their C-terminal direction (Fig. 15.3). The staggered stacking mode allows the positively charged groups of the dimer to move apart a greater distance. In addition, the staggered stacking geometry moves the monomer units of the dimer apart and may reduce some unfavorable steric effects in the dimer complex. This mode of stacking, however, apparently requires specific N-terminal substituents, such as a formamido group, for favorable stacking energetics and dimer formation.

The shift in stacking behavior of side-by-side dimeric polyamides has direct consequences on their sequence recognition properties. The different DNA recognition possibilities can be seen with the PyImIm and the f-PyImIm polyamides since these polyamides will recognize quite different sequence types in the overlapped and staggered stacking modes (Fig. 15.3). Using the established DNA base pair recognition rules, an overlapped polyamide dimer with the sequence PyImIm will bind preferentially to the sequence CCGG, while a staggered dimer will bind preferentially to the sequence CTGGsm that contains a single T-G mismatch (Fig. 15.3). Clearly, both stacked complexes are possible and analysis of binding affinity

with both possible DNA recognition sequences is required to define the level of recognition specificity. A variety of polyamides and their associated DNA recognition sequences, with careful and systematic binding studies for the polyamides, can provide results to define the preferred stacking modes and the influence of substituents.

To systematically investigate the influence of terminal groups on stacking and DNA sequence recognition, polyamide trimers with and without terminal formamido groups were synthesized [39]. The interactions of these polyamides with predicted DNA recognition sequences in the two different stacking modes were evaluated by using surface plasmon resonance (SPR) detection with immobilized DNA hairpin duplexes. Since the polyamides have relatively weak spectroscopic signals SPR methods with immobilized DNAs to investigate binding affinity, stoichiometry, and cooperativity offer significant advantages. Both equilibrium and kinetics results from SPR clearly show that a terminal formamido group has a pronounced effect on the affinity, sequence specificity, cooperativity, and rates of DNA–dimer complex formation. As predicted, the formamido f-PyImIm polyamide binds preferentially in the staggered stacking mode and recognizes CCGG, while the unsubstituted PyImIm analog binds in the overlapped mode with specificity for CTGGsm. The mismatched base pair recognition capability of these polyamides as well as others are described in more detail below.

Another stringent test for the overlap and staggered modes of polyamide binding to Watson–Crick sequences of DNA under the influence of the terminal formamido group is provided by the ImPyPy polyamide sequence. The formamido polyamide, f-ImPyPy, in the predicted staggered stacked binding mode should bind best to the sequence (A/T)GC(A/T) where (A/T) indicates that either an A·T or T·A base pair is allowed. The ImPyPy polyamide in the predicted overlapped mode, however, should bind best to an (A/T)G(A/T)C sequence. With this polyamide two Watson–Crick base-paired DNA sequences with no mismatched base pairs will be recognized by the ImPyPy polyamide sequence in the two different stacking modes. To test this hypothesis SPR binding studies of the two polyamides to DNA hairpin duplexes containing the possible recognition sequences TGCA and TGTC were conducted. As predicted, the formamido compound binds best to TGCA while the non-formamido compound binds best to TGTC in agreement with staggered and overlapped stacking modes, respectively. The quantitative results for binding of ImPyPy to the TGTC sequence are also in excellent agreement with the values obtained previously by Dervan and collaborators with high MW DNA by quantitative footprinting analysis [25]. This agreement provides a validation of the SPR method for polyamide–DNA interaction studies.

Affinities for all DNA recognition sequences increase by a factor of around 100 when a terminal formamido is added to a polyamide and the preferred sequences recognized are also different. SPR kinetic studies show that both the association and dissociation rates are slower for the formamido derivatives but the effect is larger for the dissociation kinetics. The formamido group, thus strongly affects the affinities for the interaction of polyamides with DNA and can change the preferred DNA sequence that is recognized by a specific polyamide stacked dimer [39].

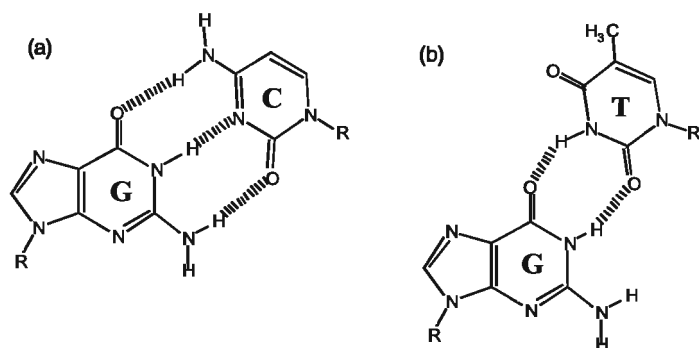


Fig. 15.4. Structures of (a) C-G Watson-Crick and (b) T-G mismatch base pairs. The availability of the G amino group in the minor groove can be easily seen in this view.

15.2

T-G Mismatched DNA Base Pairs and their Biological Relevance

Mutations in DNA that go unrepaired lead to the polymorphic nature of individual genomes. Such mutations can lead to evolutionary variations and advantages, but they can also lead to disease states or tendencies. T-G mismatched base pairs in DNA (Fig. 15.4b), for example, are responsible for most of the common mutations leading to the formation of tumors in humans. Human bladder carcinoma is an example where a G-C to A-T transition at the 3'-G of the GG doublet in codon 12 of the *Ha-ras* and *Ki-ras* proto-oncogene converts them to oncogenes [41–43]. A C to T transition to produce a T-G mismatch is often introduced by spontaneous deamination of 5-methylcytosine and can arise from errors in replication [44]. Although only about 3% of cytosine residues in the human genome are methylated, mutations in 5-methylcytosine account for about one-third of the single-base mutations that have been observed in inherited human diseases [44]. The rate of C to T transitions has been estimated to be $5.8 \times 10^{-13} \text{ s}^{-1}$ at 37°C, and with the size of the human genome this would result in an average of two T-G mismatches created per cell each day [44]. While specific T-G mismatch repair systems exist in cells, such as thymine DNA glycosylase a 55 kDa nucleoprotein in mammalian cells [45, 46], some T-G sequences escape from being repaired. For example, mismatch repair is impaired in some colon cancers that are refractory to conventional drugs. In these cells, there is a higher level of T-G mismatches in their genomes than in sensitive cells [47].

T-G mismatched base pairs most commonly appear as single nucleotide polymorphisms (SNPs) in the human genome and they account for much of our individual differences [48]. SNPs, which occur approximately every 1000 base pairs, are the most common genetic variation. They are of major importance in many areas, including population genetics, pharmacogenomics, and other similar areas that have taken on increasing importance with the sequencing of the human genome.

It has been proposed that specific SNPs, by defining individual variations, could serve as a diagnostic tools to ensure prescription of the most appropriate drug at the optimum level for each patient [49, 50]. Development of methods to rapidly and accurately characterize individual SNP patterns is thus an area of current importance. As a result, there is significant interest in developing small molecules capable of selectively recognizing mismatched base pairs, and research to discover and develop such agents is a high research priority. For example, it was shown using SPR-biosensor methods that dimers of naphthyridine immobilized on BIACORE sensorchips were capable of selectively detecting G-G mismatches existing in PCR amplification products of a 652-nucleotide sequence of the *HSP70-2* gene [51].

15.3

Potential Applications for Recognition of Mismatched Base Pairs

SNPs can be identified as mismatched base pairs by hybridization of individual genomic DNA with standard DNA sequences. Several procedures are in use to search such hybridized duplexes for mismatches by using methods such as chemical, enzymatic, or denaturation methods [52]. Automated methods that can be conducted at normal temperatures have significant advantages over methods that rely on precise selection of an elevated temperature for detection of differences in stability between Watson–Crick and mismatched base pairs. SPR methods with compounds that can selectively recognize and capture mismatches thus offer advantages if appropriate compounds can be identified.

Beyond their usefulness in genetic diagnosis, compounds which selectively recognize base mismatches can also serve as novel chemotherapeutic drugs in some selected disease states. The failure of mismatch repair (MMR) has been associated with certain forms of cancer, in particular hereditary nonpolyposis colorectal cancer. These tumor cells accumulate mismatches in their DNA because they lack the mechanisms to correct the T-G mispairs [47]. Agents that can bind to such mismatches could preferentially target such cancerous tissues, making them ideal chemotherapeutic drugs for MMR-deficient cancers, which are usually resistant to many chemotherapeutic drugs [47].

15.4

Structure of T-G Mismatched Base Pairs

Because of the biological importance of the T-G mismatch, the structural and physical properties have been extensively investigated. NMR analysis of 5'-CGTGAATTCGCG-3', containing symmetrical T-G mismatches, indicates that the mismatches adopt a wobble conformation (see Fig. 15.4b), and structural perturbations are mainly in the vicinity of the mismatch and the nearest neighbor [53]. These localized perturbations in T-G mismatch containing oligonucleotides are

supported by an X-ray analysis of 5'-CGCGAATTTGCG-3' [54]. Thermodynamic studies on 39 oligonucleotides that contain T-G mismatches demonstrated that the stability of T-G mismatches depends on their nearest neighbors [55]. Duplex sequences with 5'-C and 3'-C neighbors of the G in the T-G mismatch are the most stable [56]. The sequence TGA at the G of the mismatch is very destabilizing, but interestingly, an NMR structure of this sequence did not reveal any unusual structural perturbations [56]. The T-G pair in this sequence adopted a wobble conformation with the G base projecting into the minor groove as observed with the more stable T-G-containing DNA duplexes.

15.5

Binding of Imidazole-containing Polyamide Analogs to T-G Mismatches through a Dimeric Binding Motif – Structural Studies

In 1999, Lee, Wang and co-workers demonstrated using NMR spectroscopy and molecular dynamic studies that a triimidazole analog of distamycin, AR-1-144 or f-ImImIm (Fig. 15.5a) was able to bind to a self-complementary CCGG-containing decamer 5'-GAACCGGTTTC-3' [19]. This observation is in agreement with the polyamide pairing rules described in the previous section. According to the NMR structure, f-ImImIm formed a 2:1 side-by-side antiparallel dimer and bound to four base pairs (Fig. 15.5B) with the guanine N2 amino group forming a bifurcated hydrogen bond to a side-by-side Im/Im pair. On the basis of the model of binding depicted in Fig. 15.5b, Lee and collaborators proposed that the Im/Im pair could be

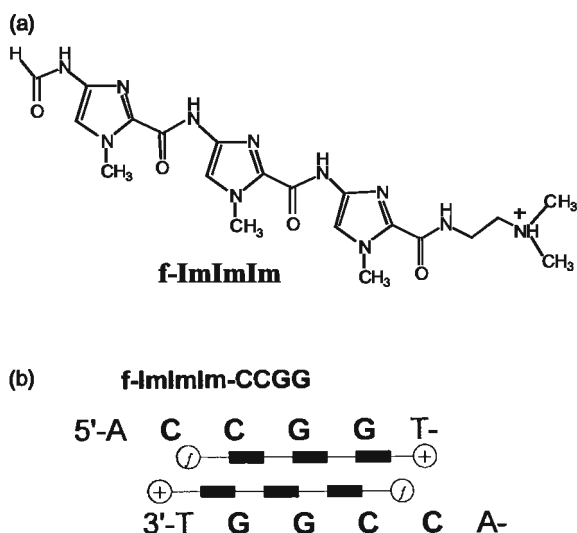


Fig. 15.5. (a) Structure of f-ImImIm and (b) model of a 2:1 complex of f-ImImIm with CCGG, with the triamide stacking in a staggered fashion.

a good motif for recognition of a T·G mismatch. Unlike in a G·C base pair, the G-N2 amino group in a T·G wobble mismatched base pair is not involved in base pairing with thymine (Fig. 15.4). Therefore, the free amino group projects into the minor groove and can form two separate hydrogen bonds to a side-by-side Im/Im pair.

To test the hypothesis, high-resolution NMR was used to probe the binding of f-ImImIm to various DNA sequences containing T·G mismatches. f-ImImIm was titrated with the three self-complementary DNA sequences, 5'-GAACTGGTTC-3', 5'-GAACTGTTC-3', 5'-GAATCGTTC-3'. Each duplex contains two T·G mismatches and complex formation was monitored with NMR methods [27]. The 5'-GAACTGGTTC-3' duplex oligomer has a 5'-***CTGG***-3'-5'-***CTGG***-3' core with adjacent T·G mismatches (the bold and italicized bases represent the mismatched sites). For the 5'-GAACTGTTC-3' duplex, the 5'-***CGTG***-3'-5'-***CGTG***-3' core also has two adjacent T·G mismatched base pairs, but in this case the order of the mismatches is reversed. For the remaining oligomer, the two T·G mismatches within 5'-***TCCG***-3'-5'-***TCCG***-3' core are not adjacent to each other. When compared to the control sequence, 5'-GAACCGGTTC-3', f-ImImIm was found to bind more strongly to CTGG than to CCGG. While it took a ratio greater than 3:1 for f-ImImIm to CCGG to fully form a 2:1 side-by-side ligand–DNA complex [19], it required only a 2:1 ratio of f-ImImIm and CTGG to produce a similar complex [27]. The results show that the Im/Im pair is better at recognizing T·G/G·T mismatches than C·G/G·C base pairs [27]. Even though f-ImImIm binds strongly to CTGG, it interacts weakly to the reverse sequence, CGTG, and more than one set of resonances are observed in the NMR spectra.

In the case of TCCG, no binding was observed when f-ImImIm was added to the solution. These results suggest that the side-by-side dimer of f-ImImIm must adopt a unique structure that matches the shape and conformation of the minor groove of CTGG, but does not fit well into the minor groove of the other two sequences. SPR studies described below, however, do show that f-ImImIm can bind strongly to DNA sequences with a single T·G mismatched base pair.

Two-dimensional (2D) NOE spectroscopy (NOESY) was used to resolve the model of f-ImImIm binding to 5'-GAACTGGTTC-3' (see Fig. 15.6a). All resonances of the 2:1 complex of f-ImImIm and GAACTGGTTC were assigned using 2D NOESY and total correlation spectroscopy (TOCSY) data. Comparison of the f-ImImIm/GAACTGGTTC spectrum with that of f-ImImIm/GAACCGGTTC showed that many of the important intermolecular NOE crosspeaks are conserved in both complexes, e.g. ARH1–C4H1' (peak a), ARH1–T5H1' (C5H1' in the CCGG complex) (peak b), and ARH5-2–G7H3 (peak c). These NOEs plus the ones between the dimethylamine tail and DNA indicate that f-ImImIm binds symmetrically in the DNA minor groove from residue 4 to 8 in the f-ImImIm/GAACTGGTTC complex (Fig. 15.6b).

The T·G mismatch adopts a wobble conformation in the complex that is similar to that seen in isolated T·G mismatches. The T5 moves into the major groove, whereas G16 stays in essentially the same position as that in the f-ImImIm/GAACCGGTTC Watson–Crick complex. The N2 amino group of G16 is not

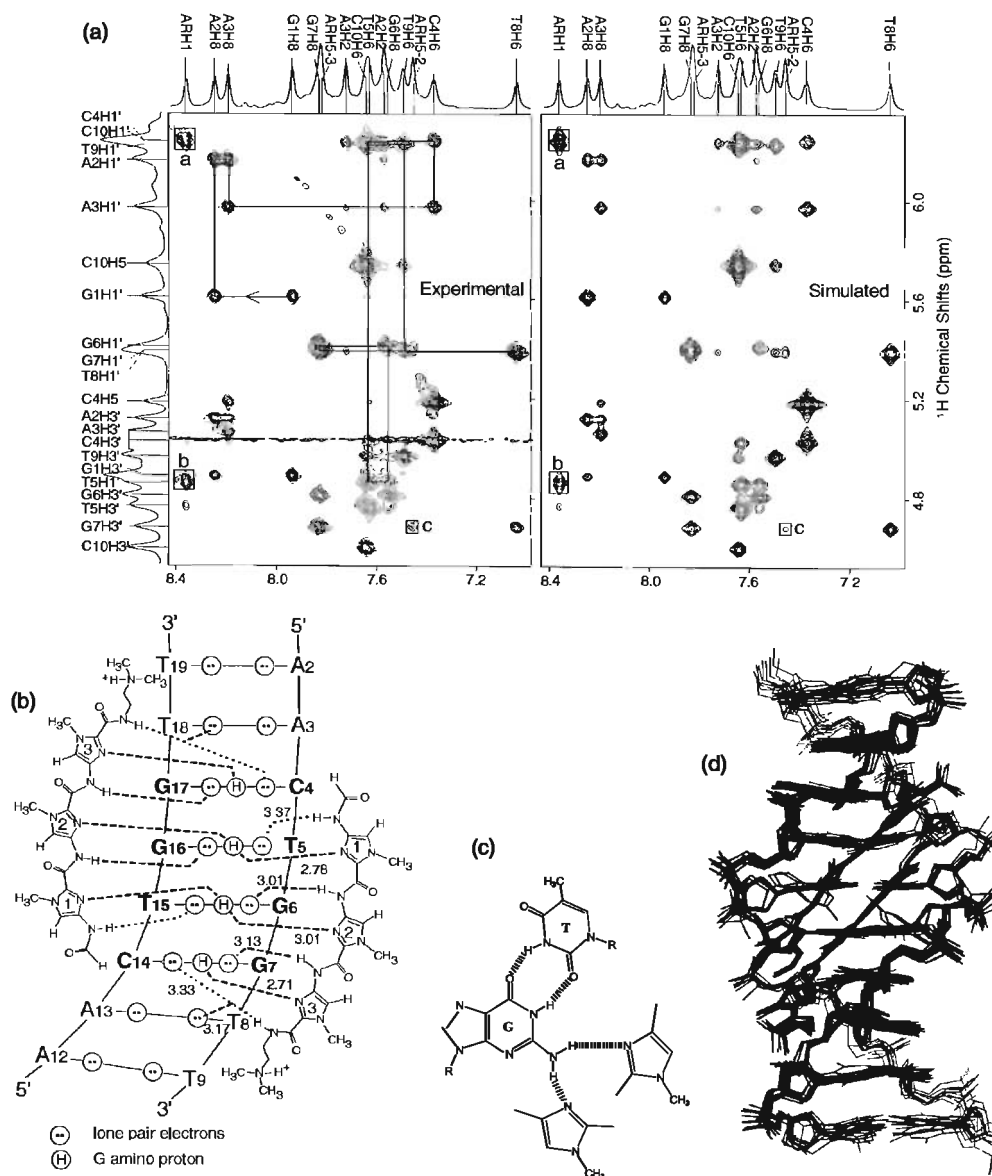


Fig. 15.6. (a) NOESY spectrum of a 2:1 complex of f-ImImIm with CTGGdm. (b) Alignment of the side-by-side complex of f-ImImIm with CTGGdm. (c) A molecular complex of an Im/Im pair with a T-G mismatch. (d) Ten overlaid NOESY restrained molecular dynamics model of the 2:1 complex.

(e)

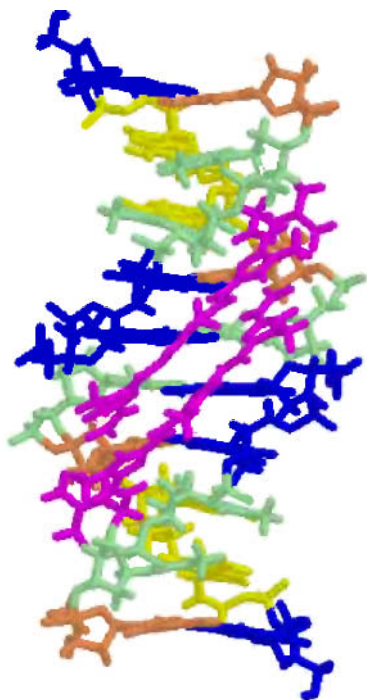


Fig. 15.6. (e) Color view of the average of multiple structures such as those shown in (d). In the color view the polyamide is purple, A bases are yellow, T bases are green, G bases

are dark blue, and C bases are orange. The center of the view is into the minor groove and the GGTC sequence with two T-G mismatched base pairs.

involved in base pairing and forms two hydrogen bonds, each with an imidazole of the Im/Im pair (Fig. 15.6c). In order to maintain stacking between the two f-ImImIm molecules, the imidazole ring on the T5 side cannot go all the way into the cavity generated by T5 moving into the minor groove. This results in the angle of the hydrogen bond between the imidazole nitrogen on the T5 side and one of the G amino protons not being optimized. The Im/Im pair forms two hydrogen bonds with the T-G base pair, instead of one weak bifurcated hydrogen bond as seen in the f-ImImIm/GAACCGGTTC complex [27]. This additional hydrogen bond significantly increases the binding affinity between drug and DNA, making the Im/Im pair a better motif for the recognition of T-G mismatches than for the recognition of C-G. An overlay of 10 refined structures from molecular dynamics calculations with NOE constraints is shown in Fig. 15.6d for the complex of f-ImImIm/GAACTGCTTC [27]. As can be seen, the stacked polyamide dimer fits tightly into the minor groove and is quite well defined except for the terminal base pairs. A color view of the average structure from models such as shown in Fig. 15.6d is shown in Fig. 15.6e to help visualize the DNA minor groove with the stacked f-ImImIm dimer.

15.6

Binding of Imidazole-containing Polyamides to T·G Mismatches through a Dimeric Binding Motif: Thermodynamic and Kinetic Studies

The structural results described above indicate that a side-by-side Im/Im pair could recognize T·G or G·T, thus adding to the pairing rules reported by Dervan and co-workers (Fig. 15.2) [24]. However, the molecular specificity for discrimination of T·G or G·T from Watson–Crick sequences or other mismatches by a side-by-side Im/Im pair cannot be determined from the structural studies. Thermodynamics and kinetics studies can provide the basis for the definition of new recognition rules for polyamides, in this case extended to mismatches in DNA. It is also important for our understanding of molecular recognition of mismatched base pairs to carefully define the energetic and kinetic basis of polyamide-mismatch complexes. The energetic basis of discrimination between the specific mismatch and other mismatches as well as Watson–Crick base pairs will help to predict how well the agent can select the mismatch in the context of a larger DNA sequence.

In this section, the interaction of f-ImImIm and three other imidazole-containing analogs (f-ImIm, f-ImPyIm, and f-PyImIm, Fig. 15.7a) with Watson–Crick and mismatch base pair containing DNA sequences (Fig. 15.7b) is described. The DNA binding properties of the polyamides were determined using a combination of BIACORE surface plasmon resonance (SPR)-biosensor, thermal melting (T_m), and CD titration studies [38, 39]. The use of SPR for studying the interactions of polyamides with DNA is advantageous because the interactions are studied in real-time. Both thermodynamics (binding constants) and kinetics (k_a and k_d) results can be obtained from each experiment. The kinetic information is particularly valuable and until now has been difficult to obtain for polyamides due to their weak spectroscopic signals [57].

The hairpin DNA sequences depicted in Fig. 15.7b were selected to satisfy several criteria. Upon folding they contain a GC-rich core of four base pairs that can be recognized by the Im/Im, Py/Im, or Im/Py pairs depending on the polyamide and DNA sequences. This DNA core is flanked by A·T and T·A base pairs that favor interactions of the cationic tails of the dimer complexes that point away from the heterocyclic stack. CCGG [19] and CTGGdm [27] have been used in the structural studies described above with f-ImImIm, and they serve as excellent reference sequences for the quantitative analysis of polyamide–DNA complexes. Studies with oligomers that contain a single T·G mismatch were designed to determine the level of selectivity of f-ImImIm for single and double T·G mismatched base pairs. The A·G and G·G mismatched sequences were selected to determine how well the Im/Im pair was able to distinguish T·G from other G containing mismatches.

15.6.1

Stoichiometry of Complexes

The stoichiometry of binding of f-ImImIm to CG-containing sequences was determined as 2:1 as expected for a stacked dimer [19, 22, 23, 24]. Titration of f-ImImIm

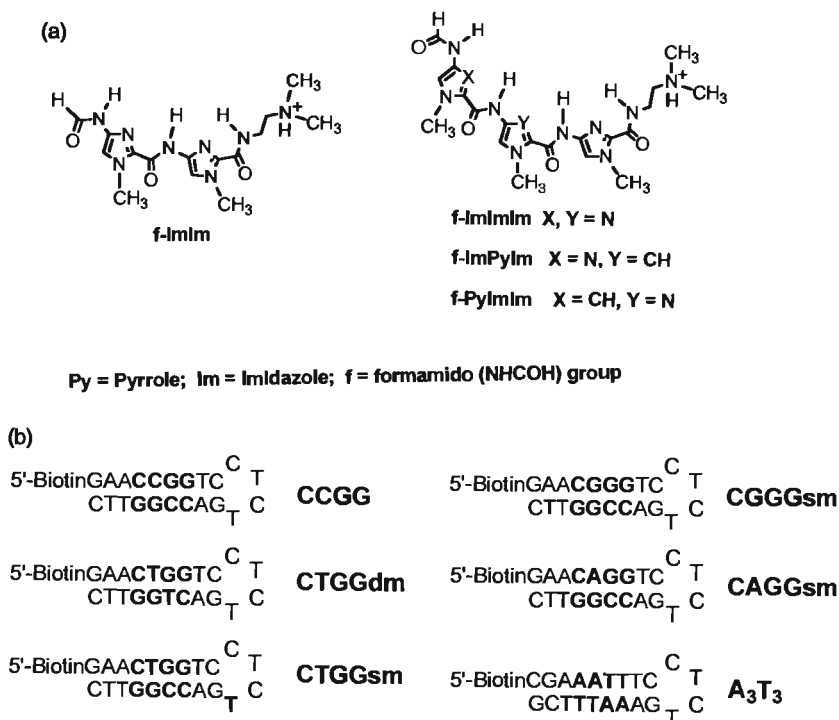


Fig. 15.7. (a) Chemical structures are shown for the polyamides f-ImImIm, f-ImPyIm, f-PyImIm, and f-ImIm. (b) Sequences of the DNA hairpins used in this study are shown:

CCGG and A₃T₃ are Watson–Crick sequences; CTGGdm contains two T–G mismatches; CTGGsm, CCGGsm, CAGGsm contain T–G, G–G, and A–G mismatches, respectively.

to CCGG and CTGGdm gave induced CD bands above 300 nm (data not shown), and such bands are indicative of minor groove binding. However, the intensity of the induced band was significantly larger for CTGGdm than CCGG at similar concentration of ligands and DNA. Plots of our CD results with CCGG and CTGGdm (Fig. 15.8a) clearly show a break at a 0.5 ratio of DNA to compound as expected for a 2:1 complex of polyamide to DNA.

SPR results with CCGG, CTGGsm, CTGGdm, CAGGsm, and CGGGsm (see Fig. 15.8b) have saturation response values that are twice the expected value for monomer complexes, again confirming the 2:1 stoichiometry with these DNA sequences. Similar results are observed in SPR experiments for the interaction of f-ImPyIm and f-PyImIm with CCGG, CTGGsm, CAGGsm, and CGGGsm. Binding affinities for the sequences vary widely and saturation was not reached for some complexes until high concentration. The set of sensorgrams in Fig. 15.8b at 5 μ M f-ImImIm, for example, shows that at this concentration each sequence reaches a different level of binding. The signal is larger at this fixed concentration for the sequence with higher affinity, CTGGdm, as expected. However, at significantly higher concentrations of f-ImImIm each of the sequences reaches satura-

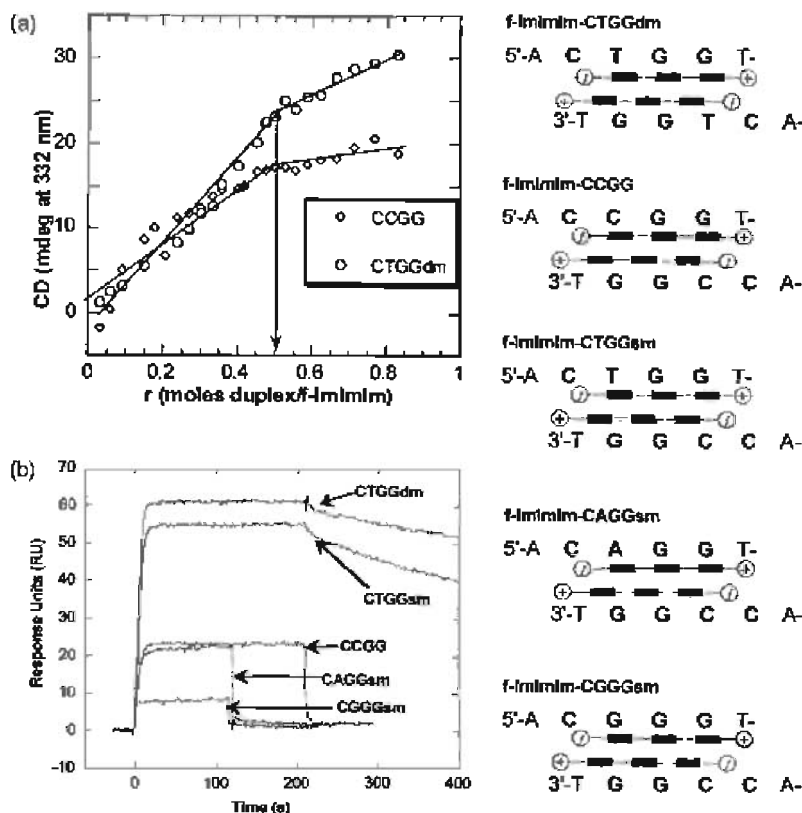


Fig. 15.8. (a) Titration curves generated by plotting the induced CD signal at 332 nm after addition of the CCGG and CTGGdm duplexes to an f-ImImIm solution. The solutions were prepared in phosphate buffer (10 mM sodium phosphate pH 7.2, 50 mM NaCl, 0.25 mM EDTA). (b) Comparative SPR sensorgrams for the interaction of f-ImImIm with CTGGsm, CTGGdm, CCGG, CAGGsm, and CGGGsm. The experiments were done at 25°C and the

concentration of f-ImImIm was 5×10^{-6} M in MES20 (0.01 M 2-(*N*-morpholino) ethanesulfonic acid pH 6.25, 0.001 M EDTA and 0.2 M NaCl). The injection volume for CAGGsm and CGGGsm was 20 μ L, while for the other sequences it was 35 μ L. The right-side panel shows models for the dimeric side-by-side binding of f-ImImIm to different DNA sequences in the staggered conformation.

tion with similar final SPR response values in RU [39]. The maximum response obtained for these sequences corresponds to twice the response predicted for binding of one monomer of the polyamide, again as expected for 2:1 binding stoichiometry.

Thermal melting experiments also agree with these results. Melting curves are biphasic and monophasic at ratios 1:1 and 2:1 moles of compounds per mole of DNA hairpin for those compounds showing significant increase in melting temperature [39]. Distamycin showed 2:1 binding to A₃T₃ in agreement with previous reports [4]. Both SPR and thermal melting experiments thus support a dimer

binding model and 2:1 binding of the polyamides to their DNA recognition sequences.

15.6.2
Binding Constants and Cooperativity

From the results obtained in SPR experiments, the DNA-binding constants for the compounds with six different hairpin oligonucleotides were determined by fitting the data as previously described [38, 39]. The square root of the product of the macroscopic association binding constants ($K_1 \ast K_2$) or the K_1 (when the best fit was a one-site model) are shown. Because of correlation between K_1 and K_2 , the error in fitting individual values of K_1 and K_2 is larger than for the product, $K_1 K_2$, for dimer binding. For the most accurate comparison of the binding of polyamides to DNA in this work, $(K_1 \ast K_2)^{1/2}$ values are reported in Tab. 15.1. By reporting the square root, all results are reported on a per bound molecule basis and comparison between binding of monomers and dimers, as well as comparison with literature results, can be made directly.

The results presented in Tab. 15.1 are striking, and they show that f-ImImIm interacts with the highest affinity and specificity for the T·G mismatch-containing

Tab. 15.1. Equilibrium association constants,^a K (M^{-1}), for binding of polyamides to the DNA sequences shown in Fig. 15.1.

Compound	CCGG	CTGGdm	CTGGsm	CAGGsm	CGGGsm	A ₃ T ₃
f-ImImIm	2.1×10^5	1.2×10^7	6.5×10^6	1.9×10^5	7.8×10^4	5.9×10^3
f-ImPyIm	2.4×10^5	2.0×10^4	1.3×10^5	1.6×10^4	3.7×10^4	3.8×10^4
f-PyImIm ^c	8.3×10^5	2.5×10^4	2.3×10^5	2.4×10^5	6.1×10^4	2.5×10^4
PyImIm ^{b,c}	9.3×10^2		2.8×10^3			$< 9 \times 10^2$
Distamycin	4.1×10^4	4.7×10^3	1.2×10^4	1.5×10^4	1.2×10^4	1.7×10^7
f-ImIm	6.0×10^3	6.9×10^3	1.0×10^4	3.4×10^3	2.3×10^3	3.1×10^3

^aThe equilibrium constants reported here are defined as the square root of the product of equilibrium association constants, $K = (K_1 \ast K_2)^{1/2}$. K_1 and K_2 were obtained by fitting the SPR binding curves at the steady-state level versus concentration of polyamide [38]. The results were obtained from SPR experiments using a BIACORE 2000 at 25°C in MES ([2-(*N*-morpholino)ethanesulfonic acid] buffer, pH 6.25, containing 0.2 M NaCl. The errors for the constants in this table are <10% for the constants over 1×10^5 , 20% for the ones from 1×10^5 to 3×10^4 and 20–40% for the ones below 3×10^4 .

^bDue to low binding of PyImIm to these DNA sequences very high concentrations of the compound were necessary to obtain a response and a limited number of points were available for analysis. In this case the binding constants were approximated by calculating the microscopic binding constant using the model for equivalent and independent sites where the number of sites is equal to two. We took the average of the results corresponding to about five different concentrations.

^cThe binding constants of f-PyImIm and PyImIm to CCGG are $61.3 \times 10^3 M^{-1}$ and $< 9 \times 10^2 M^{-1}$, respectively.

sequences. The f-ImImIm-binding constants to T-G mismatch sequences are similar to binding constants for the binding of distamycin to the canonical A₃T₃ sequence. The binding of f-ImImIm is highly cooperative, with K_1 ($2.9 \times 10^6 \text{ M}^{-1}$) about 16 times lower than K_2 ($4.9 \times 10^7 \text{ M}^{-1}$) for CTGGdm, and 10 times lower for CTGGsm, as determined by steady-state SPR measurements. The polyamide clearly prefers to bind to its recognition sequence with T-G mismatch base pairs as a stacked dimer.

f-ImImIm binds more strongly to CTGGdm and CTGGsm, over 50- and 30-fold more strongly, than to the target canonical sequence CCGG. It also binds almost 60 and 200 times more tightly to CTGGdm than to the A-G (CAGGsm) and the G-G (CGGGsm) mismatch-containing sequences, respectively. Finally, f-ImImIm binds more than 2000-fold and 1100-times tighter to CTGGdm and CTGGsm, respectively, than to the Watson-Crick A₃T₃ sequence, showing the preference of the side-by-side Im/Im pair for a T-G mismatch over C-G, G-C, T-A, or A-T base pairs and over A-G or G-G mismatches. In evaluating the influence of the terminal formamido group in the binding of polyamides to both T-G mismatch as well as Watson-Crick base-paired DNA, it is observed that the imidazole-rich polyamides containing this group prefer to stack in a staggered mode (Fig. 15.3). Polyamides lacking the formamido group stack, however, in the overlapped dimer motif [39]. In both stacking modes the heterocyclic rings of one molecule tend to stack over the amide groups of the other molecule of the dimer (Fig. 15.3).

Results from SPR studies on a diimidazole compound, f-ImIm, provide important information on the number of heterocyclic units required to selectively recognize T-G base pairs. Comparison of the binding constants of the diimidazole and the triimidazole shows that at least three imidazole rings per monomer are necessary to bind strongly to the T-G mismatch containing sequences studied here. On the other hand, f-PyImIm and f-ImPyIm show very low affinity for any of the mismatches, but they have better affinity to Watson-Crick sequences. f-ImPyIm and f-PyImIm demonstrate good affinity for the CCGG sequence with binding constants ~ 1.2 and ~ 4 times higher than the corresponding binding of f-ImImIm, but their binding affinities decrease as the number of mismatches increases. The results strongly suggest that polyamides containing a terminal formamido group, f-ImPyIm and f-PyImIm, prefer to stack in a staggered mode that produces Py/Im and Im/Py pairing to recognize C-G and G-C base pairs, respectively. It is worth noting that f-ImPyIm and f-PyImIm could physically stack in the completely overlapped mode forming Im/Im pairs similar to the non-formamido analog (Fig. 15.3) thereby gaining some binding affinity to the CTGGsm sequence. However, this mode of DNA interaction must not be thermodynamically favorable for binding to the CTGGsm oligomer because the binding constants are about 2- to 3.5-times weaker than those for binding to CCGG.

The high preference of the tripyrrole, distamycin, for AT-rich sequences is well known [1, 6]. Our SPR results show that distamycin binds strongly as a dimer to A₃T₃ with negative cooperativity. The imidazole polyamide, f-ImImIm, binds with positive cooperativity to the mismatched sequences CTGGdm, CTGGsm as well as to the matched sequence, CCGG. The cooperativity can be seen in Scatchard plots

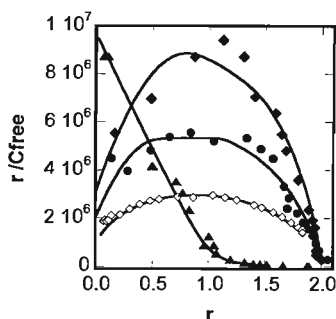


Fig. 15.9. Scatchard plot (r/C_{free} versus r) for the interaction of f-ImImIm with CTGGsm (filled circles), CTGGdm (filled diamonds), CCGG (open diamonds), and the interaction of distamycin with A_3T_3 (solid triangles). Values of r correspond to moles of compound per mole of DNA hairpin and were calculated on the basis of SPR data. C_{free} corresponds to the concentration of compound injected through

the sensorchip flow cells. Values of r/C_{free} for distamycin- A_3T_3 and f-ImImIm-CCGG complexes are multiplied by factors of 0.05 and 20, respectively, to allow visualization on the same plot. The smooth curves correspond to the data simulated on the basis of the equilibrium constants obtained by direct fitting.

(see Fig. 15.9) and by comparison of the binding constants K_1 and K_2 obtained from fitting of binding results. While K_2 ($4.9 \times 10^7 \text{ M}^{-1}$) is greater than K_1 ($2.9 \times 10^6 \text{ M}^{-1}$) by 16 times for the binding of f-ImImIm to CTGGdm, in the binding of distamycin to A_3T_3 , K_2 ($8.0 \times 10^5 \text{ M}^{-1}$) is ~ 390 times lower than K_1 ($3.1 \times 10^8 \text{ M}^{-1}$). This different cooperative behavior can be explained in terms of sequence-dependent minor groove width. It has been shown that AT-rich sequences of DNA have a narrower minor groove than GC-rich sequences [59–62]. When the first molecule of distamycin binds to A_3T_3 it fits nicely in the minor groove, but to bind the second molecule the minor groove has to widen, as observed previously [2, 4], with an associated energetic penalty and negative cooperativity. On the other hand, when the first molecule of f-ImImIm binds to any of the GC-rich sequences the molecule is not thick enough to make optimum van der Waals contacts with both walls of the minor groove. Upon binding of the second molecule of f-ImImIm the contacts are more favorable and a very tight complex with positive cooperativity is formed.

15.6.3

Kinetic Studies

Figure 15.10 shows typical examples of the SPR sensorgrams obtained for the interaction of f-ImImIm with three DNAs, the Watson–Crick CCGG sequence and the sequences with single and double T–G mismatches. A dramatic difference in rates of association and dissociation from the mismatch relative to the matched base pair sequence is easily visualized by eye (note the different time scales in the plots). Figure 15.11 also shows a visual comparison of the kinetic behavior for the

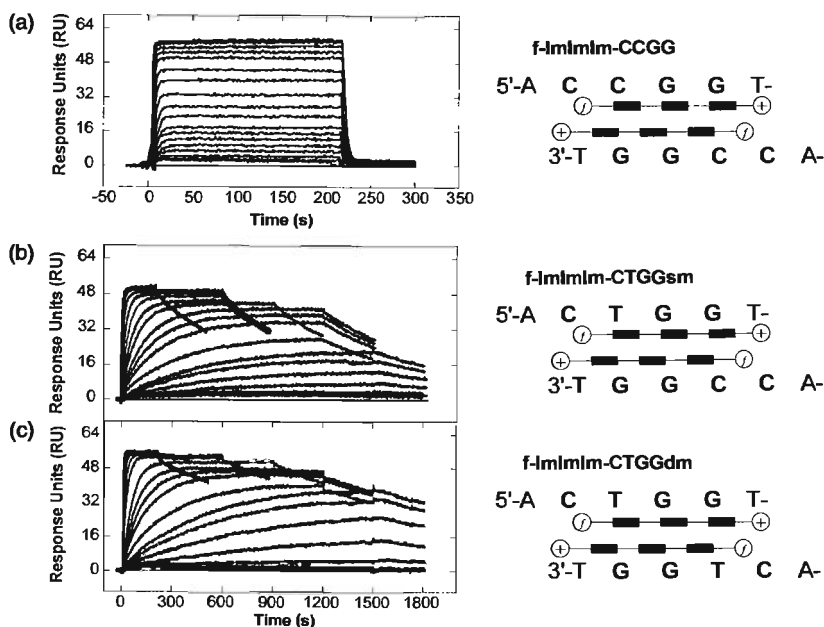


Figure 15.10. SPR sensorgrams for the interaction at 25°C of f-ImImIm with CCGG (a) CTGGsm (b), and CTGGdm (c). The concentration of the unbound polyamide varied from 7.5×10^{-7} M to 2.6×10^{-5} M in (a), from 1.0×10^{-9} M to 2.0×10^{-6} M in

(b), and 4.0×10^{-6} M in (c). All the experiments were done in MES20. The right-side panel shows the models for the dimeric side-by-side binding of f-ImImIm to CCGG, CTGGsm, and CTGGdm in the staggered (preferred) conformations.

binding of f-ImImIm to different DNA sequences. The binding behavior of different compounds with the sequence containing two TG mismatches, CTGGdm is compared in the figure. At low polyamide concentrations the association of f-ImImIm to both CTGGdm and CTGGsm is slow. At high concentration of the polyamide the association phase is much faster as expected for multimolecular kinetics. The dissociation from CTGGdm is not affected by concentration as would be expected for first order dissociation kinetics for the trimolecular complex. It can also be observed that both the association and dissociation reactions of f-ImImIm with CCGG are fast at all concentrations of polyamide showing that this is not a preferred recognition sequence for the compound. Similarly, f-PyImIm and distamycin show fast kinetic behavior when binding to CTGGdm and a relatively low RU response is observed at concentrations as high as 100 μ M (see lines in green and red in Fig. 15.11).

It is evident from these SPR results that the enhanced binding affinity of f-ImImIm to T-G mismatches is, in large part, a result of the slow dissociation of f-ImImIm from DNA oligomer duplexes that contain T-G mismatches. This highly stable f-ImImIm-T-G complex presumably is due to an optimum fit with favorable hydrogen bonding interactions between the exocyclic guanine N2 amino

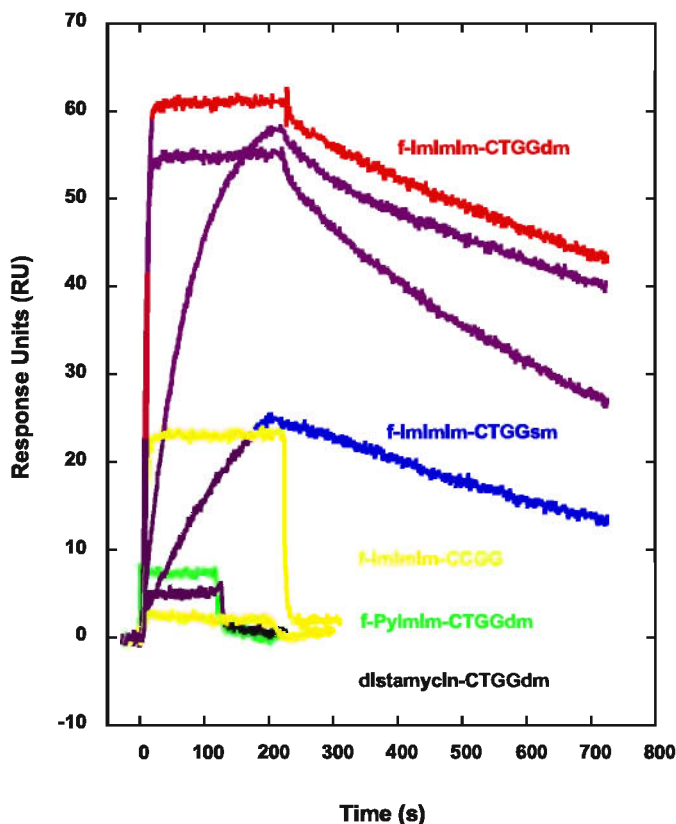


Fig. 15.11. Kinetic behavior of f-ImImIm binding to different sequences and different polyamides binding to CTGGdm. The lines in red show the behavior of f-ImImIm at high (5 μ M) and lower (0.75 μ M) concentration when binding to CTGGdm. The lines in blue and

yellow show the corresponding curves for the binding of this compound at the same concentrations to CTGGsm and CCGG, respectively. The lines in green and black show the binding of f-PyImIm and distamycin at 100 μ M to CTGGdm.

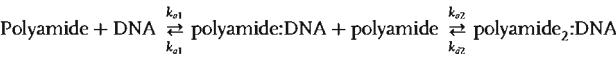
group and the imidazole moieties as shown in the structural studies described above. The results given in Tab. 15.2 illustrate that f-ImImIm has similar kinetic behavior in binding to CTGGdm and CTGGsm. The dissociation rate is slightly faster and the binding constant correspondingly lower for the latter sequence with a single mismatch. The dissociation of f-ImImIm from T·G mismatch-containing oligomers is so slow that at the end of each SPR experiment it is necessary to inject a solution of 10 mM glycine at pH 2 to promote complete dissociation of the compound from the surface through disruption of the DNA hairpin structure.

The association of the first molecule of the f-ImImIm polyamide into its dimer complex with T·G DNA sequences is slower than the first molecule of distamycin complex binding as a dimer to its AT recognition sequence. This is compensated, however, by an increase in the rate of association of the second molecule of

Tab. 15.2. Kinetics association and dissociation rate constants^a for the interaction of polyamides with DNA.

Compound	DNA sequence	$k_{a1}(M^{-1} s^{-1})$	$k_{d1}(s^{-1})$	$k_{a2}(M^{-1} s^{-1})$	$k_{d2}(s^{-1})$	$K_{eq}(M^{-1})^b$
f-ImImIm	CCGG ^c		>1		>1	
	CTGGdm	8.1×10^4	0.59	2.6×10^5	0.0006	7.7×10^6
	CTGGsm	8.2×10^4	0.70	3.5×10^5	0.0013	5.6×10^6
f-PyImIm	CCGG	7.0×10^4	0.58	2.3×10^5	0.030	9.9×10^5
Distamycin	A ₃ T ₃ ^d	2.3×10^5	0.002	$\sim 3.5 \times 10^4$ ^e	0.020	

^a k_a and k_d are the association and dissociation rate constants and are defined according to the following model:



^bEqual to the square root of the product of equilibrium association constants, $(K_1 * K_2)^{1/2}$ calculated from the kinetic parameters (k_a and k_d).

^cThe complex is totally formed/dissociated in under 10 s and the process is too fast to be followed by this technique.

^d k_d was obtained by fitting the dissociation phase of the sensorgrams with the 1:1 (Langmuir) binding model in the BIA evaluation program. k_a was calculated from the slope of k_{obs} (observed association rate) versus concentration. k_{obs} was calculated by fitting the association phase with the same model.

^eEstimated as the product of K_2 (macroscopic association constant for the second molecule) from steady-state measurements and k_{d2} .

f-ImImIm into the TG dimer. Because of the cooperative stabilization of the f-ImImIm–T-G dimer complex described above, the first f-ImImIm to dissociate from the dimer has a lower dissociation rate constant than the first molecule of distamycin to dissociate from its dimer complex with the A₃T₃ duplex. The slow dissociation kinetics result in a very stable complex of f-ImImIm with DNA sequences that contain T-G mismatched base pairs. Distamycin sensorgrams reveal that at ratios lower than one, under 20 response units in the sensorgram, there is only one slow phase of dissociation while at higher ratios there is a fast phase followed by the slower phase [39]. This agrees with previous observations that distamycin forms a 1:1 complex with this sequence at ratios lower than one and the 2:1 complex is predominant at high concentration corresponding to ratios higher than one. Comparison of the sensorgrams corresponding to f-ImImIm binding to mismatch sequences relative to Watson–Crick sequences (Figs 15.8b and 15.10) reveals the stability of the complexes and their very slow dissociation rates. This is a factor of great advantage in the competition of a potential drug with cellular components. Clearly the kinetics of f-ImImIm binding to DNA recognition sequences with T-G mismatched base pairs are unique.

Fast association and dissociation rates are observed in the interaction of f-ImImIm with all other sequences, including the A-G and G-G mismatch-containing sequences (Fig. 15.7b). f-PyImIm, f-ImPyIm, and distamycin associate

and dissociate very rapidly with CTGGdm and CTGGsm, and in general from all the other non-recognition DNA sequences. In the cases where there is a match between the polyamide and DNA, f-PyImIm with CCGG and distamycin with A₃T₃, for example, the kinetics of dissociation are significantly slower than with non-cognate DNA sequences. The f-ImImIm polyamide complex with T·G mismatch-containing DNA sequences is an unusual case with exceptionally slow kinetics of dissociation. Clearly, this complex is very stable with many favorable contacts such that even the relatively small polyamide trimer has very slow dissociation kinetics.

15.7

Developing Molecules Capable of Recognizing Mismatches in DNA

Two recent publications show the development of molecules capable of recognizing DNA mismatches [51, 52]. One compound is a cleaving agent that recognizes destabilized regions on the DNA [51] and was able to recognize a single mismatch in a 2725-base-pair linear plasmid. However, compounds with more specificity for the different mismatches are needed to readily identify single nucleotide polymorphisms. Another new agent is capable of recognizing G·G mismatches and the compound has low affinity for the other known mismatches [51].

The data presented in this chapter show that a side-by-side Im/Im pair can specifically recognize T·G mismatched base pairs in DNA; thus, adding to the recognition rules of the polyamides and adding a new powerful agent for the identification of one of the most common DNA mismatches [55]. When one Im/Im pair is formed during the stacking of polyamides, the stacked dimer can recognize a T·G mismatched base pair and distinguish it from Watson–Crick G·C, C·G, A·T, or T·A base pairs, as well as G·G and G·A mismatches. Another feature that is worth noting is that the polyamides should have at least three heterocycles in order to show satisfactory affinity for DNA sequences. This observation is in agreement with results from other polyamides systems [58]. The inclusion of one extra imidazole unit in f-ImImIm versus f-ImIm increases the affinity by ~35-fold for CCGG, and by ~650-fold for CTGGsm. Thus, both the affinity and specificity of f-ImImIm are improved relative to f-ImIm.

Our results suggest that the compounds that do not form Im/Im pairs in the staggered stacking mode (f-ImPyIm and f-PyImIm) are not able to recognize the T·G mismatch and their preference is for canonical DNA. Small molecules that have the potential for incorporation into an automated analysis for SNPs have been developed recently. Very different compounds that recognize G·G [51] or T·G [38] mismatches are now ready for development as agents in automated SNP analysis for these two mismatched base pairs. Even though these two mismatches account for formation of a large number of SNPs in the human genome, however, additional compounds are needed to selectively recognize other mismatches for SNP identification. The success of these initial compounds suggests that research in this area will yield additional mismatch recognition agents in the near future.

15.8

Use of the T-G Recognition Motif by Im/Im Pairs to Probe the Effects of the Terminal Head Group on the Stacking of Polyamides

As described above, examination of 2:1 complexes of polyamides and DNA revealed that the nature by which the heterocyclic units stack can directly impact their sequence recognition [39]. As described above, a terminal formamido group can shift stacking in the polyamide system from fully overlapped, without a formamido, to an offset or staggered motif (Fig. 15.3). This shift in stacking affects the DNA sequence recognized by polyamides and it is essential to define the stacking mechanisms in detail in order to understand mismatch base pair recognition by polyamides. As shown in Fig. 15.3, when the stacking of PyImIm shifts from fully overlapped to staggered, the DNA sequence that is recognized changes completely. This will give two entirely different sets of DNA sequences recognized for polyamides of the same composition and order of heterocycle units. This has both fundamental significance for understanding molecular recognition and practical significance for development of compounds to recognize specific sequences of DNA. The binding of both the formamido and nonformamido compounds to the CCGG and CTGG sequences was evaluated in real time using surface plasmon resonance (SPR) methods to test the overlap and staggered models in Fig. 15.3a.

Figure 15.12a shows typical SPR sensorgrams for f-PyImIm binding to CCGG. As with the f-ImImIm results described above, RU values obtained at high concentration with f-PyImIm reveal that binding to CCGG reaches saturation when two polyamide molecules are bound. This is consistent with 2:1 stacked polyamide complex as expected for GC containing DNA sequences. As illustrated in Fig. 15.12b, it is clear that the non-formamido trimer, PyImIm, binds very poorly to CCGG but significantly more strongly to the mismatch sequence. This result is consistent with the formation of an overlapped stack dimer for the non-formamido polyamide (Fig. 15.3). Typical SPR binding isotherms and best fit binding curves for the interaction of f-PyImIm with CCGG and CTGGsm are shown in Fig. 15.12c. The equilibrium binding constants were determined by fitting the response units (RU) in the steady-state region as described [38, 39]. Consistent with the preferred staggered motif of f-PyImIm (Fig. 15.3), the results given in Tab. 15.1 show that it bound more tightly to the matched CCGG than the mismatched CTGGsm sequence. It should be noted that f-PyImIm could recognize CTGGsm by sliding to an overlap stack with an Im-Im pairing. The enhanced binding of f-PyImIm to CCGG over CTGGsm strongly supports its preference for the staggered stacking mode. However, for PyImIm, the enhanced binding to CTGGsm over CCGG, due to the favorable interaction between an Im-Im pairing and a T-G mismatch, is consistent with its preference for an overlapped stacking mode as shown by Dervan and co-workers with many non-formamido polyamides. Neither the formamido nor the non-formamido derivatives are predicted to bind well to a CCGG sequence and weak binding to that sequence is observed.

The fitting results also demonstrated that the binding of f-PyImIm to CCGG is cooperative with K_2 about 20 times larger than K_1 . This is in agreement with the

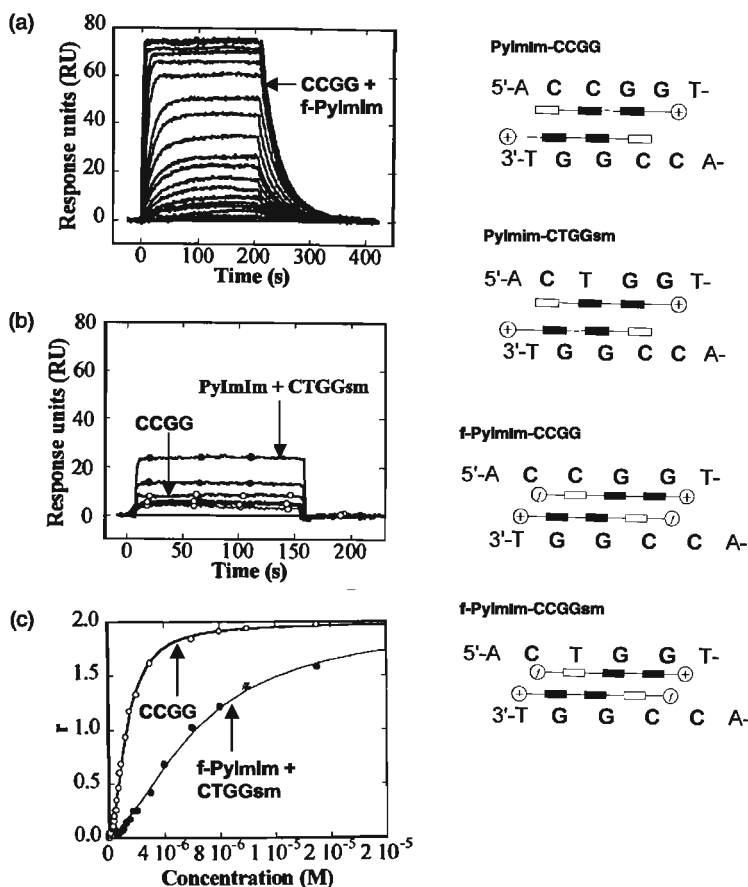


Fig. 15.12. (a) SPR sensograms for the interaction of f-PylmIm with the CCGG sequence in MES20 and at 25°C. The concentration of unbound f-PylmIm varies from 0.04 μ M to 20 μ M. (b) Interaction of PylmIm with CCGG (open circles) and CTGGsm (black dots) at 60, 120, and 200 μ M. (c) Best fit for r (moles of

compound per mole of hairpin) values for CCGG (open circles) and CTGGsm (black dots) versus the concentration of the unbound f-PylmIm. The smooth lines in (c) were obtained by non-linear least-square fit of the data to a two-site binding model.

studies described above and with minor groove-stacked systems that have shown cooperative binding in dimer DNA complexes [19, 21, 63]. The formamido group can stack with the other polyamide units as well as interact with DNA through H-bonds and van der Waals interactions, and it is clearly positioned well at the N-terminus of the polyamide to form such interactions. It is thus not surprising that the formamido group drastically increases the affinity of polyamides for DNA. The SPR results are also supported by changes in melting temperature (T_m). f-PylmIm produced a 3–4°C T_m increase with CCGG but no detectable T_m increase with

CCGG. PyImIm did not give any significant T_m increase with CCGG, CGCG, or CTGGsm.

In summary, all of our results clearly demonstrate that the presence of a terminal formamido group in polyamide analogs of distamycin strongly affects their DNA sequence recognition and binding affinity properties. As proposed in Fig. 15.3, our experimental results shown that while non-formamido polyamides prefer an overlapped stacking mode, the *N*-formamido polyamides prefer to bind to DNA in a staggered mode. The DNA sequences recognized by polyamides can, thus, change completely depending on whether the polyamide has a terminal formamido group or not.

15.9

Future Directions

15.9.1

Polyamide–DNA Complexes

Polyamides, from natural products such as distamycin and netropsin to newer synthetic agents, are a very successful family of compounds for the sequence-specific recognition of DNA. The ability of these compounds to form stacked dimers in the DNA minor groove allows them to read sequence information from both strands of the double helix and greatly expands their sequence recognition capability. The emphasis to date in the development of these compounds has been on optimizing methods to link the two component molecules of the dimer, for example, to form hairpin polyamides that have been extensively studied by the Dervan group.

There are, however, many important questions about the stacked dimer motif that remain to be answered. For example, the *N*-terminal substituents clearly have a very significant influence on both polyamide stacking and DNA sequence recognition and a full understanding of polyamide DNA recognition requires a better understanding of substituent effects on dimer formation and stacking. It seems quite likely that substituents on the polyamide system, such as the hydroxy group added to the pyrrole heterocycle to provide enhanced recognition of AT base pairs, can be used to “tune” the stacking and recognition of DNA by these molecules.

In the same manner, the DNA recognition specificity of the polyamides is critically dependent on their heterocyclic components, but to date only a few different heterocycles have been successfully incorporated into the polyamides and studied in detail in complex with DNA. Exploration of a much broader heterocycle space in the polyamide context may lead to significant improvements in their ability to select specific DNA sequences as well as identify mismatched base pairs and SNPs in genomic DNA.

15.9.2

Mismatched Base Pair Recognition

As described in this chapter, there are now quite successful agents for the identification of T-G and G-G base pair mismatches in the context of Watson-Crick DNA base pair sequences. These agents have great potential for development of methods to identify SNPs in human populations but such development will require automation of the detection methods. The agent for G-G recognition has been successfully attached to BIACORE sensor chips and this provides a development route for rapid and automated sample analysis. The f-ImImIm polyamide described in this chapter provides excellent T-G mismatch base pair recognition in a Watson-Crick context.

In order to use the f-ImImIm polyamide in an automated manner, several factors must be addressed. First, the two f-ImImIm units of the dimer must be linked to form a covalent dimer. This will prevent dissociation of the dimer during analysis as well as providing enhanced affinity for T-G mismatch base pairs. Second, the ability of the f-ImImIm polyamide to recognize single T-G base pairs in Watson-Crick sequences must be probed in a broader sequence context. The question of recognition specificity in many different sequence contexts must be addressed for application of the agent to human populations. Third, methods to automate T-G detection with the f-ImImIm dimer must be developed. Covalent linking of an f-ImImIm dimer to BIACORE sensor chips is an attractive possibility that would allow automated analysis of samples. Clearly development of these reagents is an important priority as our knowledge of gene sequences and their variations increases.

Although the T-G base pair is the most common mismatch leading to mutation in human DNA, development of agents to recognize other mismatched base pairs is essential. The compound that selectively recognizes G-G mismatches does so through direct stacking in the double helix and formation of hydrogen bonds with the two G bases of the mismatch. Groups on the G bases that are normally involved in pairing with C are used in complex formation with the synthetic agent. The f-ImImIm polyamide described in this chapter recognizes T-G base pairs in a double helix through its ability to fit into the T-G wobble base pair geometry and hydrogen bond with the guanine N2 amino group in the minor groove. These very different recognition mechanisms have similarities to classical intercalation for G-G recognition and to minor groove binding agents for T-G base pair recognition. More detailed analysis of these reagents and their complexes will hopefully suggest additional variations on these themes or entirely new agents for selective recognition of mismatches.

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Dedication of this Chapter to Professor J. William Lown on the Occasion of his Retirement

A symposium to honor Professor J. William Lown was co-organized by Professors William H. Gmeiner of Wake Forest University School of Medicine and Moses Lee of Furman University and held March, 2001 on the campus of Wake Forest University School of Medicine. Both Professors Gmeiner and Lee were post-doctoral fellows in the laboratory of Professor Lown in the Chemistry Department of the University of Alberta. Among the distinguished scientists who attended the Lown Symposium were Professor Peter Dervan (California Institute of Technology), Professor Richard Dickerson and Mary Kopka (UCLA), Professor Sidney Hecht (University of Virginia), Professor Michael Waring (Cambridge University), Professor David Wemmer (UC Berkeley), Professor David Boykin (Georgia State University), Professor John Hartley (University of London), Professor David Thurston (University of London), Professor Luis Marky (University of Nebraska Medical Center), Dr Chris Micjeda (NCI), Professor Ramaswamy Sarma (University at Albany), and Professor Terry Beerman (Roswell Park Cancer Institute).

Professor J. William Lown was born in Blyth, England and received the PhD and DIC degrees in organic chemistry at the Imperial College of Science, Technology and Medicine under the direction of Dr Lloyd Jackman (models for NADPH hydrogen transfer). After this he was a lecturer at Imperial College then, on a leave of absence, was a postdoctoral research fellow with Professor Raymond U. Lemieux at the Department of Chemistry, University of Alberta, Canada, where he worked on the synthesis and conformational characteristics of nucleotide-based coenzymes. He then spent a year in the Biophysics Division of Walter Reed Research Institute in Washington, D.C. Dr Lown joined the faculty of the Department of Chemistry, University of Alberta in 1964 and began his independent research career. His early training in nucleoside chemistry and NMR-based structural studies led to his long-term interest in DNA-targeted anticancer agents. Dr Lown developed polyamides as anticancer and anti-HIV-I agents and has examined the cellular uptake and subcellular distribution of polyamides. He studied the relation of the DNA sequence selectivity of the polyamides to their biochemical effects, *inter alia* inhibition of topoisomerases, and, in turn, their anticancer cytotoxicity. The research effort is multidisciplinary and encompasses computer-based drug design, synthesis, footprinting, bioassays, and structural analyses of drug-receptor complexes. Such complementary techniques have been applied to the study and development of more effective glycopeptide anticancer agents in the treatment of human head and neck carcinomas and the design of novel protein kinase C inhibitors.

Dr Lown was appointed Professor of Organic Chemistry in 1974, was Alberta Heritage Foundation for Medical Research Professor-in-Residence for 1984–85 and was appointed McCalla Research Professor in 1988. He has delivered over 250 invited lectures and has published over 400 research papers in refereed journals. Professor Lown was awarded the Paul Ehrlich Prize in 1992 by the Association de Recherches Scientifiques. This distinction rewards a scientist or team of scientists having made in the past 10 years an outstanding contribution in the field of Me-

dicinal Chemistry. He was elected Fellow of International Union Against Cancer in 1992. Professor Lown was selected as the 1996 winner of the Hoffman-La Roche Award. This award recognizes a distinguished contribution to the field of medicinal chemistry through research involving biochemical or organic chemical mechanisms. Professor Lown was selected by the McGill Chemical Society as the 1996–97 BioMega/Boehringer-Ingelheim Lecturer and was appointed Killam Professor, 1996–97. He serves as Associate Editor of *Cancer Research* and is on the editorial boards of *Anti-Cancer Drug Research*, *Free Radical Biology and Medicine*, *Journal of Cellular Pharmacology*, and *Heterocyclic Communications*, in addition to being a member of the Editorial Academy of the *International Journal of Oncology* and a consultant to governmental agencies and industry.

Together with the many scientists interested in understanding DNA structure and in developing novel chemical approaches to target DNA for therapeutic intervention, we wish Professor Lown joy in his “retirement,” contentment and pride in his substantial accomplishments and scientific contributions, and peace of mind that he has influenced and helped to train scores of younger scientists who continue to work with the same enthusiasm he displayed for understanding DNA structure and developing novel chemical strategies to rationally intervene in DNA-mediated processes.

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16

Dicationic DNA Minor Groove Binders as Antimicrobial Agents

Richard R. Tidwell and David W. Boykin

16.1

Introduction

DNA is an attractive target for drug design due to the large amount of detailed structural information and its role as the source of genetic information [1]. The strategy of using small molecule DNA binders to alter gene expression was suggested some time ago and it has been continually refined [2–4]. The completion of numerous genomic projects now adds greater significance to this approach. Recent *in vivo* experiments with fruit flies demonstrate that synthetic minor groove binders can alter gene expression [5, 6]. Ideally, targeting of DNA requires both highly selective interactions at crucial sequences and exquisite differentiation between host and pathogen DNA. The interaction of small molecules with DNA has been extensively studied during the past four decades and information is emerging which should allow the design of selective DNA binders.

Aromatic diamidines (dicationic molecules) which have been shown to bind to AT-rich regions of the DNA minor groove have a long history as antimicrobial agents [7]. Dicationic molecules of this type were initially studied for their activity against African trypanosomes in the 1930s [8, 9]. Aromatic diamidines have been reported to have broad-spectrum antimicrobial activity and certain ones have been shown to be effective against *Acanthamoeba* [10], *Aspergillus* sp. [11–13], *Babesia* sp. [14, 15], *Candida albicans* [11–13, 16, 17], *Cryptococcus neoformans* [11–13], *Cryptosporidium parvum* [18, 19], *Giardia lamblia* [20], *Leishmania* sp. [21–25], *Mycobacterium tuberculosis* [16], *Plasmodium* sp. [21, 23, 26], *Pneumocystis carinii* [27–30], *Toxoplasma gondii* [31], and *Trypanosoma* sp. [32–37]. Despite the broad-spectrum activity of this class of compounds, only pentamidine has been found to be of significant use in the clinic to date, where it has been used against primary stage African trypanosomiasis, antimony-resistant leishmaniasis and AIDs-associated *Pneumocystis carinii* [38–40]. Pentamidine is currently being used against these three pathogenic organisms despite the fact that it is not effective when given orally and it displays several untoward clinical effects including hypotension, dysglycemia, and renal and hepatic toxicity [41–43]. The mechanisms of pentamidine

toxicity are poorly understood, in part, because it entered into clinical use in a period during which the rigorous preclinical and clinical studies now considered routine for potential new drug entities were not required. Metabolism studies suggest that at least some of pentamidine's toxicity arises from its metabolites [44, 45].

The potential of DNA-binding molecules with broad-spectrum biological activity makes it important to understand their interaction with DNA. The interactions of small molecules that bind to DNA are typically characterized as falling into one of three main classes: (1) electrostatic binders, (2) intercalators, and (3) groove binders. The binding of small molecules to DNA can involve one or a combination of these types of interactions. The most commonly encountered electrostatic binders to DNA are metal ions (typically sodium ions) which interact with the negatively charged phosphate groups of the DNA backbone. Electrostatic interactions are generally weak and non-specific. Consequently, drug design based on electrostatic interactions alone is unlikely to be rewarding. Intercalators and groove binders, however, are discussed below.

16.1.1

Intercalation

Molecules that bind to DNA by intercalation represent an important class of DNA binders, which have seen significant use as drugs. The binding of this class of molecules to DNA is characterized by the insertion of planar aromatic rings in between DNA base pairs. This interaction can be quite strong despite the fact that energy is consumed to unwind the helix and unstack the base pairs to allow complex formation. Contributing to the stability of intercalation complexes are van der Waals, hydrophobic, and electrostatic forces. Intercalation is generally independent of base pair sequence. Two major types of intercalation binding modes have been described: (1) classical intercalation and (2) threading intercalation. Binding by the classical mode is typified by the much-studied DNA stain ethidium and the anti-malarial quinacrine (Fig. 16.1) [45, 46]. An important contributor to the binding affinity of ethidium bromide and quinacrine to DNA is the stacking interaction of the respective, heteroaromatic rings with the DNA base pairs. In the case of quinacrine, the cationic side chain contributes to complex stability by electrostatic interactions with the negative phosphate units of the groove.

A number of other classical intercalators have found use as antitumor agents including the anthracyclines and actinomycins [47–50]. Because threading intercalators typically have two side chains on opposite sides of a planar aromatic ring system, the process of complex formation with DNA is more complicated. In such cases one of the side chains must slide through the intercalation cavity in order to form a complex. Favorable interactions of the side chains with both the major and minor grooves contribute to the complex stability of threading intercalators. The significant affinity and base pair specificity of the antineoplastic agent nogalomicin (Fig. 16.1) has been attributed to the presence of the side chains in each groove in the DNA complex [50]. The molecular details of the intercalation process and the

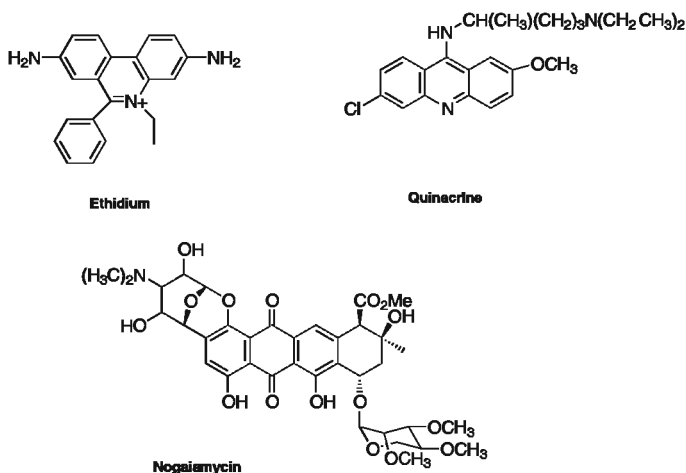


Fig. 16.1. Structures of DNA intercalators.

structural requirements of the small molecule intercalators are now well understood and these fundamental aspects have been carefully and comprehensively reviewed [45, 46].

16.1.2

Minor Groove Binding

Groove binders are another major class of small molecules that bind to DNA and play important roles in drug development. In principle, molecules can bind to either the major or minor groove of DNA. Due to the great differences in dimensions of the two grooves, targeting them requires vastly different shaped molecules. The major groove, as the name implies, is much wider than the minor groove; the groove width values for averaged-sequence B-form DNA are 11.6 and 6.0 Å, respectively [51]. Reflecting this dimensional difference, the major groove is the site of binding of many DNA interacting proteins [52]. To date there are limited reports of non-protein molecules that bind to the DNA major groove [53]. The minor groove dimensions are more amenable to small molecule binding and small molecule–minor groove interactions have been the focus of many investigations [for recent reviews see Refs 51, 54–57]. It has been speculated that the evolution of antibiotic minor groove binders that target the DNA of competing organisms is related to the more attractive dimensions of the minor groove for small molecules [58]. Minor groove binding usually involves greater binding affinity and higher sequence specificity than that of intercalator binding.

Minor groove binding has been demonstrated for neutral, mono-charged, and multicharged ligands. The focus of this chapter is dicationic ligands and the reader is referred to other recent reviews for the properties of other types of minor groove binders [51, 54–57]. A clear and detailed discussion of the mechanism of minor groove binding has appeared [54].

The forces that dominate small molecule–minor groove binding interactions are electrostatic, van der Waals, hydrophobic, and hydrogen bonding. Sequence specificity is often attributed to key hydrogen bonds between the base pairs and the small molecule. Another crucial structural requirement of the small molecule is that it has a crescent shape that is complementary to that of the minor groove (radius of curvature). Frequently, minor groove binders show pronounced AT selectivity. Several factors are thought to contribute to this commonly noted preference. The electrostatic potential is higher in AT-rich grooves than in GC-filled ones [59]. The electrostatic potential factor is thought to be an important contributor to the minor groove selectivity of the dicationic molecules to be discussed here. The dimensions of the minor groove at AT sites differ from those at GC locations. On average, AT-rich grooves are narrower and deeper than GC ones [60, 61]. A major contributor to this dimension difference has been attributed to the differences in ion interactions in the two types of base pair tracts [62]. The topology at AT sites allows for easier filling and greater van der Waals contacts by small molecules. Furthermore, the amino group of G in GC locations protrudes into the groove, thus prohibiting van der Waals contacts comparable to those achievable at AT sites [63].

The more intensely studied dicationic minor groove binders include the natural product netropsin and the synthetic molecules DAPI, berenil, and pentamidine (Fig. 16.2)

16.1.3

Netropsin

Netropsin is a dissymmetric tripeptide isolated from *Streptomyces netropsis* that contains two different terminal cationic centers: one an amidine and the other a guanidine. Netropsin shows experimental antiviral and antitumor activity that has been attributed to its ability to bind to DNA [64]. This tripeptide recognizes the minor groove of DNA at AT sites and occupies 5–6 base pairs [64]. The many bio-

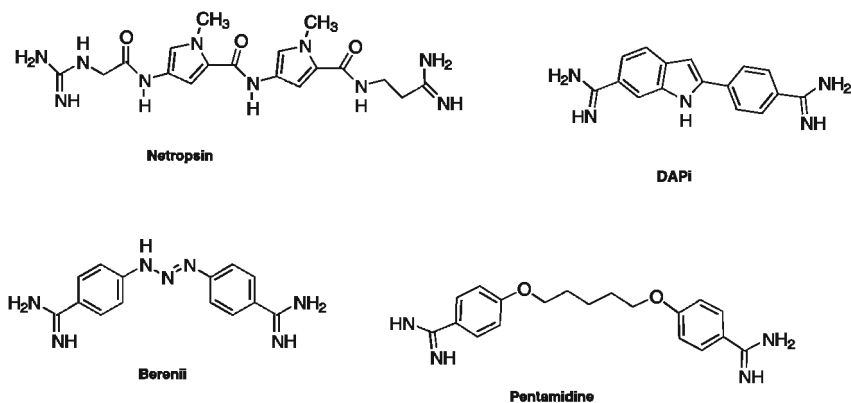


Fig. 16.2. Structures of well-studied DNA minor groove binders.

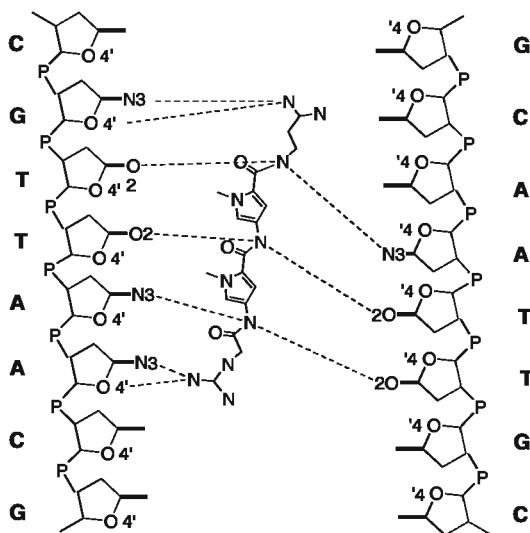


Fig. 16.3. Schematic representation of important hydrogen bonding interactions between netropsin and $d(CGCAATTGCG)_2$ based upon crystal structure results. Dashed lines represent hydrogen bonds.

physical studies of the binding of netropsin to DNA have been carefully reviewed [64]. Several crystal structures of the tripeptide with various oligomers with AT-binding sites have been reported [65–68]. The tripeptide in these complexes is essentially planar and fits snugly in the narrow minor groove, providing close contacts between the walls of the groove and the pyrrole and amide groups. Hydrogen bonds are formed between each amide NH and the base pairs. Significant hydrogen bonds between the guanidinium and amidinium groups and the base pairs are also found for these complexes (Fig. 16.3). In general, the guanidino end seems to be more firmly anchored than the amidino one by the hydrogen-bonding network [66–68].

The structures of the complexes of netropsin and various oligomers have been divided into two categories [66]. Class I structures have the three amide groups located approximately midway between the four AT bases and each participates in a bifurcated hydrogen bond array to thymine and adenine bases on opposite DNA strands. In class I complexes the pyrrole rings are located near C2 of adenines and it is that positioning that is believed to play a major role in determining AT specificity. The argument is that in a GC pair the N2 amine of G would prevent the pyrrole group from fitting snugly in the groove. The class II structures involve more disorder, no regular patterns of bifurcated hydrogen bonds are seen and the pyrrole rings are located between the base pairs. The netropsin location is shifted by approximately one-half base pair such that the amide units lie in the plane of

the base pairs in class II structures. Reanalysis of original diffraction data calls into question the validity of class II structures [66].

16.1.4

DAPI

DAPI (4',6-diamidino-2-phenylindole) is a synthetic diamidine that exhibits significant *in vivo* antitrypanosomal activity [34], but is much more widely known for its use in the molecular biology community as a fluorescence indicator for chromosomal DNA [69]. DAPI binds in the minor groove of DNA at AT sites [70]. A second, weaker binding interaction has been ascribed to intercalation at GC sites [71–74]. An alternate explanation for the weaker binding interaction has been offered [75], however NMR studies clearly indicate intercalation at GC sites [73, 74]. X-ray structures have been reported for complexes between DAPI and two different oligomers, both of which contain AATT-binding sites [76, 77].

The structures show that DAPI binds in the minor groove and is associated with approximately four base pairs. DAPI in the complex with d(GGCC AATTGG)₂ is displaced by one base pair in the 3' direction in the complex compared to that with the d(CGCGAATTCGCG)₂. Although there are differences in the two structures they both show important hydrogen bonds involving both the amidinium groups and the indole NH to the base edges (albeit to different bases). The hydrogen-bonding array for the DAPI complex with d(CGCGAATTCGCG)₂ is illustrated in Fig. 16.4 [76]. The structure includes hydrogen bonding between the 4'-amidinium and sugar 4'O atoms on opposite strands of the DNA. The indole NH bonds with two O2 atoms of thymines, also on opposite strands. The 6-amidinium unit has a tightly associated water molecule and is bonded to a thymine O2 and an adenine

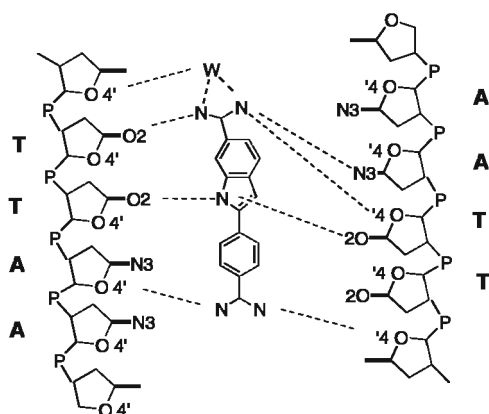


Fig. 16.4. Schematic representation of the hydrogen bonding array of the complex between DAPI and d(CGCGAATTCGCG)₂ based upon the crystal structure. Dashed lines represent hydrogen bonds.

N3 on opposite strands. Important van der Waals contacts between the walls of the groove and the aryl rings of DAPI are also noted [76].

16.1.5

Berenil

Berenil (also known as diminazene; 1,3-bis(4-amidinophenyl)triazene) is a synthetic diaryldiamidine which is widely used in Africa for the treatment of trypanosomal infections in animals [7]. It is also used to treat animal babesial infections [78]. The topical use of berenil to treat human cutaneous leishmaniasis has been reported [79]. Similar to results with DAPI, berenil has been found to bind strongly to the DNA minor groove at AT sites and a second weaker interaction has been attributed to intercalation [80, 81]. X-ray crystallography studies for berenil complexes with two different oligomers $d(CGCGAATTCGCG)_2$ and $d(CGCAAATTTGCG)_2$ have been reported [82, 83].

The data show that berenil occupies between three and four base pairs, which is consistent with its length. Depending upon the oligomer, the complexes show hydrogen bonding between the amidinium groups and the thymine O2 or adenine N3 atoms on opposite DNA strands. Figure 16.5 illustrates the hydrogen-bonding array for the berenil complex with $d(CGCGAATTCGCG)_2$ [82]. It can be seen that at one end of berenil a water molecule serves as a hydrogen bond bridge to effectively lengthen the molecule so that it recognizes an N3 atom on the base edge.

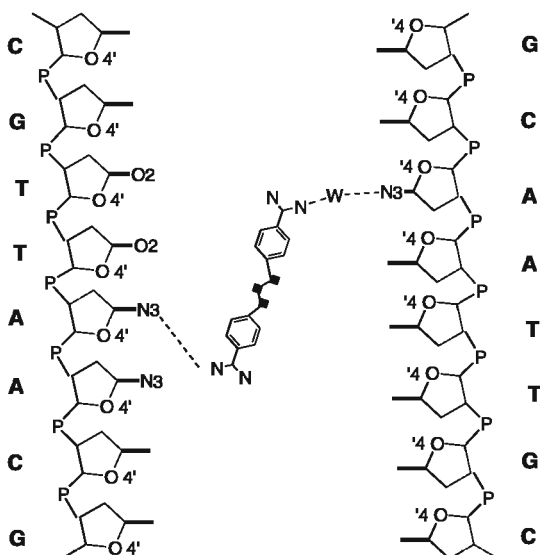


Fig. 16.5. Schematic representation of the hydrogen bonding array of the complex between berenil and $d(CGCGAATTCGCG)_2$ from the crystal structure. Dashed lines represent hydrogen bonds.

Just as is the case with DAPI, for berenil the phenyl groups lie parallel to the walls of the groove, achieving close van der Waals contacts. No important contacts between the triazine linker and DNA are noted.

16.1.6

Pentamidine

Of all the minor groove-binding compounds studied to date, pentamidine (1,5-bis(4-amidinophenoxy)pentane), a synthetic molecule, has seen the most clinical use. Pentamidine has a long history of human use, starting in the 1940s when it was used to treat African trypanosomiasis and leishmaniasis [7, 9, 37, 38]. More recently, during the HIV/AIDS epidemic, it has seen use as a secondary drug for treating AIDS-related *P. carinii* pneumonia [40–42]. Currently the drug is used to treat primary-stage African trypanosomiasis, antimony-resistant leishmaniasis, and it remains the secondary drug of choice for *P. carinii* pneumonia. Footprinting and X-ray crystallographic studies have demonstrated that pentamidine binds to the minor groove of DNA at AAAAAT-sites and that it occupies approximately four base pairs [84, 85].

The structure of the complex with d(CGCGAATTCGCG)₂ shows important hydrogen bonds between the amidinium groups and both the thymine O2 atoms and the adenine N3 atoms on opposite DNA strands [85]. For each amidinium the shorter hydrogen bonds are to the N3 atoms of adenine (Fig. 16.6). There are also

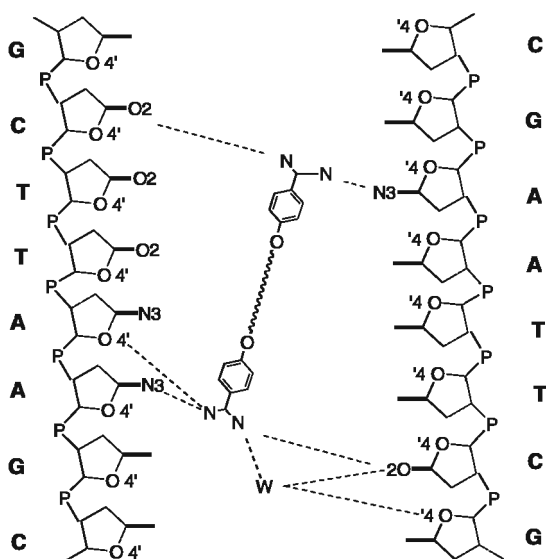


Fig. 16.6. Schematic representation of the hydrogen bonding array of the complex between pentamidine and d(CGCGAATTCGCG)₂ from the crystal structure. Dashed lines represent hydrogen bonds.

other close contacts with the adenine sugar O4' atoms, but these "bonding" angles deviate significantly from linearity. Consistent with the length of pentamidine, the molecule extends somewhat beyond the AATT-binding site as illustrated by the weaker hydrogen-bonding interactions with O2 atoms of Cs on opposite strands. The two phenyl groups are twisted by approximately 35° relative to each other such that these rings lie parallel to the groove walls and provide close van der Waals contacts. On comparison of the distance between the phenoxy *ipso* carbons in the crystal structure of free pentamidine to that in the complex it is seen that the distance is compressed from 9.75 to 9.13 Å [85]. The compression is ascribed to deviations in torsion angles of the linker from the normal staggered value of 180° [85]. This seems to be one of the structural accommodations made by pentamidine to fit the AATT-binding site. Striking changes in the details of minor groove binding are observed with relatively minor changes in the linker between the benzamidine units of pentamidine [86–88].

γ -Oxapentamidine (1,5-bis(4-amidinophenoxy)-3-oxapentane), which differs from pentamidine only by replacement of the central methylene by an oxygen atom, does not bind as strongly to DNA and the crystal structure of its complex with d(CGCGAATTCGCG)₂ differs from the similar complex for pentamidine [87]. Amongst dicationic minor groove binders, the complex for γ -oxapentamidine is unusual in that there are essentially no direct contacts between the amidinium units and the bases. The major hydrogen-bonding interactions are between the amidinium units and the O4' sugar atoms of adenines on opposite DNA strands. The lower DNA binding affinity of γ -oxapentamidine is attributed to repulsive interactions of the γ -oxygen atom and the thymine O2 atoms and to the lack of strong amidinium–base hydrogen bonds [87].

16.2

Dicationic Carbazoles and Analogs

16.2.1

Introduction

As a continuation of our search for novel dicationic antimicrobial compounds we synthesized and tested a series of 40 3,6- and 2,7-disubstituted carbazoles and related dibenzothiophenes and dibenzofurans [89–91] (Fig. 16.7). These compounds were tested for antimicrobial activity against *Pneumocystis carinii* pneumonia in a rat model [89–91], a neonatal mouse model of *Cryptosporidium parvum* infection [18] and *in vitro* against a number of pathogenic fungi [13]. Since it had been previously suggested that the antimicrobial activity of similar dicationic compounds was related to their ability to bind in the minor groove of DNA [92, 93], the carbazoles and their analogs were also examined extensively for their mode and strength of DNA binding [94, 95].

This series of compounds not only produced a number of excellent antimicrobial compounds but also proved to have very interesting DNA-binding characteristics. It

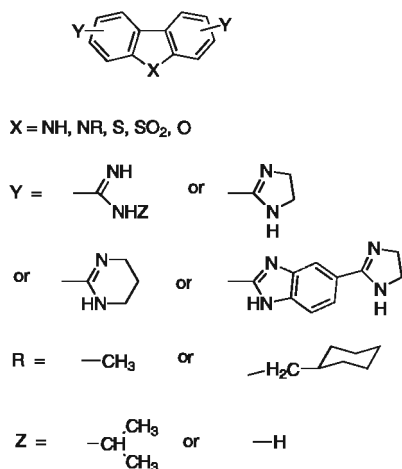


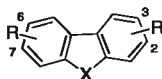
Fig. 16.7. Dicationic carbazoles, dibenzothiophenes, and dibenzofurans.

was initially thought that these carbazole compounds looked more like classical fused-ring intercalating systems than like the unfused aromatic dications or peptide compounds that have been identified as AT-specific minor groove-binding agents. However, they proved to be the first known fused-ring systems that formed strong minor groove complexes with AT sequences. The compounds also proved interesting in that attempts to make orally bioavailable amidoxime pro-drugs were successful for the dibenzothiophenes and dibenzofurans, but not the carbazoles.

16.2.2

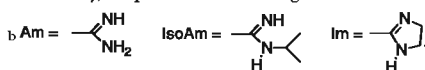
DNA Binding of Dicationic Carbazoles and Analogs

The carbazoles and related analogs were originally tested for DNA-binding affinity with calf thymus DNA by determination of changes in thermal melting (ΔT_m values) [89–91]. The ΔT_m values for selected compounds are shown Tab. 16.1. The group as a whole produced a number of high-affinity DNA-binding agents with the carbazole derivatives appearing to be stronger binders than the corresponding dibenzothiophenes and dibenzofurans. The most potent binder as measured by ΔT_m was 3,6-bis(2-imidazolinyl)-9-methylcarbazole (compound 12, Tab. 16.1), giving a ΔT_m of 24.0°C. Whether the cationic moieties were amidines or imidazolines appeared to have only minor effects on DNA binding. However, N-substitution of amidine groups with an isopropyl moiety (compounds 1, 2, and 3 compared to 13, 14, and 15) significantly reduced DNA-binding affinity. Also notable is the loss of nearly all DNA binding when the dibenzothiophene sulfur atom is oxidized from the sulfide to sulfone state (compare compounds 2–8). The resulting 2,8-bis(2-imidazolinyl)-5,5-dioxodibenzothiophene gave a ΔT_m of only 2.3°C. It was speculated that the oxygen atoms of the sulfone protruding perpendicular from the plane of the benzothiophene ring hindered the ability of the molecule to snugly fit into the

Tab. 16.1. DNA binding affinities of selected compounds as measured against calf thymus DNA and determined by changes in thermal melting.

Compound	Position of R ^a	R ^b	X	DNA binding ΔT_m (°C)
1	3,6	Am	N-H	17.2
2	3,6	Am	S	12.4
3	3,6	Am	O	14.5
4	3,6	Am	N-H	19.0
5	3,6	Am	S	15.0
6	3,6	Am	O	12.7
7	3,6	Am	N-CH ₃	19.5
8	3,6	Am	SO ₂	2.3
9	3,6	Im	N-H	19.5
10	3,6	Im	S	19.0
11	3,6	Im	O	15.0
12	3,6	Im	N-CH ₃	24.0
13	3,6	IsoAm	NH	9.6
14	3,6	IsoAm	O	8.1
15	3,6	IsoAm	S	6.4
16	2,7	Im	NH	18.6

^afor clarity, all positions of R are given with carbazole numbering.



minor groove. Viscosity determinations from these initial studies also suggested that the molecules were binding in the minor groove rather than intercalating as was initially expected for these fused-ring systems.

Since carbazoles and related molecules could, in principle, bind to AT sequences by intercalation and there were no known fused-ring systems that formed strong minor groove complexes with AT sequences, in depth studies were performed to gain an understanding of the interaction of these molecules with DNA [94, 95]. The first study characterized and compared the DNA interactions of 3,6- and 2,7-bis(2-imidazolyl)carbazole (compounds 4 and 16 in Tab. 16.1), utilizing UV-vis absorbance, fluorescence, kinetic measurements, and circular dichroism in conjunction with NMR spectroscopy [94]. All the results from the studies with the carbazoles complexed with poly[d(A-T)]₂ or the oligomer d(A-T)₇ were in agreement and pointed to minor groove complex formation.

The following evidence for minor groove complex formation was cited: (1) relatively small absorbance changes in the carbazole spectrum on the addition of the AT polymer; (2) large positive-induced CD spectral changes in the carbazole region above 300 nm on complex formation; (3) a slope of nearly 1.8 in a plot of log k_d

versus $-\log(\text{Na}^+)$ for dissociation of carbazole-poly[d(A-T)]₂ complexes; (4) small, mixed upfield and downfield NMR chemical shift changes on complex formation; and (5) NOEs from carbazole aromatic protons to specific protons in the minor groove [94].

Another interesting finding of the initial study was that despite having different structures and vastly varying radii of curvature (with respect to the amidines and the carbazole ring system) both the 2,7- and 3,6-substituted carbazoles formed tight complexes in AT regions of the minor groove. The results of NOE experiments for the two carbazoles bound to d(GCGAATTCGC)₂ indicated that the carbazole NH on the 3,6-derivative pointed out of the groove with the imidazoline groups nestled in the bottom of the groove for hydrogen bond formation. However, these experiments indicated that the 2,7-carbazole had the opposite orientation. In the case of the latter molecule, one imidazoline group and the carbazole NH were speculated to form H-bonds at the floor of the groove while the second imidazoline group is close to the edge of the groove with the possibility of electrostatic interaction with DNA phosphate groups. A schematic contrasting the binding modes of the two carbazole derivatives is shown in Fig. 16.8 and a stereoview is shown in Fig. 16.9.

In an attempt to test and refine the proposed models for the binding of 3,6- and 2,7-carbazoles, a second study was undertaken which investigated the DNA-carbazole complexes of the original two compounds along with five new carbazole derivatives (Tab. 16.2) [95]. The new study applied all the techniques carried out previously with the addition of DNA footprinting of the carbazole-DNA complexes and surface plasmon resonance biosensor analysis. Particularly important to the new study was the addition of the new carbazole derivatives. For instance, the authors proposed that since their models of DNA-carbazole complexes indicated that the carbazole nitrogen contributed to complex formation in the case of the 2,7-

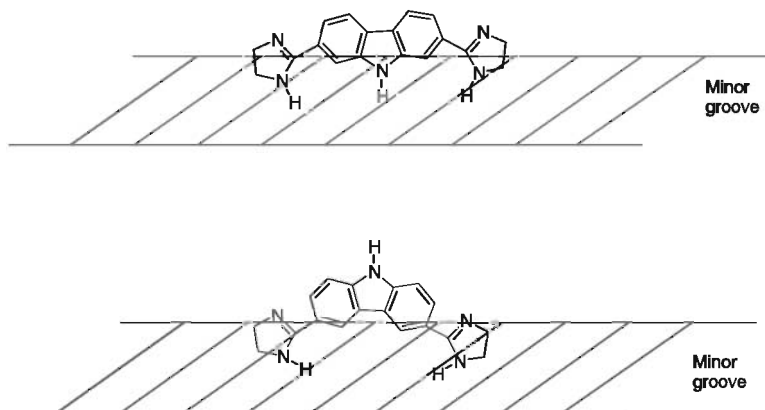


Fig. 16.8. Schematic showing 2,7-bis(2-imidazolyl) carbazole bound in the minor groove (top) with the carbazole nitrogen pointing down into the groove and 3,6-bis(2-imidazolyl) carbazole bound in the minor groove (bottom) with the carbazole nitrogen pointing away from the groove.

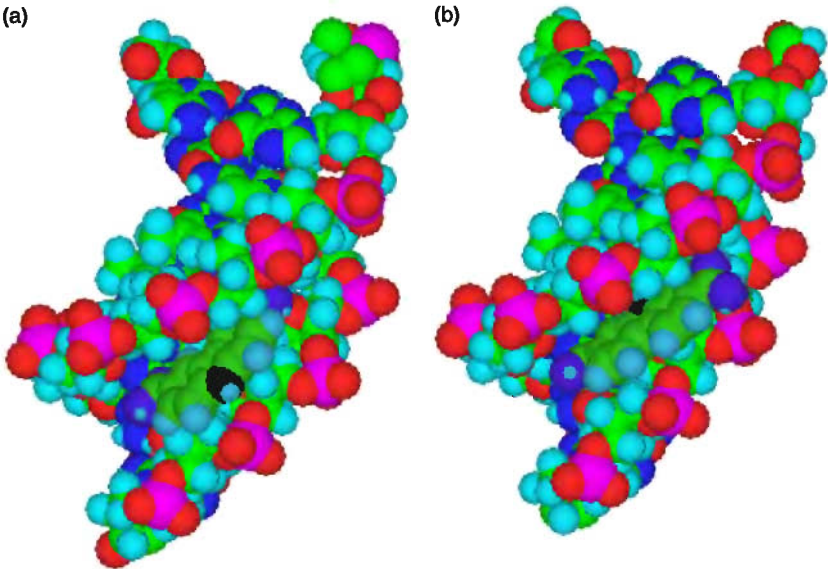


Fig. 16.9. View of 3,6-bis(2-imidazolyl)carbazole (a) and 2,7-bis(2-imidazolyl)carbazole (b) bound to the central (AATT)₂ region of d(CGCGAATTCGCG)₂, illustrating the binding of the 2,7-carbazole with the carbazole nitrogen pointing into the minor groove and the 3,6-carbazole with the

carbazole nitrogen pointing out of the groove. Only the central region base pairs are shown for clarity. The colors assigned to atoms are: C = green; N = dark blue (except carbazole nitrogen which is black); H = light blue; P = magenta; O = red.

Tab. 16.2. Structures of carbazoles used in second study and the ΔT_m values of the carbazoles with d(CGCGAATTCGCG)₂.

Rc1ccc2c(c1)c(c[nH]2)R

Compound	Position of R ^a	R ^b	X	DNA binding ΔT_m (°C)
1	2,7	Am	H	10.9
2	3,6	Am	H	8.2
3	2,6	Am	H	6.5
4	3,6	Am	CH ₃	7.5
5	3,6	Im	H	7.8
6	2,7	Im	H	12.0
7	2,7	Im	CH ₃	6.3

See Table 16.1 for explanation of footnotes.

derivatives but not the 3,6-analogs, comparison of the binding affinities of the derivatives with methyl substitutions on the carbazole nitrogens (compounds 4 and 7 in Tab. 16.2) should provide further evidence to support or refute their model. As can be seen from Tab. 16.2, the ΔT_m values of the molecules with the oligomer support the proposed model. Methylation of the carbazole nitrogen of the 3,6-derivative (compare compounds 2 and 4 in Tab. 16.2) resulted in only a small variation in the ΔT_m value while the same substitution for the 2,7-derivative (compare compounds 6 and 7 in Tab. 16.2) caused a reduction of nearly half in the ΔT_m value.

Studies also included DNAase footprinting of the carbazoles on 117, 155, 160, and 265 base pair restriction fragments. Analysis of the protected sequences indicated a broad AT specificity of carbazole binding and patterns in agreement with a minor groove complex. Surface plasmon resonance (SPR) studies of carbazole binding to an oligomer further confirmed the proposed model. SPR biosensor analysis of carbazole binding to an oligomer with an AATT central sequence indicated that compounds 1 and 6 (Tab. 16.2) had the largest binding constants. Compounds 2 and 4 (Tab. 16.2) bound with similar equilibrium constants but considerably less than compounds 1 and 6, while compound 7 had the lowest binding constant of all carbazoles studied.

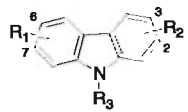
In conclusion, all the evidence strongly supports that the dicationic carbazoles bind specifically in the minor groove at AT sequences. In addition, all the studies support the proposed model with the carbazole nitrogen pointed into the groove in the 2,7-complex and out of the groove in the 3,6-complex. Final confirmation of the proposed model will have to await the formation and study of crystals of complexes between carbazoles and oligomers.

16.2.3

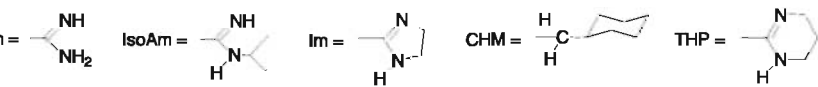
Antimicrobial Activity of Carbazoles and Related Analogs

A number of carbazole derivatives have been shown to be highly effective in two animal models of infection and effective *in vitro* against several other pathogenic microbial organisms. Initial antimicrobial studies of the carbazoles against *Pneumocystis carinii* pneumonia in a rat model of the disease [89] and *Cryptosporidium parvum* in a neonatal mouse model [18] clearly showed several of the compounds to be highly active. The data for 13 compounds against the two organisms are shown in Tab. 16.3 along with their binding affinity for calf thymus DNA as measured by change in thermal melting point. It is clear from the data that there is no correlation between strength of DNA binding and antimicrobial activity. However, it is doubtful even if such a correlation existed that it could be observed in a complex animal model due to other effects such as metabolism, distribution, and uptake by the organism. This lack of direct correlation between the antimicrobial effect of the compounds and their binding strength has also been observed in *in vitro* systems for other classes of dicationic minor groove binders [27, 41]. Also, we have never found a compound in the dicationic series that does not bind to DNA yet has antimicrobial activity despite the numerous non-DNA-binding molecules

16.3. Activity of carbazoles against a rat model of *Pneumocystis carinii* pneumonia and a neonatal mouse model of *Cryptosporidium parvum* infection.



Compound	<i>R</i> ₁ , <i>R</i> ₂ Position	<i>R</i> ₁ ^a	<i>R</i> ₂ ^a	<i>R</i> ₃ ^a	<i>P. carinii</i>		<i>C. parvum</i>		DNA binding Δ <i>T</i> _m (°C) ^f
					Dose ^b	% Saline control ^c	Dose ^d	% Saline control ^e	
Saline control						100.00			
Metamizole					10.0	3.09			10.7
Zinc							100.0	42.3	
Chromomycin							50.0	1.2	
	3,6	Am	Am	H	5.0	0.56	19.0	2.2	17.2
	3,6	Am	Am	CH ₃	5.0	0.43	17.0	3.0	19.5
	2,6	IsoAm	IsoAm	H	5.0	0.10	20.0	94.0	9.6
	3,6	Im	Im	H	5.0	0.04	20.0	34.8	19.5
	3,6	Im	Im	CH ₃	5.0	0.12	17.0	36.9	24.0
	3,6	Im	H	CHM	5.0	25.23	17.0	108.5	16.8
	3,6	THP	THP	CH ₃	5.0	0.10	17.0	90.8	13.6
	3,6	THP	THP	CHM	5.0	2.89			7.3
	2,7	Am	Am	H	5.0	0.12	19.0	0.8	19.0
	2,7	Im	Im	H	5.0	0.35	14.3	54.4	18.6
	2,7	Im	Im	CH ₃	5.0	0.35	20.0	50.3	19.1
	2,7	Im	OCH ₃	H	5.0	321.04	17.0	102.9	1.3
	2,7	Im	OCH ₃	CH ₃	5.0	200.20	17.0	129.0	3.9



Dose in mg kg⁻¹ body weight, compounds given by i.v. injection daily for 14 days.

Cysts per g of lung tissue treated over control, cysts per g lung counts were 51.5 (×10⁶) for the saline control group.

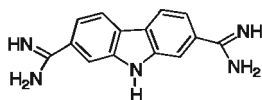
Dose in mg kg⁻¹ body weight, compound given p.o. daily for 6 days.

Mean number of oocysts in treated are expressed as percentages of the mean number of oocysts in controls.

Change in melting point determined on calf thymus DNA.

Acetyloxy-*N*-(5-nitro-2-thiazolyl)benzamide.

tested. It is interesting to note that the two monocations (compounds **12** and **13**) did not significantly bind to DNA and were devoid of antimicrobial activity. It has been reported that DNA binding is the first step in the antimicrobial process, followed by the inhibition of DNA-directed enzymes such as topoisomerases [92] or endo/exonucleases [96]. Therefore, while the ability to bind to DNA is key to antimicrobial activity, the strength of binding is not as important as the nature and selectivity of the binding.

Tab. 16.4. *In vitro* antimicrobial activity of 2,7-diamidinocarbazole against selected organisms.

Organism	Activity	
<i>Aspergillus fumigatus</i>	3.12	MIC ^a (μg mL ⁻¹)
	25.0	MFC ^b (μg mL ⁻¹)
<i>Candida albicans</i>	0.78	MIC ^a (μg mL ⁻¹)
	1.56	MFC ^b (μg mL ⁻¹)
<i>Cryptococcus neoformans</i>	1.56	MIC ^a (μg mL ⁻¹)
	6.25	MFC ^b (μg mL ⁻¹)
<i>Leishmania donovani</i>	7.92	IC ₅₀ ^c (μM)
<i>Plasmodium falciparum</i>	0.025	IC ₅₀ ^c (μg mL ⁻¹)
<i>Trypanosoma brucei rhodesiense</i>	0.005	IC ₅₀ ^c (μg mL ⁻¹)

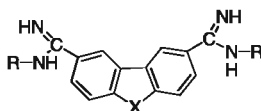
^aMinimum inhibitory concentration (80%).^bMinimum fungicidal concentration.^cInhibitory concentration 50%.

In addition to their activity in the above animal models, carbazoles have demonstrated activity *in vitro* against *Leishmania donovani* [25], *Aspergillus fumigatus* [13], *Candida albicans* [13], and *Cryptococcus neoformans* [13]. The compounds were also found to be active against trypanosomes, *Mycobacterium*, and malaria (R. R. Tidwell, unpublished results). The *in vitro* activity of 2,7-diamidinocarbazole against several organisms is shown in Tab. 16.4. In general, the sulfur and oxygen isosteres of the carbazoles demonstrated similar antimicrobial profiles, but were somewhat less potent than the corresponding carbazoles [90, 91]. While there were no benefits of the benzothiophenes and benzofurans over the carbazoles with regard to antimicrobial activity or toxicity, there was one very important pharmacological advantage that is discussed below.

16.2.4

Pro-drugs of Carbazoles and Related Analogs

Because aromatic amidines are strongly basic (pK_a between 10 and 12) there seems very little chance that they will be absorbed from the gastrointestinal tract and therefore they do not make good candidates as oral drugs. Chemical modification of biologically active compounds to form new molecules (pro-drugs), usually biologically inactive, which can be converted *in vivo* to the parent molecule can potentially improve physico-chemical properties such as water solubility and lipophilicity, transport of drug to the site of action, presystemic degradation, and oral bioavailability [97, 98]. While numerous reports exist on the pro-drug modification of many functional groups [99–103], very few reports have appeared describing

Tab. 16.5. Comparison of the antimicrobial effect of selected dicationic carbazoles, dibenzothiophenes, and dibenzofurans in a rat model of PCP.

Compound	R	X	i.v.		Oral	
			Dose ^a $\mu\text{mol kg}^{-1}$	% Control ^b	Dose ^a $\mu\text{mol kg}^{-1}$	% Control ^b
1	H	N	14.6	0.13	77.1	98.21
2	HO	N	22.0	58.23	33.0	126.87
3	H	S	10.0	0.10	33.0	68.01
4	HO	S	10.0	0.82	33.0	0.85
5	H	O	10.0	7.15	NT ^c	NT ^c
6	HO	O	10.0	14.76	33.0	45.06

^aDosing was carried out daily for 14 days.^bCysts per g lung tissue for the saline control groups were pooled across experiments. Scores for each compound are reported as % of the pooled saline.^cNot tested.

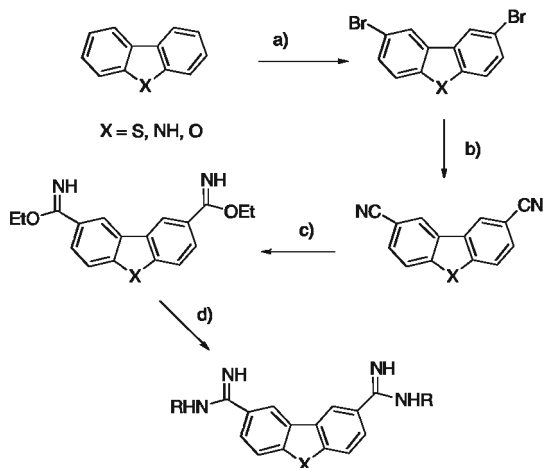
pro-drug approaches for aryl amidines [104, 105]. It has been reported by our laboratories and others that amidoximes can be used as orally active pro-drugs for the corresponding diamidines [44, 106–108]. The amidoximes are true pro-drugs in that they are inactive *in vitro* against microorganisms and must be converted to amidines to exert an antimicrobial effect. Aromatic amidoximes have pK_a values in the range of 2–6 and are readily absorbed from the gastrointestinal tract and then metabolically converted to the active amidines [106–108]. The conversion of amidoxime-type pro-drugs to amidines is discussed in more detail in the following section on furamidines.

On i.v. administration, several diamidinocarbazoles were very effective against *P. carinii* pneumonia in an animal model. The dicationic carbazoles were inactive when given orally; however, they were considered good candidates for amidoxime pro-drugs [44, 106, 108]. Unexpectedly, as can be noted from Tab. 16.5 the carbazole amidoxime pro-drug was not active against *P. carinii* pneumonia either when given orally or by i.v. injection. Despite this set back, studies were done on amidoxime pro-drugs of the corresponding dibenzothiophenes and dibenzofurans [90, 91]. In contrast to the carbazole results, both the dibenzothiophene and dibenzofuran amidoxime pro-drugs exhibited activity in the *P. carinii* animal model (Tab. 16.5). 2,8-Bis(*N*-hydroxyamidino)dibenzothiophene was very active against the infection when given both by i.v. injection and oral gavage. Investigations are currently under way in attempt to understand this unexpected variability among the isosteres in pro-drug activity.

16.2.5

Synthesis of Carbazoles and Related Analogs

The syntheses of 3,6-disubstituted carbazoles and 2,8-disubstituted dibenzothio-phenes were carried out in a similar manner from their corresponding unsubstituted parent ring systems [89–91]. A typical synthetic route is shown in Scheme 16.1. For *N*-alkylated products the alkylation step was carried out on the dibromo or dinitrile intermediates.

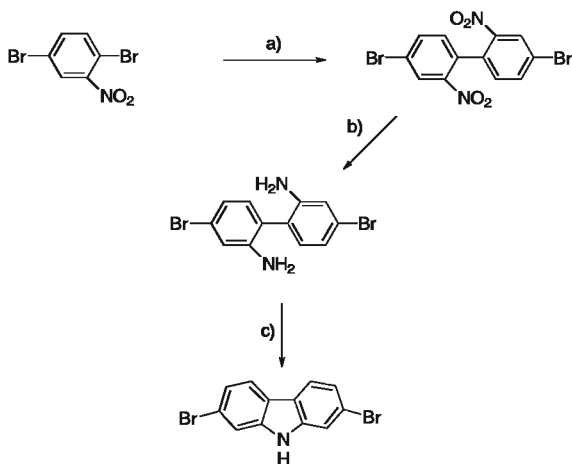


a) Br_2 , CS_2 b) CuCN , DMF, reflux c) EtOH , HCl , 1,4-dioxane d) RNH_2 , EtOH

Scheme 16.1

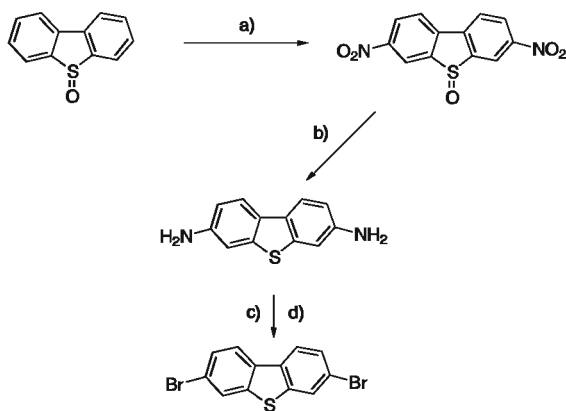
The 2,7-disubstituted carbazoles were prepared in a similar manner as the 3,6-analogs from the corresponding dibromocarbazole. 2,7-Dibromocarbazole was prepared in a three-step synthesis as outlined in Scheme 16.2 [109, 110]. The synthesis involved an Ullmann reaction of 2,5-dibromo-nitrobenzene to give the biphenyl, reduction of the nitro groups on the biphenyl with stannous chloride and a deaminative ring closure catalyzed by phosphoric acid.

As described above, all 3,7-disubstituted dibenzothio-phenes were prepared from 3,7-dibromodibenzothio-phenes. This compound was prepared according to the method outlined in Scheme 16.3 [91]. 3,7-Dibromodibenzothio-phenes was prepared by dinitration of dibenzothio-phenes oxide, reduction of the nitro groups and the oxide using a palladium catalyst, diazotization of the amine group and reaction of the diazonium salt with cuprous bromide and hydrogen bromide. 3,7-Dibromodibenzofuran, the precursor for all corresponding disubstituted dibenzofuran analogs, was prepared according to the method outlined in Scheme 16.4 [90]. The starting material was prepared according to the method described in Scheme 16.2. The product of the Ullmann coupling reaction underwent selective nucleophilic



a) Cu, DMF b) SnCl_2 , $2\text{H}_2\text{O}$, EtOH c) H_3PO_4 , 200°C

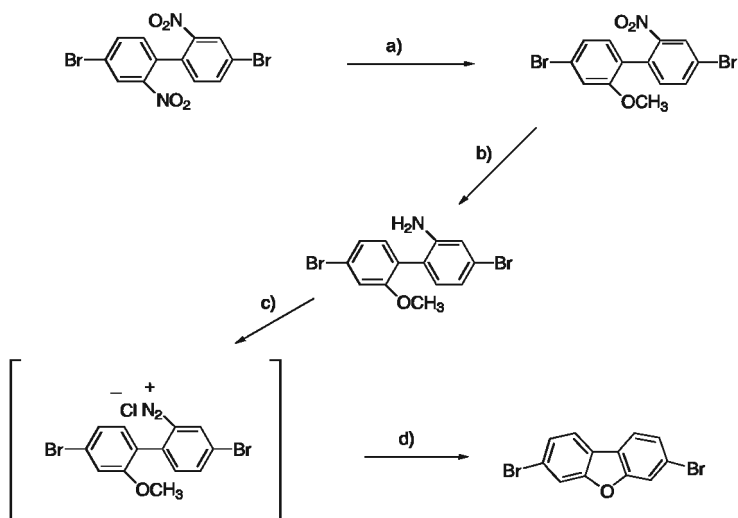
Scheme 16.2



a) HNO_3 , H_2SO_4 b) H_2 , 10% Pd/C c) NaNO_2 , HCl d) CuBr, HBr

Scheme 16.3

substitution by reacting with sodium methoxide to give 2-methoxy-2'-nitro-4,4'-dibromodiphenyl. The nitro group was then reduced to the corresponding amine using 5% ruthenium on carbon as the catalyst. The amine group was diazotized with sodium nitrite and aqueous sulfuric acid solution. The diazonium salt, obtained *in situ*, was heated to 70°C to give 3,7-dibromobenzofuran.



a) NaOCH_3 , CH_3OH , DMF b) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, EtOH , 5% Ru/C c) NaNO_2 , H_2SO_4 d) reflux

Scheme 16.4

16.3

Dicationic Furans

16.3.1

Introduction

2,5-Bis[4-amidinophenyl]furan (17, furamidine, DB75, Fig. 16.10) was reported some time ago to be effective against trypanosomes in murine and simian models [32, 33]. More recently, furamidine was shown to be highly effective in animal models against *Pneumocystis carinii* and *Cryptosporidium parvum* [18, 27]. The quite low bioavailability of these highly charged molecules is not unexpected and is evidenced by the dramatically reduced activity of pentamidine as well as furamidine on oral administration [27, 33, 107] (J. E. Hall, unpublished results). Many of the infections for which aryl diamidines offer the potential of being useful therapeutic

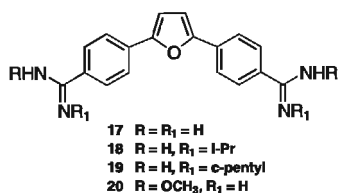


Fig. 16.10. Furamidine and analogs.

agents occur extensively in rural areas of developing countries where health care systems are often limited. Therefore, for these types of compounds to have the greatest public health impact it is essential that they are effective on oral administration. Consequently, we have undertaken a parallel study of using pro-drug approaches as a means to effectively deliver these dicationic molecules on oral administration [106, 108, 111]. Specifically, we have demonstrated that amidoxime and phenylcarbamate derivatives of furamidine are effective when given by oral administration in a rat model for *Pneumocystis carinii* pneumonia [108, 111] and amidoximes are effective in a mouse model for trypanosomiasis (J. E. Hall, unpublished results). One of these pro-drugs, 2,5-bis[4-(methoxyamidino)phenyl]furan (20, DB289) has satisfactorily completed phase I clinical trials [112, 113] and is currently in phase II trials versus African trypanosomiasis. This section will review the biology and chemistry of furamidine and its analogs.

16.3.2

DNA Binding of Furamidine and Analogs

The Wilson, Neidle, and Bailly laboratories have performed extensive biophysical studies including NMR, X-ray crystallographic, and footprinting investigations which demonstrate that the primary mode of DNA binding for furamidine is at AT-rich sites of the minor groove [114–121] and that its weaker secondary binding occurs at GC sites. Similar dual binding modes have been demonstrated for the structurally related diamidines berenil and DAPI [73, 74, 88, 122–127]. The mode of binding of furamidine at GC sites has been the subject of debate [126, 127], nevertheless the data assembled to date strongly indicates that the weak binding interaction is by intercalation [117, 128]. Crystal structures of furamidine and related *N*-alkyl furan analogs [27, 117–120] bound at the AATT site of $d(\text{CGCGAATTCGCG})_2$ have given insight into the important molecular features of complex formation for this class of compounds (Fig. 16.11). It can be seen that the curvature of the diarylfuran system complements that of the DNA minor groove. In the complex, both amidinium groups NH groups of furamidine and analogs are positioned on the floor of the minor groove, with the amidine NH groups within hydrogen-bonding distance of thymine carbonyl groups on opposite strands of the AATT sequence.

Figure 16.12 shows that furamidine displays essentially symmetrical hydrogen bonding to thymine carbonyls on opposite DNA strands. Water molecules (not shown) also contribute to the hydrogen bond network of furamidine bound in the minor groove. Whereas berenil occupies essentially only three base pairs, furamidine binds to four base pairs. Similar to the berenil complex, no specific interaction between the linker (specifically the furan oxygen) and DNA is noted. Highly ordered water structure in the minor groove is also associated with several of these complexes [117, 119]. Extensive van der Waals contacts between the aryl rings of furamidine and the walls of the minor groove are apparent. Interestingly, relatively large alkyl groups (e.g. as in 18 and 19) placed on the amidino nitrogen can be

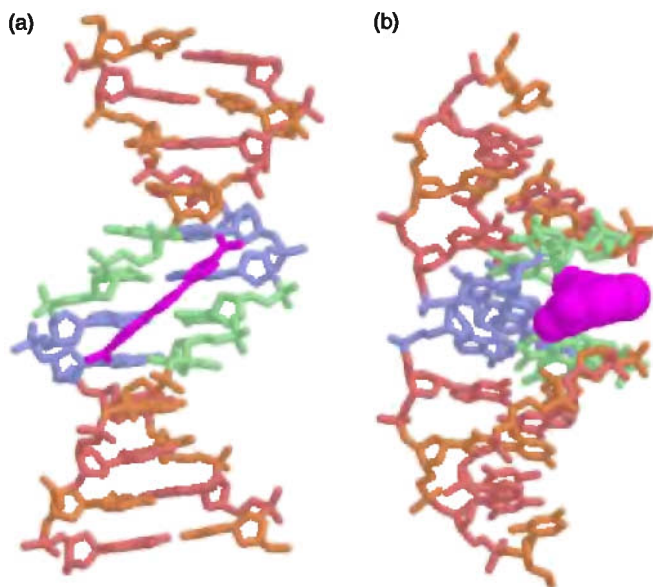


Fig. 16.11. Views of DB75 (in purple) in the AATT minor groove site of the d(CGCGAATTCGCG) duplex are shown. (a) The minor groove is in the center of the figure; (b) the model is tilted back approximately 30° to show the groove width and the fit of DB75. The compound is shown as a stick model in (a) and as space-filling in (b). DNA is shown in

the stick representation in both models with GC bases in orange, A in blue, and T in green. The hydrogen bonds between the DB75 amidines and the T carbonyl groups can be identified on the left and the twist of the DB75 structure is seen on the right. These models are from coordinates in file 227D of the Protein Data Bank [120, 159].

accommodated and seem to add to the favorable van der Waals and hydrophobic interactions as noted from the increased ΔT_m values and binding equilibrium constants for these derivatives [117].

The precise role of DNA binding in the antimicrobial action of furamidine and related aryldiamidines is unclear, but remains under investigation. The biologically active molecules of this class of compounds show strong affinity for the DNA minor groove, whereas close structural analogs (e.g. monocations) which bind only very weakly, if at all, to DNA are inactive. As previously mentioned, these observations have led to the working hypothesis that active compounds first form a complex with the DNA minor groove and after complex formation one or more DNA-dependent enzymes or control proteins are inhibited. Several different enzymes from different organisms have been implicated as possible targets [92, 96, 116, 129, 130]. It is also possible that the dications may act by direct inhibition of transcription [3, 131]. Despite the fact that no unifying mechanism across organisms has yet emerged, the potent and broad-spectrum activity of furamidine and related dicationic molecules and their selective DNA affinity has stimulated us to

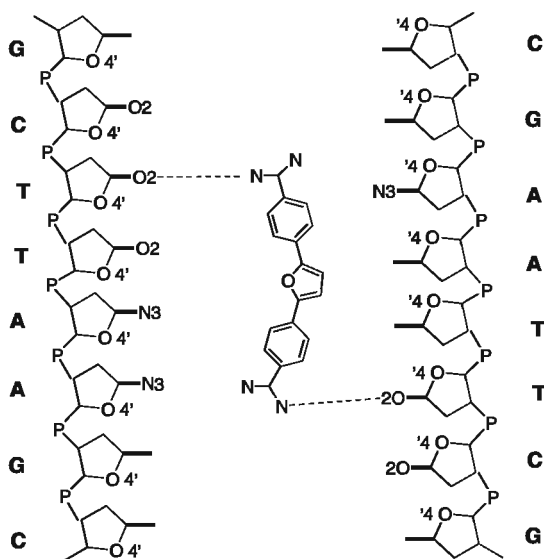


Fig. 16.12. Schematic representation of the hydrogen bonding array of the complex between furamidine and $d(CGCGAATTCGCG)_2$ from the crystal structure. Dashed lines represent hydrogen bonds.

design other DNA minor groove binders in an effort to develop useful antimicrobial agents.

The fluorescence properties of furamidine and analogs have been used to demonstrate that they enter cells and bind to the nucleus as illustrated in Fig. 16.13 (see p. 438) [132]. Precisely how furamidine enters cells is presently unknown, however it seems likely that transporters which are responsible for uptake of other related diarylamidines may be involved [133].

16.3.3

Antimicrobial Activity of Furamidine and Analogs

As previously noted, furamidine has been shown to be effective against three different organisms in animal models. The effectiveness against trypanosomes was described sometime ago in both murine and simian models [32, 33]. More recently, Hall and co-workers have demonstrated the efficacy of furamidine using one intravenous dose of $2.5 \mu\text{mol kg}^{-1}$ against a mouse model of the disease (see summary in Tab. 16.6). In this study mice were infected with *T. brucei brucei* S427 clone 22 and were treated 72 h post infection. Mean survival time of untreated controls was 5.8 days and treated animals surviving 63 days were deemed cured (J. E. Hall, unpublished results).

A 14-day intravenous dosing regimen of 13.3 mol kg^{-1} of furamidine in an immunosuppressed rat model of *Pneumocystis carinii* pneumonia (PCP) was quite

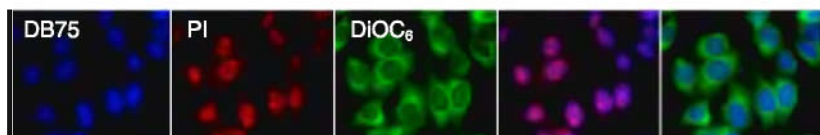
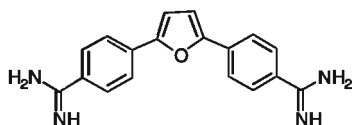


Fig. 16.13. Fluorescence micrographs of B16 melanoma cells stained with furanidine (DB75). Intracellular distribution of DB75 (2 mM) in B16 melanoma cells visualized by fluorescence microscopy ($\times 63$) after 18 h incubation. DB75 has blue fluorescence and can be seen localized in the cell nuclei. The second panel is for propidium which has red fluorescence and also localizes in the nuclei. The green images correspond to the

subsequent incubation of the same cells with 20 nM DiOC₆, which stains the cytoplasm, and the images on the right side correspond to the superimposed blue + green and blue + red fluorescence. The authors thank Dr Christian Bailly and Dr Amélie Lansiaux for providing this figure. Used with permission from *J. Med. Chem.*, **2002**, 45, 1994–2002. Copyright 2002 American Chemical Society.

Tab. 16.6. Summary of the biological activities of furanidine.



Organism	Dose	Result
<i>In vivo</i> Results		
<i>Pneumocystis carinii</i> (rat) ^a	13.3 ($\mu\text{mol kg}^{-1} \text{ day}^{-1}$) (14 days)	2.1% untreated control (cysts)
<i>Trypanosoma</i> (mouse) ^b	2.5 ($\mu\text{mol kg}^{-1} \text{ day}^{-1}$) (1 day)	6/6 survivors
<i>Cryptosporidium parvum</i> (mouse) ^c	33.0 ($\mu\text{mol kg}^{-1} \text{ day}^{-1}$) (7 days)	5.1% untreated control (cysts)
<i>In vitro</i> Results		
<i>Leishmania donovani</i> ^d		2.5 μM IC ₅₀
<i>Plasmodium falciparum</i> ^e		20.0 nM IC ₅₀
<i>Giardia lamblia</i> ^f		0.2 μM IC ₅₀
<i>Candida albicans</i> ^g		6.25/25.0 $\mu\text{g mL}^{-1}$ (MIC/MFC)
<i>Cryptococcus neoformans</i> ^g		12.5/25.0 $\mu\text{g mL}^{-1}$ (MIC/MFC)
<i>Mycobacterium tuberculosis</i> ^h		64.0 μM IC ₅₀

^a Ref. [27].

^b Dr J. E. Hall, unpublished results.

^c Ref. [18].

^d Ref. [25].

^e Dr K. Werbovetz, unpublished results.

^f Dr C. C. Dykstra, unpublished results.

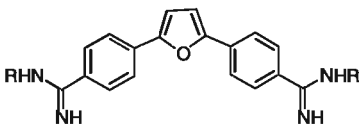
^g Ref. [25].

^h Dr S. Franzblau, unpublished results.

effective as judged by the reduction of lung cyst counts to about 2% of controls [27]. When furamidine is given orally in a neonatal mouse model of the gastrointestinal pathogen *Cryptosporidium parvum* at 33 mol kg⁻¹ for 7 days a reduction of cyst counts to about 5% of controls is observed [18]. In view of this activity and the absence of an approved drug to treat *C. parvum* infections, furamidine merits pre-clinical evaluation as a potential anti-cryptosporidial drug. Although furamidine has excellent broad-spectrum antimicrobial activity when administered intravenously, it is not orally effective against PCP or trypanosomes. Thus, it is against *C. parvum* that it is conceivable to see its direct use by virtue of its oral effectiveness against this organism. Furamidine shows interesting activity, but ultimately probably has limited usefulness against several other organisms, including *Candida albicans* [25], *Cryptococcus neoformans* [13], *Giardia lamblia*, *Leishmania donovani* [25], *Plasmodium falciparum*, and *Mycobacterium tuberculosis* as shown by the *in vitro* data in Tab. 16.6.

During the past several years we have explored several approaches to improve the antimicrobial efficacy of furamidine-type minor groove binders. Several investigations have suggested that groups that would increase the van der Waals interactions of minor groove binders with the walls of the groove might increase the DNA affinity of these types of molecules [134, 135]. Despite the fact that no direct relationship between DNA affinity and antimicrobial effect has been noted for dicationic compounds, we made a series of bis-*N*-alkyl analogs and evaluated their effectiveness on intravenous injection in the immunosuppressed rat model for *P. carinii* pneumonia in an effort to increase efficacy of these furamidine types. Table 16.7 contains data for selected compounds from this study [29]. Most of the *N*-alkyl-substituted compounds exhibit a higher DNA affinity than furamidine as judged by an increase in ΔT_m values. Not only does the minor groove accommodate some rather bulky groups at the termini of the diarylfuran unit but also the

Tab. 16.7. *In vitro* activity of 2,5-bis(4-amidinophenyl)furans versus PCP.^a



Compound	R	ΔT_m (poly dA-dT)	ΔT_m (oligo) ^b	Dosage (μmol kg ⁻¹)	% of control (lung tissue cysts)
Saline					100.0
Pentamidine		12.6	4.8	22.0	2.3
17	H	25.0	11.7	2.7	5.65
18	i-Pr	>28	14.4	2.2	8.4
19	c-pentyl	>28	15.8	0.9	0.11
21	c-Pr	>28	12.4	10.8	0.03
22	2-pentyl	20.4	7.7	4.8	24.4

^a Ref. [29].
^b oligo = d(CGCGAATTCGCG)₂.

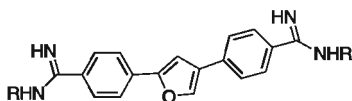


Fig. 16.14. 2,4-Diaryl furan analogs of furamidine.

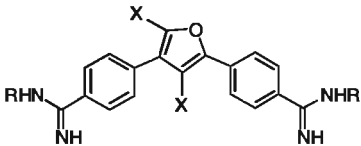
presence of these groups leads to an increase in the DNA affinity. The results are consistent with the view that van der Waals interactions are enhanced by the greater surface contact with the walls of the groove [117]. The biological activity of the more effective compounds in this study range from 10 to 20 times that of furamidine. The cyclopentyl compound **19** appears to be a good candidate for further evaluation against *P. carinii* pneumonia, although a regime involving intravenous injection would be required since it is not effective when given orally.

In addition to modification of the terminal amidino units we have replaced the central furan ring with a number of other heterocyclic ring systems. Analogs of furamidine in which the furan ring has been replaced by pyrrole [136], thiophene [136], and other five-membered ring units have been studied [137]. We have also replaced the central ring with six-membered rings including pyridine [138], pyrimidine [139–141], pyridazine [137], and triazine [142]. While these modifications have led to a number of compounds with both significant DNA affinity and antimicrobial activity, especially in the pyrimidine series, none appeared to have significant advantages over the furan analogs.

As part of the search for compounds with improved efficacy we prepared a series of 2,4-bis(4-amidinophenyl)furans (see Fig. 16.14) [30]. The 2,4-substitution on the core furan ring was expected to modestly increase the twist of the system, which may enhance the isohelicity of these molecules and could result in better binding affinity. Such an effect could be offset by the fact that the hydrogen atom on carbon 3 of the core furan ring points to the floor of the groove and repulsive interactions may counter any gain in isohelicity. Also, the twist could conceivably alter the hydrogen bonding array and electrostatic interactions. Methyl groups were placed on the 3- and 5-positions to induce significant out-of-plane twist to further increase these effects. Table 16.8 contains data from selected compounds from this study [30].

When the ΔT_m values of the compounds in the 2,4-series are compared with those of their 2,5-isomers, one notes that the 2,4-compounds generally exhibit ΔT_m values which are lower by 10–20% of that of the 2,5-series. Thus it seems that the 2,4-array introduces modestly unfavorable interactions with the DNA minor groove. Despite the moderately lower DNA affinities, the anti-PCP activity of the 2,4-series is generally somewhat higher and the toxicity is somewhat lower than those of the 2,5-series. It is noteworthy that the compounds with 3,5-dimethyl groups, the ones with the greater torsional twist, show lower DNA affinities and biological activities, suggesting that optimal isohelicity had been exceeded. The 2,4-diaryl series of compounds also provides some good candidates for additional evaluation versus PCP, however these compounds also are limited by lack of oral activity.

In another approach that should increase van der Waals interactions, modify the

Tab. 16.8. *In vitro* activity of 2,4-bis(4-amidinophenyl)furans versus PCP.^a


The chemical structure shows a central furan ring with substituents X at the 2 and 5 positions. At the 3 and 4 positions of the furan ring, there are two phenyl rings. Each phenyl ring is substituted at the para position with an amidine group, represented as RHN-C(=NH)-NH₂.

Compound	R	X	ΔT_m (poly dA-dT)	ΔT_m (oligo) ^b	Dosage ($\mu\text{mol kg}^{-1}$)	% of control (lung tissue cysts)
Saline						100.0
Pentamidine			12.6	4.8	22.0	2.3
17			25.0	11.7	13.3	2.1
23	H	H	19.0	9.1	10.0	0.3
24	i-Pr	H	17.6	10.9	10.0	0.04
25	c-Pr	H	23.2	10.9	10.0	0.01
26	c-pentyl	H	20.4	13.6	10.0	0.16
27	H	CH ₃	17.0	6.5	10.0	0.9
28	i-Pr	CH ₃	12.5	5.4	10.0	24.4

^a Ref. [30].

number of base pairs recognized, and improve binding affinity, we made analogs of furamidine with extended aromatic systems. The modifications to the furamidine system are: (a) one phenyl ring was replaced with a benzimidazole ring, (b) both phenyl rings were replaced with benzimidazole rings, and (c) benzimidazole rings were added as an extension to the phenyl rings [143]. As representative of this approach, the parent diamidines, in each of the three series are shown in Fig. 16.15.

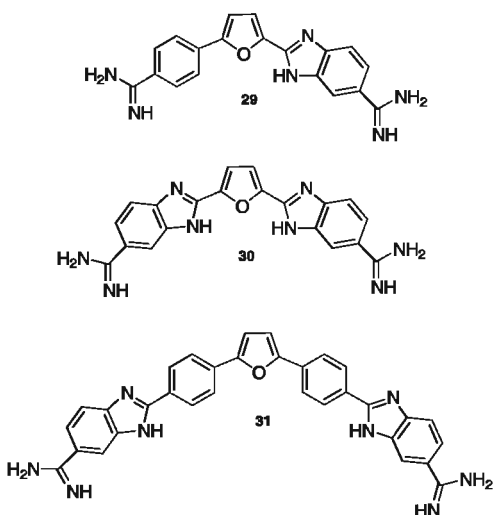


Fig. 16.15. Furamidine analogs with extended base pair coverage.

Because the length of these target molecules varies significantly it was necessary to compare the DNA binding of these molecules to oligomers of varying AT-tracts. Table 16.9 contains comparisons of ΔT_m values for furamidine (17) and its three extended analogs 29–31 with three different oligomers with 4,6- and 8-AT base pair binding sites. Subsequently, 29 has been found to bind to DNA in an unusual dimer motif which has potential for development of a new gene-regulation paradigm [144–146]. It can be seen from the data that, in general, as the aromatic stacking surface was extended, the DNA-binding affinity, as measured by ΔT_m , was increased. For example, in reviewing the data for $d(CGCGAATTCGCG)_2$ it is noted that the phenylbenzimidazole 29 has greater affinity than furamidine and that the bis-benzimidazole 30 has greater affinity than 29. However, the longest molecule 31 causes precipitation of the complex. A similar trend for 17 to 29 to 30 was noted for the interactions with $d(CGAAATTTAGC)_2$, however in this case 31 caused a destabilization of the oligomer. For the study with $d(GAAAATTTTCGAAAATTTTC)_2$ the binding affinity increased with increasing length of the diamidine: $17 < 29 < 30 < 31$. It is thought that the behavior of 31 with the two oligomers with shorter AT-tracts is likely due to self-stacking interactions being greater than drug–DNA interactions.

When the binding site is enlarged to accommodate the entire molecule, which appears to require eight AT base pairs, this effect is not observed. The results for the *in vivo* testing of these compounds versus PCP are also included in Tab. 16.9. Only the intermediate-sized molecule 30 shows significant activity against PCP. In some of the longer compounds (data not shown here) increased toxicity was observed. It was concluded that, generally, extending the length of the compound did not increase the antimicrobial efficacy of this class of compounds [143].

We have shown that 2,5-diphenylfuran and 2,4-diphenylfuran diamidines are highly effective antimicrobial agents in several animal models and that they show promising activity *in vitro* against a number of pathogenic organisms. As another approach to improve upon these antimicrobial activities we decided to investigate the effect of more fundamental structural variations on the cationic centers as

Tab. 16.9. PCP results and DNA affinities (ΔT_m) for 17, and 29–31 with oligomers with varying AT-tracts.^a

	17	29	30	31
$d(CGCGAATTCGCG)_2$	8.3	10.0	11.7	ppt
$d(CGAAATTTAG)_2$	12.6	14.1	17.1	–1.5
$d(GAAAATTTTCGAAAATTTTC)_2$	7.3	14.8	18.0	29.6
% of control(cyst'g of lung) (PCP)	2.1 ^b	51.0 ^c	1.8 ^d	180 ^e

^a Ref. [145].

^b 13.3 $\mu\text{mol kg}^{-1}$.

^c 5.0 $\mu\text{mol kg}^{-1}$.

^d 10.0 $\mu\text{mol kg}^{-1}$.

^e 7.1 $\mu\text{mol kg}^{-1}$.

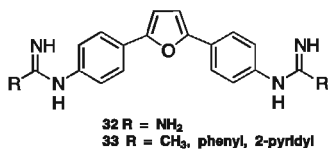


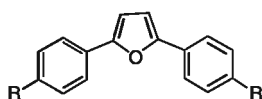
Fig. 16.16. Guanidino and reversed amidine analogs of furamide.

contrasted to previous *N*-alkylation of the amidine. The guanidino group (as in **32** in Fig. 16.16) is another amidine-related cationic unit that due to its different number and array of atoms has the potential to recognize different base sequences and consequently is a functional group worthy of study. There are reports of antimicrobial activity of guanidino compounds [7, 147] and, of course, netropsin, which contains one guanidino group, is a much studied minor groove binder. However, the guanidino class of cationic compounds has not been evaluated as extensively as the amidino class, and has not been previously studied in the diarylfuran system. The synthesis, DNA-binding affinities, and the antimicrobial properties of 2,5-bis[alkyl (or aryl) imino] aminophenylfurans (as in **33** in Fig. 16.16) are also being studied [16].

These compounds have the imino group of the amidine attached to an “anilino” nitrogen in contrast to the original amidino furans in which the imino groups are directly attached to the phenyl rings. We refer to these compounds as “reversed” amidines. It should be noted in the case of the 2,5-bis[(aryl) imino] aminophenylfurans that the amidino unit has two aryl groups directly attached which will lower the pK_a of the compounds and may improve their oral bioavailability. For example the pK_a of benzamidine is reported as 11.2 and the pK_a of *N*-phenylbenzamidine is 7.7; a lowering of the pK_a by approximately 3.5 pK units [148]. An initial report that describes the synthesis and some of the biological properties of these two classes of compounds has appeared [16].

Tables 16.10–16.12 contain DNA-binding affinities and antifungal activities for selected guanidino and reversed amidino compounds. Table 16.10 contains molecules that vary only in the structure of the cationic centers. The parent diguanidino compound **32** shows a strong affinity for DNA as judged by the ΔT_m values for both poly dA-dT (21.6) and the dodecamer (10.8) (Tab. 16.10). These values compare well to those for furamide (25 and 11.7, respectively), suggesting little net difference in the affinity of the interaction of the amidine and guanidine cationic centers with the minor groove of DNA when using the 2.5-diarylfuran framework. The reversed diamidine **35** bearing a phenyl terminal group showed an increase in affinity over that of the parent diguanidino **32**. The ΔT_m value for the compound **34** with a terminal 2-pyridyl group is significantly lower than that for its phenyl counterpart **35**. The lower affinity of **34** suggests a different binding mode or different base pair selectivity for these two closely related analogs.

The introduction of a methyl group (**36**) as the terminal group led to a significant drop in binding affinity. Placement of a single substituent on each of the two phenyl rings of the 2,5-diphenylfuran system produced differences in ΔT_m values for both the diguanidine and reversed diamidine series. For the diguanidine series

Tab. 16.10. *In vitro* antimicrobial activities and DNA-binding results for 2,5-diarylfuran dicationic molecules.^a

Compound	R	<i>C. albicans</i>		<i>A. fumigatus</i>		<i>M. tuberculosis</i>	DNA affinity ^c	
		MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	ΔT_m ($^{\circ}\text{C}$)	ΔT_m (oligo)
32	NHC(=NH)NH ₂	12.5	25	100	>100	3.13	21.6	10.8
34	NHC(=NH)-2-Pyr	nt	nt	nt	nt	1.56	19.6	7.5
35	NHC(=NH)Ph	25	50	nt	nt	1.56	28.6	15.0
36	NHC(=NH)CH ₃	>100	nt	100	>100	nd ^b	15.9	6.0
Furamidine		6.25	25	nt	nt		25	11.7
Fluconazole		0.25	NA					
Rifampin						0.062		

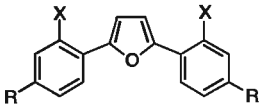
^a Ref. [16].^b No activity observed at 6.25 $\mu\text{g mL}^{-1}$.^c AT = poly dA-dT; oligo = d(CGCGAATTCGCG)₂. nt = not tested; nd = not determined.

(Tab. 16.11), placement of single substituent of approximately the same size but of differing electronic properties (e.g. Me, OMe, Cl) on the phenyl rings resulted in a lowering of the ΔT_m values; compare the values for oligomer binding of 32 with 37–39. The ΔT_m values for a series of reversed diamidines with terminal 2-pyridyl groups (Tab. 16.12) showed a different sensitivity to substituents. In this case, the introduction of a substituent on each of the two phenyl rings of the 2,5-diarylfuran system caused only a modest effect on the ΔT_m values when the substituent was methyl (40) or methoxy (43). However, introduction of a chloro group (44) resulted in significant reduction of the value, perhaps in part due to a pK effect.

Interestingly, the ΔT_m -lowering effect of the chloro group was greater for the pyridyl derivative 44 than for the analogous guanidine 39, possibly due to the lower basicity of the reversed amidine relative to the guanidine. The reversed amidine system is also sensitive to structural change at the terminal location. For example, replacement of the terminal 2-pyridyl with a 2-quinolyl group (41) resulted in a significant lowering of the ΔT_m value; clearly there are size and shape limitations for the terminal group. In contrast, introduction of a methyl group on the terminal pyridyl ring (42) slightly enhanced the binding affinity.

The antimicrobial data for these compounds are also included in Tabs 16.10–16.12. The diguanidino compound that showed the most promising activity was 37, which was effective *in vitro* against both *C. albicans* and *M. tuberculosis*. Compound 37 gave MIC values of approximately 1 $\mu\text{g mL}^{-1}$ against both organisms, and was fungicidal against *C. albicans*. Four of the compounds with terminal pyridyl groups (34, 40, 42, and 43) showed promising activity against *M. tuberculosis* (MIC values from 1.0 to 2.0 $\mu\text{g mL}^{-1}$). Compounds 42 and 43 also showed good

16.11. *In vitro* antimicrobial activities and DNA-binding results for diguanidino 2,5-diarylfurans.^a



Compound	X	R	<i>C. albicans</i>		<i>A. fumigatus</i>		<i>M. tuber-</i> <i>culosis</i>	DNA affinity ^c	
			MIC	MFC	MIC	MFC	MIC	ΔT_m	ΔT_m
			($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)	(ΔT)	(oligo)
	X=H	NHC(=NH)NH ₂	12.5	25	100	>100	3.13	21.6	10.8
	X=CH ₃	NHC(=NH)NH ₂	1.04	2.08	33.4	33.4	≤1	17.8	6.9
	X=OCH ₃	NHC(=NH)NH ₂	10	100	100	100	16	15.2	2.8
	X=Cl	NHC(=NH)NH ₂	10	100	100	100	4	26.1	4.7
amidine			6.25	25	nt	nt		25	11.7
conazole			0.25	nf					
mpin							0.062		

f. [16].
^a = poly dA-dt; oligo = d(CGCGAATTCGCG)₂.
= not tested; nf = not fungicidal.

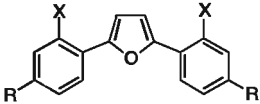
activity at the MIC level of $\leq 1.0 \mu\text{g mL}^{-1}$ against *C. albicans*, and 43 was similarly active versus *Aspergillus fumigatus*.

The data presented indicate that both the diguanidines and the reversed diamidines in the 2,5-diarylfuran series have significant DNA-binding properties that are dependent on structure. Substituents on both the central phenyl rings of the 2,5-diarylfuran system and on the terminal cationic centers effect the DNA-binding affinity. It is possible that the different series bind to DNA by different modes; an understanding of any such differences awaits the results of biophysical studies that are underway. Compounds from both of these two new classes of DNA-binding agents show promising *in vitro* antimicrobial activities.

16.3.4
Pro-drug Approaches for Furamidine

As noted in a previous section arylamidines are quite basic and exhibit pK values of approximately 11 [148]. Consequently, the low oral bioavailability of these type molecules is not unexpected. Nevertheless, very few reports have appeared describing pro-drug approaches for aryl amidines [104, 105]. Weller and co-workers have demonstrated that both amidoxime and carbamate derivatives of mono-amidines are effective pro-drugs and provide significantly improved oral bioavailability for fibrinogen antagonists [104]. It appears that there are no carbamate-specific enzymes in mammals [149], nevertheless, several activated alkyl carbamates synthesized from amines have been reported to behave well as pro-drugs and their conversion to the parent drug has been attributed to enzymatic hydrolysis [150].

Tab. 16.12. *In vitro* antimicrobial activities and DNA-binding results for 2,5-diarylfuran “reversed” amidines.^a



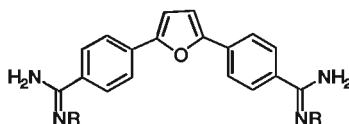
Compound	X	R	<i>C. albicans</i>		<i>A. fumigatus</i>		<i>M. tuber-</i>	<i>DNA</i>	
							<i>culosis</i>	<i>affinity</i> ^b	
			MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	MFC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	ΔT_m ($^{\circ}\text{C}$)	ΔT_m (oligo)
34	X = H	NHC(=NH)- 2-Pyr	nt	nt	nt	nt	1.56	19.6	7.5
40	X = CH ₃	NHC(=NH)- 2-Pyr	10	10	10	100	≤ 1	22.6	8.9
41	X = CH ₃	NHC(=NH)- 2-Qu	100	100	100	nt	nt	7.5	1.1
42	X = CH ₃	NHC(=NH)- 2-Pyr-5-CH ₃	≤ 1	10	10	> 100	2	24.3	10.8
43	X = OCH ₃	NHC(=NH)- 2-Pyr	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	19.0	7.8
44	X = Cl	NHC(=NH)- 2-Pyr	100	> 100	> 100	nt	8	5.2	0
Furamidine			6.25	25	nt	nt		25	11.7
Fluconazole			0.25	nf					
Rifampin							0.062		

^a Ref. [16].
^b AT = poly dA-dT; oligo = d(CGCGAATTCGCG)₂.
nt = not tested; nf = not fungicidal.

Reports of pro-drugs of diamidines are even more limited. The *in vivo* conversion of pentamidine dioxime to pentamidine has been noted [107]. However, on oral administration of pentamidine dioxime in the immunosuppressed rat model for PCP it was only moderately effective [106]. We have reported that the bis-amidoxime and bis-O-methylamidoxime of furamidine (17) were quite effective anti-PCP agents in the rat model on both oral and intravenous administration (see Tab. 16.13) [108]. However, the bis-O-ethylamidoxime of 17 was ineffective, demonstrating a remarkable sensitivity to structure.

We have also prepared a number of carbamates, including some which are double pro-drugs, of 17 as part of our continuing effort to develop pro-drug approaches for diamidines to achieve better oral activity (J. E. Hall, unpublished results). Table 16.13 also includes the data for the most promising of these carbamates, which are of comparable effectiveness to the oxime pro-drugs in the rat model for *P. carinii*. Thus, we have developed two effective pro-drug types for furamidine. As previously noted, one of these 2,5-bis[4-(methoxyamidino)phenyl]furan (20, DB289), is currently undergoing phase II clinical trials.

Tab. 16.13. Pro-drugs of furamidine: oximes and carbamates versus PCP.



Compound	R	Dose ($\mu\text{mol kg}^{-1}$)	Cysts per g of lung (% of control)
Pentamidine ^a		i.v. 22.0	2.0
		oral 33.0	133.1
Pentamidine dioxime ^a		i.v. 22.0	0.11
		oral 33.0	17.3
17 ^b	H	i.v. 13.3	0.8
		oral 39.8	42.6
45 ^b	OH	i.v. 22.0	0.7
		oral 33.0	3.1
20 (DB289) ^b	OCH ₃	i.v. 22.0	14.3
		oral 33.0	1.8
46 ^c	COOPh(<i>p</i> OMe)	i.v. 22.0	0.02
		oral 33.0	2.1
47 ^c	COOPh(<i>p</i> F)	i.v. 22.0	0.02
		oral 33.0	2.2

^a Ref. [106].^b Ref. [108].^c Ref. [111].

An understanding of the bioconversion of **20** to furamidine (**17**) is important for its clinical development as well as to provide fundamental information needed to aid in the development of new amidine pro-drugs. Wide-ranging studies of the metabolism of **20** are underway [112, 151–153]. Preliminary results suggest that the major metabolism pathway is a multistep one that is outlined in Fig. 16.17. Interestingly, initial results suggest that the first and key step in the biotransformation of **20** is demethylation of the methamidoxime to form an amidoxime. The amidoxime units appear to be the functional groups that are ultimately reduced to form amidino groups found in the parent drug furamidine. Two pathways from **20** to **17** are feasible as illustrated in Fig. 16.17.

16.3.5

Synthetic Approaches for Furamidine and Analogs

Our original synthesis of furamidine is outlined in Scheme 16.5 [32] and is essentially the approach used by Dann [36]. The process begins with a Friedel–Crafts reaction between bromobenzene and fumaryl chloride. The reaction proceeds well when bromobenzene or carbon disulfide is used as the solvent, but gives very poor yields with other solvents such as nitrobenzene. The reduction of the dibenzoylthene can be achieved with Zn/HOAc or with stannous chloride, however the latter is now the preferred reagent [153]. Furan ring formation is readily achieved under

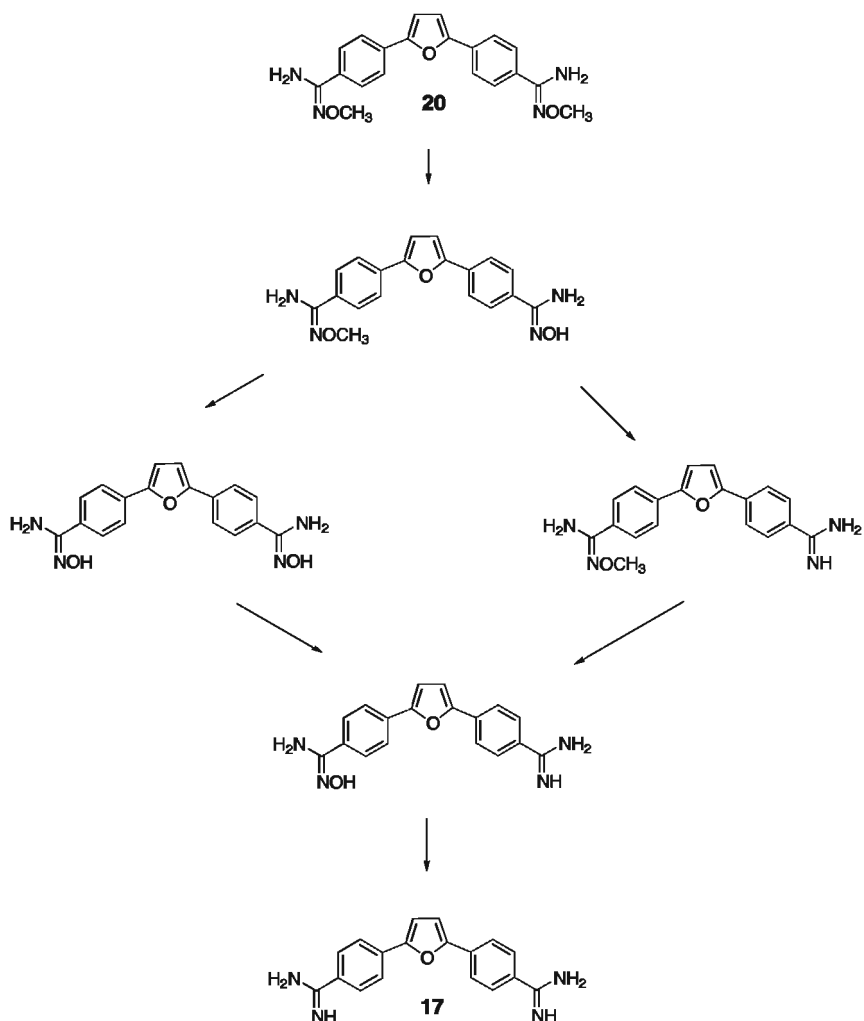
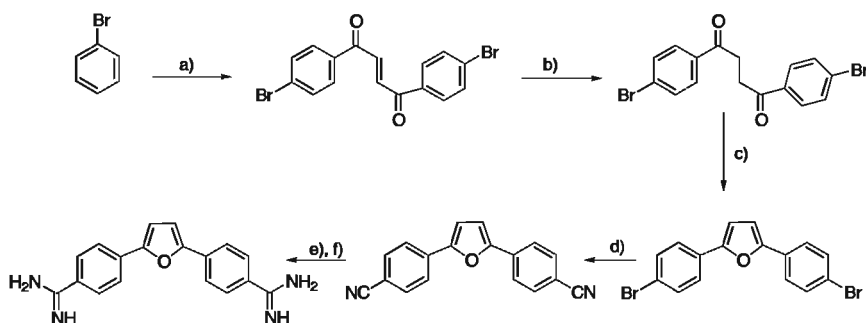


Fig. 16.17. Bioconversion of **20** to **17**.

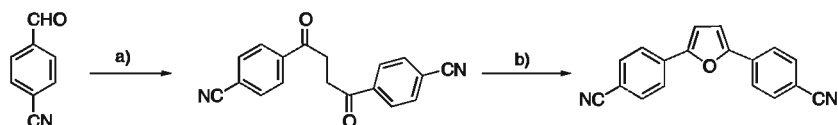
dehydrating conditions. 2,5-Bis[4-bromophenyl]furan is converted into 2,5-bis[4-cyanophenyl]furan by the action of copper (I) cyanide. The bis-nitrile is converted to furamidine by a classical Pinner sequence which involves first formation of the bis-imidate ester employing anhydrous ethanol and hydrogen chloride followed by reaction with ammonia to yield the diamidine [32]. While the sequence outlined in Scheme 16.5 works well it involves several steps. The original synthesis also requires the use of carbon disulfide or bromobenzene in excess in the first step, either of which is disadvantageous for large-scale synthesis. Consequently, we explored other approaches to make these types of compounds by seeking a more efficient route to prepare 2,5-bis[4-cyanophenyl]furan.

Scheme 16.6 outlines an approach to make 2,5-bis[4-cyanophenyl]furan based on



a) fumaryl chloride, AlCl_3 , CS_2 b) SnCl_2 , EtOH, HOAc c) H_2SO_4 , Ac_2O d) CuCN , DMF e) HCl , EtOH f) NH_3 , EtOH

Scheme 16.5



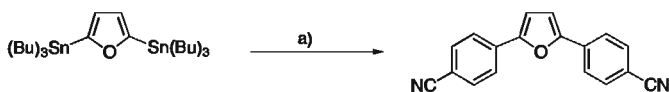
a) divinylsulfone, NaOAc , thiazolium catalyst, EtOH b) H_2SO_4 , Ac_2O

Scheme 16.6

Setter methodology. In this case the thiazolium catalyzed reaction of divinyl sulfone and 4-cyanobenzaldehyde gave the saturated 1,4-diketone in one step [154]. However, the yield for this reaction was consistently only in the 40–50% range. Conversion of the saturated 1,4-diketone to the furan was readily achieved in high yields under similar conditions as noted previously in Scheme 16.5.

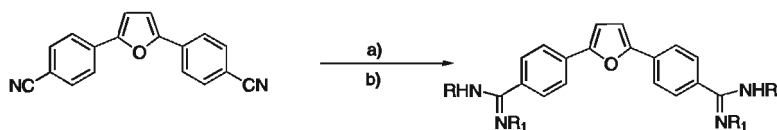
Another route for preparation of 2,5-bis[4-cyanophenyl]furan is outlined in Scheme 16.7. In this case Stille coupling of 2,5-bis[tri-*n*-butylstannyl]furan with 4-bromobenzonitrile gave the desired bis-nitrile in a 70% yield [155]. This approach has been used in the bulk drug scale-up GMP synthesis of **20** (DB289) for clinical trials [156].

Scheme 16.8 summarizes the Pinner approach for the conversion of 2,5-bis[4-cyanophenyl]furan into the corresponding 2,5-bis[4-(*N*-alkylamidino)]furans and the structurally related bis-amidoximes. The Pinner approach is arguably the most



a) 4-bromobenzonitrile, $\text{Pd}(\text{PPh}_3)_4$, 1,4-dioxane

Scheme 16.7



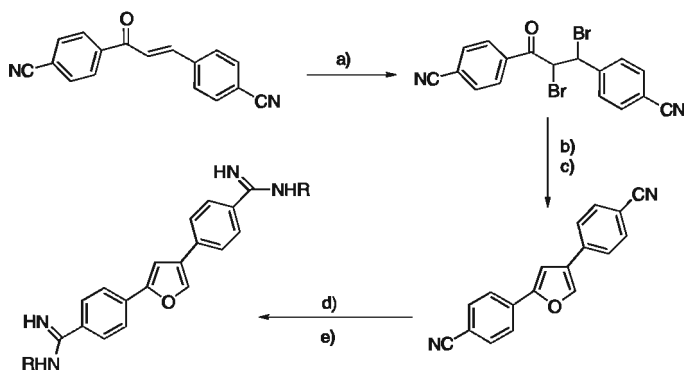
a) HCl, EtOH b) NHR, or NH_2OR_1 , EtOH

Scheme 16.8

widely used method for preparation of amidines. Generally, this method works quite well, however it requires the rigorous exclusion of water [27, 29, 32]. A recently reported method for conversion of a nitrile group to an amidine, involving the formation of and subsequent reduction of an amidoxime, is quite attractive as it does not require rigorous water exclusion [157]. However, this method cannot be readily used for preparation of *N*-alkylamidines.

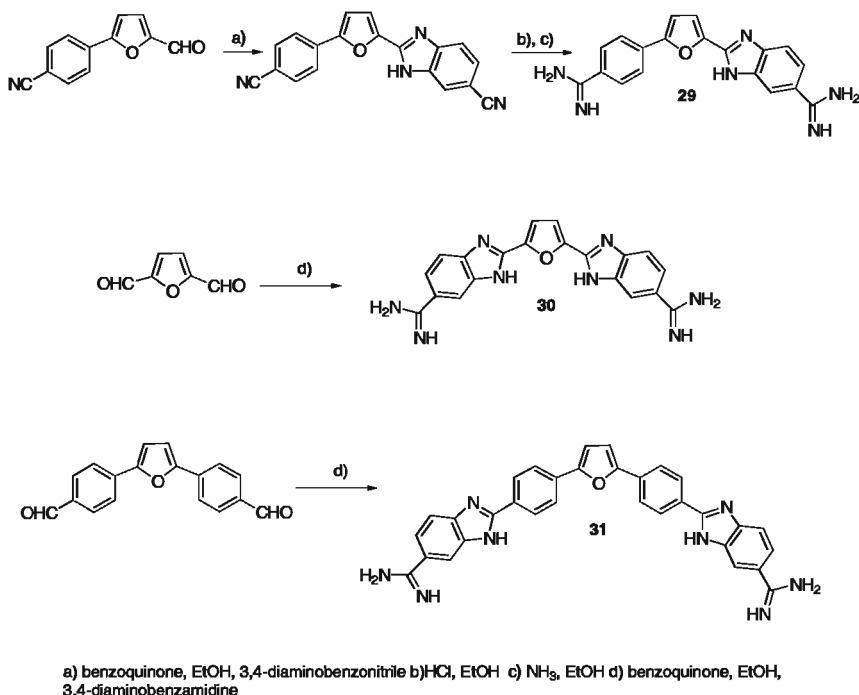
The synthesis of the 2,4-bis[4-amidinophenyl]furans is summarized in Scheme 16.9 [30]. Bromine was added to the bis-cyanochalcone in a conventional manner to give the expected dibromo analog. The dibromo analog was converted into the corresponding enol ether by the action of excess sodium methoxide. The enol ether was, without characterization, treated with dimethyl sulfonium ylide generated in dimethylsulfoxide. The insertion reaction yielded the expected 2,4-bis[4-cyanophenyl]furan. An alternate synthetic route into the 2,4-bis[phenyl]furan system was later developed [158]. 2,4-Bis[4-cyanophenyl]furan was converted into the corresponding amidines using the Pinner approach.

The syntheses of the three benzimidazole-containing analogs of furamide 29–31 are outlined in Scheme 16.10 [143]. In the synthesis of each analog, oxidative coupling of the appropriate aldehyde with a phenylenediamine is employed for the key step of benzimidazole ring formation. In the case of the synthesis of 29 the bis-nitrile was first prepared and Pinner methodology was used to convert it



a) Br_2 , CHCl_3 b) NaOCH_3 , CH_3OH c) NaH , DMSO , THF , $(\text{CH}_3)_3\text{SiI}$ d) HCl, EtOH e) NH_3 or RNH_2 , EtOH

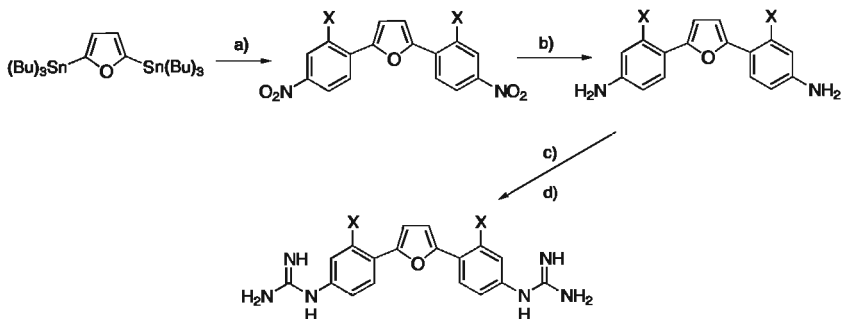
Scheme 16.9



Scheme 16.10

into the bis-amidine. Both **30** and **31** were prepared, in one step, by coupling 3,4-diaminobenzamidine with the appropriate dialdehyde. The use of benzoquinone as the oxidizing agent in these coupling reactions gave the respective benzimidazoles in good yields (52–79%).

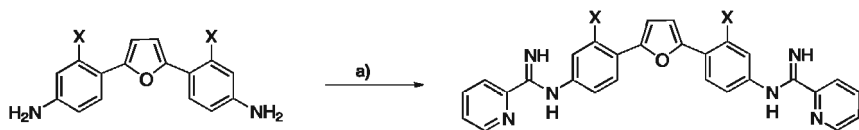
Scheme 16.11 outlines the approach employed for synthesis of the bis-guanidino compounds of type **32**. The preparation of the key intermediate 2,5-bis[4-amino-



Scheme 16.11

phenyl]furans was achieved in two steps [16]. The first step employs Stille coupling between 2,5-bis(tri-*n*-butylstannyl)furan and a substituted 4-bromonitroarene to form the corresponding 2,5-bis(4-nitrophenyl)furans. This reaction typically gave good yields (65–88%) of the dinitro compounds [155]. In the second step, reduction of the 2,5-bis(4-nitrophenyl)furans was achieved either by catalytic hydrogenation or by the action of stannous chloride, and generally produced the desired diamino compounds in excellent yields. The diguanidino analogs of type **32** were prepared in a two-step process from the 2,5-bis[4-aminophenyl]furans. The first step involved reaction with Boc-protected *S*-methylthiourea in the presence of mercuric chloride. The Boc-protected guanidine analogs were deprotected using anhydrous HCl in dichloromethane/EtOH to give the bis-guanidines in good overall yield [16].

Scheme 16.12 outlines the approach employed for synthesis of the reversed amidines compounds of type **33**. The reaction of the 2,5-bis[4-aminophenyl]furans with a *S*-(2-naphthylmethyl)-thioimide in EtOH/MeCN gave the desired reversed amidines [16]. This reaction gave good yields (60–80%) when the diamine was unsubstituted or substituted with electron-donating groups; however, when the diamine was substituted with the deactivating chloro group, the yield was greatly reduced (25%).



a) *S*-(2-naphthylmethyl)-2-pyridylthiocarboximidate, CH₃CN, EtOH

Scheme 16.12

16.4

Conclusions

Carbazoles and related dibenzothiophenes and dibenzofurans proved to be an interesting series of compounds both in their DNA-binding characteristics and biological activity. The series was unique in that they were the first example of fused ring systems forming strong minor groove complexes with AT sequences. Characterization of the complexes with DNA was determined for the carbazoles since they appeared to be stronger DNA-binding agents than the corresponding isosteres. These studies determined that there were different binding modes for 3,6- and 2,7-bis(2-imidazolynyl)carbazole. The 3,6-analog formed a complex with the minor groove of d(CGGAATTCGCG)₂ with the carbazole nitrogen pointing away from the groove, while the 2,7-compound complexed to the decamer duplex with the carbazole nitrogen facing into the minor groove. DNase footprinting studies indicated that carbazoles complexed in the minor groove with broad AT specificity and formed patterns in agreement with minor groove binding. These series of

compounds produced a number of excellent antimicrobial agents. In addition to being stronger DNA-binding agents, the carbazoles, as a whole, appeared to have better antimicrobial activity than corresponding dibenzothiophenes and dibenzofurans. Amidoxime pro-drugs for the dibenzothiophenes and dibenzofurans were effective, however those for the carbazoles were not. Studies are currently underway to determine the cause of this difference in pro-drug activity.

The more active compounds from both the 2,4-bis[4-*N*-alkylaminophenyl]furan and 2,4-bis(4-amidinophenyl)furan series showed increased efficacy and lower toxicity when compared to pentamidine and furamidine on intravenous administration in the immunosuppressed rat model for *P. carinii*. These compounds also exhibit significant DNA-binding affinity. However, these dicationic compounds are not effective when given orally. The furamidine analogs with extended aromatic systems show strong DNA-binding affinities and the longer molecules recognize AT tracks with eight base pairs. Generally, these extended molecules are less effective and more toxic than furamidine. The “reversed” amidines (2,5-bis[[arylimino]aminophenyl]furans) and the 2,5-bis(4-guanidinophenyl)furans show significant DNA-binding affinities and several of them showed promising *in vitro* antimicrobial properties. The ultimate usefulness of these compounds will depend upon results from studies in animal models, which are in progress. Diamidoxime and dicarbamate pro-drugs for furamidine, which are effective on oral administration in the immunosuppressed rat model for *P. carinii*, demonstrate the potential for this approach to capitalize on the antimicrobial activities of these types of DNA minor groove binding agents and has led to clinical trials for one of these compounds.

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17

Energetics of Anthracycline–DNA Interactions

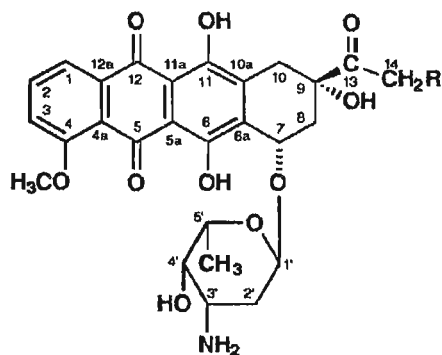
Jonathan B. Chaires

17.1

Introduction

The anthracycline antibiotics have been mainstays of cancer chemotherapy for over 30 years [1–6]. Daunorubicin (daunomycin) and doxorubicin (adriamycin) are the parental compounds of this class of drugs. Both are natural products, and are synthesized by particular species of *Streptomyces* [7, 8]. Daunorubicin is primarily used now in the treatment of leukemias. Doxorubicin has a broad spectrum of activity, and is used alone and in combination to treat a variety of cancers. Doxorubicin differs from daunorubicin at only the C13 position, where a hydroxyl group is substituted for a simple hydrogen atom. Figure 17.1 shows the structure of daunorubicin. Table 17.1 provides a comparison of key chemical and physical properties for daunorubicin and doxorubicin. The added hydroxyl substituent renders doxorubicin more polar than daunorubicin, as manifested by a larger polarizability and a smaller non-polar solvent accessible surface area. As a result, although the two drugs are similar in size and structure, their chemical and physical properties differ, exemplified by strikingly different water-to-octanol partition coefficients.

DNA is a key target for daunorubicin in the cell. The biological effects of the drug on cells are, however, pleiotropic [6, 9, 10]. Membrane interactions and robust free radical chemistry certainly contribute to anthracycline antibiotic cytotoxicity. Inhibition of topoisomerase II is currently thought to be the primary mechanism by which daunorubicin and doxorubicin kill cells [11–13]. Intercalation is necessary for topoisomerase II inhibition by anthracyclines, emphasizing the key role of DNA as a target [11]. Perhaps the most convincing evidence for DNA as a primary target for the anthracyclines comes from quantitative structure–activity relationship (QSAR) studies [14]. A QSAR was built for 29 anthracycline analogs whose antitumor activity against B-16 melanoma cells was measured. Inclusion of a term for the DNA interaction energy of each analog led to a statistically more significant QSAR, indicating that DNA binding is a key component of the mechanism of action of these anthracyclines. The anthracyclines are the best structurally characterized intercalators, with 22 high-resolution structures on deposit in the Nucleic Acid Structure Database [15]. While it was hoped that such information would fuel



- 1 - R=OH -- Doxorubicin
 2 - R=H -- Daunorubicin

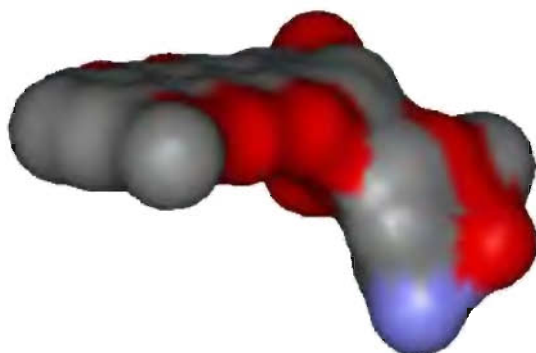


Fig. 17.1. Structure of daunorubicin (daunomycin). The bottom image is the van der Waals surface rendering, colored according to atom type.

rational, structure-based design of improved anthracyclines, it has not. Structure-based design strategies, in general, have not lived up to their initial promise and hyperbole [16].

In part, this failure arises from the neglect of thermodynamics. Structures alone are incapable of defining the molecular forces that drive a particular association reaction, and tend to emphasize the static features of the final complex. Hydrogen bonds, in particular, are often heavily emphasized in discussion of the structures of drug–DNA complexes, when in fact they may contribute only marginally to the overall free energy of complex formation. Rational drug design efforts must integrate thermodynamic and kinetic data into the process, along with structural information, in order to succeed.

Tab. 17.1. Physical and chemical properties of daunorubicin and doxorubicin.

Property	Daunorubicin	Doxorubicin
$P_{w \rightarrow o}$	3.6	0.8
Log P_{exp}	0.56	−0.09
Log P_{calc}	0.46	−0.05
$\text{SASA}_{\text{Total}} (\text{\AA}^2)$	790.9	794.3
$\text{SASA}_{\text{Nonpolar}} (\text{\AA}^2)$	550.9	518.2
$\text{SASA}_{\text{Polar}} (\text{\AA}^2)$	240.0	276.1
Dipole moment (D)	4.98	6.11
Polarizability ($\text{\AA}^3$)	46.51	46.17
Log S	−2.97	−2.28

$P_{w \rightarrow o}$, water to octanol partition coefficient. Log P_{exp} , experimental log $P_{w \rightarrow o}$ value. Log P_{calc} , calculated log $P_{w \rightarrow o}$ value. $\text{SASA}_{\text{Total}}$, total solvent accessible surface, calculated using a 1.4 Å probe radius. $\text{SASA}_{\text{Nonpolar}}$, non-polar (“hydrophobic”) solvent accessible surface area. $\text{SASA}_{\text{Polar}}$, Polar (“hydrophilic”) solvent accessible surface area. Log S , calculated solubility. Log P_{calc} was calculated using the CLOGP program (BioByte Corp., Claremont, CA, USA). Solvent accessible surfaces area values were taken from Ref. [35]. All other calculated values were computed using QikProp software (Shrodinger, Inc., Portland, OR, USA).

The purpose of this contribution is to provide a detailed and integrated overview of thermodynamic studies of the DNA binding of anthracycline antibiotics, with particular emphasis on daunorubicin. Years of studies from this laboratory on the daunorubicin–DNA interaction have provided perhaps the most detailed set of thermodynamic data available for any intercalator. These data will be summarized and integrated here.

17.2

Binding Free Energy

Binding free energies may be obtained from experimentally determined binding constants by using the standard Gibbs relationship $\Delta G = -RT \ln K$, where K is the equilibrium binding constant, R the gas constant, and T the temperature. Accurate determination of binding constants and stoichiometries is not easy. Proper determination of complete binding isotherms demands that over a 100-fold range in free ligand concentration be covered – a daunting task [17]. Fortunately, the anthracyclines have distinctive changes in their intrinsic absorbance and fluorescence upon binding to DNA that provide convenient signals to monitor binding interactions over such a range [18]. Figure 17.2 shows experimental data that define the interaction of daunorubicin with DNA under a particular solution condition (0.2 M Na^+ , pH 7.0, 20°C). The method of continuous variations [19, 20] was used to construct the Job plot shown in Fig. 17.2a. That plot shows that the site size on the DNA lattice for daunorubicin is 3–4 bp.

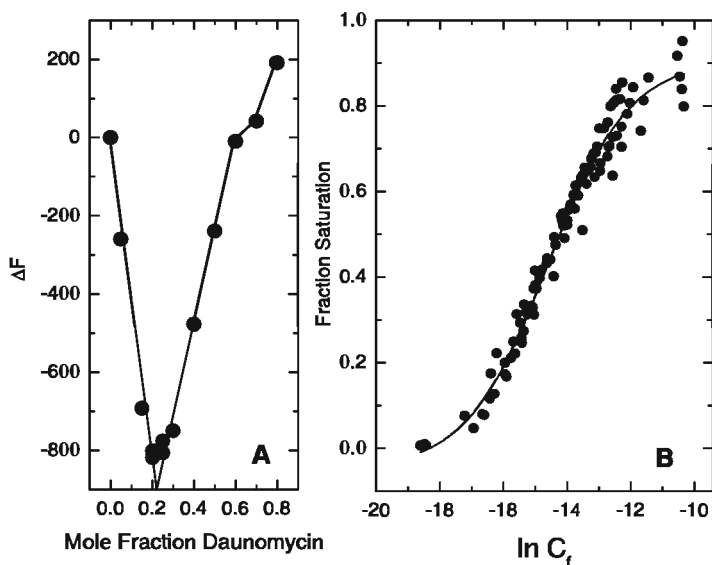


Fig. 17.2. Binding of daunorubicin to calf thymus DNA. (a) Job plot for the interaction of daunorubicin and calf thymus DNA. The total concentration (drug + base pairs) was maintained at 50 μM . ΔF is the difference in fluorescence emission intensity relative to drug

alone at the identical concentration. (b) Binding isotherm for the interaction of daunorubicin with calf thymus DNA. The fractional saturation (calculated assuming a site size of 3.4bp) is shown as a function of free drug concentration.

Figure 17.2b shows the binding isotherm for the daunorubicin–DNA interaction. This isotherm is as complete as any that is available for a drug–DNA interaction, and almost fully covers complete saturation of the DNA lattice by the drug. For a complete binding isotherm such as this, it is possible to obtain a model-free estimate for the free energy required to saturate DNA-binding sites with drug by using Wyman's concept of the median ligand activity [21, 22]. The free energy of ligation (ΔG_X) to go from a state where no ligand is bound to a degree of saturation of $\bar{X}$ is given by the equation

$$\Delta G_X = RT \int_0^{\bar{X}} \ln C_f \delta \bar{X}$$

where RT has its usual meaning. The pronounced advantage of this equation is that it provides an estimate of the free energy needed to attain any degree of saturation without recourse to any specific binding model. Numerical integration of the data in Fig. 17.2b yields an estimate of $\Delta G_X = -7.8 \text{ kcal mol}^{-1}$ for the full ligation of a daunorubicin-binding site. Free energies derived from binding constants obtained by curve fitting to specific models must agree with this model independent value if the model is reasonable.

The binding data of Fig. 17.2b may also be fit to specific binding models to obtain a binding constant. Full discussions of the non-linear least squares fitting

methods and binding models used in our laboratory for extracting binding constants may be found in Refs [18, 23, 24]. Analysis of the data using the standard neighbor exclusion model yields a binding constant $K = 6.6 (\pm 0.2) \times 10^5 \text{ M}^{-1}$ and a neighbor exclusion parameter $n = 3.3 \pm 0.1$. The binding free energy calculated using this binding constant is $-7.8 \text{ kcal mol}^{-1}$, in excellent agreement with the model free analysis presented above. Because of the assumptions inherent in the neighbor exclusion model, this binding free energy refers to the binding of a drug molecule to an isolated site (3.3 bp long) on the DNA lattice.

The meaning of the DNA binding constant and free energy is complicated by daunorubicin's sequence selectivity [25, 26]. DNase footprinting studies showed that daunorubicin binds preferentially to triplet sequences of the type 5'(A/T)CG and 5'(A/T)GC, where (A/T) means that either A or T may occupy the position. However, binding to these triplets is not absolute, and daunorubicin can and does interact with other triplet sequences with a variety of affinities. A quantitative analysis of available footprinting data revealed a distribution of binding constants for various triplet sequences that ranged from $0.4 \times 10^6 \text{ M}^{-1}$ to $2.4 \times 10^6 \text{ M}^{-1}$ under the conditions of the footprinting experiment [27]. That range corresponds to an approximately 2 kcal mol^{-1} difference in binding free energy. Such heterogeneity alters the interpretation of the binding constant obtained for daunorubicin's interaction with calf thymus DNA by invoking the neighbor exclusion model.

Formally, the neighbor exclusion binding constant refers to drug binding to identical, non-interacting sites along the DNA lattice. If heterogeneity exists, the exact value of the binding constant obtained is a complex weighted average, weighted by the frequency of triplet sites and the microscopic binding constant for interaction with that sequence. Indeed, the binding data of Fig. 17.2b can be analyzed and fit to a 10-site model in which each unique dinucleotide site is assumed to have a unique binding constant [24]. In spite of these complexities, the overall binding constant and free energy values obtained above remain invaluable quantitative measures of drug–DNA interaction, and provide firm insights into the forces that drive the association reaction.

17.3

Salt Dependency of Daunorubicin Binding to DNA

Daunorubicin binding to DNA is strongly dependent on the bulk salt concentration, as shown in Fig. 17.3a. The salt dependency of the binding constant shown may be interpreted by the polyelectrolyte theories of Manning [28] and Record [29]. Briefly, the salt dependence arises from a thermodynamic linkage between binding of the positively charged drug molecule and condensed sodium counterions surrounding the DNA duplex. Binding of the drug releases condensed counterions into solution, providing an entropically favorable contribution to the binding free energy. A full discussion of the analysis and interpretation of salt-dependent drug–DNA binding constants may be found in Ref. [30]. Experimentally, the binding constant is measured as a function of salt concentration to provide the quantity

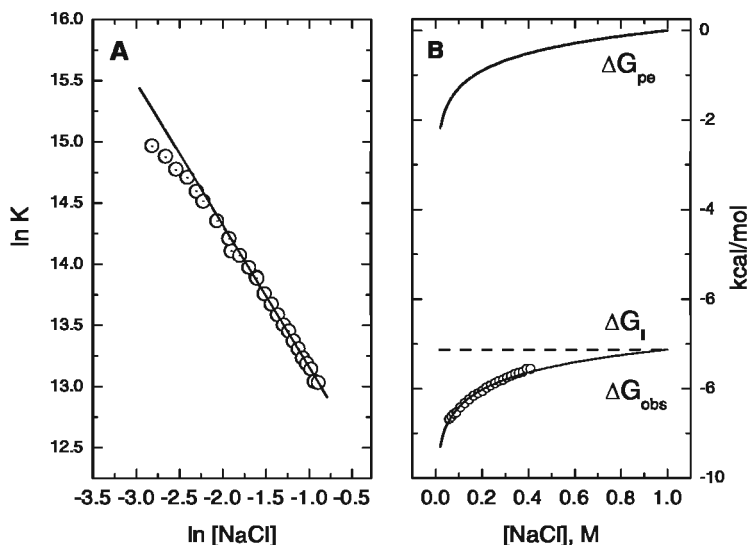


Fig. 17.3. Salt dependency of daunorubicin binding to DNA. (a) Plot of the natural logarithm of the binding constant as a function of logarithm of NaCl concentration. The solid line is the linear least squared fit to the data

over the range 0.1–0.4 M NaCl. (b) Parsing of the binding free energy into the polyelectrolyte (ΔG_{pe}) and non-polyelectrolyte (ΔG_l) contributions as a function of NaCl concentration.

$(\delta \ln K / \delta \ln M^+) = -Z\Psi$. (M^+ is the monovalent cation concentration; Z is the charge on the ligand; Ψ is the extent of counterion concentration and is constant at 0.88 for duplex DNA.) The data in Fig. 17.3a yield a slope of -1.08 , slightly greater than the value -0.88 expected for the binding of a ligand with a charge of $+1$.

Wilson and Lopp [31] and Friedman and Manning [32] both showed that for charged intercalators slopes greater than -0.88 should be expected, due to additional counterion release resulting from the increased phosphate spacing forced by intercalation. Determination of $Z\Psi$ is valuable because it allows observed binding free energies to be partitioned into two contributions, the polyelectrolyte (ΔG_{pe}) and non-polyelectrolyte (ΔG_l) components [30]. The former is the free energy contribution arising from counterion release, and is entirely entropic in origin. The latter is the free energy arising from all other molecular forces that stabilize complex formation. The simple equation for these terms is

$$\Delta G_{obs} = \Delta G_l + \Delta G_{pe}$$

The value of the polyelectrolyte contribution is $\Delta G_{pe} = RT(Z\Psi) \ln[M^+]$. Figure 17.3b shows the salt dependence of the free energy components for the binding of daunorubicin to DNA. The magnitude of ΔG_{pe} varies from over -2 kcal mol^{-1} to 0, depending on the NaCl concentration. ΔG_l is, of course, independent of salt concentration. The polyelectrolyte contribution to the overall observed binding free

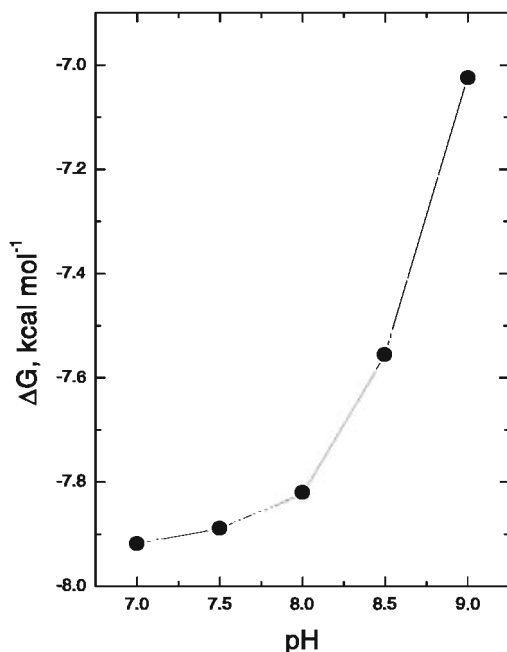


Fig. 17.4. pH dependency of daunorubicin binding to DNA.

energy in 0.2 M NaCl is $-1.0 \text{ kcal mol}^{-1}$, corresponding to about 12% of the total binding energy.

The polyelectrolyte contribution to the binding free energy arises from the positive charge on daunorubicin. The charge on the drug is due to the protonated amine on the daunosamine moiety, which has $pK_a = 8.2$ [2]. If the drug were uncharged, its DNA binding free energy should be reduced by an amount equal to ΔG_{pe} . Figure 17.4 shows that such is the case. Figure 17.4 shows the pH dependency of the DNA binding free energy for daunorubicin. At pH 7, the drug is fully protonated, with a net charge of +1. At pH 9, the drug is only 14% protonated, and the overall binding free energy was reduced by $0.9 \text{ kcal mol}^{-1}$. In 0.2 M NaCl, $\Delta G_{pe} = -1.0 \text{ kcal mol}^{-1}$. Removing the charge on daunorubicin reduces the free energy by the amount expected from the polyelectrolyte contributions. The results in Fig. 17.4 do not arise from linkage of protonation and drug binding – protons are not taken up or released upon complex formation. Over the pH range shown in Fig. 17.4, no DNA groups are titratable, so the observed effects can be safely attributed to the effect of drug charge on binding. Values of pH greater than 9 could not be studied because of the lability of the aglycone–sugar linkage under such alkaline conditions [2].

Apart from the general polyelectrolyte effect described above, it is possible that ions might participate in specific interactions with drug–DNA complexes. For example, certain crystal structures of daunorubicin–DNA complexes showed a Na^+

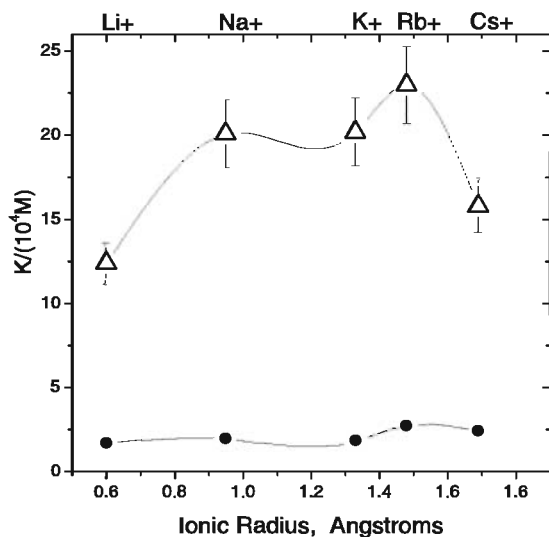


Fig. 17.5. Dependency of the non-polyelectrolyte portion of the binding free energy on cation type. Triangles refer to data obtained for daunorubicin. Solid circles refer to data obtained for ethidium bromide.

ion specifically coordinated in the major groove [33]. Substituents on both daunorubicin and DNA formed the putative coordination site, suggesting that complex formation led to the formation of a specific ion binding site. If a ternary drug–DNA– Na^+ complex in fact formed in solution, the binding constant might depend on cation type, since cations differ in size and might fit to differing extents into the putative coordination site. Figure 17.5 shows that measured binding constants show little dependency on cation type. Binding constants for daunorubicin binding to DNA were measured as a function of salt concentration for LiCl, NaCl, KCl, RbCl, and CsCl. The non-polyelectrolyte portion of the binding constant was extracted as described above, and plotted as a function of the ionic radius. The non-polyelectrolyte portion of the binding constant was selected because it should be sensitive to any specific ion binding. Figure 17.5 shows that this part of the binding constant changes by less than a factor of 2 for the different cation types, an essentially negligible effect. We conclude that specific ion binding to the daunorubicin–DNA complex in solution either does not occur, or, if it does, is energetically unimportant.

17.4

Binding Enthalpy and the Temperature Dependence of Binding

In order to partition the binding free energy into its enthalpic and entropic components, calorimetric studies or studies of the temperature dependence of the

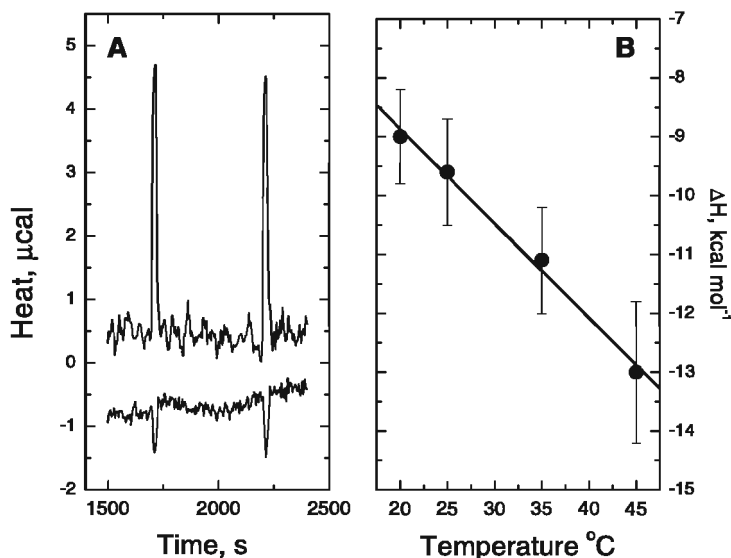


Fig. 17.6. Isothermal titration studies of daunorubicin binding to calf thymus DNA. (a) Examples of primary data from an ITC experiment in which 3 μL of a 1 mM daunorubicin solution was injected into 1 mL of a 1 mM calf thymus DNA solution at 300 s

intervals. The lower trace shows injection of drug into buffer alone. (b) Temperature dependency of the daunorubicin DNA binding enthalpy. The slope yields $\Delta C_p = -160 \pm 33 \text{ cal mol}^{-1} \text{ K}^{-1}$.

binding constant are needed. Calorimetric studies are the most direct means of obtaining binding enthalpy values and are much preferred over the indirect, model-dependent, van't Hoff analysis. The standard relationship

$$\Delta G = \Delta H - T\Delta S$$

defines the enthalpy (ΔH) and entropy (ΔS) contributions to the free energy. Figure 17.6 shows some results from the direct determination of the binding enthalpy for the daunorubicin–DNA interaction by isothermal titration calorimetry. By using the “model-free ITC” protocol adopted by our laboratory [34, 35], ΔH values may be determined as directly as is possible for daunorubicin binding to calf thymus DNA. At 20°C, a value of $\Delta H = -9.0 (\pm 0.8) \text{ kcal mol}^{-1}$ was determined. Combining this ΔH value with the ΔG value obtained above, $T\Delta S$ is calculated to be $-1.2 \text{ kcal mol}^{-1}$. For daunorubicin binding, then, $\Delta S = -4.1 \text{ cal mol}^{-1} \text{ K}^{-1}$ at 20°C. The negative sign indicates that entropy *decreases* upon binding, and is *unfavorable*. Daunorubicin binding to DNA is thus driven by a large, favorable enthalpy term, and is opposed by an unfavorable entropy term. Table 17.2 shows a summary of the thermodynamic parameters that describe the daunorubicin–DNA interaction.

Figure 17.6b shows that the daunorubicin binding enthalpy is strongly dependent upon temperature. This is a consequence of a heat capacity change in the

Tab. 17.2 Thermodynamic properties of daunorubicin and doxorubicin binding to calf thymus DNA.

Parameter	Daunorubicin	Doxorubicin
ΔG (kcal mol ⁻¹)	-7.9 ± 0.3	-8.9 ± 0.3
ΔH (kcal mol ⁻¹)	-9.0 ± 0.8	-7.4 ± 0.6
$T\Delta S$ (kcal mol ⁻¹)	-1.1	+1.5
ΔC_p (cal mol ⁻¹ K ⁻¹)	-160 ± 33	-149 ± 33
$\delta \ln K / \delta \ln 1/57$	-1.08 ± 0.1	-0.97 ± 0.1
ΔG_t (kcal mol ⁻¹)	-6.9	-8.0
ΔG_{pe} (kcal mol ⁻¹)	-1.0	-0.9

system upon binding, since $\Delta C_p = \delta \Delta H / \delta T$. The slope of the data in Fig. 17.6b yields $\Delta C_p = -160 (\pm 33)$ cal mol⁻¹ K⁻¹. Heat capacity changes are very difficult to determine, and only a few accurate values are available for drug–DNA interactions [35–38]. Current interpretations of heat capacity changes are centered on correlations between ΔC_p values and the removal of non-polar solvent accessible surface area upon binding [35–37, 39, 40]. Record and co-workers [39] have related heat capacity changes to the hydrophobic contribution to the binding free energy and showed that $\Delta G_{\text{hydrophobic}} = 80 \Delta C_p$. For daunorubicin binding to DNA, this means that a large, favorable, contribution to binding of -12.8 kcal mol⁻¹ comes from the hydrophobic transfer of the drug from aqueous solution into the intercalation site.

Prior to the ready availability of sensitive calorimeters, binding enthalpies were routinely determined from the temperature dependence of binding constants and subsequent analysis using the van Hoft relationship, $\delta \ln K / \delta T^{-1} = -\Delta H_{\text{VH}} / R$. Figure 17.7 shows a van't Hoff plot for the daunorubicin–DNA interaction. By this approach, and assuming $\Delta C_p = 0$, we reported a binding enthalpy $\Delta H_{\text{VH}} = -12.8$ kcal mol⁻¹ [18]. We now believe this value to be incorrect, because ΔC_p is not zero. Detailed Monte Carlo simulations showed that ΔC_p values of less than [200] cal mol⁻¹ K⁻¹ could bias the slopes of van't Hoff plots *without* producing curvature that is detectable within the noise of typical experiments [41]. Figure 17.7 shows curves calculated for ΔC_p values of 0, -50, -100, and -200 cal mol⁻¹ K⁻¹, obtained by fixing ΔH to the calorimetrically determined value at 20°C (Tab. 17.2). These curves show that the data in fact are slightly curved, and that a non-linear fit might be more appropriate than the usual linear fit. By fitting the data in Fig. 17.7 to a more appropriate equation that includes ΔC_p as an adjustable parameter [41], while constraining ΔH to the calorimetrically determined value (Tab. 17.2), a value of $\Delta C_p = -170$ cal mol⁻¹ K⁻¹ is obtained. Proper error analysis of this estimate [42], however, yields upper and lower bounds of -49 and -290 cal mol⁻¹ K⁻¹, respectively. The estimate of ΔC_p obtained from non-linear fitting of the data in Fig. 17.7 is in excellent agreement with that obtained more directly by isothermal titration calorimetry (Fig. 17.6b), but the large uncertainty in the estimate attests to the general difficulty of obtaining reliable heat capacity changes from van't Hoff plots.

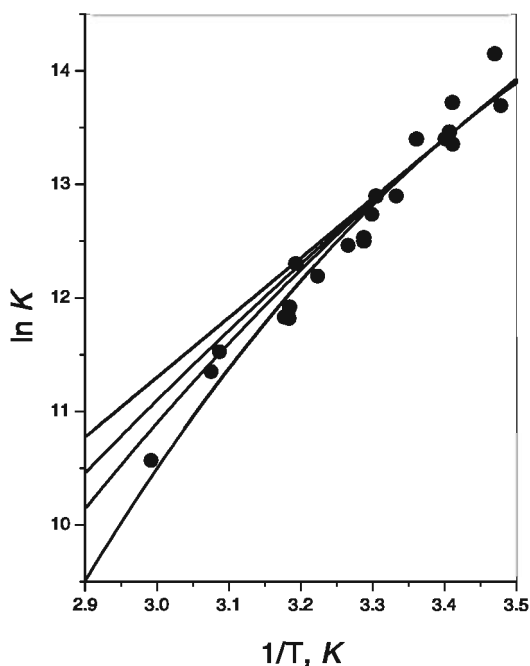


Fig. 17.7. Van't Hoff plot for the daunorubicin–DNA interaction. The solid circles are experimental data. The solid lines are calculated curves, assuming $\Delta H = 9.0 \text{ kcal mol}^{-1}$ at 20°C and the following values of ΔC_p (from top to bottom): 0, -50 , -100 , $-200 \text{ cal mol}^{-1} \text{ K}^{-1}$.

Regardless, enthalpy estimates for the daunorubicin–DNA interaction obtained by direct calorimetric measurements or by van't Hoff analysis are in full agreement when heat capacity changes are taken into account.

17.5

Thermodynamic Profile for Daunorubicin Binding to Calf-Thymus DNA

From these studies of the salt and temperature dependence of the daunorubicin–DNA binding constants and the calorimetric studies, it is possible to derive the complete thermodynamic profile of the drug–DNA interaction. Figure 17.3b shows the salt dependency of the binding free energy under isothermal conditions. Figure 17.8 shows the temperature dependence of ΔG , ΔH , and $T\Delta S$. This plot shows that all three parameters are temperature dependent, because ΔC_p is non-zero. Comparatively small free energy changes result from enthalpy–entropy compensation. The entropic term opposes binding over most of the temperature range shown, and daunorubicin binding is therefore enthalpically driven.

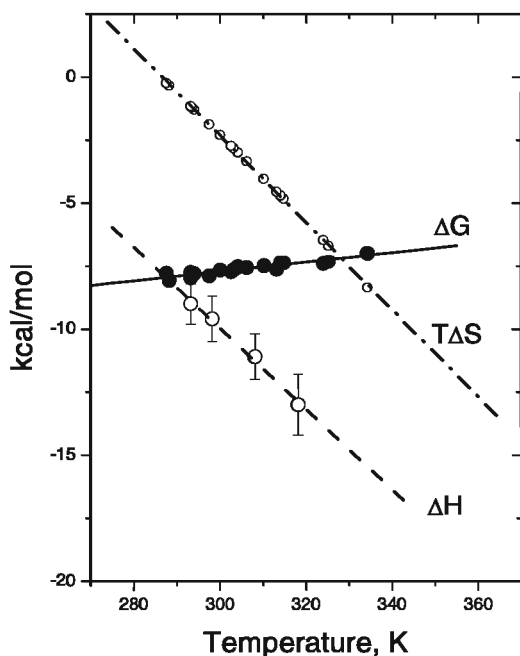


Fig. 17.8. Complete thermodynamic profile of the daunorubicin–DNA interaction.

A complete thermodynamic profile would require enthalpy and entropy estimates as a function of salt concentration and temperature. An attempt was in fact made to obtain ΔH and ΔS values as a function of NaCl concentration [43]. While the results of those early studies were highly reproducible and were independently verified in another laboratory [44], they now must be considered suspect because of their reliance on van't Hoff analysis and their neglect of heat capacity changes.

17.6

Hydration Changes

Water is a key participant in biochemical reactions [45, 46], but has received little attention in drug–DNA interaction studies. In part this is because of the lack of suitable experimental methods for unambiguously identifying hydration changes that may occur upon drug binding to DNA. The osmotic stress method alleviates the problem. In one useful version of the osmotic stress method, small neutral solutes (“osmolytes”) are added to macromolecule solutions to alter the water activity [45, 46]. Conformational transitions or binding reactions may then be studied as a function of water activity, and the results analyzed and interpreted by the well-established principles of linkage thermodynamics. Assuming that there is no direct interaction of the osmolytes with DNA or the drug, the change in hydration is given by the equation

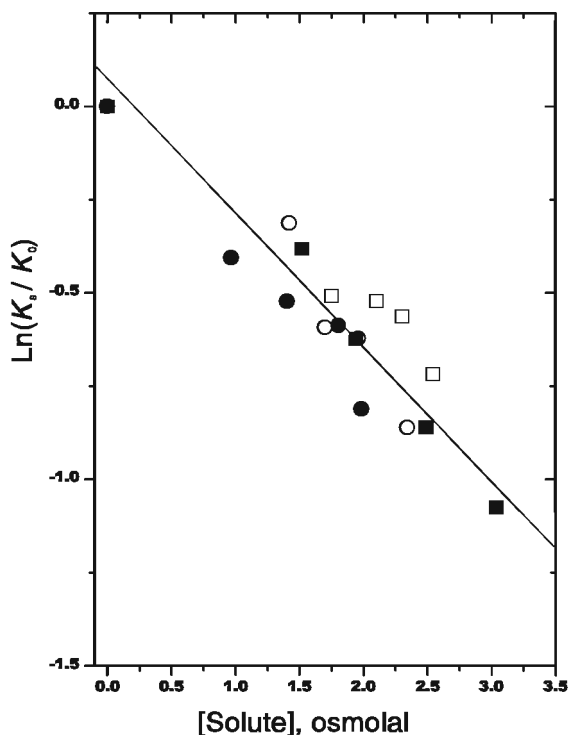


Fig. 17.9. Osmotic stress measurements of daunorubicin binding to DNA. The different symbols refer to binding studies using different osmolytes (sucrose, betaine, and triethylene glycol). The line is the global linear least squares fit to the data with a slope of -0.324 , yielding $\Delta n_w = 18.0 \pm 0.3$.

$$\partial \ln(K_s/K_0)/\partial [\text{Osm}] = -\Delta n_w/55.5$$

where $\ln(K_s/K_0)$ is the change in binding free energy, “Osm” is the osmolality of the solution, and Δn_w is the difference in the number of bound water molecules between the complex and the free reactants [45]. Figure 17.9 shows experimental results for daunorubicin binding to DNA. The negative slope indicates first of all that Δn_w is positive in sign, meaning that water is taken up upon formation of the daunorubicin–DNA intercalation complex. From the slope of the line, a value of $\Delta n_w = +18.0 \pm 0.3$ may be calculated. This means that net of 18 water molecules are taken up when daunorubicin binds to DNA. This is a surprising finding, since removal of hydrophobic surface upon complex formation should, at first glance, result in water release. However, high-resolution crystal structures of daunorubicin–DNA complexes often show specifically bound water molecules within the complex. Specific water molecules form hydrogen bond bridges to anchor the drug to the DNA, and networks of water molecules are often organized around drug

substituents in either the minor or major grooves. Whatever the underlying microscopic mechanism, the macroscopic thermodynamic measurements, surprising as it is, unambiguously show that water is taken up when daunorubicin binds to DNA.

Comparative data are sparse, but water appears to participate to different extents for different intercalation reactions [47]. Ethidium binding to DNA, for example, is characterized by $\Delta n_w = 0.25 \pm 0.5$, indicating no water uptake or release. Actinomycin binding, though, is characterized by $\Delta n_w = 32.0 \pm 3.0$. The exact value of Δn_w thus appears to be a unique, distinguishing property for different intercalators [47].

Anion effects on binding provide another probe for hydration changes in intercalation complex formation [48]. Sidorova and Rau [48] showed that measurement of binding constants in the presence of different anions in the Hofmeister series could provide an independent estimate for the stoichiometry hydration changes. Anion effects were examined for the DNA binding of ethidium and daunorubicin (Tab. 17.3). The qualitative results are clear. For ethidium, for which there is no change in hydration upon complex formation, there is little effect of different anions on its binding constant. In contrast, different anions alter the magnitude of the binding constant for daunorubicin, for which substantial hydration changes were found. Sidorova and Rau [48] showed that hydration changes could be approximated from the slopes of plots of $\ln K$ versus ΔS_w (the partial molal water entropy for each anion, with units of $\text{cal mol}^{-1} \text{K}^{-1}$). The approximate relationship they derive is

$$c \ln(K)/\partial \Delta S_w \approx -\Delta n_w c / 55.5 R$$

where c is the anion concentration and R is the gas constant. From the data in Tab. 17.3, a value of $\Delta n_w = +17$ may be calculated for daunorubicin, in excellent agreement with the value of +18 obtained by the osmotic stress method (Tab.

Tab. 17.3. Anion effects of F^- , Cl^- , and ClO_4^- on ethidium and daunomycin binding to calf thymus DNA.^a

Anion	$\Delta S_w/\text{mole anion}$	$K/10^5 \text{ (M}^{-1}\text{)}$	
		Ethidium	Daunomycin
F^-	−13.88	1.0	10.2
Cl	0.39	1.0	6.2
ClO_4^-	7.04	0.9	5.8

^aFluorescence titration were carried out in a buffer consisting of 6 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1 mM Na_2EDTA , and 0.185 M NaX ($\text{X} = \text{F}^-$, Cl^- , or ClO_4^-). Data were obtained and analyzed as described in Ref. [58]. The anion-specific partial molal water entropies, $\Delta S_w/\text{mole anion}$, were obtained from Ref. [48]. The fitting errors were within 15%, as evaluated by a Monte Carlo analysis [58].

17.2). For ethidium, the data in Tab. 17.3 yield $\Delta n_w \approx +2$, consistent with little change in hydration upon its binding to DNA.

Taken altogether, studies of daunorubicin binding to DNA in the presence of osmolytes and different anions show clearly that water is an important participant that has heretofore been ignored. Exactly what contribution water binding makes to the binding free energy is difficult to quantitatively evaluate at present. Water immobilization surely makes an unfavorable entropic contribution [49], perhaps one source of the overall unfavorable entropy term that is measured.

17.7

Substituent Contributions

Thermodynamic studies can provide quantitative information about the contribution of specific drug substituents to the binding free energy [30, 37, 50]. Rather detailed structure–affinity relationships may then be examined. The approach is as follows. For a series of drug analogs synthesized to contain the substituent changes of interest, complete binding isotherms are obtained at several salt concentrations. From these primary data, observed binding free energies are obtained, which are then parsed into their polyelectrolyte and nonpolyelectrolyte contributions using the procedures outlined above. The non-polyelectrolyte contribution is then used to evaluate the energetic contribution to the free energy, with reference to the parent compound. An example for a series of anthracycline derivatives is shown in Fig. 17.10 [50].

The advantages of this approach are many. First, by subtracting out the polyelectrolyte contribution, the possible effects of charge are eliminated, and both charged and uncharged derivatives may be directly compared. Second, the non-polyelectrolyte portion of binding free energy contains the most information about the contribution of specific molecular interactions. From Fig. 17.10, different derivatives bind with a wide range of free energies. Molecules with single substituent changes (hydroxyrubicin, daunorubicin, 9-deoxydoxorubicin) lose about 1 kcal in binding free energy relative to doxorubicin. In these cases, the altered substituents were all implicated in hydrogen-bonding interactions by high-resolution structural studies. Thermodynamics support that conclusion. More drastic modifications have more drastic energetic consequences. Complete removal of the daunosamine sugar to yield adrimycinone costs over 2 kcal in binding free energy. That attests to the key role of interactions of the daunosamine moiety in the DNA minor groove in stabilizing the complex. Finally, stereochemical changes drastically alter binding affinity. The β -anomer of doxorubicin, with alteration of the positioning of the daunosamine, binds to DNA with a free energy change some 3 kcal less than doxorubicin.

This approach is laborious, both in the synthesis of specific derivatives for study and in the determination of the requisite number of binding isotherms. It does, however, yield the most definitive and quantitative information about structure–affinity relationships that is possible.

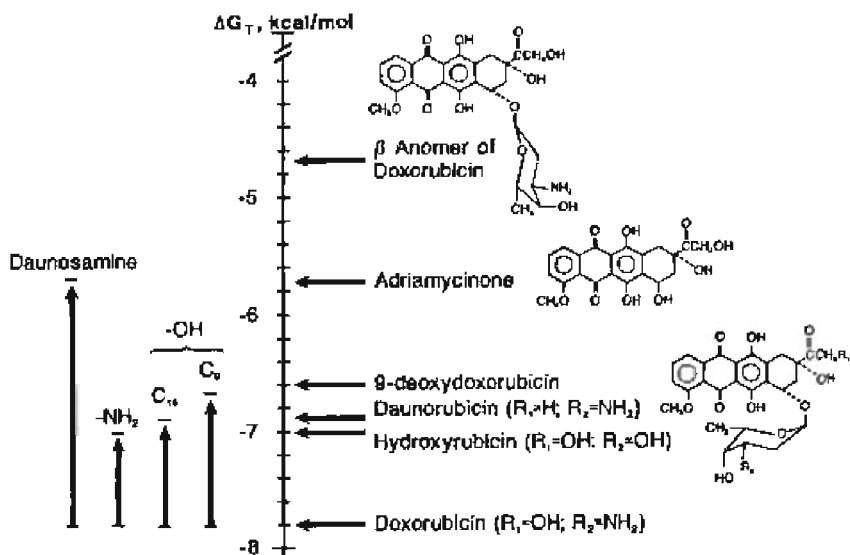


Fig. 17.10. Effect of substituent changes on DNA binding energetics. The nonpolar portion of the DNA binding free energy is shown for a number of anthracycline antibiotics. Free energy differences relative to doxorubicin are shown on the left side for specific substituent changes.

17.8

Isostructural is not Isoenergetic

Ideally, complete thermodynamic profiles should be obtained for the binding of all drug derivatives of interest. The labor in that task is enormous, and had been undertaken for only a few cases. For the anthracyclines, we have managed to complete a comparison of only daunorubicin and doxorubicin. The results are shown in Tab. 17.2 and Fig. 17.11. These results lead to a key conclusion. High-resolution crystal structures of daunorubicin or doxorubicin bound to DNA show essentially no differences between the complexes [33]. The structures of the two drugs are virtually superimposable, and the molecular interactions that can be visualized are equivalent. At the most, subtle variations in hydration and ion binding are reported. Daunorubicin and doxorubicin complexes are essentially isostructural. The doxorubicin DNA-binding constant, however, is nearly an order of magnitude higher than that found for daunorubicin. The underlying energetic basis for this is shown in Tab. 17.2 and Fig. 17.11. Daunorubicin and doxorubicin show strikingly different thermodynamic profiles. The difference is most striking in the entropy of binding. Whereas daunorubicin binding to DNA is *opposed* by entropy, Doxorubicin binding is *favoured* by entropy. The binding enthalpy of doxorubicin is significantly less than that of daunorubicin. Because of the favorable entropic contribution, however, the overall binding free energy is greater for doxorubicin relative

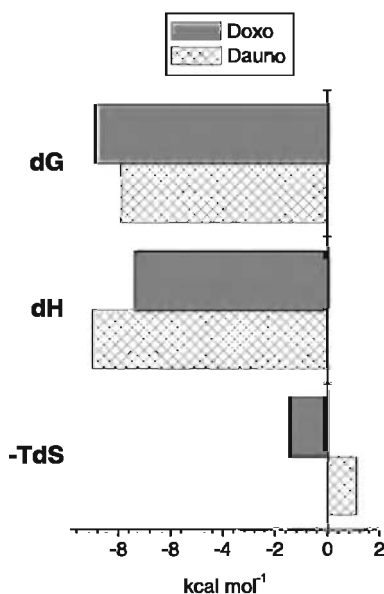


Fig. 17.11. Comparison of the binding energetics of daunorubicin and doxorubicin.

to daunorubicin. Thermodynamics reveals clear differences between the two drugs that are invisible in structures of even high resolution. Isostructural does not, therefore, mean isoenergetic.

17.9

Parsing the Binding Free Energy

Once a detailed thermodynamic profile is obtained, it is possible to attempt parsing of the observed binding free energy for an intercalation process to reveal the underlying contributions from various molecular interactions [34, 36, 37]. The observed binding free energy (ΔG_{obs}) contains contributions from at least five component terms:

$$\Delta G_{\text{obs}} = \Delta G_{\text{conf}} + \Delta G_{\text{r+t}} + \Delta G_{\text{pe}} + \Delta G_{\text{hyd}} + \Delta G_{\text{mol}}$$

In this equation, ΔG_{conf} is the contribution from conformational transitions in the DNA and intercalator, $\Delta G_{\text{r+t}}$ is the free energy cost for the restriction of rotational and translational freedom, ΔG_{pe} is the polyelectrolyte contribution, ΔG_{hyd} is the hydrophobic contribution, and ΔG_{mol} is the contribution of all other molecular interactions (e.g. van der Waals interactions, H-bonding, etc.). A detailed discussion of the contributor terms and their magnitudes is published elsewhere [34, 37].

Binding of both daunorubicin and doxorubicin to DNA is opposed by two terms, ΔG_{conf} and $\Delta G_{\text{r+t}}$. Since both drugs undergo little conformational change upon

binding, formation of the intercalation site is the primary contributor to ΔG_{conf} . The best estimate for the energetic cost of forming an intercalation site is $\Delta G_{\text{conf}} = +4.0 \text{ kcal mol}^{-1}$ [34, 37]. The most appropriate value of $\Delta G_{\text{r+I}}$ is a matter of some debate [51–56]. Estimates range from +5 to +15 kcal mol^{-1} . The larger estimate results from use of the Sakur–Tetrode equation, and is the value that seems most appropriate for intercalation reactions [34, 37]. In order to form a stable complex with DNA, both daunorubicin and doxorubicin must overcome nearly +20 kcal mol^{-1} from these unfavorable contributions. In 0.2 M NaCl, both drugs have a favorable polyelectrolyte contribution from the release of counterions, with $\Delta G_{\text{pe}} \approx -1 \text{ kcal mol}^{-1}$ (Tab. 17.2). The largest favorable free energy contribution comes from the hydrophobic effect. From the measured heat capacity changes (Tab. 17.2), values for ΔG_{hyd} of $-12.8 \text{ kcal mol}^{-1}$ and $-11.9 \text{ kcal mol}^{-1}$ may be calculated for daunorubicin and doxorubicin, respectively. The hydrophobic transfer of the drugs from solution into the intercalation site is the single most favorable driving force for the binding reaction. In order to account for the observed binding free energies of daunorubicin and doxorubicin, an addition contribution of -13 to $-15 \text{ kcal mol}^{-1}$ must come from ΔG_{mol} . This term is from all of the other molecular interactions (van der Waals, electrostatic, hydrogen bonds) that occur with the intercalation complex. This term is the hardest to evaluate quantitatively. In the previous section, individual substituents were seen to contribute 1–2 kcal of favorable free energy to binding. All of these would presumably sum to provide the total needed to account for ΔG_{mol} .

As tentative and approximate as this parsing exercise is, it is still valuable since it provides a realistic glimpse into the multiple forces that drive the intercalation of anthracyclines into DNA, along with their relative importance. The overall picture is complex, and binding arises from the balance of opposing forces. Formation of the intercalation site and the entropic cost of forming a bimolecular complex are energetically unfavorable and oppose complex formation. Favorable energetic contributions from the polyelectrolyte effect, the hydrophobic effect, and from the formation of molecular interactions within the intercalation site balance and overwhelm the opposing forces. The single largest favorable driving force comes from the hydrophobic effect, a fact commonly ignored by structural biologists.

17.10

Summary

The anthracycline antibiotics daunorubicin and doxorubicin are now perhaps the best-characterized DNA intercalators. They are, in fact, not simple intercalators, but rather more complex DNA-binding ligands with distinct intercalating and groove binding moieties. The detailed and comprehensive thermodynamic data summarized here serve to complement the wealth of structural data available for this class of drug. Together, the structural and thermodynamic data provide a clearer picture of the forces that drive DNA binding, and the role of specific substituents that help anchor and stabilize the complex. Such information is essential

and fundamental to rational drug design efforts. The anthracyclines should serve as a useful paradigm for the design of new generations of DNA-targeted drugs.

Acknowledgements

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18

Acridine-4-carboxamides and the Concept of Minimal DNA Intercalators

William A. Denny

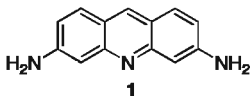
18.1

DNA Intercalation

18.1.1

Definition

There are three generally recognized ways in which molecules can bind reversibly to double-stranded DNA. First, the recognition sequences of regulatory proteins primarily interact in the major groove, recognizing a unique pattern of H-bond donor and acceptor functions in a 10–15 base pair, usually GC-rich, cognate sequence. Secondly, cationic small molecules composed of repeated aromatic units with an annular topology similar to that of the DNA bind in the minor groove in AT-rich regions, utilizing electrostatic, hydrophobic and entropic contributions to binding strength [1, 2]. Finally, the better-described mode of small molecule binding to DNA is by intercalation between the base pairs, a mode of reversible binding that was first described by Lerman [3] for the acridine proflavine (**1**). Intercalation is now understood to be the preferred binding mode of virtually any flat polyaromatic ligand of sufficiently large surface area and suitable steric properties. The driving forces for intercalative binding are primarily stacking (charge-transfer and dipole-induced dipole) interactions between the ligand chromophore and the base pairs, with entropic factors (dislodgement of ordered water around the DNA, particularly by appended side chains) of significant but lesser (and variable) importance [4].



18.1.2

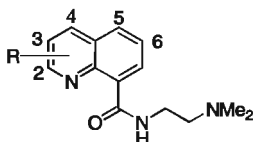
Effect of Chromophore Size

One important criterion determining binding mode is the size and nature of the intercalating chromophore [5]. A number of studies suggest that fused two-ring systems (naphthalene-type) are the minimum necessary for intercalative binding

[6, 7]. NMR shift and line width changes show no evidence for intercalation of isolated benzene rings over a range of different compounds [6]. They also suggest that two-ring naphthalene-type ligands only intercalate if they possess an appended cationic side chain, and have similar affinities irrespective of the presence of charged or uncharged nitrogen atoms in the aromatic system.

Studies with a series of phenylquinolines support this, showing that the quinolinecarboxamide (2) does not intercalate DNA, even with an appended cationic side chain [7]. Of a series of phenyl-substituted analogs, the 2-, 3-, and 6-phenyl compounds (3, 4, 7) did intercalate, whereas the 4- and 5-phenyl derivatives (5, 6) did not (Tab. 18.1) [7]. The intercalators have DNA-binding constants about 10-fold higher than the non-intercalators, which presumably bind non-specifically to DNA. A crystal structure of 3 showed that the phenyl ring was twisted only 20° out of the plane of the quinoline [8], while the phenyl rings of 5 and 6 were expected to be more out-of-plane due to peri interactions [7]. Perhaps more importantly, CPK models of complexes of the phenylquinolines with DNA showed that derivatives 5 and 6 could not be positioned with all three aromatic rings in the intercalation site. However, plausible complexes that involved the phenyl ring in the DNA-binding site could be modeled for derivatives 3, 4, and 7 [7]. This was later confirmed in a molecular modeling study [8], where the lowest-energy complexes of 3, 4, and 7 in a DNA intercalation site had the side chains in the minor groove and all three aromatic rings participating in stacking. However, no stable complexes that engaged

Tab. 18.1. Phenylquinolines.



No.	R	Log K (AT) ^a	θ ^b	IC ₅₀ ^c	OD ^d	ILS ^e
2	H	5.12	0	>25,000	100	—
3	2-Ph	5.97	14	1300	100	++
4	3-Ph	5.68	20	2600	100	+
5	4-Ph	4.73	0	14,700	150	—
6	5-Ph	4.74	0	13,700	100	—
7	6-Ph	6.12	20	910	65	+

Data from Ref. [7].

^aLog of binding constant to poly(dA-dT) at 0.01 M ionic strength.

^bUnwinding angle (degrees) of closed circular supercoiled DNA; a measure of intercalation.

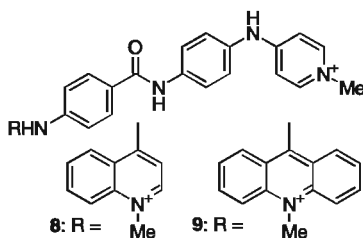
^cDrug concentration (nM) to lower the growth of L1210 cells to 50% of controls (72 h exposure).

^dOptimal drug dose (mg kg⁻¹) given intraperitoneally on days 1, 5, and 9.

^eActivity (at the optimal dose) in increasing the lifespan (ILS%) of mice inoculated intraperitoneally with 10⁶ P388 leukemia cells on day

0. ++++ substantial cures, +++ ILS > 120%, ++ ILS 120 – 70%, + ILS 70 – 30%, – ILS < 30%.

all three rings could be found for compounds 5 or 6 [8]. In contrast, fused three-ring systems such as acridines have sufficient stacking interactions to intercalate without the need for appended side chains; for example proflavine (1) [3]. Another example of the predilection of fused three-ring chromophores to intercalate is that the quinolinium derivative 8 was shown unequivocally by NMR to bind in the minor groove of an oligonucleotide [9], whereas the corresponding acridinium analog 9 is an intercalator [10].



18.1.3

Effect of Chromophore Cross-sectional Thickness

Another important criterion for intercalation is the cross-sectional thickness of the intercalating chromophore. Although all polyaromatics have a similar cross-sectional thickness (about 3.5 Å), directly attached substituents of larger cross-section also have to be considered, as first investigated by Gabbay [11]. Later work with derivatives of both amsacrine (10) [12] and proflavine (1) [13, 14] supported these findings. While amsacrine analogs with methyl, ethyl, or isopropyl substituents at the 2-, 3-, or 4-positions (11–19) were full DNA intercalators in both unwinding and helix extension assays, the corresponding *tert*-butyl analogs (20–22) were not (Tab. 18.2). Positioning of a *tert*-butyl group, with a cross-sectional thickness much larger than that of an aromatic ring (3.4 Å), anywhere on the chromophore causes an abrupt change to non-intercalative binding. This is consistent with later NMR studies of a broad range of chromophores, which found no evidence for non-classical intercalation by partial insertion [6]. Similar abrupt trends in binding mode were seen with the 2,7-dialkyl analogs of proflavine (compounds 23–26, Tab. 18.3). Both unwinding angles with closed circular supercoiled DNA [13] and the upfield shift of the DNA imino protons in the NMR spectrum [14], suggest that in this series (bearing two varying substituents) the abrupt loss of intercalative binding mode occurs with both isopropyl and *tert*-butyl substituents (compounds 25, 26).

18.2

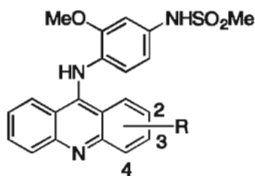
Intercalative Binding and Cytotoxicity

18.2.1

Correlation of Cytotoxicity with Mode, Strength and Kinetics of Binding

The above references [7, 12–14] demonstrate that intercalative binding is a necessary (but not sufficient) condition for useful cytotoxicity and *in vivo* antitumor

Tab. 18.2. Alkyl-substituted amsacrine.



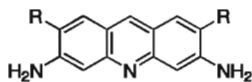
No.	R	C_{50} (AT) ^a	θ ^b	IC_{50} ^c	OD ^d	ILS ^e
10	H	10.3	20.5	32	13.3	++
11	2-Me	21	15	1400	65	+
12	3-Me	5.7	20	12	10	++
13	4-Me	0.25	19	30	20	++
14	2-Et	28	13	3400	150	—
15	3-Et	11	16	110	65	++
16	4-Et	0.41	16	88	45	+
17	2-iPr	34	14	1300	100	—
18	3-iPr	23	13	2900	100	+
19	4-iPr	0.53	15	220	100	—
20	2-tBu	43	0	4600	100	—
21	3-tBu	37	0	3000	100	—
22	4-tBu	0.68	0	6300	150	+

Data from Ref. [12].

^a Drug concentration to lower the fluorescence of ethidium bound to poly(dA-dT) by 50%; a measure of DNA binding strength.

^{b-e} As for Table 18.1.

Tab. 18.3. 2,7-Dialkylproflavines.



No.	R	C_{50} (AT) ^a	θ ^b	Slope ^c	IC_{50} ^d
1	H	4.7	17	2.8	580
23	Me	3.5	17.5	1.8	500
24	Et	3.5	16	1.8	360
25	iPr	14	<9	0.25	860
26	tBu	13	0	0.3	330

Data from Refs [13] and [14].

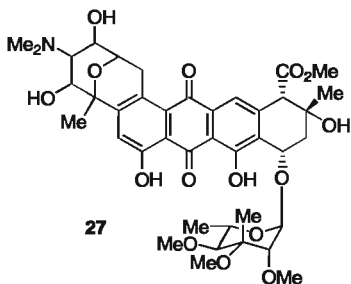
^{a,b} As for Table 18.1.

^c Slope of chemical shift:drug-base pair ratio plot (an NMR measure of intercalative binding).

^d Drug concentration (nM) to lower the growth of L1210 cells to 50% of controls (72 h exposure).

activity within 9-anilinoacridines, proflavines and quinolinecarboxamides. This general observation about the requirement of intercalative binding is supported by QSAR studies in several series that show positive quantitative correlations between the strength of intercalative binding and various measures of biological activity [15–18].

Biological activity also appears related to the kinetics of binding of intercalating agents to DNA, with longer average residence time of a ligand at a particular binding site correlating positively with biological potency. This observation is supported by stopped-flow spectrophotometric studies of the dissociation of actinomycin D analogs [19], 9-aminoacridinecarboxamides [20] anthraquinones [21, 22] and bisantrene analogs [23, 24] and NMR studies of a wide range of intercalators [25]. For mono-intercalators, one strategy for attaining longer average residence times is to flank the DNA-intercalating chromophore with two bulky side chains, situated so that one has to disengage through the helix to achieve complete dissociation of the ligand. This concept is exemplified by the natural product nogalamycin (27), which binds in such a fashion [26] and has very slow dissociation kinetics [27].



18.2.2

The Drive For Tight Binders; Chromophore and/or Side Chain Modulation

The broad positive relationship observed between high DNA binding and biological efficacy resulted in attempts to develop more tightly binding compounds, by using more than one DNA-intercalating chromophore (bis- and poly-intercalators) [28–30], more than one cationic side chain, or both [31]. However, while such approaches led to compounds that bound to DNA nearly as tightly as regulatory proteins [32, 33], no clinically useful drugs have resulted from this work.

18.2.3

Mechanism of Cytotoxicity of DNA Intercalators: Topoisomerase Poisoning

The primary mechanism of the antitumor activity of DNA intercalating agents is due to their production of DNA double-strand breaks [34], through interference with the normal functioning of the DNA enzyme topoisomerase II [35]. This discovery showed why it had not been possible to completely predict the biological

activity of these compounds in terms of the nature of their binding to DNA. It also explained the dichotomous structure–activity relationships with 9-anilinoacridines, where substituents on the (DNA-binding) acridine chromophore affected activity largely by how they altered DNA binding [14], whereas substituents on the anilino side chain affected activity independently of this (possibly by contributing to binding to the topoisomerase enzyme) [36].

18.2.4

Pharmacological Drawbacks of Tight DNA Binding: the Concept of "Minimal Intercalators"

While tight DNA binding correlates with cytotoxicity, at least within individual drug series, it also severely limits the extravascular distributive properties of drugs, since diffusion is driven by the free drug concentration. For drugs that bind to DNA, the effective diffusion coefficient (D_{eff}) that determines the rate of extravascular diffusion, is greatly lowered by DNA binding, in direct proportion with the binding constant. It is given by $D_{\text{eff}} = D/(1 + K_{\text{DNA}}S_f)$, where D is the diffusion coefficient, K_{DNA} is the DNA association constant and S_f is the concentration of available DNA-binding sites [37, 38]. Assuming a total DNA-binding site concentration of about 1 mM for a binder of low sequence specificity (about 10% of the sites in chromatin), the upper limit of K_{DNA} to keep the diffusion time reasonably rapid on the pharmacological timescale is about 10^4 M^{-1} [38], suggesting that quite moderate levels of DNA binding can limit diffusion.

Such effects of DNA binding on drug diffusion have been shown in tumor spheroids, with tightly binding compounds such as doxorubicin [39] and mitoxantrone [40] distributing poorly. Experiments using multicellular layers of defined geometry growing on porous Teflon supports also showed that less tightly binding compounds had higher rates of flux, attributed to higher free drug diffusion coefficients [41, 42]. The concept of "minimal intercalators" was proposed to incorporate the requirements of a mandatory intercalative binding mode together with the lowest possible level of binding [43], and a search was made for structures that fulfilled these requirements.

18.3

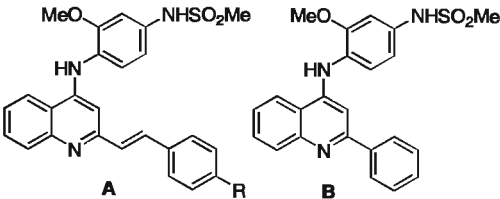
Classes of "Minimal Intercalators"

18.3.1

Styrylquinolines

Replacement of one of the aromatic rings of the tricyclic acridine chromophore of the clinical [44] anticancer drug amsacrine (**10**) with a styryl unit gave the styrylquinolines [43] (see examples **28–30**, Tab. 18.4). These were also intercalators, but bound more tightly to DNA than amsacrine, and were less effective biologically. The related phenylquinoline **31** was also an intercalator, showing similar binding strength to amsacrine, but has poor biological activity (Tab. 18.4).

Tab. 18.4. Styrylquinolines.



No.	Structure	C ₅₀ ^a	θ ^b	IC ₅₀ ^c	OD ^d	ILS ^e
10		10	20.5	32	13.3	++
28	A: R=H	6	18	1100	100	+
29	A: R=NH ₂	1.3	ND ^f	1150	150	—
30	A: R=NO ₂	4	ND	200	375	++
31	B	10	20	900	150	—

Data from Ref. [43].

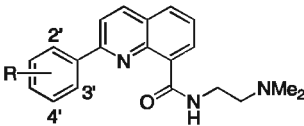
^{a-c}As for Table 18.1.

^fNot measured.

18.3.2
2-Phenylquinolines

Following studies [7] that the 2-phenylquinolinecarboxamide **3** was a DNA intercalator, a study of phenyl ring substituted compounds was undertaken [45] (Tab. 18.5). The 2'-Cl analog **32** was the weakest DNA binder, but this did not bind by

Tab. 18.5. Substituted 2-phenylquinolines.



No.	R	Log K (AT) ^a	θ ^b	IC ₅₀ ^c	OD ^d	ILS ^e
3	H	5.97	14	1300	100	++
32	2'-Cl	5.32	0	8100	100	—
33	3'-Cl	6.05	15	1200	65	+
34	4'-Cl	6.08	16	1170	100	+
35	4'-aza	5.82	ND ^f	170	45	+++
36	4'-F	6.74	ND	730	100	—
37	4'-Br	6.63	ND	600	100	++
38	4'-OMe	6.40	ND	1290	150	+

Data from Ref. [45].

^{a-c}As for Table 18.1.

^fNot measured.

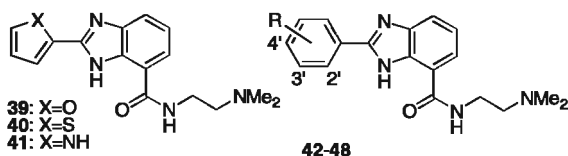
intercalation due to the 2'-substituent twisting the phenyl ring substantially out of coplanarity with the quinoline. In contrast, the 3'-Cl and 4'-Cl compounds (**33** and **34**), where the phenyl ring can lie coplanar, bound much more tightly to DNA and showed improved cytotoxicity (Tab. 18.5). Of a series of 4'-substituted analogs, of which **34**–**38** are representative examples (Tab. 18.5), no relationship could be seen between activity and substituent electronic or lipophilic properties [45]. The aza derivative **38** was the most effective biologically, and was at one time considered for preclinical development.

18.3.3

Phenylbenzimidazoles

To explore the “minimal intercalator” concept further, replacement of some of the benzene rings in the 2-phenylquinolinecarboxamide chromophore with less aromatic five-ring heterocycles was studied [46]. 2-Furoyl- (**39**), 2-thienyl- (**40**), 2-pyrrolo- (**41**), and 2-phenylbenzimidazolecarboxamides (**42**) had similarly low binding constants, but only the latter showed biological activity (Tab. 18.6). Compound **42** appeared to intercalate DNA, albeit with an unwinding angle of only 11°. Structure–activity studies for phenyl-substituted analogs of **42** showed low IC₅₀ values and poor *in vivo* activity (see representative examples **43**–**48**, Tab. 18.6), but overall there was a weak correlation ($r = 0.61$) between IC₅₀ and log*K* for binding [46]. These data suggest that the 2-phenylbenzimidazole may be close to a true “minimal” DNA-intercalating chromophore.

Tab. 18.6. Substituted 2-phenylbenzimidazoles.



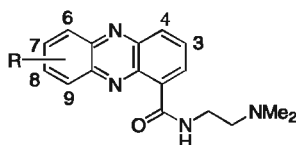
No.	R	Log K (AT) ^a	θ ^b	IC ₅₀ ^c	OD ^d	ILS ^e
39		5.18	ND ^f	40,000	150	—
40		5.39	ND	23,100	150	—
41		5.38	ND	28,200	150	—
42	H	5.41	11	22,000	100	+
43	2'-OMe	5.51	ND	8000	150	+
44	2'-Cl	5.22	ND	16,000	100	+
45	3'-OMe	5.43	ND	11,700	65	—
46	3'-Cl	6.00	ND	5,900	150	+
47	4'-OMe	5.52	ND	5,800	150	+
48	4'-Cl	5.43	ND	4,000	100	—

Data from Ref. [46].

^aAs for Table 18.1.

^fNot measured.

Tab. 18.7. Substituted phenazine-1-carboxamides.



No.	R	Log K (AT) ^a	IC ₅₀ ^c	OD ^d	ILS ^e
49	H	6.58	1400	150	++
50	3-Me	6.48	410	225	++
51	4-Me	6.78	400	100	+
52	6-Me	6.68	820	150	+
53	7-Me	6.80	1500	150	+
54	8-Me	6.43	630	150	+
55	9-Me	6.93	42	65	+
56	9-OMe	6.59	48	100	++
57	9-Cl	6.44	11	30	+
58	8,9-benz	7.12	33	30	++

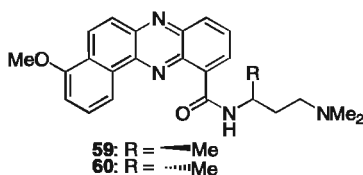
Data from Ref. [48].

^{a,c-e} As for Table 18.1.

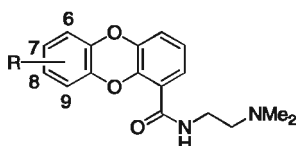
18.3.5

Phenazines

Phenazine-1-carboxamides were explored as a class of linear neutral tricyclic chromophores that bound by intercalation (an unwinding angle of 18° for the parent 49) (Tab. 18.7) [47]. While 49 showed good cytotoxicity and modest *in vivo* activity [48] (Tab. 18.7), it also has significantly higher DNA binding than the corresponding 2-phenylquinoline (log K_{AT} 6.58 compared with 5.97 for the 2-phenylquinoline). The effect of ring substituents on activity was small except at the 9-position, where small lipophilic substituents substantially increased cytotoxicity (see examples 50–58, Tab. 18.7). The more potent 8,9-benzo derivative 58 showed, as expected, the highest DNA-binding constant. This is not a minimal intercalator, but was the starting point for recent work on benzophenazine carboxamides that has resulted in the clinical agent 59 (XR 11576 [49]). This latter work is of particular note because it is the first time that a chiral side chain has been used. The *R* and *S* enantiomers (59 and 60) showed a significant difference in cytotoxicity in H69/P human small cell lung carcinoma cells (IC₅₀ 23 and 96 nM, respectively).



Tab. 18.8. Substituted dibenzodioxins.



No.	R	Log K (AT) ^a	IC ₅₀ ^c	OD ^d	ILS ^e
61	H	5.05	3000	150	++
62	6-Me	5.26	2100	45	—
63	7-Me	5.11	3000	45	—
64	9-Me	5.28	250	100	+++
65	9-OMe	5.55	380	45	+
66	9-Cl	5.48	230	100	++

Data from Ref. [50].

^{a,c-e}As for Table 18.1.

18.3.4

Dibenzodioxins

In a further attempt to develop very weakly binding tricyclic chromophores, the aromaticity of the ring system was lowered by breaking the conjugation. A survey [47] of several linear tricyclic chromophores identified the dibenzo[1,4]-dioxin (**61**) as potentially interesting, lacking a fully conjugated chromophore yet retaining planarity and binding by intercalation (unwinding angle 20°) but weakly (log K_{AT} 5.03). While the parent compound **61** was only weakly cytotoxic (IC₅₀ 3000 nM) it showed good *in vivo* antileukemic activity (Tab. 18.8) [50]. Structure–activity relationships showed that substitution of the chromophore had little effect on DNA binding or cytotoxicity except at the 9-position, where substituents increased cytotoxicity about 10- to 15-fold and also improved *in vivo* potency (see examples **62**–**66**, Tab. 18.8). This is a similar situation to that of the phenazines.

18.4

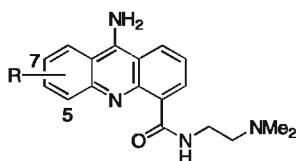
Acridinecarboxamides: the Development of DACA

18.4.1

9-Aminoacridine-4-carboxamides

The 9-aminoacridine-4-carboxamide class of compounds were originally explored as part of a broad attempt to develop acridine derivatives as anticancer drugs [51]. Because these compounds exist as dications under physiological conditions [52], they are tight-binding DNA intercalating agents (**67** has a log K of 7.08 binding to poly(dAT), Tab. 18.9). They are also potent cytotoxins with well-defined structure–activity relationships [51, 53] (Tab. 18.9). As with the other linear tricyclic dibenzo-

Tab. 18.9. Substituted 9-aminoacridinecarboxamides.

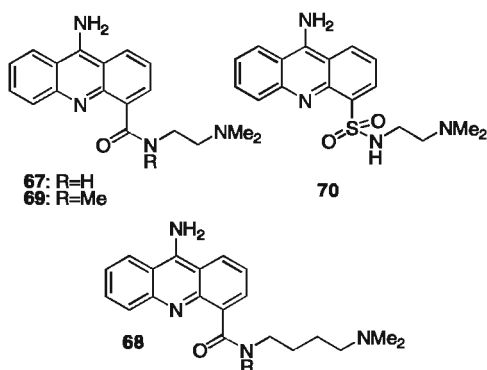


No.	R	pK_a	$\log K (AT)^a$	IC_{50}^c	OD^d	ILS^e
67	H	8.30	7.08	15	4.5	++
71	5-F	7.11	7.90	1.4	3.9	++
72	5-Me	8.01	7.55	0.47	2.6	++
73	5-Cl	6.87	7.48	2.9	2.6	++
74	5-SO ₂ Me ^f	5.15	7.32	2.8	30	+++
75	7-Cl	7.48	7.67	722	13.3	—
76	7-SO ₂ Me	6.93	7.71	3090	100	—

Data from Ref. [52].

^{a,c-e} As for Table 18.1.^f The only analog active in the Lewis lung *in vivo* model.

dioxins and phenazines, small lipophilic substituents in the same spatial position with respect to the side chain (in this case at the 5-position) enhanced both potency and DNA binding [52]. There was a requirement for the side chain at the 4-position to possess a cationic center in a tightly defined relationship to the chromophore. Attenuation of the pK_a of the side chain nitrogen or alteration of its position with respect to the chromophore by changing either the nature of the link group or the length of the linking chain abolished activity. As examples, the 4-butylcarboxamide (68), *N*-methylcarboxamide (69), and sulfonamide (70) were all inactive [51].



X-ray crystal studies [54] showed that the 4-carboxamide group in the free base of 67 is coplanar with the acridine ring, and stabilized by H-bond formation between the acridine ring N10 acceptor and the amide NH donor. Computations show that the minimum-energy conformation of the dication is also essentially planar, but

with a hydrogen bond between the protonated N10 and the amide carbonyl oxygen, placing the side chain in a different orientation. However, the barrier to rotation between these two states is so low (ca. 4 kcal/mol) that side chain rotation about the carboxamide C4–C11 bond would be virtually unhindered. Stopped-flow kinetic studies of 9-aminoacridine-4-carboxamide dissociation from calf thymus DNA were interpreted [55] in terms of a model of drug binding where the acridine chromophore intercalated and the amide NH and protonated $N^+H(Me)_2$ groups of the side chain were hydrogen bond donors to the O2 of a cytosine base adjacent to the binding site.

Later crystal structure studies on **67** and analogs bound to the hexanucleotide d(CGTACG)₂ have delineated an apparently robust binding model for this class of compounds [56–58]. The structure of the complex formed between **67** and this hexanucleotide shows the drug molecule intercalates between each of the CpG dinucleotide steps, with the side chain oriented by an internal hydrogen bond between the 4-carboxamide carbonyl and the protonated N-10 atom of the acridine ring. This places the side chain in the major groove, where the protonated dimethylamino group hydrogen bonded to either the N7 or O6 of guanine G2. A water molecule forms bridging hydrogen bonds between the 4-carboxamide NH and the phosphate group of the same guanine. Intercalation perturbs helix winding throughout the hexanucleotide compared to B-DNA. This structure allows a rationalization of the known structure–activity relationships of analogs of **67** for cytotoxicity and DNA-binding kinetics. A complex of the 5-fluoro analog **71** binding to d(CGTACG)₂ showed enhanced stacking interactions between the 5-substituent and cytosine in the CpG-binding site, providing a possible reason for the slower off-rates and higher potencies of 5-substituted analogs [58].

The 9-aminoacridine-4-carboxamides are very potent cytotoxins in cell culture (**67** has an IC_{50} of 15 nM and the 5-methyl derivative **72** an IC_{50} of only 0.4 nM) (Tab. 18.9). A series of 5-substituted analogs had good activity *in vivo* against leukemia models when dosed intraperitoneally (see examples **71–76** in Tab. 18.9) [53]. In contrast, the only analog showing significant *in vivo* activity in a remotely sited Lewis lung carcinoma model [52] was the 5-SO₂Me derivative **74**. The enhanced solid tumor activity of this compound was attributed to its very weak acridine pK_a (5.15), which allows it to exist primarily as the monocation, thus permitting more efficient distribution to remote tumor sites [52].

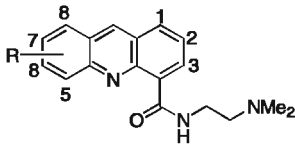
18.4.2

DACA and Other Acridine-4-carboxamides

18.4.2.1 Introduction

An equally efficient way of lowering acridine pK_a is to remove the resonant 9-amino group. The acridine-4-carboxamide (**77**, DACA) has a pK_a of only 3.54 [59] and a lower binding constant ($\log K_{AT}$ 6.12). However it was still an intercalator, and while it had lower cytotoxic potency than **67**, it and many of its chromophore-substituted analogs showed curative activity in the Lewis lung model (see examples **78–86**, Tab. 18.10) [59, 60]. This and its activity against multidrug resistant cells

Tab. 18.10. Substituted acridinecarboxamides (DACA analogs).



No.	R	pK _a	Log K (AT) ^a	IC ₅₀ (nM) ^c	P388		Lewis lung	
					OD ^d	ILS ^e	OD ^d	ILS ^e
67		8.30	7.08	15	4.5	++	4.5	—
77	H	3.54	6.12	105	66	++	100	++++
78	1-OMe	3.81	7.05	8.8	30	+++	65	++++
79	2-OMe	3.34	7.14	30	100	++	100	+++
80	5-OMe	3.87	7.96	12	65	+	45	+
81	5-Me	ND ^f	6.99	54	30	++	30	++
82	5-Cl	ND	7.63	6.9	20	++	20	++
83	6-OMe	4.59	7.28	210	100	+	100	++
84	7-OMe	3.54	6.87	640	150	++	150	+
85	7-Cl	ND	6.87	250	100	++	100	++
86	8-OMe	3.82	7.14	58	100	++	100	++

Data from Refs [59] and [60].

^{a,c-e} As for Table 18.1.

^f Not measured.

[61] led to its clinical evaluation, which required the development of a new synthesis that avoided the use of toxic mercury salts and the lachrymatory acridine-4-carboxylic acid intermediate [60, 62].

18.4.2.2 Interaction of DACA with topoisomerase

DACA stimulates the formation of cleavable complexes between topo II and DNA, inducing both DNA breakage and the formation of DNA protein crosslinks [63]. The DNA protein crosslinks formed by DACA are readily reversible, with a bell-shaped concentration dependence suggesting self-inhibition at higher drug levels, whereas induced DNA double-strand breaks continued to increase linearly with drug concentration. The results suggested that DACA interferes with the action of topo II, but by a different mechanism to that of amsacrine [64]. However, the cytotoxicity of DACA was not antagonized by preincubating cells with ICRF-187, a catalytic inhibitor of topo II, and DACA protected cells against DNA breakage induced by etoposide (an inducer of cleavable complex formation with topo II). These results indicate that DACA is a weak topo II poison, and that this enzyme is not its main target [65]. With purified topo II enzyme, DACA induced breakage of supercoiled plasmid DNA (poisoning), whereas the analog 7-chloroDACA (85) was a very weak poison. Conversely, with purified topo I, DACA exhibited no poisoning activity, but 7-chloroDACA induced topo I-mediated DNA breakage [66]. These results show that acridine substituents on DACA can substantially influence its

interaction with topoisomerases, with 7-chloro substitution suppressing topo II-dependent DNA cleavage and stimulating cleavage by topo I [67].

18.4.2.3 Cellular studies with DACA

An attractive property of DACA is its ability to overcome transport-generated multidrug resistance (MDR) [51]. Studies in P-glycoprotein-expressing CEM/VLB100 and MRP-expressing CEM/E1000 cells (sublines of the CCRF-CEM human leukemia line), and in MDR sublines of the HL60 human leukemia line, showed that DACA was much more effective than idarubicin, an anthracycline analog known for its relative ability to circumvent MDR [68].

In subcutaneous colon 38 adenocarcinomas in BDF1 mice, DACA had activity comparable to that of 5-fluorouracil and superior to that of doxorubicin, cyclophosphamide, and asulacrine. Mitoxantrone, amsacrine, etoposide, teniposide, and daunorubicin showed minimal activity in this assay. DACA also showed significant activity against NZM3 human melanoma cell xenografts in athymic mice [69]. Studies with ^3H -labeled DACA showed that the kinetics of both uptake and efflux were very rapid, with equilibrium being reached within seconds of drug addition to cultures of Lewis lung carcinoma, P388 leukemia, and multidrug resistant P/DACT cells. DACA had an uptake ratio of 550 in Lewis lung cells. It was proposed that DACA's known high affinity for membranes contributes to the rapid uptake and efflux that allows it to overcome transport-mediated multidrug resistance [70]. DACA and a series of acridine-substituted analogs showed broadly equivalent potencies in a wild-type human Jurkat leukemia line and mutant lines resistant to many topo II agents due to lower levels of the enzyme [71].

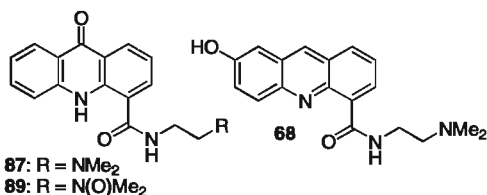
Structure-activity studies suggested that activity correlated with the steric bulk of the substituents, but was independent of their electronic properties. Most of the 5-substituted derivatives were more cytotoxic than DACA, but were less effective in the mutant lines, suggesting a mode of cytotoxicity largely mediated by effects on topo II. However, 5,7-disubstituted derivatives retained both the higher cytotoxic potency of the 5-monosubstituted analogs and the broad-spectrum effectiveness of the 7-monosubstituted analogs [72].

18.4.2.4 Metabolism and pharmacology of DACA

The primary route of metabolism of DACA is oxidation at C-9 by aldehyde oxidase to give the acridone 87, although oxidative demethylation of the side chain dimethylamino group has also been observed [73]. Comparative studies of the conversion of DACA to 87 were carried out using aldehyde oxidase preparations from human, guinea pig, and rat. The human enzyme had intrinsic clearance values (V_{max}/K_m) ranging from 0.27 to 0.35 mL min⁻¹ mg⁻¹ protein, whereas rat and guinea pig had approximately 7- and 160-fold greater intrinsic clearances. This is likely due to lower K_m values in rats (4.5 μM) and guinea pigs (0.15 μM) compared with humans (28 μM). Another DACA metabolite, 7-hydroxyDACA (88) was a potent inhibitor of the enzyme [74]. Equilibrium dialysis studies also showed significant species differences in the binding of DACA to plasma proteins, with the smallest free fraction occurring in human plasma (3.4%), followed by dog (8.1%),

mouse (15%), rat (16%), and rabbit (20%). The greatest binding to human proteins was to α 1-acid glycoprotein, followed by albumin [75]. The bioavailability of ^3H -labeled DACA in rats was low (8%) following oral administration, despite relatively high bioavailability (>58%) of total radioactivity, suggesting that high first-pass metabolism is the limiting factor [76].

^{11}C -labeled DACA was prepared and used to study biodistribution and metabolism in both rats and humans (the latter with i.v. injections of tracer-level doses), by PET scanning and rapid HPLC [77]. There was rapid clearance of ^{11}C -labeled DACA in both humans and rat plasma, with the generation of seven metabolites, one of which was identified as the acridone *N*-oxide (**89**). PET scanning in the rat showed little retention of radioactivity in major organs, with rapid excretion via the gut and kidney. Overall there was no unexpected interspecies difference in metabolism [78].



18.4.2.5 Clinical studies with DACA

The major urinary metabolite of DACA in a phase I clinical trial was the acridone *N*-oxide **89** (34% of the dose), and other minor metabolites resulting from combinations of ring and side chain oxidation. No ring-hydroxylated metabolites were detected. The major plasma metabolites were the acridone **87** and the corresponding *N*-oxide **89** [79]. The blood plasma pharmacokinetics of patients given i.v. doses of DACA ranging from 18 to 1000 mg m⁻² were studied by HPLC. Drug loss from plasma was very rapid, with an initial half-life of 0.28 h, and a terminal half-life of 2.04 h. The free drug fraction (estimated between 0.9 and 3.3%) was much lower than those measured (by equilibrium dialysis) for mice (15%) and rats (16%) [80]. A phase I trial of DACA, using a 3 h infusion regimen repeated 3-weekly, indicated a maximum tolerated dose of 750 mg m⁻², with the dose-limiting toxicity being infusional arm pain of unknown cause. No activity was seen, but calculations based on rodent models suggested this dose is subtherapeutic in humans [81, 82]. DACA was taken to extensive phase II clinical trials as XR 5000, but reports of these trials have not yet been published.

18.5

Conclusions

The studies summarized above suggest that neutral or weakly basic three-ring “2-1” type chromophores (e.g. 2-phenylquinolines, 2-phenylbenzimidazoles) bearing additional cationic side chains are probably close to the desired “minimal in-

tercalators". These compounds reliably bind to DNA by intercalation, with binding constants (to poly(dAT)) as low as $2 \times 10^5 \text{ M}^{-1}$ at low ionic strength. Linear three-ring systems (e.g. acridines) tend to bind more tightly, but multicellular layer experiments suggest that analogs such as DACA (77) can still diffuse efficiently in tumor tissue, such that transport should not be a major limitation to activity. DACA itself showed broad-spectrum antitumor activity through dual inhibition of topo I and topo II, was unaffected by P-glycoprotein-mediated multidrug resistance, and has undergone extensive phase II clinical trials.

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19

DNA Topoisomerase-targeted Drugs

M. Palumbo, B. Gatto, and C. Sissi

19.1**Introduction**

DNA-interacting anticancer drugs were introduced to clinical practice about half a century ago. The early interpretation of their mechanism of action essentially relied on the covalent or reversible structural changes generated in the nucleic acid upon drug binding, especially through intercalative mechanisms, that impaired the basic functions of rapidly proliferating cancer (and normal) cells. Although this in part holds true even in the light of the present information, it soon became apparent that, in several instances, a more complicated series of processes caused cell death and that interaction with DNA was an accompanying aspect not necessarily connected with drug potency and therapy outcome [1].

A seminal breakthrough in cancer pharmacology was the discovery that the most important cytotoxic agents efficiently interacting with DNA were able to impair the functions of a family of DNA-processing essential enzymes present in all organisms – the topoisomerases (tops) [2]. This chapter will first deal with the structure and functions of tops and then focus on several pharmacologically relevant agents interfering with their activity. Since a number of recent exhaustive reviews are available on tops and top-directed agents [3–11], we will mainly discuss the latest advances in the field.

19.2**Structure and Functions of DNA Topoisomerases**

DNA topoisomerases are involved in the regulation of DNA topology and its degree of supercoiling [12, 13]. Two types of the enzyme are known: type I tops change the degree of supercoiling of DNA by causing single-strand breaks and religation, type II tops (including bacterial gyrase) cause double-strand breaks. Top1 is dispensable for a living cell whereas top2 is not. The different roles of DNA top1 and top2 may reflect the need for fine regulation of DNA supercoiling efficiency. Top1 and top2 functions are especially crucial during DNA transcription and replication, when the DNA helix must be unwound to allow the large enzymatic machinery to

operate properly, and tops have indeed been shown to maintain both transcription and replication. Recently, other new members of the family of both types I and II have been discovered. These may contribute to even more specialized roles for topoisomerases, with some enzymes implicated in regulation of recombination events, chromatin remodeling, chromatin condensation/decondensation, recombination, segregation of newly replicated chromosomes, and DNA repair [14].

19.2.1

Type I Topoisomerases

Top1 participates in the relaxation of negative supercoils and has also been implicated in knotting and unknotting DNA and in linking complementary rings of single-stranded DNA into double-stranded rings [12].

The relaxation process catalysed by the enzyme essentially consists of a transient break of a segment of single-stranded DNA, followed by passing an intact strand of DNA through the gate (type I-A) or allowing rotation of the broken strand around the intact strand (type I-B), with final rejoining of the broken segment. Top1 has several distinct features. Unlike type II enzymes, it appears to fully perform its functions as a monomer. In addition, it does not require ATP hydrolysis to catalyze the complex topological rearrangements of DNA.

19.2.1.1 Structural features

Topoisomerases I are single polypeptides with extensive secondary structure. The crystallographic structure of both prokaryotic and eukaryotic protein is now available (Fig. 19.1), and helps understanding the mode of catalytic action [15, 16]. There is a wide (25–30 Å) hole in the center of the protein large enough to bind

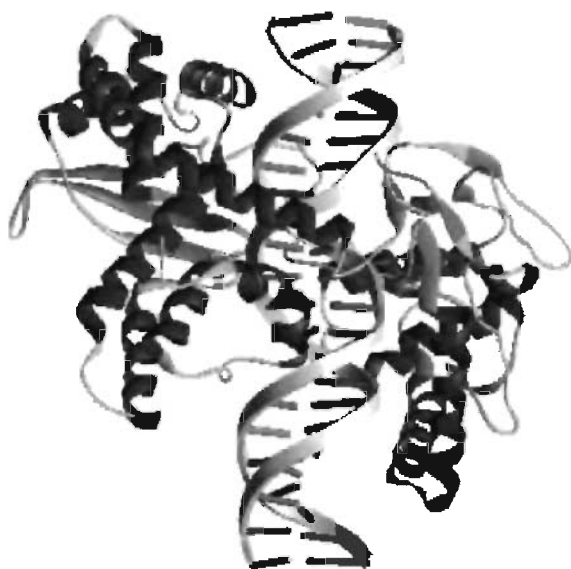


Fig. 19.1. Human reconstituted top1 in covalent complex with a 22-bp DNA.

either a single- or double-stranded piece of DNA with no steric hindrance between the DNA sugar–phosphate backbone and protein side chains within the torus. This hole exhibits numerous basic (positively charged) residues to provide efficient electrostatic interaction to the negatively charged phosphate oxygens of the DNA backbone. Thus, both the size and electrostatic character of the cavity make it a likely candidate for temporarily holding a DNA segment. The fragment consists of four structurally organized major domains (I–IV). The type of non-covalent interface between domains I, III, and IV and the rigid combination of domains II and III, suggest that the latter domains II and III may be able to move backwards and forwards from domains I and IV. Top1 would therefore be able to adopt two principal conformations: a “closed” one, characterized by an interaction interface between domain III and domains I and IV; and a “open” one, in which domain III no longer contacts its above mentioned partners. The catalytic tyrosine is located in domain III at the interface between domains I, III, and IV. The active site structure is stabilized by several hydrogen bonds and one salt bridge, which also maintains the non-covalent boundary between domains.

19.2.1.2 Catalytic process

The enzymatic cycle of top1 [13] can be divided into the following steps: (a) binding of the enzyme to DNA, (b) cleavage of a single DNA strand, (c) passage of an intact strand through the gate, and (d) DNA religation. Once the initial protein–DNA complex is formed, a transesterification process occurs between a key catalytic Tyr residue and a phospho-diester bond in the backbone of the single-stranded DNA segment. In type IA tops, the 3′ oxygen in the backbone is displaced by a tyrosine oxygen, covalently linking the tyrosine oxygen to phosphorus at the 5′ backbone nick. Thus, the 5′ nick is covalently linked to top1 in domain III. In type IB enzymes the 5′ oxygen is displaced by Tyr and the protein is bound at the 3′ backbone nick. Besides tyrosine, an important catalytic role is played by a highly conserved histidine residue [17]. Studies on mutated proteins indeed show that it does not act as a general acid to protonate the leaving 5′ oxygen during the cleavage reaction, but participates in stabilizing the pentavalent transition state through hydrogen bonding to one of the non-bridging oxygens.

The covalent linkage to DNA causes a severe structural deformation of the protein in the “closed” form, which strongly modifies the non-covalent interface between different domains containing many of the active site residues, and the rigid part formed by domains II and III moves away from the remainder of the structure, generating the “open” conformation of the enzyme. The 3′ nick remains non-covalently associated with the DNA-binding domain I, while the 5′ nick will move up with domains II and III, thus creating a gate in the single-stranded segment. Now, either the passing element travels through the transient gate or a controlled rotation of the covalent enzyme–DNA complex around an intact DNA strand occurs (Fig. 19.2), changing the relative positions of the cleaved and non-cleaved DNA fragments. At this point, the open form reverts to the closed form to allow rejoining of the broken DNA ends through nucleophilic attack of the nick phosphorus by the nick OH group. The passing DNA segment is released from the complex, completing the strand passage event. The rejoined single-stranded DNA

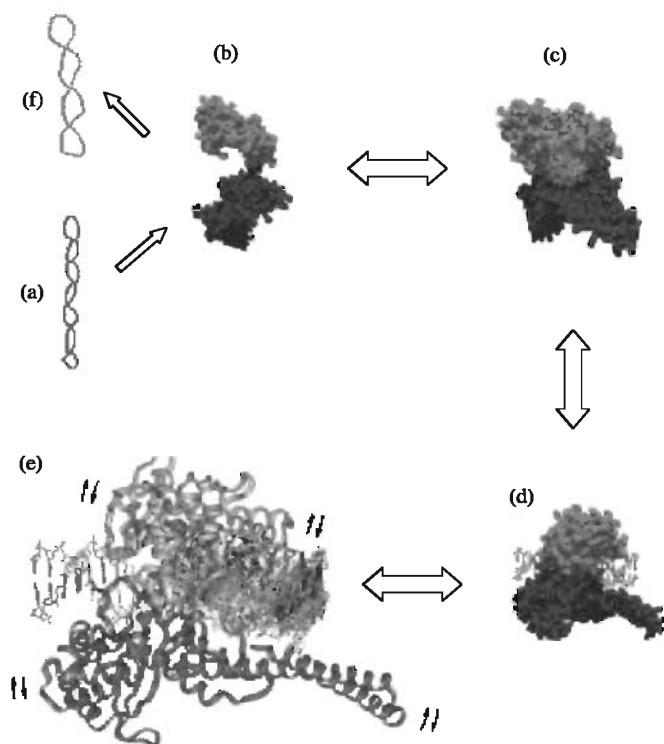


Fig. 19.2. Controlled rotation mechanism for top1 catalysis. DNA (a) binds reversibly to the enzyme (b) forming a non-covalent complex (c), which undergoes the cleavage reaction to produce the covalent complex (d). Controlled

rotation (e) produces strand passage. The new covalent complex reverts to the non-covalent complex, which finally releases the processed DNA (f). Adapted from Ref. [15].

element may remain non-covalently associated with the enzyme for reiteration of the mechanism. As mentioned above, top1 operates in the absence of ATP. Hence, the catalytic process must be driven solely by interactions within and between the enzyme and DNA. There is no defined directionality of this mechanism, so the overall process must involve the release of free energy. It seems likely that the supercoiled and distorted DNA conformations which top manipulates are highly energetically unfavorable, and thus the lower energy state of the manipulated conformation may drive the mechanism. It is finally important to notice that single- or double-stranded DNA fragments may both serve as the passing element, whereas gating has only been observed with single-stranded DNA.

19.2.2

Type II DNA Topoisomerases

Several type II tops have been identified and characterized [18]. These include the prokaryotic DNA gyrase (gyr) and topoisomerase IV (top4), enzymes from T-even

bacteriophages and the eukaryotic topoisomerase II (top2). The human enzyme htop2 is characterized by two isoforms, alpha and beta. More recently, another member of the family, archeal topoisomerase VI (top6), has been described. The various enzymes share a high level of primary sequence homology (except perhaps top6) although they may differ in the number of subunits required for generating the catalytically active form. Type II tops can transport one duplex DNA segment through another. They can catenate or decatenate duplex DNA rings and alter DNA superhelicity. DNA gyrase is unique among type II enzymes; it negatively supercoils DNA, whereas the others relax positively and negatively supercoiled DNA.

19.2.2.1 Structural features

No crystal structure of the entire enzyme is thus far available. However crucial information at the molecular level has been gained with the structure determination of the sequence 410–1202 from the *Saccharomyces cerevisiae* protein [19]. Two main domains A' and B' were identified by homology with the corresponding gyrA and gyrB proteins of the prokaryotic enzyme. The *S. cerevisiae* fragment is organized as a dimer and exhibits two subunit interfaces: one between the helical subdomains of the C-terminal A' regions, and the other between the B' domains of each monomer at the N-terminal region. The dimer structure encloses a large central hole approximately 50–55 Å in diameter that can accommodate a duplex fragment. A model of the human alpha isoenzyme is presented in Fig. 19.3. The catalytic tyrosine is located in a region of the A' domain in a positively charged curved groove, wide enough to accommodate DNA.



Fig. 19.3. Model of human reconstituted top2 in its dimeric form.



Fig. 19.4. The unique process of DNA wrapping around the DNA-gyrase tetramer.

Although information is still lacking at the molecular level for the intact enzyme, single particle electron microscopy of top2–DNA complexes has recently suggested a structure of top2 in which the ATPase domains sit atop the B'/A' DNA binding or cleavage core with DNA bound in the middle [20, 21].

To explain negative supercoiling by gyr, the enzyme is suggested to bind DNA, wrapping it around its core in a positive superhelical sense [22], as schematically shown in Fig. 19.4. In the presence of ATP the DNA tails are closely wrapped around the protein, keeping the T segment in a defined orientation. Thus, transport of either tail results in the introduction of negative superhelical turns into closed circular DNA molecules [23]. This also explains why in the presence of gyrase, intramolecular reactions (such as supercoiling) are favored over intermolecular reactions (such as decatenation) [24].

19.2.2.2 Catalytic process

The top2-mediated process has been extensively investigated over the years; an experimentally fully validated molecular mechanism is however not available as yet. All type II tops use an ATP-coupled DNA transport reaction in which the enzyme cleaves a target duplex DNA segment (the G-segment), transports a second duplex segment (the T-segment) through the gate and subsequently religates the cleaved DNA. Similarly to top1A enzymes, top2 generates phosphotyrosine linkages to the 5' ends of the DNA. However, two nicks are generated by top2s, one on each strand of the duplex, at bonds four base pairs (bp) apart. The proposed structure for top2 enzymes suggests how the catalytic process might occur. Concerted cleaving in the two strands would be regulated by the dimerization of the ATPase domains. This would then cause the conformational changes needed to form the gate in the G-segment. Then the T-segment, after crossing through the G-segment, could exit through a second gate in the A' domains. The results of electron microscopy and biochemical studies are hence consistent with top2 providing separate entry and exit doors for the transport DNA fragment [25]. Top2 utilizes covalent protein–DNA interactions to align the 5'-termini of cleaved DNA for religation, but does not form covalent bonds with the 3'-DNA termini. Current studies indicate

that the enzyme positions the 3'-DNA termini for religation essentially by generating non-covalent protein–DNA contacts [26]. Moreover, a recent theoretical investigation suggests a strand-passing model whereby top2s introduce a sharp bend in DNA. The enzymes are proposed to adopt a specific orientation relative to the bend, thus providing unidirectional strand passage and explaining decatenation and unknotting below thermodynamic equilibrium [27].

19.3

Drugs Targeted at Topoisomerases

Given the crucial role of tops in fundamental mechanisms of cell replication and maintenance, it can be easily understood how drugs interfering with these mechanisms could seriously impair cell functions and finally cause cell death.

That tops are the biological targets for a number of leading drugs was originally shown in the early 1980s [28, 29]. The drugs were grouped into two main classes: inhibitors and poisons, the latter being by far more effective.

Topoisomerase inhibitors impair the enzymes' functions by binding to them and preventing them from performing their catalytic cycle. Inhibitors do not induce DNA damage, and their effects are moderate and reversible. Therefore, much less effort is devoted to identify new top-inhibitory agents.

Poisons act in a more complex way [3–11]. If a drug is able to affect the DNA cleavage–religation process by increasing the DNA cleavage rate or by reducing the DNA religation rate, the so-called cleavage complex spans its life and DNA breaks persist longer. Stabilization of the cleavage complex converts the topoisomerase into an endogenous toxin that “freezes” DNA damage in the genome and triggers a sequence of biochemical events that finally leads to programmed cell death, called apoptosis. There are several ways in which a drug can affect the cleavage complex, but it is generally believed that a ternary complex is formed, including the drug, the nucleic acid, and the enzyme. In the ternary complex, the drug is available to contact both the DNA and the enzyme (Fig. 19.5). Hence, two pharmacophoric domains are commonly present in a poison molecule, an enzyme-directed domain and a DNA-directed domain [30]. The mechanism through which the drug produces its pharmacological effects is not simply an inhibition of the enzyme; rather it is a topoisomerase-poisoning process and the drugs are accordingly named top poisons.

Information about the structural details of the poisoning process is very important, as it can allow a rational understanding on how drugs act at the molecular level, hence providing powerful tools for novel drug design and development. It should be emphasized that, although many very effective drugs are available today, none are devoid of adverse effects like toxicity and onset of resistance. Both aspects may dramatically reduce the drugs' efficacy and eventually lead to discontinuation. Clearly, novel strategies to circumvent the above mentioned effects are needed and this is why the search for safer, more potent and selective agents has remained very active over the years.

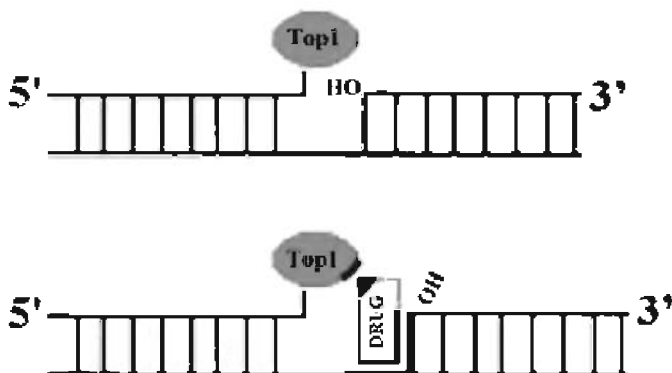


Fig. 19.5. Binding of a drug within a cleavage complex. The drug domains interacting with DNA and top (top1 in this figure) are highlighted in black.

Modern research requires the use of appropriate model systems to investigate drug effects *in vivo*. In this connection appropriately designed yeast systems have proved to be very useful for studying both top1 and top2 poisons [31, 32].

Another key area of research is aimed at understanding the complex signaling pathways that lead to programmed cell death (apoptosis) after induction of DNA damage by top poisons [33–37]. Promotion of apoptosis may potentiate the sensitivity of target cells to chemotherapeutic agents, thus improving the efficacy of pharmacological treatment. Indeed, many interconnecting apoptotic cascades and protein interactions are emerging, and each drug is likely to affect them to different extents.

19.3.1

Top Poisons

19.3.1.1 Top1 poisons

Relatively few top1 poisons have been thus far described. A clinically useful drug known for a long time is camptothecin (CPT), discovered as an antitumor alkaloid from the Chinese tree *Camptotheca acuminata* approximately 30 years ago. CPT was later shown to stabilize the covalent top1–DNA cleavage complex by preventing DNA religation. In fact, CPT both inhibits DNA relaxation and induces cleavage complexes (protein-linked DNA single-strand breaks) [38]. The drug-stabilized cleavable complex formation is a reversible molecular event. In addition, the single-strand breaks resulting from top1–CPT interaction should in principle be easily repaired. Hence, it is difficult to explain the outstanding antiproliferative activity exhibited by top1 poisons. In fact, to account for cell death stimulation, it is believed that collisions between ternary (drug–top–DNA) complexes and advancing replication forks generate double-strand breaks. These lesions are much more toxic than single-stranded breaks and, by recruiting other factors like p53, are able to trigger the cell death program [39].

Top1 is the primary target of CPT as shown by CPT-resistance in yeast cells, in which the top1 gene is deleted, and sensitivity is restored upon expression of wild-type top1. Also, eukaryotic cells selected for high levels of CPT resistance show mutations that render the enzyme insensitive to the drug. Due to its poor pharmacokinetic properties, analogs need to be developed, and some of them are now in clinical trials. Topotecan, irinotecan, 9-aminocamptothecin, and 7-*N*-methylpiperazinomethyl-10,11-ethylenedioxy-20-*S*-camptothecin are important examples (Fig. 19.6).

Since CPT is the only drug for which a detailed molecular model for the ternary cleavage complex is available, we will consider this drug (and its family) in more detail.

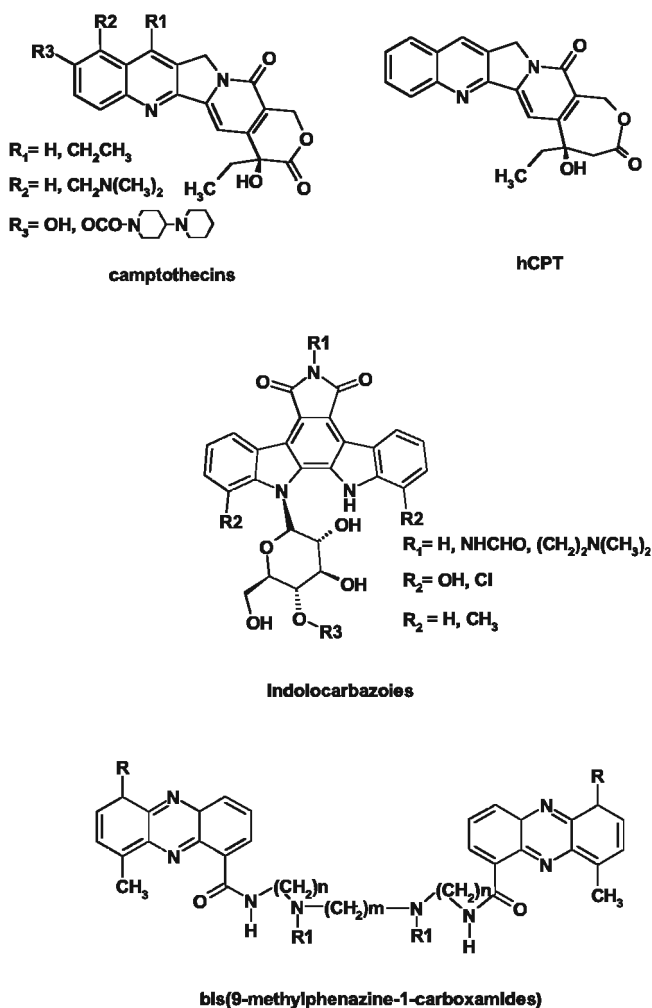


Fig. 19.6. Chemical structure of selected top1 poisons.

CPT is a planar molecule with a bent shape. In spite of its planarity, it does not appreciably intercalate into duplex DNA. Interaction with guanine (possibly from the DNA minor groove) can, however, be detected using UV activation, and a recent study suggests a direct interaction between CPT carboxylate form and DNA [40]. Another study in solution using flow linear dichroism, circular dichroism, and Raman spectroscopy suggests that the lactone (rather than the carboxylate) form of CPT derivative topotecan interacts with DNA within the DNA minor groove and confirms the preferential binding to GC-rich DNA [41].

Testing of a number of camptothecin derivatives with purified top1 has suggested the existence of a stereospecific drug-binding site in the top1 cleavage complex. Salient points of the CPT structure–activity are as follows:

1. Substitutions at positions 7 and 9 do not generally affect top1 inhibition, suggesting the absence of tight interaction between the receptor site and part of the A and B rings of CPT around positions 7 and 9. For instance, 7-ethyl substitution in SN-38 contributes to the remarkable potency of this agent.
2. Hydroxyl substitutions at position 10 increase top1 targeting.
3. Bulky substitutions at position 11 and even small substituents at position 12 inactivate CPT derivatives, suggesting a tight interaction between the top1 cleavage complex and the concave region of the drug. By contrast, addition of a methylenedioxy (or ethylenedioxy) ring increases top1 inhibition.
4. Natural CPT is in the 20-S configuration with the hydroxyl group pointing upward. The fact that 20-R-camptothecin is inactive suggests that the 20 hydroxyl residue must interact closely, possibly by hydrogen bonding with the top1 cleavage complex. Other substitutions in the E-ring generally abolish CPT activity. The 21-lactam derivative is inactive in spite of similar geometry. This difference is probably related to the resistance of the lactam derivative to ring opening.

Based on drug crosslinking experiments and on the observations described above, CPT likely interacts both with the enzyme and the DNA in the ternary complex. Fan *et al.* [42] have recently used molecular modeling and computer simulation to dock camptothecin into a hypothetical cleavage complex. In this model, camptothecin is stacked inside the top1-mediated DNA cleavage site with its concave region facing the DNA major groove. A second model has been proposed by Redinbo *et al.* [43] on the basis of the crystal structure of the top1–DNA cleavage complex. In this model CPT is stacked against the +1 purine, the +1 base is flipped out of the DNA duplex and the CPT molecule is structurally altered.

A third proposal, obtained by docking CPT into the top1–DNA cleavage complex obtained from X-ray data upon the crystal structure of the top1–DNA complex, is consistent with intercalation of the drug at the cleavage site, with the A-ring directed toward the major groove and the E-ring pointing into the minor groove. Here, the drug's orientation differs remarkably with the previous intercalation model, while it is similar in the guanine-flipping model [44].

More recently, solution of the X-ray structure of a ternary complex with top-

otecan has been announced [45]. This will perhaps give the final answer to the issue. Of course, the relevance of the solid-state structure of the cleavage complex to the real situation occurring in cell systems has to be assessed in the future.

Besides structural approaches to drug design, another powerful technique is a predictive QSAR analysis using the most advanced statistical methods and the availability of large database information (for example the NCI Drug Database). Principal methods include stepwise linear regression, principal component regression, partial least-squares regression, and fully cross-validated genetic function approximation. Hierarchical clustering, considering over 150 compounds, was successfully used for the CPT family [46].

Novel CPT congeners have been recently prepared. BN 80915, a difluoro-derivative where the six-membered α -hydroxylactone ring of CPT is replaced by a seven-membered β -hydroxylactone ring to give the homocamptothecin (hCPT, Fig. 19.6) family, is a lead compound that recently entered clinical trials [47, 48]. In spite of the modification to the crucial E-ring, it retains effective top1-poisoning activity, and is also very potent as evidenced by IC_{50} values consistently lower than those of the corresponding CPT analogs in sensitive cell lines as well as in related multidrug-resistant lines. In addition, BN80915 is a potent inducer of apoptosis in HL-60 cells. This is associated with cell cycle changes, intracellular pH, activation of caspase-3 and caspase-8, DNA fragmentation, and externalization of phosphatidylserine lipids but no significant changes in the mitochondrial membrane potential or in the expression of oncogenes like Bcl-2. A nice finding is that homologation of the lactone ring slows down hydrolysis, which, in contrast to CPT analogs, is irreversible, leading to improved plasma stability and reduced toxicity related to ring closure during excretion.

Interesting top1-poisoning potency was found in another hCPT derivative, 12-Cl-hCPT, which bears a chlorine substituent at position 12 [49]. Again, homologation of the E-ring lactone produced a noticeable loss of cross-resistance with CPT in cancer cells presenting multidrug resistance phenotype.

Among 7-substituted derivatives, a novel series of lipophilic analogs of CPT was recently described. The authors' hypothesis is that lipophilicity could promote a rapid cellular accumulation and stabilization of drug-target interactions. In particular, derivative ST1481, suitable for oral administration, exhibited an impressive response in terms of efficacy, potency, and therapeutic index when compared with the reference topotecan [50]. Even in this case, no cross-resistance to ST1481 was found in ovarian carcinoma cells overexpressing P-glycoprotein.

Other important members of the top1 poison family are the indolocarbazoles derived from rebeccamycin (Fig. 19.6), and minor groove binders such as the bis-benzimidazoles Hoechst 33258 and Hoechst 33342 [5]. The indolocarbazole family are dealt with in another chapter of this book. The Hoechst derivatives are more interesting as biochemical probes than as drug candidates.

Besides monomeric ring systems, a family of dimeric bis(9-methylphenazine-1-carboxamides), reported in Fig. 19.6, joined by several dicationic linkers of varying length and rigidity showed effective top1-poisoning activity [51]. Analogs joined by a $(CH_2)_2NR(CH_2)_2NR(CH_2)_2$ linker were extremely potent and showed selectivity

toward a panel of human cancer cell lines. Moreover, two selected compounds showed *in vivo* activity in murine and human colon tumor xenograft models.

In general, interference with top1 is characterized by reversible interactions with the drug. More complex appears to be the situation in which chemically reactive groups, such as alkylating functions, characterize the drug. In this case the cytotoxic response may arise from the sum of enzyme-mediated and non-mediated effects. A typical example is represented by the potent antitumor agent presently in clinical trials ecteinascidin 743 (Et743), a natural marine product isolated from the Caribbean sea squirt [52]. It selectively alkylates guanine N2 from the minor groove of duplex DNA and bends the DNA toward the major groove. Et743 DNA adducts have been claimed to suppress gene expression selectively and to induce top1 cleavage complexes *in vitro* and top1–DNA complexes in cell culture. The DNA damage consisted of comparable amounts of DNA–protein cross-links and DNA single-strand breaks, not associated with detectable DNA double-strand breaks. Hence, the drug was suggested to act by poisoning top1. However, by contrast with CPT, the DNA lesions persisted for several hours after drug removal. In addition, the sensitivity of CPT-resistant cells, which fail to express detectable top1, was similar to the sensitivity of the wild-type cells, suggesting that top1 was not a critical target for the drug. Indeed, it was recently demonstrated that what appeared a top1-mediated process depended instead upon a new cell-killing mechanism mediated by transcription-coupled DNA nucleotide excision repair [53]. This fact should make us careful in assessing modes of drug action.

In any event, alkylating agents can promote top1-mediated DNA damage by affecting the stability of a phosphodiester linkage close to the site of damage. In fact, methylation of position 6 of guanine by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produced an 8- to 10-fold enhancement of top1 cleavage by affecting both the rate of top1-mediated DNA religation and that of the enzyme cleavage step. Enzymatic removal of the methyl groups from guanine abolished this enhancement [54]. These results suggest a role for top1 poisoning by alkylated bases in the anti-proliferative activity of alkylating agents as well as in the DNA lesions resulting from endogenous and carcinogenic DNA modifications.

As mentioned before, a deeper insight into the mechanism of drug action can be gained by having a more precise knowledge of the underlying biochemical processes. Two interesting examples deserve attention.

The first consists of an investigation on the effects of top1-targeting drugs on F1p recombinase, an enzyme of the lambda-Int family able to introduce transient single-strand breaks in DNA by similar chemical processes [55]. Indeed, agents like CPT and NSC-314622 interfere specifically with the enzyme. Hence, F1p and other Int family recombinases may provide further model systems for dissecting the molecular mechanisms of topoisomerase I-directed anticancer therapeutic agents.

The second example deals with the amino acid residue(s) involved in top1 interaction with the drug [56]. In this study, Capranico *et al.* were able to select human top1 mutants about 30-fold more CPT-sensitive than wild-type top1 in *S. cerevisiae* cells by directed evolution of a C-terminal portion. A top1 mutant had only two

amino acid changes in domain II, one of which mapped at the domain II–III contact surface. The information arising from such drug-hypersensitive topoisomerases might prove useful in developing DNA cutters with high cell lethality.

19.3.1.2 Top2 poisons

The number of known top2 poisons greatly exceeds that of top1 poisons. Indeed different families of drugs are able to interfere with the cleavage/rejoining process mediated by top2. These include epipodophyllotoxins, anthracyclines, acridines, anthraquinones, ellipticines, bisantrene, actinomycin D, terpenoids, quinolones, and flavonoids [8, 9]. The different chemotypes hardly share similar structural features. To confirm this, some are very efficient DNA binders, yet they poorly poison top2, others do not bind to DNA to a measurable extent, but are quite effective in stimulating damage of the nucleic acid. In the case of top2 poisons, the target cleavage complex contains a 4-bp staggered double-strand DNA cut. This double lesion, as mentioned before, is very cytotoxic when stabilized by interaction with a drug and eventually triggers apoptosis in a way similar to the collision between top1-mediated single-strand breaks and the replication fork.

The principal clinically relevant targets for top2-directed agents are the human htop2 enzyme (2 isoenzymes) and the bacterial gyr and top4 enzymes.

Human top2-directed poisons Many of the most effective drugs, such as acridines, bisantrene, anthracyclines, and anthracenediones, are characterized by a planar polycyclic region with appropriately located, often charged or polar, side chains. Examples of “classical,” clinically relevant, human top2 poisons are presented in Fig. 19.7. The planar structure facilitates an insertion of the chromophore into DNA through an intercalation process. This will generate strong interactions between the aromatic portion of the drug and DNA bases in regions containing alternating purine–pyrimidine sequences. As with top1 poisons, two domains are present in these compounds (DNA-binding and enzyme binding), which can partially overlap. The relative position of the two regions plays a major role in modulating nucleic acid binding and enzyme-poisoning effects [57].

It has to be noted that more than one single cytotoxic mechanism can be promoted by drugs. This is the case of anthracenedione-based compounds, for which redox cycling represents an alternative way to cause cellular damage. A recent femtosecond dynamic investigation on the anthracycline–DNA complex has indeed shown a crucial role of drug/base/ O^{-2} in efficient and catalytic redox cycling, with possible implications in the cell-killing response [58].

Although very few studies are available on complex formation between the enzyme and the poison in the absence of DNA, useful information on specific contacts is slowly being rendered available. This is particularly important for drugs binding poorly to the nucleic acid and hence expected to interact more efficiently with top2 domains. It has become evident that top2 alpha binds etoposide specifically and that ATP interferes with this process [59]. Effective interaction has also been described between the quinolone ciprofloxacin and the A subunit of gyr, this process being mediated by divalent ions such as Mg^{2+} [60].

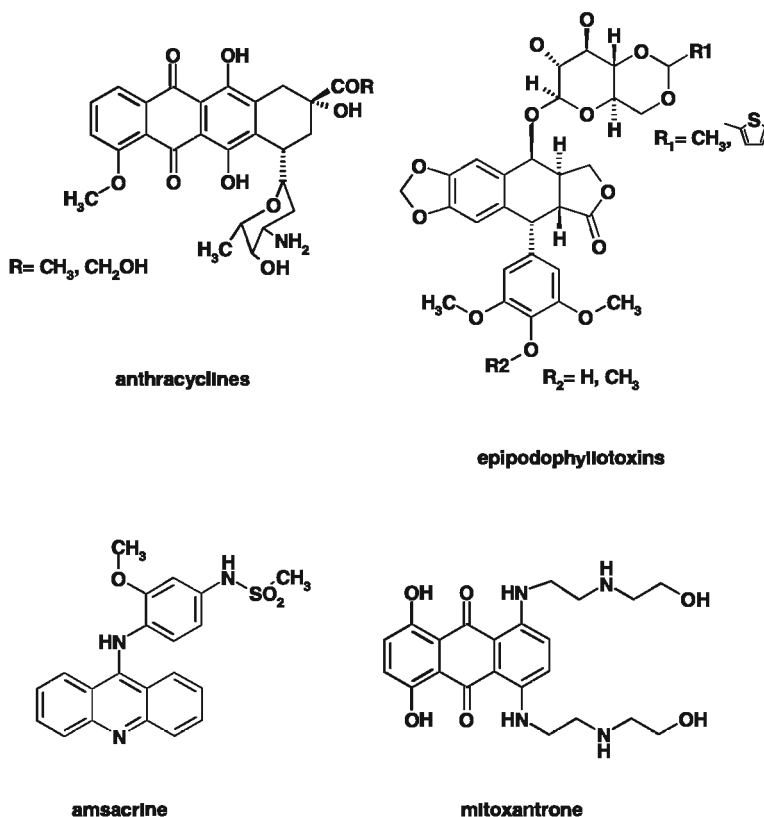


Fig. 19.7. Chemical structure of selected “classical” top2 poisons.

A further contribution to the mechanistic aspects of top2 inhibition derives from the finding that ATP-bound top2 acts as a target for antitumor drugs and discriminates between two groups of compounds: ATP-insensitive top2 poisons such as amsacrine, batracylin, and menadione that were poorly responsive to the ATP-bound conformation of top2, and ATP-sensitive top2 poisons including doxorubicin, etoposide, mitoxantrone, and amsacrine, the action of which was remarkably stimulated (30- to 100-fold) in the presence of the nucleotide triphosphate [61]. This suggested that ATP-bound top2 may be the specific target of ATP-sensitive poisons.

One of the most frequent substitutions in a lead molecule to obtain SAR information consists in examining bioisosteric congeners. The consequences of bioisosteric N–C substitution in the planar tricyclic moiety, as in aza-anthracendiones and aza-anthrapyrazoles, has been examined in detail. In general, the presence of nitrogen in the ring system reduces top2-mediated effects, suggesting the predominance of other cell-damage mechanisms, possibly redox-related [62]. These changes can usefully operate to reduce cross-resistance with other top2 poisons.

A further case of cross-resistance reduction is represented by the etoposide ana-

log TOP-53, a promising agent effective against non-small cell lung cancer in animal tumor models [63]. Although TOP-53 has top2 as its primary cytotoxic target and kills cells by acting as a top2 poison, it displays nearly wild-type activity against a mutant yeast type II enzyme that is highly resistant to etoposide. This shows that substituents on the etoposide C-ring can subtly modulate top2–drug interactions.

Isoenzyme specificity is another important issue in top2 poisoning. In fact, the two isoforms (alpha and beta) of the human top2 enzyme are expressed to variable and distinct extents during cell cycle progression [64]. Differential drug action towards individual isozyme would play a role in the cytotoxic response and eventually in the clinical outcome of drug treatment. Indeed, until now no specific isoform-targeting agent was reported, top2 poisons being in general effective on both isoforms and showing only moderate preference for one or the other.

An interesting exception is XK469, a synthetic quinoxaline phenoxypropionic acid derivative reported in Fig. 19.8 that showed remarkable preference for the beta isoform in cell-free systems [65]. In addition, top2 beta knockout cells were resis-

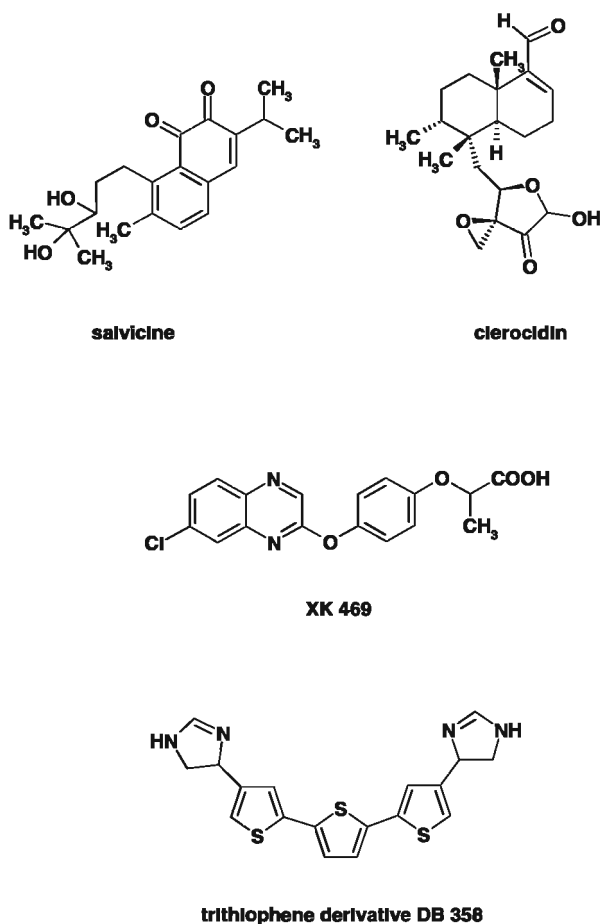


Fig. 19.8. Chemical structure of recently investigated top2 poisons.

tant to XK469 treatment. The high IC_{50} for topoisomerase I inhibition confirmed that top1 is not a significant target for this drug. More recent work, however, shows the combination of multiple pathways of cytotoxic action, including p53-dependent and p53-independent G₂-M arrest via inactivation of cdc2/cyclin B1 kinase activity [66]. Once more, the interplay of different oncogenes with topoisomerase expression and poisoning finely tunes the drug's action. The compound exhibited broad activity against murine tumors and is entering phase I clinical development.

A second drug recently recognized as a top2 beta poison is the bis-dioxopiperazine ICRF-193. It was formally considered to be a catalytic inhibitor of the enzyme, hence devoid of DNA-damaging potential. However, recent work shows dose-dependent crosslinking of human top2 beta to DNA and stimulation of top2 beta-mediated DNA cleavage by the ICRF derivative [67]. *In vivo* experiments confirmed the requirement of a chaotropic protein denaturant in the assays and selectivity for the human beta-isozyme. Other studies, however, besides confirming ICRF-193 as a top2 poison, showed that decreased top2 alpha expression confers increased resistance to ICRF-193 as well as to VP-16 in mouse embryonic stem cells [68]. Hence, also isoform alpha seems to be affected by the bis-dioxopiperazine agent, casting some doubt on isoenzyme specificity.

In addition to optimizing previously reported compounds or getting deeper insight into their poisoning mechanism, a number of novel top2 poisons are emerging in the recent literature. They can be useful to reduce side-effects or circumvent resistance. In addition, solid tumor targeting is a desired feature of valuable drugs.

Salvicine, a structurally modified diterpenoid quinone derived from *Salvia prionitis* (Fig. 19.8) has significant *in vitro* and *in vivo* activity against malignant tumor cells and xenografts, especially some human solid tumor models. This anticancer activity of salvicine is associated with its ability to induce tumor cell apoptosis [69]. Salvicine was also found to have a profound cytotoxic effect on multidrug-resistant (MDR) cell lines by downregulating the expression of MDR-1 mRNA of MDR cells. Salvicine acted as a top2 poison through its marked enhancement effect on top2-mediated DNA double-strand breaks. In contrast, no inhibitory activity was observed against the catalytic activity of top1. The drug greatly promoted top2-DNA binding and inhibited pre- and post-strand top2-mediated DNA religation without interference with the forward cleavage steps. DNA damage correlated with cell growth inhibition. Furthermore, salvicine produced alterations of the expression of selected proliferation regulatory genes, in particular a reduction in the transcription rate of *c-myc* in a dose-dependent manner and a marked induction of *c-fos* and *c-jun* [70]. These early events would then trigger cell death.

We mentioned previously that planar polycyclic structures are rather common among top2 poisons. However, compounds with extended unfused aromatic systems including trithiophene derivatives of furimidazoline (Fig. 19.8) also showed remarkable poisoning effects towards top2, strongly stimulating DNA cleavage [71]. This was not the case for a trifuran analog. Unexpected chemotypes are thus emerging as potential new drugs.

Antitumor metallocenes dihalides Cp_2MCl_2 , ($M = Ti, V, Mo, Nb$) represent another example of recently recognized DNA-damaging agents that interfere with

top2 function [72]. It should, however, be emphasized that, as previously discussed with DNA-alkylating top1-directed agents, the presence of reactive groups in a drug molecule can elicit multiple cytotoxic mechanisms.

Chemically reactive functions can be present in a top2 poison. This is the case for the diterpenoid clerocidin (CL, Fig. 19.8), an efficient DNA-damaging cytotoxic agent, exhibiting reversible as well as irreversible effects. A recent study by our group showed that CL is indeed able to alkylate non-paired guanines in DNA [73]. This reaction could represent the basis to explain the irreversible poisoning effects observed when DNA cleavage stimulation occurs at sites having a G at the -1 position. An investigation of the biological mode of action of CL using whole cell assays was also performed [74]. The authors confirm that CL recognizes top2 as its main intracellular target, but suggest binding to the enzyme prior to formation of the cleavage complex with DNA. This is difficult to explain in view of the well-documented irreversible damage to the nucleic acid at specific sites.

Various endogenous and environmental agents are able to attack DNA-producing abasic sites in the cell. Indeed, these are the most commonly formed DNA lesions. When located within a top2–DNA cleavage site, “intact” abasic sites act as top2 poisons and dramatically stimulate enzyme-mediated DNA scission. However, most abasic sites in cells are not intact. They exist as processed base excision repair intermediates that contain DNA strand breaks proximal to the damaged residue. When strand breaks are located within a top2 DNA cleavage site, they create suicide substrates that are not religated readily by the enzyme and strongly enhance DNA scission. This can generate permanent double-stranded DNA breaks [75]. Drugs producing abasic lesions could therefore be considered as “indirect” top2 poisons.

The final goal of developing new htop2 poisons is to discover and employ effective anticancer agents to cure patients and save their lives. Hence, it is interesting to compare biochemical and biological determinations with clinical response.

Topotecan and etoposide were used in combination to treat patients with recurrent or refractory non-Hodgkin's lymphoma [76]. Throughout the treatment, top–DNA covalent complex formation was measured *in vivo*, whereas top1, 2 alpha, and 2 beta mRNA expression levels were determined. Drug-induced top–DNA complex formation was always observed in blood samples of all the responding patients, whereas very few non-responding patients showed detectable formation of covalent complex. Hence, top–DNA complex formation correlated with response to treatment. In all patients, expression levels of top1 and 2 beta mRNA remained similar to pretreatment levels, whereas top2 alpha mRNA levels had decreased dramatically by the third day. This information can be very valuable in dosing drugs and scheduling treatment.

A correlation between top2 alpha expression and the chemosensitivity to top2-targeting drugs was also found in gynecological carcinomas [77]. Data suggested that the top2 alpha index of a tumor is a reflection of its chemosensitivity to top2-targeting drugs. Hence, the use of this index may enable prediction of a clinical response to chemotherapy using top2-targeting drugs in gynecological malignancies.

A link between top2 expression and prognostic factors/clinical outcome was not found, however, in childhood acute lymphoblastic leukemia when using regimens that include daunorubicin or etoposide [78]. Indeed, neither top2 alpha, nor top2 beta expression levels could predict the response to the above poisons.

Induction of top activity after ErbB2 activation and response to breast cancer were finally examined [79]. It was shown that the cytotoxicity of doxorubicin is inhibited in ErbB2+ breast cancer cells by the anti-ErbB2 antibody herceptin. This is accompanied by a decrease in top2 alpha protein levels and activity, suggesting that this is the mechanism of change in doxorubicin response. In addition, a 10- to 100-fold increase in potency of doxorubicin is seen after ErbB2 activation, along with a remarkable increase in top2 expression and activity. Besides explaining the differential benefit seen with doxorubicin- versus alkylator-based chemotherapy in ErbB2+ breast cancer, the information gathered can be used to suggest the optimal sequencing of herceptin and chemotherapy agents in ErbB2+ breast cancer.

To be able to obtain a complete view of the linkages between *in vitro* and *in vivo* studies and to identify the critical parameters to be determined, we need to develop simpler, faster, and more accurate biological assays that could be performed for a larger number of patients and that would give a statistically significant response in diagnostic and prognostic tests.

Bacterial top2-directed poisons Prokaryotic type II topoisomerases represent the targets of potent chemotherapeutic agents. Two main classes of top2-targeted antibacterial agents are known: quinolones and coumarins, having totally distinct mechanisms of action.

Fluoroquinolones (Fig. 19.9) are a very important family of antibacterial agents that are widely prescribed for the treatment of infectious diseases in humans [8]. Although the founding members of this drug class had little clinical impact, successive generations include the most active and broad-spectrum oral antibacterials currently in use.

In the cleavage complex, fluoroquinolones interact mainly with the A subunit of gyr and ParC of top4, inducing a stabilization of the enzyme–DNA cleavage complex.

In gyrA, in fact, a region spatially close to the active tyrosine and called quinolone resistance determining region has been identified. Amino acid mutations clustered within this region greatly reduce the affinity of quinolones for the enzyme–DNA complex. Key amino acids are Ser83 and Asp87 [80]. As previously pointed out, gyr acts mainly in front of the replication fork to remove positive supercoiling. Thus the drug-stabilized complex behaves as a barrier to the progression of the replication machinery. Processing of the stalled cleavage complex causes induction of bacterial SOS response [81]. This enhances DNA repair as well as mutagenesis and recombination processes that are linked to the emergence of resistance to quinolones [82]. As for other top2 poisons, resistance represents a major problem for this drug family. This is often connected to quinolone abuse or misuse.

The structural features of the quinolone–gyr–DNA complex are still not clear [83]. Experimental evidence suggests that quinolones, in the presence of proper

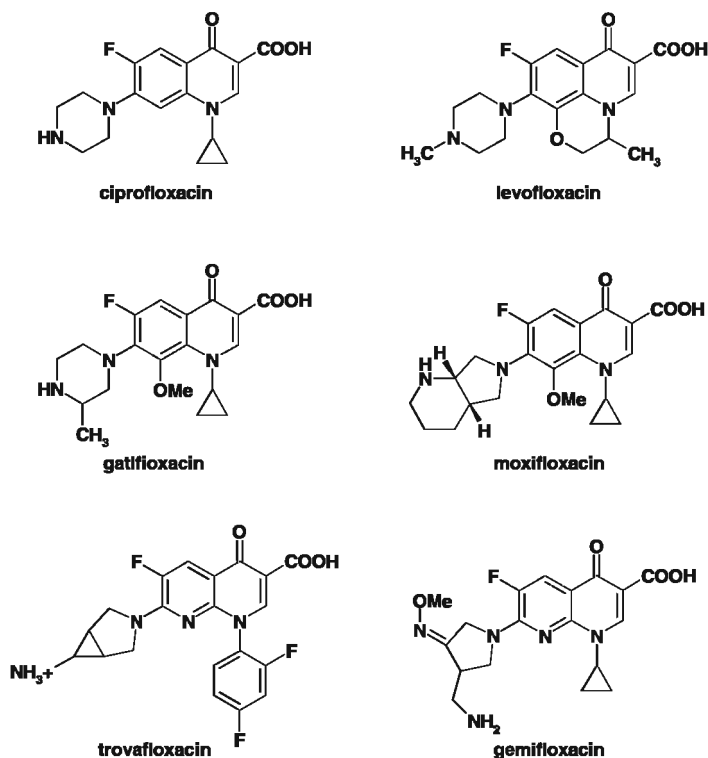


Fig. 19.9. Chemical structure of selected DNA-gyrase and top4 poisons.

metal ions, bind single-stranded DNA and gyrase A with moderate affinity [84, 60]. However they bind efficiently the gyr-DNA complex [85, 86]. DNA cleavage is not required to allow drug binding to occur. Since quinolones do not exhibit a large planar ring system to grant efficient stacking interactions as classical intercalating agents do, they interact preferentially with single-stranded or highly distorted double-stranded DNA. Structural distortion of the double helix bound to gyr and stabilized by direct interactions with protein residues can be sufficient to produce favorable interaction sites for quinolones. Double helix distortion does appear to stem from DNA wrapping as quinolones can stimulate, even if to a low extent, cleavage in DNA fragments as short as 20 bp [87, 88]. However, wrapping, which indeed plays an important role in stabilizing the enzyme-DNA complex, can modulate the sequence specificity of the observed cutting sites. More complex models of ternary complex formation by quinolones have been proposed [89]. These foresee external and intercalative binding to DNA of four drug molecules held together by magnesium bridges. On the enzyme side, binding of quinolones alters not only the structural features of gyrA but also those of the overall A₂B₂-DNA complex [60, 90] in a magnesium ion-dependent fashion.

Consistent with the fact that DNA wrapping is not the major event promoting quinolone interactions, top4 is also a good target for these antibacterial agents [91].

This is proven by a shared activity *in vitro* and similar molecular mechanisms of enzyme poisoning. *In vivo*, the situation is slightly more complicated by the fact that the two enzymes act mainly one ahead and one behind the replication fork. Thus, differential inhibition on the two enzymes can induce different biological effects. In particular, acting on the top4–DNA complex, behind the fork, replication will stop slowly, and this explains why top4-mediated killing occurs generally at lower rate and higher drug concentration than with gyr [92]. These differences can lead also to an easier repair of the top4-mediated breaks by the post-replicative repair system as well as to the involvement of different DNA repair mechanisms [93].

Additionally, many quinolones differ in their relative activities against gyr and top4 in a bacterial cell. The duality of the target plays an extremely important role during the development of resistance. In fact, despite no enzymatic mechanism of drug inactivation exists in bacteria and no indications for transfer of clinically relevant resistance exist, resistance is becoming a major problem due to the extensive use of quinolone drugs [94, 95].

A second mechanism of resistance is a modification of drug accumulation. Efflux of fluoroquinolones is the major cause of decreased accumulation of these agents, enhanced active efflux of hydrophilic quinolones being mediated by the membrane-associated proteins.

Investigations aiming at understanding the molecular mechanisms of quinolone action and resistance in more detail should provide the basis for a rational design of more potent derivatives.

To extend antibacterial activity and fight resistance phenomena, new generations of fluoroquinolones have been recently developed [96]. They preserve excellent activity against Gram-negative bacteria but, as key property, they show an increased activity towards Gram-positive bacteria compared with the parent drug ciprofloxacin (Fig. 19.9). The greatest interest in these compounds is at the moment for treatment of respiratory diseases [97].

A valuable input in the design of new effective derivatives came from the observation that compounds carrying a methoxy group at position C-8 cause fewer side effects than those with halogens at this position, while showing higher activity. A peculiar feature of this family of compounds is a comparable targeting of gyr and top4. Dual targeting is a desirable property to overcome and limit the emergence of bacterial resistance [98].

It has been proposed that the C-8 methoxy group may also lower the propensity for resistance development compared with quinolones possessing different substituents at the C-8 position. In addition, the methoxy substitution increases the activity of the drug against mutated gyrA strains. In this connection, an interaction between the C-8 group of the quinolone and the region of residues 83 and 87 (the most frequent mutations in ciprofloxacin-resistant strains) has been proposed [99].

In addition, fluoroquinolones modified at position C-7 showed that this position plays a key role in determining target selection, thus driving the drug preferentially towards DNA gyr or top4 [100]. A direct interaction of this portion of quinolone drug with the enzyme is strongly suggested.

The most recent derivatives in this group are moxifloxacin, gatifloxacin, premarfloxacin, and clinafloxacin (Fig. 19.9). Despite good bioavailability and extended half-lives, some of them have been withdrawn clinically due to the presence of adverse side effects like phototoxicity and hypoglycemia [97].

Gatifloxacin and moxifloxacin are the most promising new-generation derivatives [101–103]. In fact, they are very potent against both penicillin-susceptible and multidrug-resistant *Streptococcus pneumoniae*, while retaining activity against enterobacteria. A low cytotoxicity toward mammalian cells due to a higher selectivity for bacterial top2 and the absence of phototoxic potential contribute to produce very satisfactory clinical and bacteriologic responses in respiratory infections without clinically significant adverse effects.

Another new promising derivative is trovafloxacin (Fig. 19.9). It is probably the one that displays the greatest activity against human respiratory pathogens. Experimentally, this has been confirmed by a faster induction of top4-mediated DNA scission in comparison to other quinolones [104]. Although a clinical use of this drug is limited because of hepatotoxicity, it represents an important lead compound for future design. Indeed, a related derivative, gemifloxacin (Fig. 19.9), is currently under development as an antipneumococcal drug [105]. This quinolone acts against gyr and top4 in *S. pneumoniae*, with gyr being the preferred *in vivo* target, but with an increased affinity for top4. The marked potency of gemifloxacin against wild-type and quinolone-resistant mutants may be correlated to greater stabilization of cleavable complexes with the target enzymes [106].

As already pointed out, the preferred top2 target changes for each quinolone depending upon bacterial species. In addition, congener quinolones may show inverted preferences for the same strain. For example, older quinolones have generally greater potency against gyr than against top4 in many Gram-negative bacteria and greater potency against top4 than against gyr in many Gram-positive bacteria. With the newer derivatives this difference is reduced or inverted. Indeed, in wild-type *S. pneumoniae* the primary target of trovafloxacin, levofloxacin, and ciprofloxacin is top4, whereas the primary target of gatifloxacin is gyr.

Some quinolone-related toxic effects have been attributed to the presence of a fluorine group in the molecule. In fact, these substituents can be actively involved in photoreactions leading to photodegradation and phototoxicity. Recent studies showed that, following proper reoptimization of the substituents, fluorine can be successfully replaced by an amino group [107]. This concept led to the development of new 8-methoxy, non-fluorinated quinolones (NFQs). Overall, NFQs were less susceptible than the parent fluoro quinolones to existing mechanisms of quinolone resistance in staphylococci and *Streptococcus pneumoniae* [108]. Thus they represent additional leads in the search for novel gyr inhibitors. In fact, although these compounds are either in the discovery or early development phase, they extend the possibility of establishing unprecedented structure–activity relationships and disclose new chemotypes for DNA gyr inhibition.

Other new classes of gyrA inhibitors have a peptide structure. CcdB is a bactericidal toxin encoded by the F plasmid. The ccd module of this plasmid is composed of two genes coding for two small proteins: a 11.7-kDa protein corresponding to

the CcdB toxin and a 8.7-kDa protein corresponding to CcdA, an antidote for CcdB. When the action of the toxin is not prevented by the presence of the antidote, a reduction of DNA synthesis, an excellent induction of the SOS response, and finally cell death occur [82]. The CcdB protein acts on DNA gyrase by poisoning the gyr-DNA complex and blocking the passage of polymerases. Crystallographic data suggest that the CcdB dimer binds into the central hole of the 59-kDa N-terminal fragment of gyrA, after disruption of the head dimer interface of the subunit. Both poisoning and inactivation can be prevented and reversed by the F plasmid-encoded antidote, the CcdA protein [109].

As quinolone- and CcdB-resistant mutations map to different domains of gyrA and cross-resistance is not observed, the current hypothesis is that they trap different intermediates of the cleavage complex. Given its properties, CcdB can be seen as a valuable scaffold for the development of new inhibitors of DNA gyrase.

Finally, micromicin B17 is a small hydrophobic peptide (≈ 3.2 kDa) active against many enterobacteria. In the presence of ATP, it stabilizes a gyr-dependent DNA cleavage complex in a manner reminiscent of quinolones, Ca^{2+} or the bacterial toxin CcdB. The pattern of DNA cleavage produced by gyr in the presence of microcin B17 is different from that produced by quinolones and more closely resembles Ca^{2+} -mediated DNA cleavage [110]. The presence of heterocyclic rings in the molecule represents a unique opportunity for the use of this peptide as a model. At present, the relationships between the structure of this unusual antibiotic and its activity are not fully established. Among derivatives containing modified bis-heterocyclic sites, relative potency was shown to parallel the capacity of the compounds to prevent growth of sensitive cells [111].

A distinctive aspect of quinolones is their remarkable ability to target different top2 enzymes. In fact, some congeners of this drug family display high activity not only against bacterial top2, but also against eukaryotic top2, as well as cultured mammalian cells and *in vivo* tumor models [112]. These antineoplastic quinolones represent an exploitable source of new anticancer agents, link antibacterial and anticancer chemotherapy together and provide an opportunity to clarify drug mechanism across divergent species.

19.3.1.3 Sequence specificity of top poisoning

Top-targeted drugs exhibit considerable levels of specificity in producing DNA damage [113]. Hence, understanding sequence-specific recognition of the top-DNA complex represents a key step to direct the action of the drugs onto selected genomic sequences. We have reported in a previous paragraph that drugs can discriminate not only among different types of tops, but also among different isoenzymes. Specific stimulation of DNA damage is also observed for different poisons. In fact, well-defined preferences have been reported for DNA bases flanking the cleavage site, each drug showing an individual pattern depending upon its molecular features (dimension, shape, electron distribution) [113]. Establishing the molecular basis for DNA sequence-dependent recognition of the cleavage complex by poisoning agents would enable us to develop very effective drugs targeted at

defined loci in cancer cells. Specific targeting would reduce the drug dose needed to observe a given effect and render resistance phenomena less critical.

Drug–conjugate systems can also be devised consisting of two principal domains: a cleavage complex recognition (poisoning) domain and a DNA-sequence specific element [114–116]. The latter can direct the poisoning domain only at one (or very few) site(s) along the genome, hence producing unique DNA damage. This would turn tops into peculiar restriction endonucleases and open up new therapeutic horizons. Promising examples of specificity exhibited by top1 poisons tethered to minor and major groove ligands have been reported in recent literature.

A more detailed analysis of sequence-specific interactions of drugs interfering with the topoisomerase–DNA cleavage complex are available in a recent review article [117].

19.3.2

Top Inhibitors

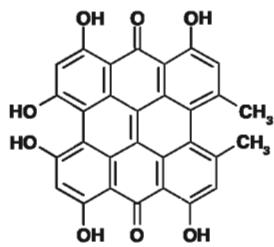
19.3.2.1 Human top2 inhibitors

Top2 inhibition is typical of compounds that bind to the enzyme and prevent it from performing its catalytic cycle by binding to it. This would impair DNA processing by top2 and cause cytostatic effects. Since it is highly preferable to induce cancer cell death than growth arrest, top2 inhibitors are not particularly appealing as drug candidates.

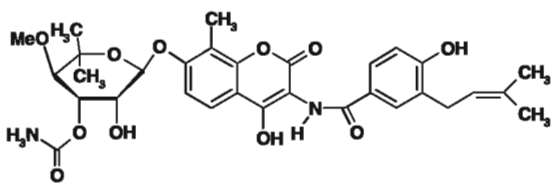
Besides sodium azide [117], which is a non-specific chemical inactivator via an ATP-sensitive process, hypericin (Fig. 19.10), a naphthodianthrone from St. John's wort (*Hypericum perforatum*), has been included among non-DNA damaging top2 catalytic inhibitors [118]. *In vitro* assays indicate that hypericin is a potent antagonist of cleavage complex stabilization by etoposide and amsacrine. This antagonism appears to be due to the ability of hypericin to interact with the DNA structure, thereby precluding top2 binding and/or DNA cleavage. Cancer patients using top2-targeted drugs should be aware of the potential antagonism of herbal extracts of St. John's wort.

19.3.2.2 Bacterial top2 inhibitors

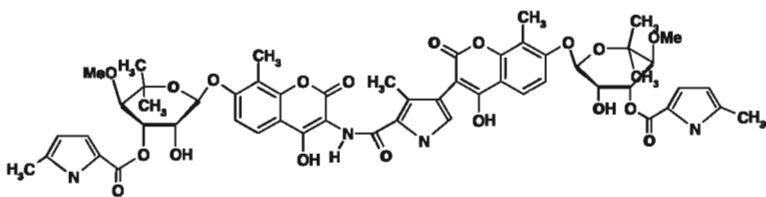
The principal gyr inhibitors are the coumarins, a family of natural products from *Streptomyces* species, the most relevant being novobiocin and coumermycin [119], characterized by the structures reported in Fig. 19.10. Although they are extremely active *in vitro*, these compounds are not used clinically due to toxicity, low solubility, and rapid development of resistance. From a pharmacological point of view, however, they are still valuable since, unlike quinolones, a complete picture of their mechanism of action at the molecular level is now available. They bind very strongly ($K_d \approx 10^{-8}$ M) the N-terminal domain of gyrB [120] by an enthalpy-driven process. Also, the stoichiometry of the process has been assessed: novobiocin binds to the protein in a 1:1 ratio, coumermycin in a 0.5:1 ratio. Biochemical studies revealed that these drugs are competitive inhibitors of the ATPase reaction of gyrB despite a lack of structural similarities.



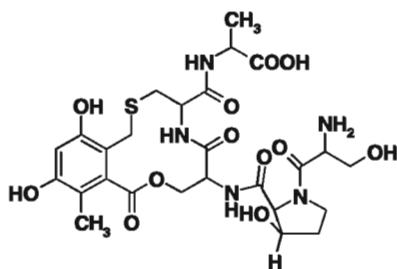
hypericin



novobiocin



coumermycin A1



cyclothialidine

Fig. 19.10. Chemical structure of selected top inhibitors.

A major breakthrough in the understanding of the mechanism was the solution of the crystal structure of coumarin complex with the 23-kDa N-terminal domain of gyrB [121, 122]. The binding sites of the two drugs are distinct, but partially overlap those of ATP. In particular, the sugar ring of the drugs binds at the same site as the adenine ring of ATP, preventing access of the nucleotide to its natural location. The crystal structure of an ATPase inhibitor of the cyclothialidine family (Fig. 19.10) has also been reported [123]. Again, an ATP site-overlapping group is represented by the resorcinol residue, which supports a comparable mechanism of action. Additionally, the distinct protein areas occupied by cyclothialidine explains why the drug is not cross-resistant with coumarins.

As previously pointed out, no clinical application is expected for these derivatives. However, due to the availability of crystallographic information and to the increased problem of resistance to quinolones, gyrB still represents a stimulating target.

In the active field of coumarin-related derivatives, attention has been recently focused on substitution on the saccharidic moiety. It has been discovered that introduction of dialkyl substituents at 5'5'-position of noviose leads to coumarin analogs with improved *in vitro* and *in vivo* antibacterial activity. These antibacterial properties are linked to a safe and satisfactory pharmacokinetic profile at least for one derivative, RU-79115 [124]. Similar results were obtained replacing the 5-methylpyrrole-2-carboxylate group of coumarin with an *N*-propargyloxycarbamate bioisostere [125].

Among cyclothialidines a number of seco-derivatives were synthesized. However, despite an high and selective activity toward bacterial DNA gyrase and toward Gram-positive bacteria *in vitro*, no satisfactory results were obtained in *in vivo* models [126].

A different approach to the production of efficient gyrB inhibitors has been recently devised. An *in silico* needle screening was performed prior to the high-throughput screen, validation, and optimization steps. This led to the identification of novel inhibitors of ATP binding. In particular a 3,4-disubstituted indazole showed promising properties, its potency against DNA gyrase being 10 times higher than novobiocin potency [127].

19.3.3

Mixed Top1/2 Poisons or Inhibitors

In addition to agents targeting type I or type II enzymes, a number of mixed poisons have been described. These compounds exhibit structural features able to stabilize the cleavage complex of both tops. Simultaneous blockage of top activity possibly results in superior cell-killing effects and lower risk of resistance. Among others, saintopin, intoplicine, aclarubicin, acridine-4-carboxamide derivatives, bis(phenazine-1-carboxamides), indeno-quinolines, acetyl-boswellic acids, and fluorinated lipophylic epipodophylloids are worth mentioning [128–133]. Selected structures are shown in Fig. 19.11. Why these compounds behave as dual poisons

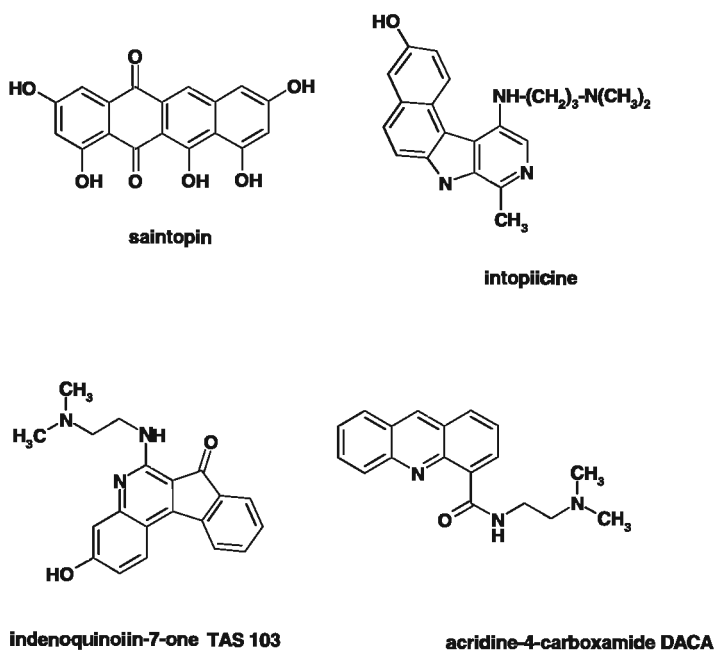


Fig. 19.11. Chemical structure of selected top1/2 mixed poisons.

is not immediately evident. Perhaps there are similarities in the structure of the top1–DNA and top2–DNA cleavage complexes that can be simultaneously recognized by the mixed poisons in terms of shape and of electronic properties.

Among the newest compounds, the bis-phenazine XR5944 demonstrated remarkable activity (in the nanomolar range) against human and murine tumor cells *in vitro* and *in vivo* [134]. Also, XR5944 was unaffected by atypical drug resistance and retained significant activity in cells overexpressing P-glycoprotein or multidrug resistance-associated protein. The bis-phenazine demonstrated antitumor efficacy in human carcinoma xenograft models generally unresponsive to chemotherapy.

The inhibitor disulfiram, a thiol-reacting drug, deserves a comment. This compound has been used in the therapy of alcohol abuse for more than 40 years. Now, it has been shown to inhibit both top1 and top2 [135]. Conceivably, the drug reacts with thiol (cysteine) groups of the enzyme, which impairs top catalytic action. Further studies are warranted to identify the protein residue(s) involved in the reaction with disulfiram and to assess the usefulness of this drug and its analogs as antineoplastic agents.

A final aspect, envisaging synergistic effects, originates from studies with combinations of DNA-binding and top2-directed agents [136]. Incubation of the DNA minor groove-binding agents Hoechst 33342 and Hoechst 33258 with nuclear extracts revealed drug binding to regions within the top2 alpha promoter and displacement of proteins binding to these elements. This resulted in increased expression of top2 alpha. Interestingly, cytotoxicity assays showed an additive effect

of incubation with Hoechst 33342 and the top2 alpha poison etoposide. This suggests that a strategy combining DNA-binding drugs which interfere with transcription factor activation of the top2 alpha promoter with top2 alpha poisons may be of therapeutic value.

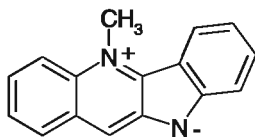
19.4

Conclusions

Although DNA topoisomerase poisons and inhibitors have been known for over two decades, a wealth of perspectives are still open to research and development. Pharmacogenomics and pharmacoproteomics are relatively young branches of modern medicinal chemistry. Indeed, thorough information about the structure and function of genes and gene products will give strong impulse to the discovery of new topoisomerase-related pharmacological targets. These could be specific DNA–protein or protein–protein complexes occurring at particular stages of cell-cycle progression, able to generate more specific and effective drug-mediated consequences. In particular, regulation of topoisomerase expression, management of the molecular events following DNA damage and combined approaches to generate drug synergies will surely yield valuable new drugs. For example, it has been recently reported that hammerhead ribozyme cleavage of telomerase mRNA sensitizes cancer cells to topoisomerase poisons, suggesting that telomerase inhibition therapy might be combined advantageously with topoisomerase-directed chemotherapy [137]. In addition, topoisomerase-directed agents were found to alter the basal transcriptional activity and DNA conformation for several genes, every gene displaying an individualized profile in response to drugs [138]. The expression changes elicited by top1 or top2 poisons are not equivalent and are also context dependent, differing between chromosomal or episomal promoters. Hence, these DNA-damaging agents may evoke a biological response determined also from transcriptional effects. This knowledge will be profitable to modulate the activity of selected genes in response to drug treatment, thus producing a better therapeutic profile.

Moreover, mechanistic studies will shed more light on the structural basis for different, and often concomitant, pathways of drug action. Considering the family of anthracyclines, nuclear pathways may include targeting not only of top2, but also of top1 and of transcriptional inhibition, whereas cytoplasmic targeting focuses on binding to protein kinase C with consequent modulation of activity [139].

Extended knowledge in the above biochemical and molecular biological areas will possibly lead to more appropriate handling of top-directed healing strategies. From the chemical point of view, the drug discovery process in the topoisomerase area will be helped by major recent advances in *in silico* methods and in combinatorial chemistry. Indeed, the power of computer-aided modeling has risen steadily, and a thorough and rapid investigation of macromolecular systems and of drugs docking to them is now feasible. In addition, dynamic studies yield fine details of the mechanisms of drug-target recognition, while theoretical pharmacokinetics



cryptolepine

Fig. 19.12. Chemical structure of cryptolepine.

approaches will allow initial screening of drug candidates. On the other hand, evolution of synthetic and naturally derived libraries will represent a powerful tool to exploit chemical diversity and identify new, yet unrecognized, structural motifs to be used for topoisomerase-directed drug development [140].

As a final remark, surprises never come to an end. The process of DNA intercalation, discovered in the early 1960s and typical of many clinically relevant top-targeted drugs as reported above, seemed to be completely dissected, with hundreds of drug–intercalator complex structures resolved by X-ray or NMR techniques. These intercalators invariably positioned themselves between alternating pyrimidine–purine sites. Now cryptolepine (Fig. 19.12), an alkaloid extract of *Cryptolepis sanguinolenta*, is reported to intercalate between non-alternating pyrimidines (cytosines) rather than between pyrimidine–purine residues of DNA [141]. Besides an intellectual interest for such an atypical intercalation between non-alternating DNA bases, the new mode of interaction might open the door to new strategies for designing drugs, including topoisomerase-directed agents, targeted at a wide range of genetic diseases, including cancer.

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20

Targeting DNA and Topoisomerase I with Indolocarbazole Antitumor Agents

Christian Bailly

20.1

Introduction

In the cell, DNA primarily exists in a supercoiled form and therefore unwinding of DNA is required prior to using it for transcription, replication, or recombination. These functions require the services of enzymes called topoisomerases, which cleave and religate one or two strands of DNA, and hence allow the DNA to unwind [1]. For more than 15 years, these enzymes have attracted considerable attention as targets for cancer therapeutics since many drugs inhibiting them, such as topotecan, adriamycin, and etoposide, are used extensively in the clinic to combat different forms of cancers, from colorectal cancer, to brain tumors and hematological malignancies.

There are three classes of topoisomerase enzymes in human cells [2]. Topoisomerase I introduces breaks in one strand of DNA and mediates DNA relaxation in a process that does not require any cofactor. In contrast, topoisomerase II cuts the two strands of the double helix and changes the topology of the DNA by catalyzing the passage of one double-stranded segment of DNA through a double-strand break produced in a second DNA segment. Topoisomerase III, which was identified more recently [3], cleaves single-strand DNA and binds covalently to the 5' end of the cleaved DNA [4]. There are, as yet, no specific inhibitors for topoisomerase III, whereas there are many for the two other enzymes. Both topoisomerases I and II have been shown to constitute critical cellular loci for a number of clinically important antitumor agents [5–7].

The plant alkaloid camptothecin (CPT) represents the archetype of the topoisomerase I poisons. The mechanism by which this natural product kills cancer cells involves the stabilization of an intermediate binary complex in which one DNA strand has undergone strand scission and is covalently attached to the enzyme. By binding to this complex and preventing religation, the topoisomerase poison precludes DNA replication and transcription, and thereby leads to the death of cells attempting to undergo these processes [8]. CPT shows little or no affinity for DNA or topoisomerase I alone but interacts strongly and specifically with the enzyme–DNA complex. It is worth remembering that for almost a quarter of a

century, CPT was an orphan drug in the sense that its primary molecular site of action was unknown. The discovery in 1985 that topoisomerase I was a specific molecular target for CPT has rekindled interest in the design of analogs. Once the target was identified and characterized, it took not less than 10 years to develop safe analogs such as the drugs topotecan (TPT, Hycamtin) and irinotecan (IRT, Camptosar) (Fig. 20.1), which are now in clinical use [9]. A variety of second- and third-generations CPT derivatives, such as lurtotecan, exatecan, rubitecan, and diflomotecan (Fig. 20.1), are currently in various stages of clinical development [10, 11].

Over the past 10 years, the number of topoisomerase I poisons has greatly expanded and now includes more than 60 members with very diverse structures and origins [12, 13]. However, only a very few have revealed significant antitumor ac-

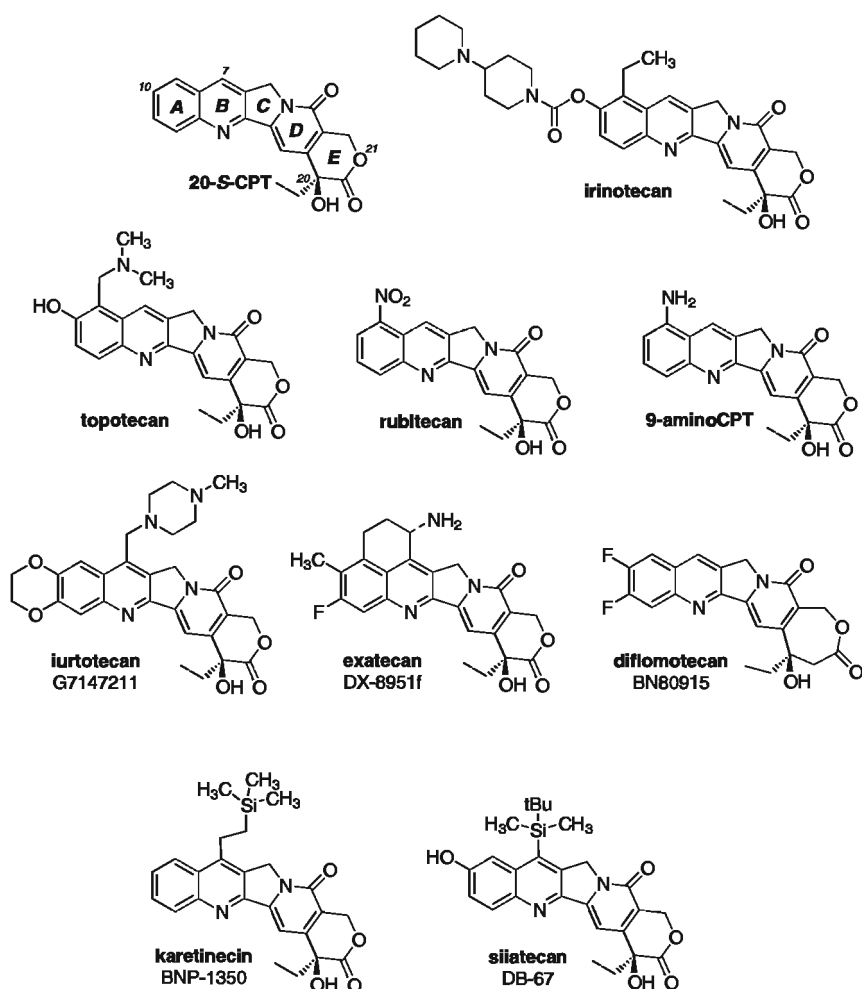


Fig. 20.1. Camptothecin (20-S-CPT) and various tumor-active analogues.

tivities *in vivo*. Apart from the CPTs, the only class of tumor-active topoisomerase I inhibitors is arguably that of the indolocarbazoles (IND) reviewed here.

20.2

Naturally Occurring Indolocarbazoles

IND is the generic name for a group of compounds possessing a planar 6-ring nucleus. The group includes several microbial metabolites, in particular K252a, AT2433-B1, and rebeccamycin. But the chief among the indolocarbazoles is the antibiotic staurosporine, a fascinating natural product, the discovery, chemical structure, and structure–activities of which have been presented elsewhere [14, 15].

The indolocarbazoles can be classified in two groups. The closed series refers to compounds for which the glycosyl residue is linked to the IND chromophore via the two indole nitrogens, and the open series refers to the compounds with one free indole NH group and the other linked to the carbohydrate. The following section describes how these naturally occurring IND were exploited for the development of novel anticancer agents targeting topoisomerase I and/or DNA.

20.2.1

Staurosporine and Analogs with a Pyranose Sugar Moiety

Staurosporine was first isolated in 1977 from *Streptomyces staurosporeus* [16] and has since been isolated from various actinomycete strains (e.g. *S. actuosus*) as well as from some marine organisms [17]. This IND alkaloid, possessing antifungal and hypotensive activities, was initially described as an inhibitor of protein kinase C (PKC) [18] by competing with the ATP binding in the catalytic domain of PKC. In fact, PKC defines a family of serine/threonine-specific protein kinases, which consists of at least 12 isoforms [19]. The conventional PKC isoenzymes, which are calcium-dependent and activated by diacylglycerol/phorbol ester (e.g. PKC α , β_1 , and β_{11}) are particularly sensitive to staurosporine but the drug also affects other protein kinases such as PKA, PKG, and protein tyrosine kinases [20, 21] as well as non-kinase proteins. For example, staurosporine potently stimulates the expression of the vasoprotective and anti-atherosclerotic enzyme NOS III and this activity seems to be unrelated to protein kinases inhibition [22].

Many synthetic derivatives of staurosporine have been designed [14] and a few of them display antitumor activities such as 7-hydroxystaurosporine (UCN-01) and 4'-N-benzoylstaurosporine (PKC412 or CGP41251) (Fig. 20.2). UCN-01 exhibits different cellular effects (cell cycle checkpoint abrogation, G₁ delay, induction of apoptosis) depending on the dose and cell type used but has no effect on topoisomerase I [23]. PKC412 is interesting because in addition to being a potent and specific PKC inhibitor, it presents the additional advantage of modulating the multidrug resistance phenotype in cancer cells [24, 25]. However, despite their low toxicity profile, both PKC412 and UCN-01 display high binding to human plasma proteins, a problem which may restrict their clinical development [26].

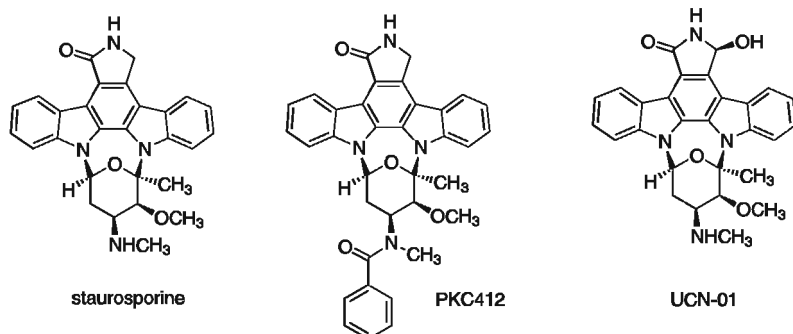


Fig. 20.2. Staurosporine and two synthetic derivatives currently undergoing clinically trials: *N*-benzoyl-staurosporine (PKC412) and 7-hydroxy-staurosporine (UCN-01).

Thus far, no poisoning activity against topoisomerase I has been reported with staurosporine, PKC412, or UCN-01. Topoisomerase I poisons generally induce cell cycle arrest at the G₂ phase and the G₂ checkpoint implicates the correct functioning of different kinases, in particular human checkpoint kinase 1 (Chk1). In inhibiting this enzyme, UCN-01 is capable of overriding the G₂ arrest induced by topotecan, therefore potentiating the cytotoxicity of this topoisomerase I inhibitor [27]. Similar effects have been recently reported with SB-218078 (Fig. 20.3), another IND derivative analogous to K252a [28]. UCN-01 sensitizes the cytotoxic action of drugs such as mitomycin C, cisplatin, 5-fluorouracil, and methotrexate

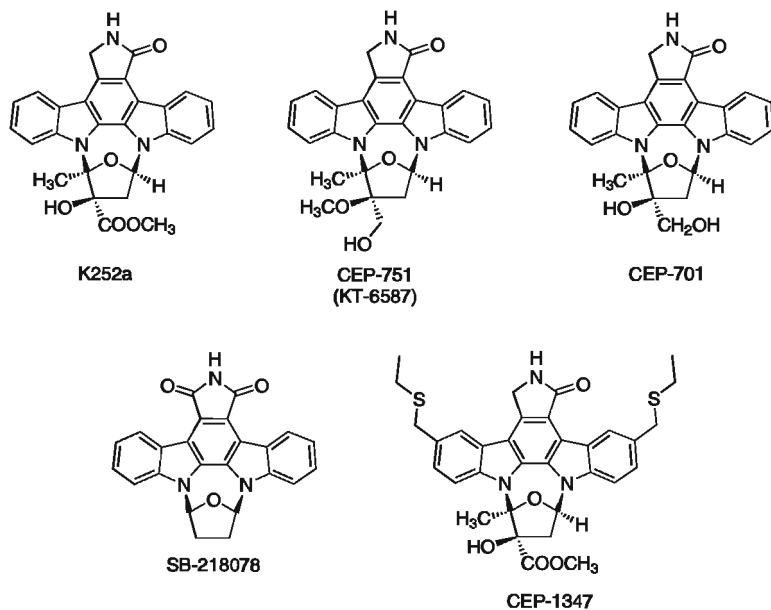


Fig. 20.3. K252a and analogs.

[23, 29] and phase II clinical trials associating UCN-01 with DNA-damaging agents and antimetabolites are currently ongoing.

20.2.2

K252a and Analogs with a Furanose Sugar Moiety

The antibiotic K252a, produced by *Nocardioopsis* species [30, 31] is also a potent protein kinase inhibitor acting through competitive inhibition of ATP-binding domains. It modulates the activity of many different kinases including PKC but it is particularly interesting for its inhibitory action on the tyrosine kinase of the neurotrophin-specific Trk receptor [32–35]. This activity has been exploited to develop Trk-specific inhibitors, leading to the discovery of potent compounds such as CEP-701 (KT-5555) and CEP-751 (KT-6587), which preferentially inhibit autophosphorylation and signaling of Trk family receptors at nanomolar concentrations *in vitro* and display potent antitumor activity *in vivo* [36–39]. Combined with androgen ablation, these IND derivatives may be particularly useful for the treatment of prostate cancers [40–42] and have also revealed significant activity against neuroblastoma and medulloblastoma [43, 44]. These tumors of the neuroectoderm, which are among the most common tumors in childhood, generally express one or more of the tyrosine kinase receptors TrkA, TrkB, and/or TrkC targeted by CEP-751. However, in addition to their anti-Trk activity, these analogs of K252a also interfere with other proteins such as the platelet-derived growth factor, epidermal growth factor receptor, and probably other as yet unidentified tyrosine, serine, or threonine kinases [37].

CEP-751 and CEP-701 are candidates for cancer treatment and a phase I clinical trial for solid tumors in adults has been reported with CEP-701 [45]. But further modifications of the K252 structure can lead to compounds with a totally different spectrum of activity. For example, the K252a analog CEP-1347 displays a broad neuroprotective profile and is currently in clinical trials for Parkinson's disease. This 3,9-bis-ethyl-thiomethyl derivative (Fig. 20.3) has no effect on PKC and TrkA but is a very potent inhibitor of the *c-jun* N-terminal kinases (JNK) signaling cascade acting via an inhibition of the mixed lineage kinases (MLK), a family of serine/threonine kinases [46–48]. Targeting MLK with K252a-derived IND may be an effective strategy for blocking neurodegeneration associated with Parkinson's disease and very recently, various analogs of CEP-1347 possessing different alkylthiomethyl groups at positions 3,9 have been described [49].

Unlike staurosporine and rebeccamycin, K252a exhibits taxol-like functional activity. K252a and K252b both support the growth of taxol-dependent Tax 2-4 cells and synergize with taxol (paclitaxel), suggesting that they could specifically inhibit kinases that contribute to the destabilization of microtubules [50].

K252a shows no effect on DNA topoisomerases and has no significant interaction with DNA. However, in the course of a screening program Yamashita and co-workers found two semisynthetic derivatives named KT6006 and KT6528 (Fig. 20.4) which can stimulate DNA cleavage by topoisomerase I [51]. KT6006 is a 3,9-bis-hydroxy derivative of K252a and, as will be discussed below, it is now well

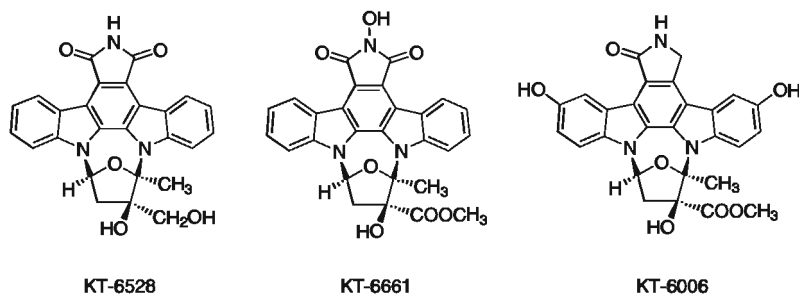


Fig. 20.4. Topoisomerase I inhibitors derived from K252a.

established that the presence of OH groups on the IND framework markedly changes the capacity of the drug to interact with DNA and topoisomerase I. KT6528 bears a CH_2OH group on the furanose ring instead of a COOCH_3 group common to K252a and KT6006 as well as the analog KT6661. In addition, the drugs differ by their functionality of the upper ring. K252a and KT6006 have an amide function as for staurosporine whereas KT6528 bears an imide, as with rebeccamycin and the bis-glycosyl antibiotic AT2433. DNA relaxation experiments indicated that KT6528 strongly unwinds supercoiled DNA with a potential comparable to that of drugs like adriamycin (a classical anthracycline intercalator) whereas its analog KT6006 showed little effect in this type of relaxation assay. These observations lead to the conclusion that KT6528 bearing the *N*-hydroxy maleimide ring was a strong DNA intercalator in contrast to KT6006 considered as a weak intercalator. These two compounds exhibit different binding affinities for DNA but are equally potent at stabilizing topoisomerase I–DNA covalent complexes. Thus this pioneer work revealed that there is no direct relationship between the strength of DNA interaction and the capacity of these IND derivatives to inhibit topoisomerase I [51].

This observation can now be considered as a general rule within the IND series; DNA binding and poisoning of topoisomerase I are clearly two distinct aspects, not necessarily correlated. Interestingly, the two topoisomerase I poisons KT6006 and KT6528 showed antitumor activity in the murine P388 leukemia model *in vivo*, whereas K252a and the compound KT6661 (Fig. 20.4), both inactive against topoisomerase I showed no significant antitumor activity *in vivo*. Therefore, the possibility that the anticancer activity was mediated by topoisomerase I was raised and this observation has obviously greatly encouraged the search for tumor-active IND derivatives targeting topoisomerase I. But thus far, in the closed series no other potent topoisomerase I has been reported.

20.2.3

Rebeccamycin

Rebeccamycin is another glycosylated IND which has profoundly inspired the development of antitumor agents, especially those targeting topoisomerase I, although this natural product is relatively weakly active against this enzyme

[52]. Rebeccamycin was originally isolated from the actinomycete strain C-38,383 (ATCC 39243) which is considered to be a member of the species *Saccharotrix aerocolonigenes*, previously known as *Nocardia aerocolonigenes* [53]. The structure of the drug was solved by NMR and X-ray analysis [54] and the absolute configuration was determined by a total synthesis [55]. Unlike the aforementioned IND such as staurosporine and K252a, its glycosyl moiety is linked to only one of the two indole nitrogens and this is the central point for its mechanism of action. The *N*-glycoside is a β -D-4-methoxyglucose residue which is orientated at right angles to the plane of the planar aromatic ring. In addition, the symmetric heterocyclic ring system of rebeccamycin is substituted with chlorine atoms at positions 1,11. These chlorines originate from sodium chloride present in the fermentation broth because the replacement of NaCl by NaBr produced the 1,11-deschloro-1,11-dibromo-rebeccamycin [56]. The drug has no effect on PKC and DNA topoisomerase II. Its antitumor activity seems to be rather correlated to its inhibitory potency against topoisomerase I. Rebeccamycin is highly toxic to tumor cells and its anticancer activity can be further enhanced by introducing fluoro substituents on the IND moiety. Recently, three new fluoroindolocarbazoles (compounds A–C in Fig. 20.5), obtained biosynthetically by feeding fluorotryptophan to culture of *S. aerocolonigenes* ATCC 39243, were found to be more potent than rebeccamycin against P388 leukemia in mice [57, 58].

Rebeccamycin is essentially insoluble in water ($<1 \mu\text{g mL}^{-1}$) and, therefore, soon after its discovery, chemists from Bristol-Myers Squibb initiated the design of analogs with an improved water solubility profile. A diethylaminoalkyl side chain was introduced on the N6 imide and the water-soluble analog BMS 181176 (NSC655649) was shown to possess antitumor activity similar to that of rebeccamycin [59]. This first designed rebeccamycin analog was shown to bind to duplex DNA and to unwind supercoiled plasmid DNA, providing evidence for an intercalative mode of binding [60]. Later, additional spectroscopic measurements fully confirmed that in general (but there are a few notable exceptions) rebeccamycin and its analogs behave as typical DNA intercalating agents [61].

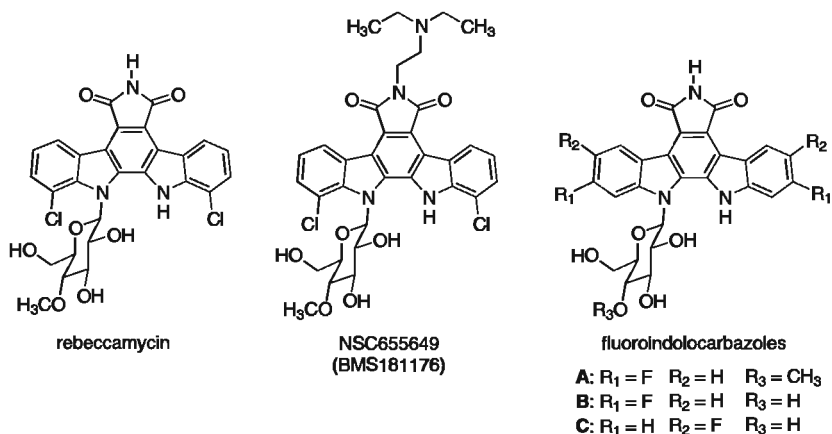


Fig. 20.5. Rebeccamycin, NSC655649 and fluoro analogs.

NSC655649 exhibits a broad antitumor activity *in vitro* against pediatric solid tumors [62]. This compound has been described as a catalytic inhibitor of topoisomerase II (without inducing enzyme-mediated DNA double-strand breaks) [58] and it has been evaluated in phase I clinical trials in both adult and pediatric patients [63, 64]. A *N*-de-ethylated metabolite, presumably produced by the cytochrome P450 3A4 isoenzyme, has been isolated in patients treated with NSC655649 [65]. This compound is currently undergoing phase II clinical trials for the treatment of hepatobiliary and gall bladder cancers [58].

20.2.4

AT2433

The antitumor antibiotic AT2433-B1 (Fig. 20.6) was originally isolated from culture broth of *Actinomadura melliaura* [66, 67]. It contains a unique disaccharide consisting of a methoxyglucose and an amino sugar subunit, 2,4-dideoxy-4-methylamino-L-xylose [68]. This bis-glycosyl IND derivative does not inhibit topoisomerase I but it binds strongly to DNA and interacts preferentially with GC-rich sequences. DNase I footprinting and surface plasmon resonance studies, as illustrated in Fig. 20.6, revealed that the sequence 5'-AACGCCAG provides a privileged binding site for AT2433-B1, whereas interestingly the related indolo[2,3-*a*]carbazole diglycoside iso-AT2433-B1 interacts 15 times more weakly with this sequence than the parent compound [69]. Iso-AT2433-B1 is a diastereoisomer of the natural aminodisaccharide and corresponds to the incorrect structure originally proposed for AT2433-B1. The absolute stereochemistry of the amino sugar is 3*S*,4*S* in AT2433-B1 and 3*R*,4*R* in iso-AT2433-B1 [70]. Therefore, a subtle modification of the amino sugar moiety has a profound impact on the drug-DNA recognition process. The amino sugar subunit (2,4-dideoxy-4-methylamino-L-xylose) of AT2433-B1, which is reminiscent of minor groove binding oligosaccharide portions found in the enediyne antibiotics calicheamicin γ_1 and esperamicin A₁ [71, 72], represents the primary structural determinant for binding to DNA and the basic element for GC-rich sequence selectivity [69].

AT2433-A1 is the 11-chloro analog of AT2433-B1. The presence of a chlorine atom on the IND chromophore, as in rebeccamycin, is detrimental to DNA binding but nevertheless the chlorinated compound AT2433-A1 is more cytotoxic to P388 murine leukemia cells than the AT2433-B1. The presence of a chlorine atom on the IND chromophore apparently alters the distribution profile of the drug in the cell, i.e. the amount of drug molecules found in the cytoplasm versus the nucleus, as judged from fluorescence microscopy studies (unpublished data).

20.3

Synthetic Indolocarbazole Derivatives Targeting DNA and Topoisomerase I

Over the past 10 years a large diversity of open-form IND derivatives have been synthesized. Two groups can be distinguished: analogs derived from rebeccamycin and those issued from the aglycone antibiotic BE13793C.

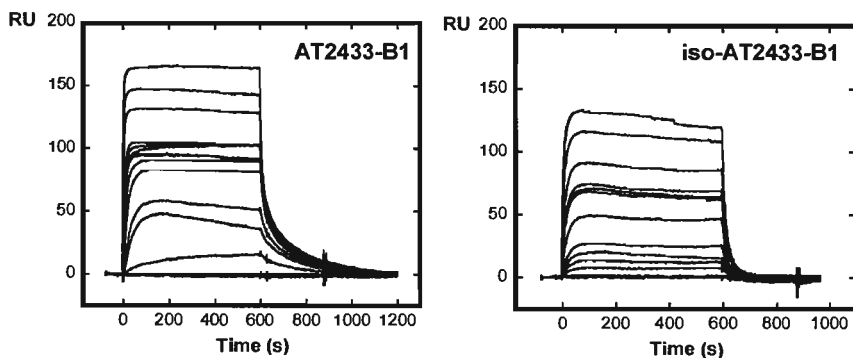
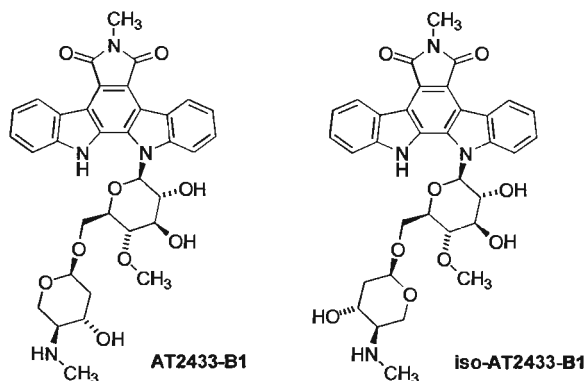


Fig. 20.6. Surface plasmon resonance (SPR) sensorgrams for binding of AT2433-B1 and iso-AT2433-B1 to the sequence 5'-AACGCCAG. The unbound ligand concentrations in the flow solution range from 10 nM in the lowest curve to 4 μ M in the top curve [69].

20.3.1

Influence of Chloro and Bromo Substituents on the IND Chromophore

As mentioned above, rebeccamycin is characterized by the presence of chlorine atoms at positions 1,11 on the IND chromophore. Therefore, one of the first studies in this area was to evaluate the influence of these chloro substituents. Dechlorinated rebeccamycin, obtained upon hydrogenolysis of the natural product with Raney nickel in aqueous sodium hydroxide [73], remains inactive against protein kinases A and C, and its inhibitory activity against topoisomerase I is only slightly superior to that of rebeccamycin. The removal of the chlorine atoms significantly decreases the cytotoxicity of the drug. Dechlorinated rebeccamycin was found to be 10 and 36 times less toxic to murine P388 leukemia cells and B16 melanoma cells, respectively, compared with rebeccamycin [51]. The chloro groups of rebeccamycin reinforce the chemical stability of the F-ring imide heterocycle

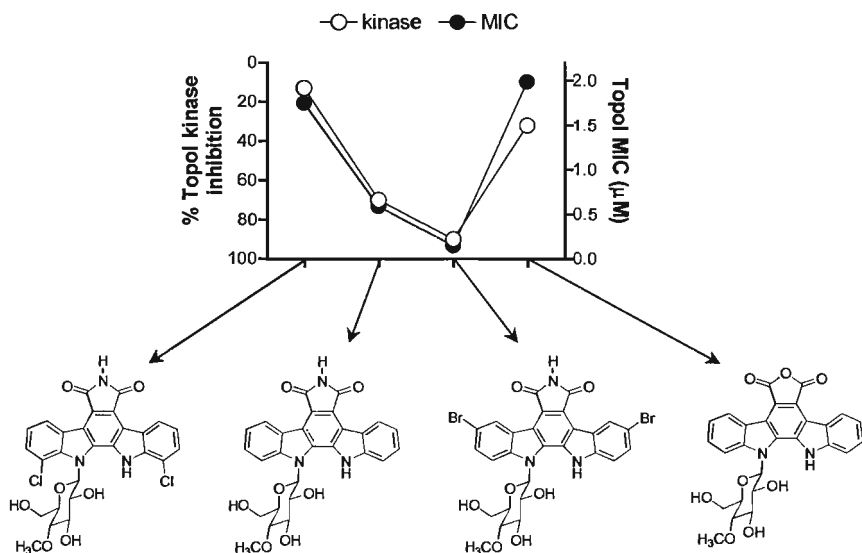


Fig. 20.7. Correlation between inhibition of the kinase activity and minimum inhibitory concentration (MIC, μM) required to inhibit DNA cleavage by topoisomerase I by rebeccamycin and three analogs [75].

[74] and also play a role in the cellular distribution of the compounds, but they are detrimental to the interaction with DNA. Dechlorinated rebeccamycin has significant interaction with DNA and behaves as a typical intercalating agent, whereas rebeccamycin shows little affinity for DNA. As in the AT2433 series mentioned above, the presence of the chloro groups prevents the drug from inserting its IND chromophore between two consecutive DNA base pairs [61].

Brominated rebeccamycin analogs have been synthesized and, interestingly, a 3,9-dibromo derivative was found to potently inhibit both the DNA relaxation and the kinase activities of topoisomerase I [75]. Tazi and collaborators have established that topoisomerase I can phosphorylate splicing factors of the SR protein family, such as SF2/ASF (see Section 20.4.6) [76]. The dibrominated imide compound represented in Fig. 20.7 strongly prevents the phosphorylation of SF2/ASF by topoisomerase I even in the absence of DNA, whereas rebeccamycin itself shows little effect. A marked inhibition of protein phosphorylation was also observed with dechlorinated rebeccamycin but not with its *N*-methyl imide derivative or the anhydride derivative. It is worth noting at this point that inhibition of the kinase activity of topoisomerase I has also been observed with camptothecin but only in the presence of DNA [76]. The kinase activity of topoisomerase I requires ATP and it is conceivable that the IND inhibitors bind directly to the ATP-binding site of the enzyme. However, the kinase site of topoisomerase I must be different from the ATP-binding site of protein kinase C which is not inhibited by these compounds [75]. The capacity of the four compounds shown in Fig. 20.7 to inhibit the kinase

activity of topoisomerase I is directly comparable to their capacity to inhibit the DNA cleavage activity of the enzyme. The dibrominated compound is a potent inhibitor of both the kinase activity and the DNA cleavage activity of topoisomerase I. The search for IND compounds capable of interfering selectively with the kinase activity of the enzyme is actively pursued.

20.3.2

Modification of the Imide Heterocycle

A series of *N*-methyl imide, *N*-methyl amide, and anhydride derivatives have been synthesized to evaluate the role of the imide nitrogen of rebeccamycin in the inhibition of topoisomerase I and cytotoxicity [74]. A good correlation between topoisomerase I inhibition and cytotoxicity was observed with the imide and anhydride compounds but not with the amide derivatives (Fig. 20.8). Incorporation of a methyl group on the indole nitrogen reduces considerably the capacity of the drug to inhibit topoisomerase I as well as the cytotoxicity (compare activities of compounds 1, 3, and 5 to compounds 2, 4, and 6 in Fig. 20.8). In contrast, no correlation was observed with the amide compounds 8.8' and 9.9', which are potent

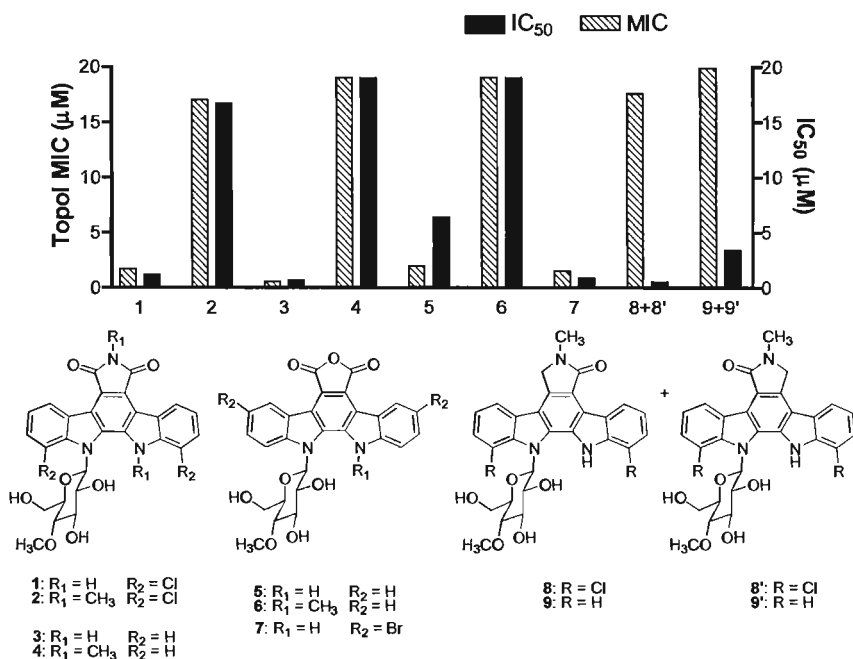


Fig. 20.8. Correlation between cytotoxicity against P388 leukemia cells and minimum inhibitory concentration (MIC, μM) required to inhibit DNA cleavage by topoisomerase I for a series of imide, anhydride and amide

derivatives of rebeccamycin. IC₅₀ values correspond to the drug concentration that inhibits murine P388 cell growth by 50% after incubation in liquid medium for 96 hours [74].

cytotoxic agents but they inhibit neither topoisomerase I nor the PKC enzymes. The anhydride derivative 5 is significantly less cytotoxic (but still active against topoisomerase I) compared with its imide derivative 3. This is an important observation because the anhydride is one of the main metabolic forms of these glycosylated rebeccamycin derivatives, as discussed below with the antitumor drug NB-506 (see Section 20.4.7).

20.3.3

Halogenoacetyl Derivatives

Some of the aforementioned compounds, in particular the imide derivative 3 and the two amide derivatives 8,8' and 9,9' were found to potently inhibit the reverse transcriptase activity associated with virus particles released from CEM-SS cells infected with the HIV-1 virus, but the molecular basis of this antiviral activity is not yet known [74]. Significant anti-HIV-1 effects were also observed with a series of rebeccamycin derivatives bearing a halogenoacetyl substituent either on the sugar moiety or on the nitrogen imide. Compounds such as 4 and 6 shown in Fig. 20.9 are weak inhibitors of topoisomerase I and weakly active against HIV-1. In contrast, the potent topoisomerase I poisons such as 2, 5, and 8 strongly inhibit the HIV-1 reverse transcriptase activity. On the basis of these observations, it has been postulated that topoisomerase I contributes, at least partially, to the anti-HIV-1 properties of these compounds. The literature data showing that topoisomerase I activates HIV-1 reverse transcriptase activity [77] and that this activation can be inhibited by a CPT derivative like topotecan [78] also support this hypothesis. The discovery that such glycosylated IND derivatives inhibit HIV-1 proliferation provides novel opportunities to design anti-AIDS drugs.

20.3.4

Glucose and Galactose Derivatives: Stereospecific DNA Recognition

The carbohydrate moiety of rebeccamycin derivatives is absolutely essential to both DNA recognition and topoisomerase I inhibition. Attempts have been made to replace the methoxy-glucose residue of rebeccamycin with another sugar such as a glucose, a galactose, or a fucose. Little differences were observed between the glucose and galactose derivatives in terms of DNA recognition and topoisomerase I inhibition. The orientation of the 4'-hydroxyl group on the sugar residue can be inverted without altering the drug-DNA recognition process. In contrast, the removal of the 6'-OH group (which is supposed to engage a hydrogen bond with the indole NH) reduces considerably the strength of the interaction between the drug and DNA. The fucose analog of rebeccamycin (lacking the 6'-OH group) was found to be less active than the glucose analog [79].

In the naturally occurring IND such as rebeccamycin and AT2433, the carbohydrate moiety is linked to the chromophore via an equatorial β -N-glycosidic bond which orients the sugar in a suitable position for DNA recognition. The inversion of the configuration at the C-1' of the carbohydrate residue has a major impact on

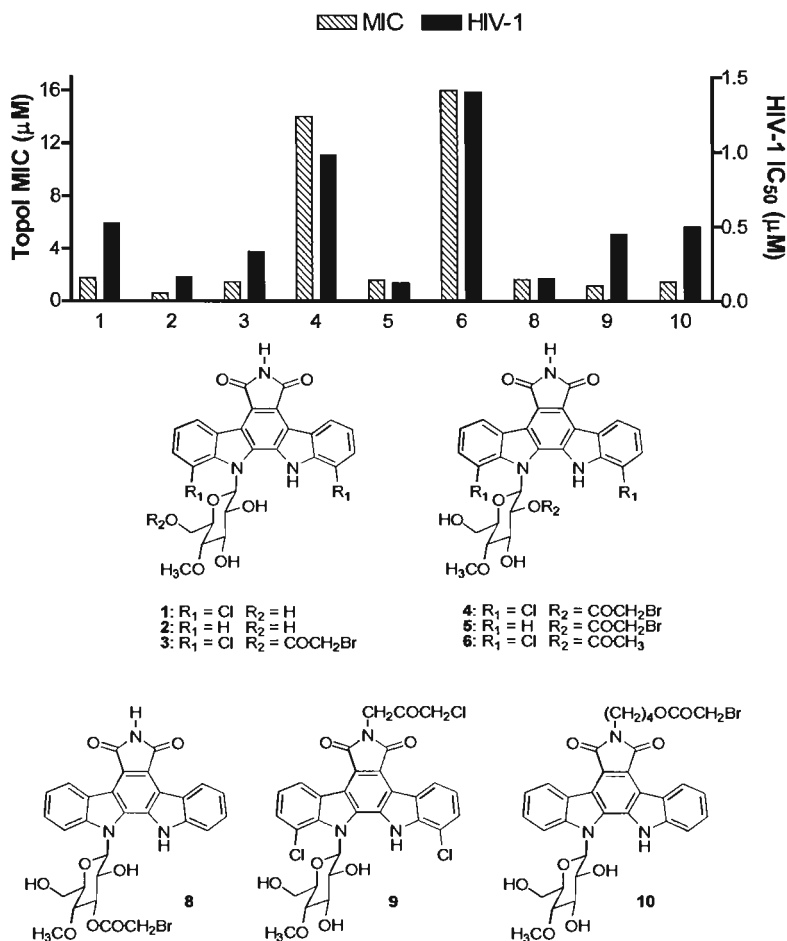


Fig. 20.9. Correlation between anti-HIV-1 activity and minimum inhibitory concentration (MIC, μM) required to inhibit DNA cleavage by topoisomerase I for a series of imide, anhydride and amide derivatives of

rebeccamycin. The anti-HIV-1 activity was measured by quantification of the reverse transcriptase activity associated with virus particles released from HIV-1 Lai-infected CEM-SS cells [74].

the capacity of the drugs to bind to the target. The comparison of the DNA-binding properties of the α - and β -glucose, galactose, and fucose derivatives of rebeccamycin showed that only the compounds having the sugar in the β -conformation inhibit the relaxation of supercoiled DNA by topoisomerase I and intercalate in the DNA double helix, whereas the α -isomers have no effect on topoisomerase I. The β -N-glycosidic linkage is a key element for topoisomerase I inhibition and recognition of GC-rich sequences in DNA. But, the cytotoxicity measurements showed little differences between the α and β isomers, suggesting therefore that in this series, DNA binding and topoisomerase I poisoning contribute only partially to the cytotoxic activity [79].

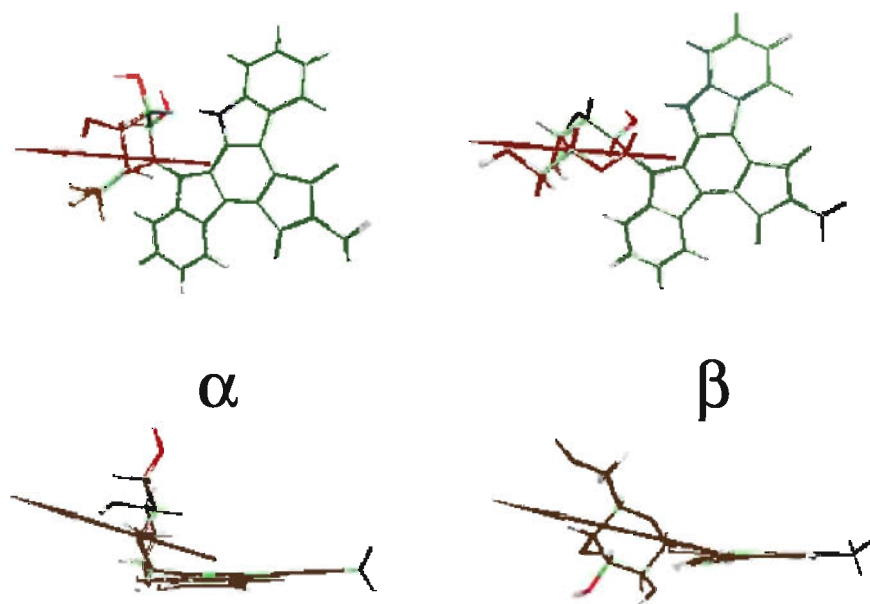


Fig. 20.10. Top and side views of energy minimized models of the α and β anomers of the galactose analog of rebeccamycin. In each case, the dipole moment vector is indicated ($\mu = 6.711$ D and 6.745 D for the α and β anomers, respectively).

The study of the DNA-binding properties of the two rebeccamycin analogs containing a galactose residue attached to the IND chromophore via an α (axial) or a β (equatorial) glycosidic linkage has revealed that the α isomer has lost both the capacity to intercalate into DNA and the property to induce topoisomerase I-mediated cleavage of DNA. In the α isomer, the orientation of the galactose residue at a right angle with respect to the planar chromophore provides steric constraints that limit the insertion of the IND between the base pair. In the β isomer, the sugar adopts a more extended configuration (Fig. 20.10) allowing the formation of a very stable and stereochemically feasible intercalation complex with the oligonucleotides $d(\text{CGCGCG})_2$ and $d(\text{CGTGCG})\text{-(CGCACG)}$. This work has revealed that the interaction of rebeccamycin analogs with DNA is controlled to a large extent by the sugar residue and that the isomery of the glycosidic linkage is essential [80]. Often the binding of glycosylated drugs (e.g. anthracyclines, aminoglycosides, pluramycins, bleomycins) to nucleic acids depends essentially on the structure of their saccharide unit.

20.3.5

From Rebeccamycin to Staurosporine-type Analogs

The 2'-OH group on the sugar residue of rebeccamycin can be selectively tosylated in the presence of *p*-toluenesulfonylchloride and potassium carbonate. The treat-

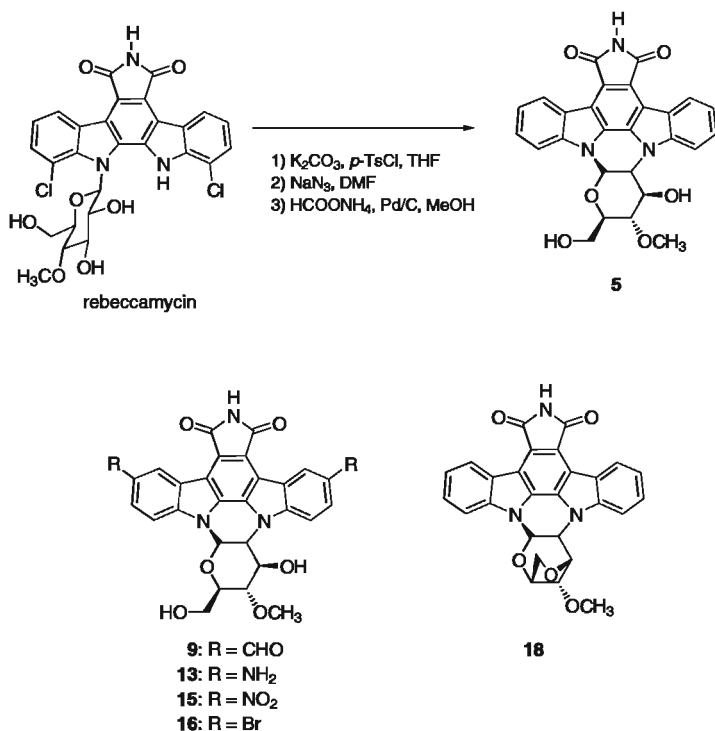


Fig. 20.11. Synthesis of the closed-type compound **5** from rebeccamycin and structures of other cyclic molecules.

ment of the resulting *O*-tosyl ester with sodium azide gives a cyclic product whereby the sugar residue is linked to the IND via the two indole nitrogens, together with a 3'-azido side product. Dechlorination of the cyclic product with ammonium formate and palladium on activated carbon finally provided the desired staurosporine-like compound (**5** in Fig. 20.11) with a rigid tetrahydropyrazine ring [81]. This hemisynthetic product is totally inactive against PKC but was found to bind to DNA and to potently inhibit topoisomerase I. It is considerably more toxic to P388 leukemia cells (IC_{50} 0.7 μM) than to the equivalent camptothecin-resistant cells expressing a mutated topoisomerase I (IC_{50} > 20 μM), suggesting therefore a direct contribution of the enzyme in its mechanism of cytotoxicity. This compound has provided a useful model for the preparation of other related cyclic products, such as the 3,9-disubstituted analogs shown in Fig. 20.11 recently synthesized [82]. For example, the dibromo derivative **16** showed a potent cytotoxic potential against different types of tumor cell lines (K562 and L1210 leukemia, A549 and HT29 carcinoma) whereas the diformyl analog **9** displayed a selective toxicity toward the L1210 leukemia cell line. The diamino derivative **15** was found to be inactive whereas the dinitro analog **13** was strongly cytotoxic to the two leukemia cell lines but had no effect on the growth of carcinoma cells. In general,

these compounds induced a significant arrest of the L1210 cells in the G₂/M phase, with the notable exception of the anhydro compound 18 producing a marked arrest in the G₁ phase of the cell cycle. The molecular targets of these compounds are not precisely known, through topoisomerase I and certain protein kinases are likely involved in the cytotoxic action [82].

20.3.6

Amino Sugar Derivatives

It is now well established that DNA intercalation is not necessary for inhibition of topoisomerase I by rebeccamycin [83], but initially a number of rebeccamycins were designed with the aim of increasing DNA affinity. The observation that chlorination of the IND nucleus leads to an important decrease of anti-topoisomerase I activity supported the development of dechlorinated analogs [84]. One of the most convenient strategies for promoting DNA interactions consists of incorporating a positively charged group either directly on the IND chromophore or on the sugar residue. The replacement of the 4'-methoxy-glucose residue of the rebeccamycin analog R-2 (Fig. 20.12) with a 2'-amino-4'-methoxy-glucose sugar reinforces the interaction of the drug with DNA by a factor of about 3. However, the amino group introduced on the glycoside residue of compound 85 (Fig. 20.12) did not appear to participate in any specific molecular contacts with DNA. The energetic contribution of the amino group of the rebeccamycin analog was found to be weaker than that of the sugar amino group of the anthracycline daunomycin, possibly because the indolocarbazole derivative is only partially charged at neutral pH. Nevertheless, the amino sugar does not prevent the drug from inhibiting topoisomerase I and maintains the cytotoxic potential [80].

20.3.7

Methylation of the Indole Nitrogen

By definition for the open series, one of the indole nitrogens is linked to the carbohydrate and the second indole nitrogen is not substituted. This NH free group is absolutely essential for topoisomerase I inhibition. Substitution of this indole nitrogen with a methyl group is not compatible with topoisomerase I inhi-

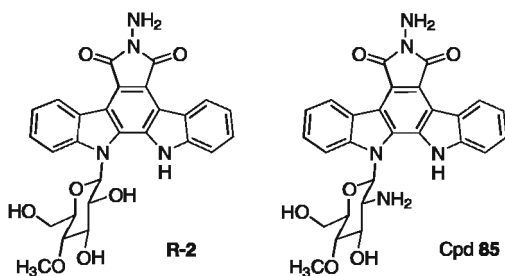


Fig. 20.12. R-2 and compound 85.

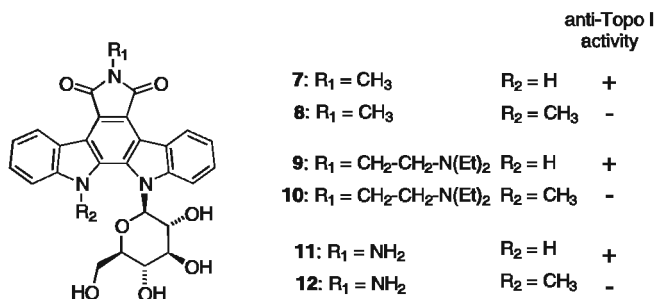


Fig. 20.13. Structures of NH and *N*-methyl IND derivatives.
Only the NH derivatives were found to inhibit topoisomerase I.

bition. For example, the rebeccamycin derivatives **1**, **3**, and **5** shown in Fig. 20.8 potentially inhibit topoisomerase I with MIC (minimal inhibitory concentration) values of 1.7, 0.6, and 2 μ M, respectively, whereas concentrations higher than 16 μ M were required to observe an effect with the corresponding *N*-methyl analogs **2**, **4**, and **6** [74]. In addition, these *N*-Me compounds were found to be inactive against P388 leukemia and B16 melanoma cells whereas the NH compounds exhibit potent cytotoxic activities. In this case, a relationship between topoisomerase I inhibition and cytotoxicity was observed. Topoisomerase I may also contribute, at least partially, to the antiviral activity (especially the anti-HIV-1 effect) of these compounds [74].

A recent study with another series of NH (**7**, **9**, **11**) versus *N*-methyl (**8**, **10**, **12**) rebeccamycin analogs (Fig. 20.13) fully confirmed that a free indole NH group is required to enable topoisomerase I inhibition. Here again, compounds **7**, **9**, **11** were found to stimulate DNA cleavage by the enzyme whereas no effect was observed with the corresponding methylated analogs **8**, **10**, **12** (C. Bailly and D. L. Van Vranken, unpublished data). The formation of a hydrogen bond between the indole NH and one of the oxygens of the adjacent β -glycoside is expected to maintain the sugar in an optimal conformation for enzyme inhibition. The existence of a hydrogen bond between the indole NH and the 6'-OH group on the sugar was initially postulated, essentially because a fucose analog of rebeccamycin specifically lacking this 6'-OH group was found to be considerably less active than the glucose and galactose analogs [79]. But latter, NMR studies by Van Vranken and collaborators revealed that in fact the H-bond implicates the pyranose oxygen [85]. Using IND glycosides lacking the imide F-ring (as in the ttipanazoles), the authors clearly showed that the compounds are balanced between two conformations: a closed conformation containing a cyclic hydrogen bond and an open conformation which is favored by intermolecular hydrogen bonding (Fig. 20.14). For these compounds as well as for rebeccamycin itself, the closed conformation is clearly the dominant form, which can be observed both in the NMR and X-ray structures [85].

It is interesting to note also that in this study, the authors obtained NMR evidence for the formation of a bifurcated hydrogen bond implicating the 6'-OH

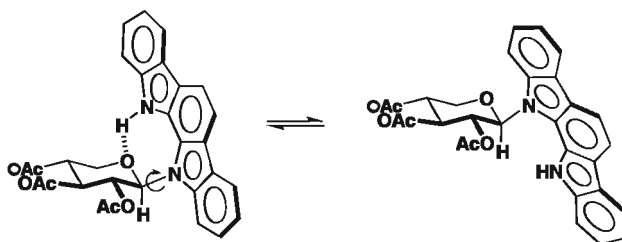


Fig. 20.14. Representation of the open and H-bond closed conformations adopted by IND glycosides [85].

group in the case of the non-chlorhydrated compounds, as depicted in Fig. 20.12. But the fact that compounds like rebeccamycin and AT2433-B1 cannot adopt this bifurcated conformation for steric reasons suggests that a bifurcated arrangement is necessary neither for topoisomerase I inhibition, nor for the cytotoxic activity.

The different IND glycoside natural products, such as rebeccamycin and AT2433-A1, are substituted in such a way as to strengthen pre-organization in the closed conformation. Therefore, the classification mentioned above of the IND compounds into the open and closed series is *a priori* perhaps not so judicious, as in fact they can all adopt a closed conformation, covalently closed for the staurosporine and K252 derivatives and H-bond closed for the rebeccamycin and AT2433 derivatives. A classification between rigid and flexible IND might be better, but in fact the open conformation must be considered because it is entirely plausible that when a rebeccamycin-type drug stabilizes the DNA–topoisomerase I covalent complex, the indole NH does not interact with the glycoside residue of the drug but engages a H-bond with the macromolecule, possibly the deoxyribose of DNA.

20.3.8

Indolo[2,3-*c*]carbazole

All naturally occurring IND derivatives, such as rebeccamycin and AT2433-B1, contain an indolo[2,3-*a*]carbazole nucleus with the five rings in line along the same axis. An attempt to modify the geometry of the IND skeleton was made by synthesizing the curved indolo[2,3-*c*]carbazole system [86]. Although the resulting glucose derivative 8 (Fig. 20.15) retained the capacity to intercalate into DNA, this compound showed no effect on topoisomerase I and was essentially non-toxic to murine L1210 leukemia cells ($IC_{50} = 10.4 \mu M$ compared with $0.11 \mu M$ for dechlorinated rebeccamycin). The bis-glucosyl analog 7 (Fig. 20.15) reminiscent of the 11-O-glucuronide biliary metabolite of the tumor-active compound NB-506 (see Section 20.4) was also inactive against topoisomerase I and leukemia cells. The replacement of the linear indolo[2,3-*a*]carbazole framework with a crescent-shaped indolo[2,3-*c*]carbazole system *a priori* does not appear to be a profitable route to the design of tumor-active IND compounds.

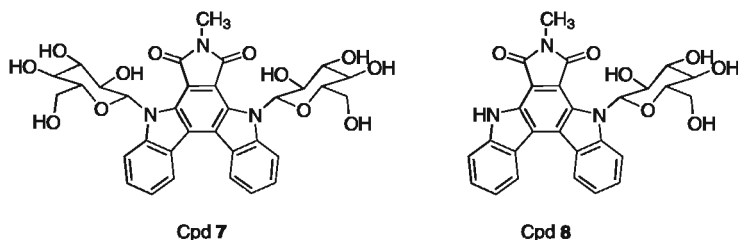


Fig. 20.15. Structures of the mono and bis-glucosyl indolo[2,3-c]carbazole derivatives.

20.3.9

Rebeccamycin Dimers and Conjugates

Various other strategies have been tested to elaborate IND derivatives with an improved pharmacology profile. A recently published approach consisted of replacing the neutral sugar residue of rebeccamycin with a positively charged amino acid or a short peptide designed to increase the water solubility of the compound and to promote its interaction with DNA. Along this idea, IND derivatives bearing a lysine residue or the metal-chelating tripeptide Gly-His-Lys (GHK) linked to one of the indole nitrogens via a propylamino chain were prepared. Rebeccamycin analogs possessing a lysine substituent on the carbohydrate unit at 2' or 3' positions were also synthesized [87]. In some cases the designed compounds were effectively bound to DNA more tightly than the parent compounds but this is at the expense of sequence recognition and topoisomerase I inhibition. These compounds are poorly cytotoxic but a derivative bearing a lysine at the 2' position exhibits strong antimicrobial activities against Gram-positive bacteria and the fungus *C. albicans*, providing an opportunity for the development of antimicrobial agents [87].

In another approach recently investigated the rebeccamycin unit was duplicated in order to obtain compounds that could potentially bis-intercalate into DNA. This strategy has been very fruitful in the anthracycline series with the design of the bis-daunomycin analogs WP631 and WP652, which have exceptional DNA-binding and anticancer activities [88]. Unfortunately, the dimeric compounds A and B shown in Fig. 20.16, obtained by joining two molecules of dechlorinated rebeccamycin via the imide nitrogen with a flexible aminoalkyl linker or a rigid xylylene linker, were not very cytotoxic [89].

Rebeccamycin derivatives have also been used for a gene-targeting strategy aimed at directing the cleavage of DNA by topoisomerase I to specific sequences. The linkage of an IND derivative to a triple helix-forming oligonucleotide reinforces the stability of the triplex and the conjugates were able to recruit topoisomerase I for DNA cleavage at two juxtaposed sites proximal to the triple helix binding site [90, 91]. This topoisomerase I-based gene-targeting strategy, essentially developed with camptothecin derivatives as potent topoisomerase I poisons [92], now appears as a promising approach to control gene expression.

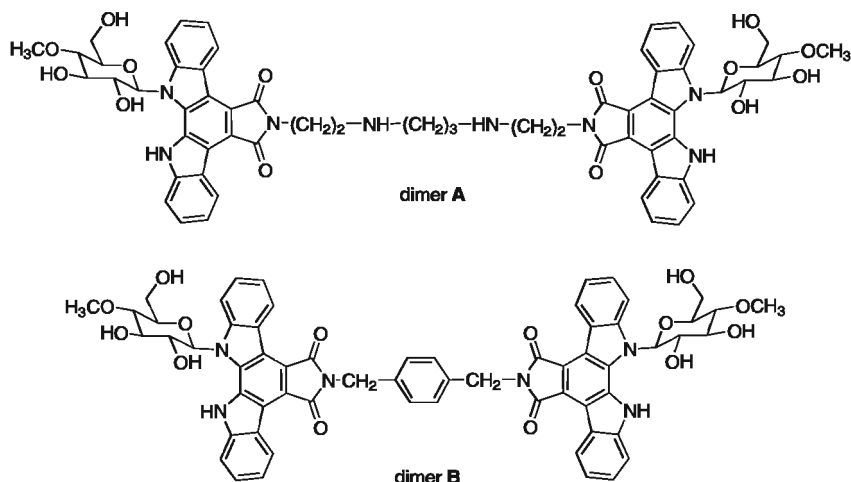


Fig. 20.16. Rebeccamyacin dimers.

20.4

Design of a Tumor-active Compound: NB-506

So far, despite the discovery of several highly potent inhibitors, none of the topoisomerase I-targeted IND analogs derived from rebeccamyacin has produced a compound sufficiently potent against experimental tumors to justify the initiation of clinical trials. But in another series, closely related to rebeccamyacin but nevertheless issued from a different antibiotic (BE-13793C), the researches have led to the synthesis of a promising anticancer agent (NB-506) [93] which has been evaluated in the clinic.

20.4.1

From BE13793C to ED-110 and NB-506

The IND antibiotic BE-13793C (J-104303) (Fig. 20.17) was isolated from a culture broth of *Streptomyces mobaraensis* BA13793, a strain found from a soil sample from Seto (Aichi Prefecture, Japan) by Kojiri and collaborators from the Japanese company Banyu Pharmaceuticals [94]. The key elements of this natural product are the two hydroxyl groups symmetrically placed at positions 1 and 11 on the IND chromophore, which contribute importantly to its anti-topoisomerases activity. BE-13793C exhibits potent cytotoxic activities against different tumor cell lines, including those resistant to doxorubicin and vincristine, and as such it provided a useful lead for the development of synthetic analogs endowed with improved pharmacological profiles. A very successful chemistry program was launched at the Banyu Tsukuba Research Institute leading rapidly to the design of the tumor-active analog ED-110 (J-109384).

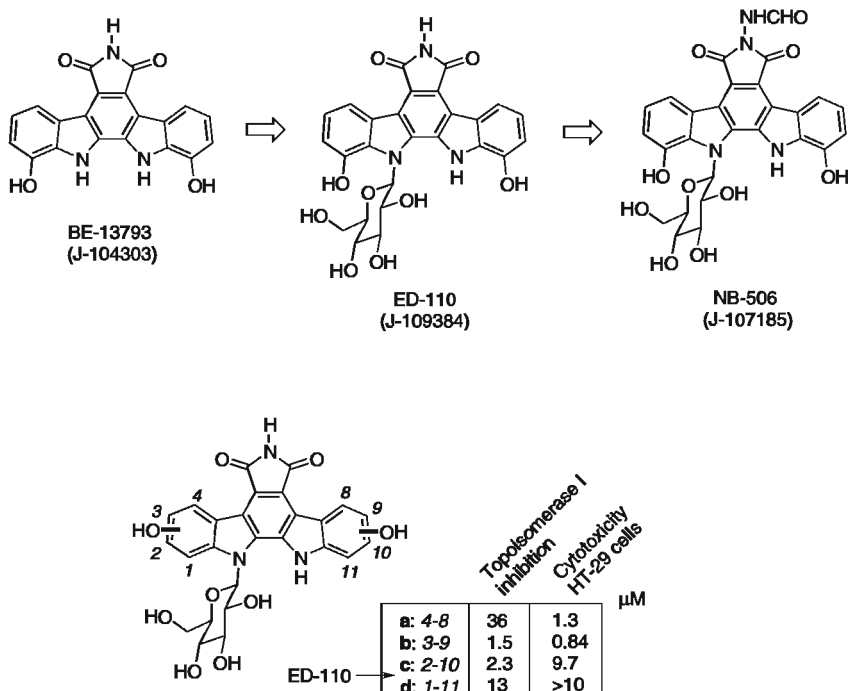


Fig. 20.17. Structures of NB-506 and its parent products. Influence of the position of the OH groups on the IND chromophore on anti-topoisomerase I activity and cytotoxicity [98].

By analogy with rebeccamycin, one of the two indole NH group of BE-13793C was substituted with a β -D-glucopyranose residue in order to improve the water solubility of the molecule [95]. The resulting compound ED-110 (Fig. 20.17) showed remarkable anticancer activities *in vivo* against human solid tumors transplanted in mice and topoisomerase I was identified as the main target of this glycosyl IND derivative [96]. Unlike BE-13793C, ED-110 showed no effect on DNA topoisomerase II but exhibited characteristic topoisomerase I-type toxicity in P388 murine leukemia cells: (1) stabilization of topoisomerase I–DNA covalent complexes with an efficiency equivalent to that of the reference inhibitor camptothecin, (2) inhibition of nucleotide synthesis rather than protein synthesis, and (3) G₂/M-specific cell cycle arrests [97].

The semisynthetic IND derivative ED-110 was further exploited to develop more potent analogs. The four symmetrical regioisomers of ED-110 containing the two hydroxyl groups at positions 1–11, 2–10, 3–9, and 4–8 have been prepared by total synthesis and, as indicated in Fig. 20.17, the 3,9-dihydroxy isomer proved to be 10 times more potent than ED-110 at inhibiting topoisomerase I and its cytotoxicity was also considerably enhanced [98]. This elegant work showed most clearly that

the relocation of the hydroxyl groups on the IND chromophore has a profound impact on the cytotoxic action of the compounds.

Among the various substituents introduced on the free nitrogen imide of ED-110, the formylamino derivative proved to be sufficiently soluble for use in *in vivo* studies. Compound NB-506 (J-107185) (Fig. 20.15) was synthesized from the *N*-methyl derivative of ED-110 converted into the corresponding anhydride upon treatment with aqueous potassium hydroxide. The heating of the anhydride in formic hydrazide in DMF afforded the desired formylamino compound [99, 100, 101].

20.4.2

DNA Binding and Topoisomerase I Inhibition

Unlike staurosporine, NB-506 shows no effects on protein kinases A and C and the EGF receptor protein tyrosine kinase. Its cytotoxic action was initially attributed to its capacity specifically to inhibit topoisomerase I, without inhibitory activity against topoisomerase II [102]. NB-506 induces a high level of topoisomerase I-dependent DNA single-strand break both with the purified enzyme in the test tube and in a cellular context [93, 103].

NB-506 is a DNA intercalating agent. The planar IND chromophore inserts between two consecutive base pairs, preferentially the G-C pairs, locating the appended carbohydrate residue in one of the two helical grooves, probably the minor groove as is the case with the anthracyclines and other DNA-binding antibiotics. However, binding studies using a related 2'-amino derivative of rebeccamycin suggested that the drug can adopt different orientation within the DNA helix, with the sugar occupying either the minor groove or the major groove, depending on the target sequence [104]. A recent biophysical study showed that NB-506 binds almost equally well to both A-T and G-C base pairs in DNA and the binding affinity ($K = 10^5 \text{ M}^{-1}$) is similar to that of certain classical intercalators such as amsacrine and bisantrene. The binding of NB-506 to DNA is enthalpy driven ($\Delta H = -7.2 \text{ kcal mol}^{-1}$). A similar binding enthalpy was measured for NB-506 and the tumor-active drug doxorubicin but the DNA interaction processes for the two drugs differ markedly in terms of entropy and ΔG . The free energy of NB-506 binding to DNA is considerably less favorable than that of doxorubicin [105]. Surprisingly, NB-506 was also found to bind preferentially to the DNA triplex poly(dA)·(poly(dT))₂ but the significance of this unexpected structural selectivity is unknown at present [106].

20.4.3

Cytotoxicity and Apoptosis

NB-506 exhibits marked cytotoxic effects towards a variety of tumor cell lines from different origins. A cell line-dependent cytotoxicity profile was observed with little or no correlation between IC₅₀ values and the anti-topoisomerase I activity in the different cell tumor lines examined. But a linear relationship was observed

between IC_{50} values and the extent of drug accumulation into cells. Cells highly sensitive to NB-506, such as K562 leukemia cells and HCT116 colon cancer cells, appear to accumulate the drug molecules more efficiently than the resistant cells [102]. Interestingly, the drug is highly effective against metastatic cells such as IMC carcinoma cells metastasized to the liver [107]. In the human small cell lung cancer cell line SBC-3, the combination of NB-506 and cisplatin was found to be synergistic. The local perturbation of the DNA helix (unwinding, kinking) induced by the platinum crosslinks seems to increase the inhibition of topoisomerase I by NB-506. In contrast to the camptothecin derivative SN-38, NB-506 increases the formation of DNA interstrand crosslinks by cisplatin, thereby enhancing the antitumor activity [108].

The mechanism by which NB-506 kills tumor cells remain unclear. At first sight, it is conceivable that inhibition of topoisomerase I by NB-506 represents the first stimulus that initiates a cascade of cellular dysfunctions. In different leukemia cell lines, such as HL-60 human promyelocytic cells, NB-506 was found to promote apoptosis with an activation of caspase-3 and caspase-8 [109]. Important cell cycle perturbations were observed. In the P388 murine leukemia model, the drug induces a concentration-dependent accumulation of the cells in the G_2/M phase, as illustrated in Fig. 20.18. A significant proportion of these P388 cells stained positively with annexin V, suggesting that they have engaged the apoptotic pathway. Similar effects were observed with camptothecin-resistant P388CPT5 cells but in this case, 3- to 5-fold higher concentrations of NB-506 are required to detect the arrest of the cells in the G_2/M phase of the cell cycle. This resistant cell line carries

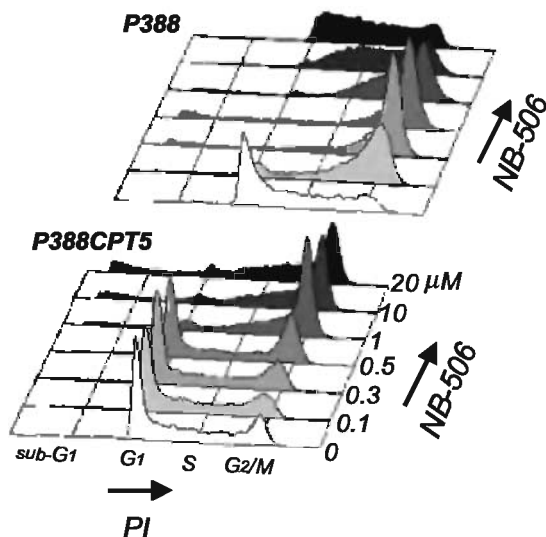


Fig. 20.18. Cell cycle analysis of P388 and P388CPT5 leukemia cells, sensitive and resistant to camptothecin, respectively, treated with graded concentrations of NB506 for 24 hours.

two point mutations in conserved regions of the *top1* gene (Gly361Val and Asp709Tyr) which confer partial resistance to NB-506 [110].

The role of topoisomerase I in the cytotoxic action of NB-506 was investigated further with the use of three cell lines with known topoisomerase I alterations. DU145/RC1 prostate carcinoma cells (mutation R364H), CEM/C2 leukemia cells (mutation N722S) and DC3F/C10 Chinese hamster fibroblasts (mutation G503S), all resistant to camptothecin, were found to be cross-resistant NB-506, suggesting thus that the two drugs CPT and NB-506 share a common binding site in the topoisomerase I–DNA complex [111]. This idea was initially developed in another study with the IND derivative R-3 (Fig. 20.19), an analog of NB-506 lacking the OH groups at positions 1,11. The recombinant topoisomerase I enzyme F361S resistant to CPT was also cross-resistant to R-3 [112]. A recent study using different mutated enzymes expressed in yeast strains has confirmed that topoisomerase I is the cellular target of R-3 but also revealed that distinct drug–enzyme interactions at the active site are required for efficient poisoning by this IND derivative and camptothecin [113].

The two cell lines RPMI8402/K5 and PC-7/CPT, which are resistant to CPT-11 (irinotecan) [114], are also resistant to NB-506 [115]. However, topoisomerase I is probably not the unique target of NB-506 because the resistance indices measured with this IND compound are always considerably lower than those measured with CPT. For example, the resistance ratio measured with the DU145/RC1, CEM/C2, and DC3F/C10 and the corresponding non-mutated cell lines are 11, 5, and 4, respectively, whereas they reach 361, 540 and 84 with CPT [111]. The *top1* mutations confer a high resistance of the enzyme but a limited resistance of the cells to NB-506. The antiproliferative activity of NB-506 must therefore result from interference with cellular processes other than topoisomerase I inhibition.

20.4.4

NB-506-resistant Cell Lines

The development of drug resistance is generally a major limiting factor in determining the clinical efficacy of new antitumor agents. It is therefore of primary importance to evaluate the cytotoxicity of NB-506 toward multidrug-resistant cell lines and to characterize NB-506-resistant cells.

SBC-3 small cell lung cancer cells are particularly sensitive to NB-506 and therefore provide a good model for establishing drug-resistant sublines. The clone SBC-3/NB#9 showed a high resistance to NB-506 ($\times 454$) which was attributed, at least partially, to a marked decrease in the topoisomerase I content and activity [115]. In a later study, other NB-506-resistant clones were identified using the P388 murine leukemia cell line [116]. The clone P388/F11, resistant to NB-506 ($\times 73$) and to a lower extent to CPT ($\times 3.5$), showed a reduced topoisomerase I level compared with the parental cell line due to the expression of an abnormal-sized 170-kDa (1357 amino acids) topoisomerase I protein. A large portion of the *top1* gene (from codon 21 to codon 609) was duplicated in its coding region. The re-

arranged topoisomerase I from these F11 cells is catalytically active but resistant to NB-506 [117].

The aforementioned NB-506-resistant cell lines display altered topoisomerase I activity but this may be sufficient to explain the high resistance indexes. However, other mechanisms can be invoked. A specific transporter responsible for resistance to NB-506 and the related IND derivative J-107088 has been identified. The ATP-binding cassette transporter BCRP/MXR/ABCP (BCRP) was found to be over-expressed ($\times 31$) in a LY/NR2 mouse fibroblast cell line resistant to NB-506 [116]. NB-506 is a good substrate for this transporter BCPR (which is generally little expressed in human tumors) but is not a substrate for the conventional and widely expressed transporters [102, 118]. Indeed, the expression of cell membrane efflux pumps such as the P-170 glycoprotein (Pgp-170) or multidrug-resistance protein (MRP) does not confer resistance to NB-506. For example, the MRP-expressing human HT1080/DR4 fibrosarcoma cell line, which is highly resistant to doxorubicin (resistance factor = 250), is poorly sensitive to NB-506 (resistance factor = 0.7) [119]. Passive diffusion through the plasma membrane has been suggested for the uptake of rebeccamycin into cells [120].

20.4.5

Antitumor Activity

NB-506 is remarkably potent at inhibiting tumor growth *in vivo*. The drug caused significant regression of nodules of human PC-13 lung cancer and MKN-45 stomach cancer cells when injected i.v. at a dose of 90 mg m⁻². In addition, NB-506 showed a lower cumulative toxicity than classical drugs like etoposide and cisplatin. The potent anticancer effects in transplanted tumors in mice coupled with its low cumulative toxicity have justified the setting of clinical trials with this drug. A first phase I study was conducted in 1994 in Japan using single doses of NB-506 ranging from 37.5 to 180 mg m⁻² [121]. In 1995, a second phase I was performed using a 5-day infusion of NB-506 given for 1 h i.v. repeating every 4 weeks and a dose level of 330 mg m⁻² was recommended [122]. Some secondary effects were observed (nausea, vomiting, liver dysfunction) [123] and apparently the clinical trials with NB-506 have been interrupted. Recently, clinical trials with an analog of NB-506, referred to as compound J-107,088 have been initiated.

20.4.6

Inhibition of the Topoisomerase I Kinase Activity

Unlike the antibiotics staurosporine and K252a, rebeccamycin-type compounds such as R-3 and NB-506 have no significant effects on the different PKC isoenzymes. However, the possibility that they interfere with other types of protein kinases has been considered. For example, Pollack and co-workers have reported that NB-506 selectively potentiates the activation by neurotrophin-3 of the nerve growth factor receptor TrkA which belongs to a family of transmembrane tyrosine receptor kinases [124]. The authors suggested that the IND drug could interact

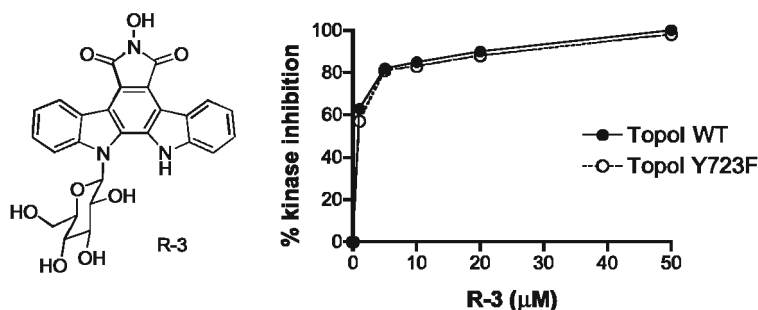


Fig. 20.19. Inhibition of kinase activity of wild type (WT) topoisomerase I and the mutated Y723F topoisomerase I by compound R-3 [125].

with the extracellular domain of the TrkA receptor so as to promote its interaction with the neurotrophin NT-3. A direct NB-506–protein interaction has also been suggested with topoisomerase I.

Topoisomerase I exerts a kinase activity in addition to its primary action at the DNA level. Tazi and collaborators have demonstrated that topoisomerase I can phosphorylate specific SR proteins, such as the human splicing factor 2/alternative splicing factor (SF2/ASF) protein [76, 125]. The kinase domain of the enzyme is located at the N-terminus part of the protein (from amino acids 139 to 175), at the opposite end to the DNA-binding/cleaving domain located at the C-terminus. The catalytic tyrosine Y723 responsible for the DNA nicking activity is absolutely not implicated in the kinase activity. The wild-type enzyme and Y723F (Tyr → Phe substitution) mutated enzyme can both phosphorylate SR proteins [76, 126, 127]. The IND drug R-3 is a potent inhibitor of the kinase activity of topoisomerase I. A concentration of 1–2 μM suffices to reduce the phosphorylation of the bacterially expressed recombinant SF2/ASF protein by 50% (Fig. 20.19). Under identical conditions, camptothecin showed no effect even at a concentration as high as 500 μM. Inhibition of the topoisomerase I kinase activity by camptothecin was only observed in the presence of DNA, whereas with R-3 the inhibitory activity is detected even in the absence of DNA. This suggests that the two activities of the enzyme, DNA cleavage and kinase, proceed independently [128].

NB-506 also potently inhibits the topoisomerase I kinase action with a comparable efficiency compared to R-3. It is remarkable that these two IND compounds specifically inhibit the phosphorylation of the SF2/ASF protein by topoisomerase I but not by the related kinases SPRK1 and Cdc2. There are good arguments to suggest that NB-506 binds at a specific site near or at the N-terminus kinase domain of topoisomerase I but not in the ATP-binding site of the enzyme [110]. The effect at the kinase level has direct consequences on the splicing activity. NB-506 prevents the formation of spliceosome which is a dynamic ribonucleoprotein structure where splicing takes place. As a result, the drug alters the mRNA levels and/or splicing patterns of different genes such as the anti-apoptotic gene Bcl-X and the CD44 gene implicated in extracellular matrix attachment and tumor

metastasis [110]. Therefore, drugs like R-3 and NB-506 must not be solely viewed as conventional topoisomerase I poisons stabilizing the covalent complexes with DNA but also as topoisomerase I kinase inhibitors. Both mechanisms likely contribute to their antineoplastic activities.

20.4.7

A Novel Clinical Candidate: J-107088

In the course of their structure–activity relationship studies, chemists at the Banyu Tsukuba Research Institute (Japan) identified the compound J-107388 as a highly potent topoisomerase I poison [129]. This new compound differs from NB-506 in two aspects. First, the formylamino substituent on the imide F-ring nitrogen, supposed to establish a direct contact with topoisomerase I [130], has been replaced by a more polar NH-CH-(CH₂OH)₂ group. This chain, positively charged at neutral pH, increases the aqueous solubility of the molecule and reinforces its DNA-binding interaction without penalizing the action on topoisomerase I [130]. But most importantly, the newly introduced substituent contributes to limit the hydrolysis of the compound into its inactive anhydride form, referred to as compound ED-551. A metal-dependent catabolic pathway has been postulated to explain the conversion of NB-506 into its deformylated amino product and then its anhydride derivative [131, 132]. In human plasma, the transformation of J-109404 into the metabolite ED-551 occurs much more slowly compared to J-107185 (Fig. 20.20). Capping the nitrogen F-ring with a NH-CH-(CH₂OH)₂ chain preserves the intact imide function that is essential for topoisomerase I inhibition.

Second, the two hydroxyl groups on the IND chromophore have been shifted from positions 1,11 to positions 2,10. The relocation of the OH groups considerably reduces the capacity of the drug to bind to DNA but affects neither its capacity to stabilize topoisomerase I–DNA complexes nor its ability to inhibit the SR protein kinase activity of the enzyme. Compound J-109382 (Fig. 20.21) does not inter-

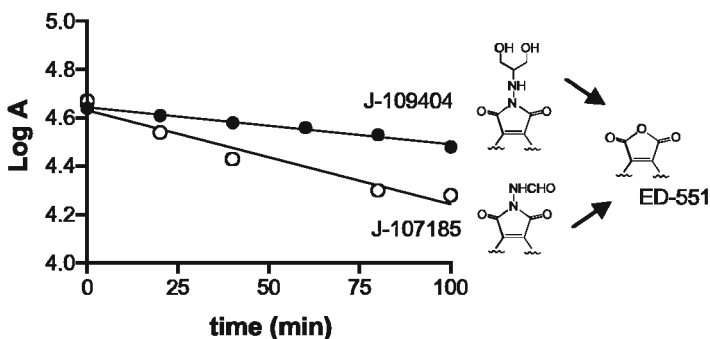


Fig. 20.20. Time-dependent conversion of (open circle) J-107185 (NB-506) and (bullet) J-109404 in the corresponding anhydride metabolite ED-551 in human plasma ultrafiltrate at 37°C. Peak areas (log scale) were measured by HPLC (two order rate reaction).

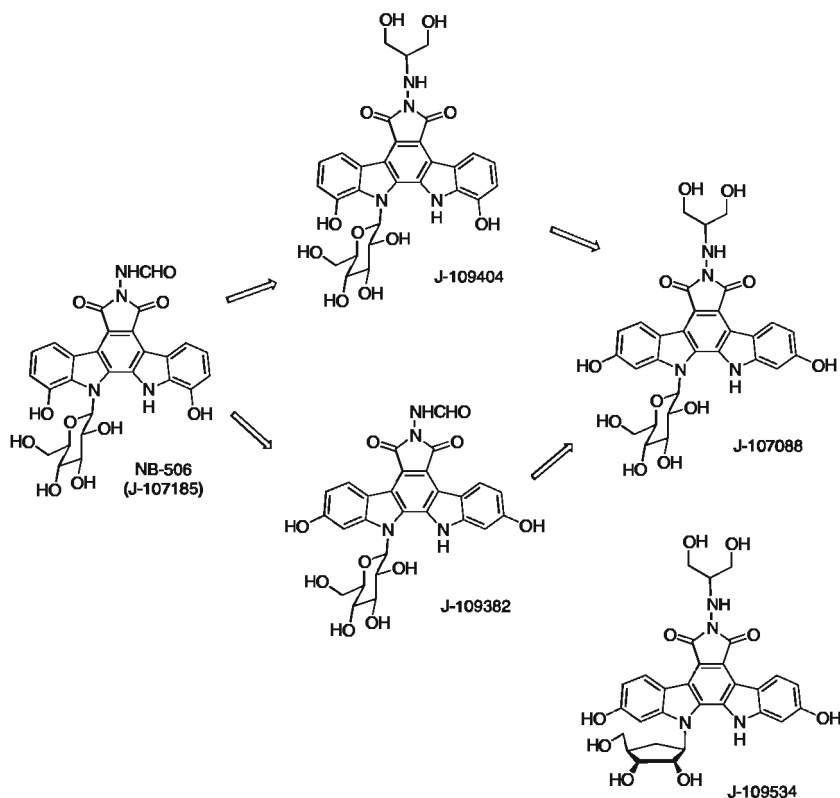


Fig. 20.21. Structures of the tumor-active derivative J-107088 and analogs.

calate into DNA but still potently directs topoisomerase I-mediated cleavage at sites identical to those seen with camptothecin [83]. Both types of structural changes reinforce the cytotoxic potential of the compound. The $\text{NHCHO} \rightarrow \text{NH-CH-}(\text{CH}_2\text{OH})_2$ substitution and the $\text{OH-1,11} \rightarrow \text{OH-2,10}$ shift decrease IC_{50} values by a factor of about 4 and 10, respectively, on different cell lines [83, 130, 133, 134]. J-107088, which combines these two types of structural changes (Fig. 20.21) has shown remarkable antitumor activities *in vivo* on murine and human tumors transplanted in mice and it was found to be active in a much wider range than NB-506 [135].

Moreover, J-107088 is active against different primary solid tumors (e.g. complete inhibition of IMC-HM tumor growth at 300 mg m^{-2}) as well as against micro-metastases in the liver. Pronounced anticancer effects have also been reported with five pediatric and adult central nervous system tumor xenografts [136]. This augurs well for the clinical development of J-107088, which has been recently initiated in Japan.

Attempts to further improve the antitumor potency of J-107,088 were recently reported with the synthesis of sugar modified analogs. The β -D-glucopyranose

moiety was replaced by a β -allo, β -manno and β -galacto-pyranose but the anti-topoisomerase I activity was not significantly improved. In contrast, interesting results were observed with synthetic analogs for which a furanose was substituted for the pyranose moiety of J-107088. The β -ribofuranose analog J-109534 (Fig. 20.21) was 6 times more potent than J-107088 at stimulating DNA cleavage by topoisomerase I [137]. Unfortunately, the increased potency at the enzyme level did not confer enhanced tumor activity *in vivo*. J-109534 showed reduced activity against MKN-45 xenografts in mice compared with J-107088. So far the extensive structure-activity relationships studies have not produced compounds superior to J-107088.

20.4.8

Biosynthesis

The antibiotic BE13793C (J-104303) has provided the initial microbial product from which all NB-506 type compounds were derived. A β -D-glucose residue was introduced chemically on the nitrogen indole ring to obtain the tumor-active compound J-109384 (ED-110) [95]. Recently, it was shown that this glycosylation step can be carried out biosynthetically in *Streptomyces mobarensis* BA13793 via an *N*-glucosyltransferase encoded by the *ntg* gene. Ohuchi *et al.* [138] have cloned this gene and expressed the corresponding *N*-glucosyl transferase also capable of introducing a D-glucose moiety into 6-*N*-methylarcyriaflavine (Fig. 20.22). This novel

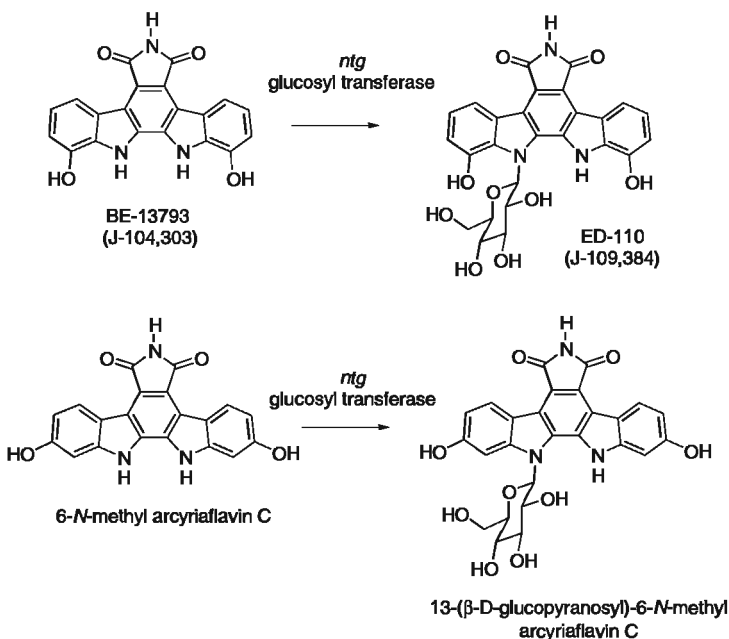


Fig. 20.22. Biosynthesis of glucosylated IND derivatives.

bioengineered pathway opens the door to the design of a variety of biosynthetic glycosylated products.

20.5

Conclusion

Naturally occurring IND antibiotics have been intensively exploited for the design of antitumor drugs. Staurosporine and K252a were essentially used to produce protein kinase inhibitors whereas BE13793C and rebeccamycin have mainly produced topoisomerase I inhibitors. The bisglycosyl antibiotic AT-2433-B1 is now also used as a template for the design of DNA-targeted drugs. In each series, the structure–activity relationships are complex and a subtle modification of the glycosyl moiety or the IND chromophore can often change significantly the mechanism of action of the drug. In most cases, these type of compounds do not bind to a single target but often affect several signaling pathways in different compartments of the cell. It is also relatively rare to observe that a molecule, such as R-3 and NB-506, can interfere with a specific molecular target such as topoisomerase I by two distinct mechanisms, inhibiting both the DNA cleavage and the kinase activity of the enzyme. The diversity of the mechanisms of action of this type of compound complicate the development of effective antitumor agents in the IND series. The elucidation of detailed structure–activity relationships is still required in order to optimize the anticancer activities of these compounds.

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Defining the Molecular Interactions that are Important for the Poisoning of Human Topoisomerase I by Benzimidazoles and Terbenzimidazoles

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21.1

Human DNA Topoisomerase Type I

Human DNA topoisomerase type I (hTOP1) is one of three type I DNA topoisomerases that have been identified in human cells, the other two being topoisomerase III α and topoisomerase III β . hTOP1 (a type IB topoisomerase) is a 100-kDa monomeric protein of 765 amino acids that is encoded by a gene located on human chromosome 20q12–13.2 [1, 2]. It functions as a swivel during DNA replication and transcription, as well as in chromosome condensation and segregation [3–7]. hTOP1 achieves this function by cutting and religating one of the two strands of the target DNA duplex [4, 6]. Recent studies on both *Drosophila melanogaster* and knockout mice have revealed that TOP1 is an essential enzyme [7–9]. The catalytic cycle of the enzyme entails the formation of a transient covalent enzyme–DNA complex (termed the cleavable complex) in which the active site tyrosine residue at position 723 is covalently linked to the 3'-phosphate terminus of the transiently cleaved DNA strand [4, 6, 10]. The enzyme consists of four primary domains [11, 12]: (1) the N-terminal domain, which consists of residues 1 to ~210, is highly charged, and is not required for catalytic activity; (2) the core domain, which consists of residues ~211 to ~635; (3) a linker region, which encompasses residues ~636 to ~712 and contributes to but is not strictly required for catalytic activity; and (4) the C-terminal domain, which consists of residues ~713–765 and contains the active site tyrosine residue (Tyr723).

Although not strictly required for the catalytic function of the enzyme, the N-terminal domain contains several nuclear-targeting signals and has been implicated in nucleolar localization [13–16]. It also contains binding sites for several nuclear proteins, including T antigen, nucleolin, and Topors [13, 14, 17–19].

hTOP1 does not exhibit a great deal of sequence preference with respect to its target cleavage sites [20–22]. Significantly, however, eukaryotic TOP1 enzymes are covalently linked to thymine more than any other nucleotide (i.e. with a frequency of greater than 9 in 10) [20–22]. DNase I footprinting and modification interference studies have demonstrated that TOP1 protects a region of 16–20 base pairs, in which the cleavage site is centrally located [23, 24]. A core of approximately six base

pairs upstream from the cleavage site is of particular importance for the enzyme–DNA interaction [23, 24]. This observation has been confirmed by recent X-ray crystallographic studies on hTOP1 in both covalent and non-covalent complex with DNA [12]. The cleavage efficiency of TOP1 is modulated by ionic strength, with lower ionic strength resulting in greater cleavage efficiency [25, 26].

21.2

Topoisomerase I as a Target for Anticancer Drugs

Studies on the antineoplastic agent camptothecin (CPT) have led to the identification of hTOP1 as a target for anticancer drugs (reviewed in Refs [6, 10, 27, 28]). In 1966, Wall and co-workers first identified CPT as the antitumor agent in an extract from the tree *Camptotheca acuminata* [29]. The cytotoxic activities of CPT and its analogs are derived from their abilities to bind the TOP1–DNA interface and trap the TOP1–DNA complex in its cleavable (covalent) state by inhibiting the religation step of the TOP1 catalytic reaction [25, 26, 30]. CPT derivatives exhibit remarkable antitumor activities in animal models [31–35]. However, their antineoplastic activities in humans have been compromised by several factors: (1) CPT derivatives are chemically unstable, exhibiting a pH-dependent equilibrium between their active lactone and inactive carboxylate forms [36–38]. (2) The CPT carboxylate binds to human serum albumin with a very high affinity, thereby reducing the effective concentration of CPT lactone in the blood plasma [39, 40]. (3) Several CPT analogs are substrates of the adenosine triphosphate (ATP)-binding cassette family of drug transporters [41]. (4) Glucuronidation of CPT analogues is associated with enhanced drug clearance and resistance of human tumor cells [42, 43].

These limitations on CPT antitumor activity highlight the need for the development of new classes of TOP1-targeting anticancer drugs. Toward this end, numerous new classes of TOP1-poisoning agents have been identified, including nitidine [44, 45], the indolocarbazoles [46–49], the protoberberines [50–54], the benzimidazoles [55], and the terbenzimidazoles [56–60]. These TOP1 poisons are identified and characterized based on their abilities to trap the TOP1–DNA cleavable complex. Unlike CPT, many of the recently identified TOP1 poisons are known DNA-binding compounds [44, 49, 54, 58, 60, 61]. However, the role, if any, that DNA binding plays in the poisoning of TOP1 by such compounds remains unclear. This chapter focuses on the molecular interactions that are important the poisoning of hTOP1 by benzimidazole-containing compounds, with particular emphasis on derivatives that contain either one or three benzimidazole functionalities (benzimidazoles (BZs) and terbenzimidazoles (TBs), respectively).

21.3

Benzimidazoles

21.3.1

The Extent to which Benzimidazoles Stimulate hTOP1-mediated DNA Cleavage Depends on their Structure

A number of BZ analogs that differ with respect to their substituents at their 2-, 5-, and/or 6-positions (see structures in Fig. 21.1) were synthesized and evaluated for

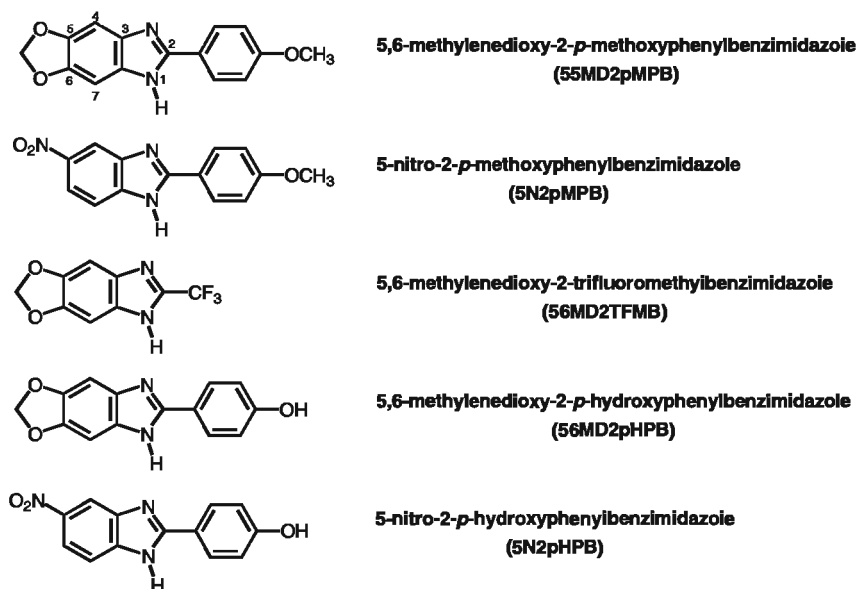


Fig. 21.1. Chemical structures of various benzimidazole derivatives. The atomic numbering is indicated in the structure of 56MD2pMPB (top).

their abilities to poison hTOP1. The relative TOP1-poisoning activities of these BZ compounds were determined by measuring the relative extents to which they stimulate hTOP1-mediated cleavage of end-labeled YEpG DNA. In these measurements, the DNA was incubated with various concentrations of ligand in the presence of TOP1, and was subsequently alkali-denatured prior to gel electrophoresis. This procedure reveals single-strand DNA breaks at specific sites on the DNA, with the resulting DNA cleavage fragments appearing as fast-migrating species in the gel.

Figure 21.2 shows representative gels comparing the DNA cleavage patterns induced by three different BZ analogs, which differ with respect to the substituents on their 5,6- and/or 2-*para*-phenyl positions. Note that all three BZ compounds stimulate hTOP1-mediated DNA cleavage in a dose-dependent manner. However, the extents to which they stimulate DNA cleavage differ markedly. Specifically, these extents follow the hierarchy 5N2pHPB > 5N2pMPB > 56MD2pMPB. Further inspection of Fig. 21.2 reveals that the BZ analogs stimulate hTOP1-mediated DNA cleavage at similar sites as CPT (see Fig. 21.2a). This observation suggests that the BZ analogs and CPT have similar modes of interaction in the ternary drug–DNA–TOP1 cleavable complex. In fact, as discussed below, our spectroscopic, viscometric, and computational studies suggest that both the BZ and CPT families of compounds trap and stabilize the cleavable TOP1–DNA complex via an intercalative mode of interaction. Note that for the three BZ analogs characterized in Fig. 21.2, a concentration of $\geq 10 \mu\text{M}$ is required to stimulate hTOP1-mediated DNA cleavage to the extent induced by only $0.1 \mu\text{M}$ of CPT. Thus,

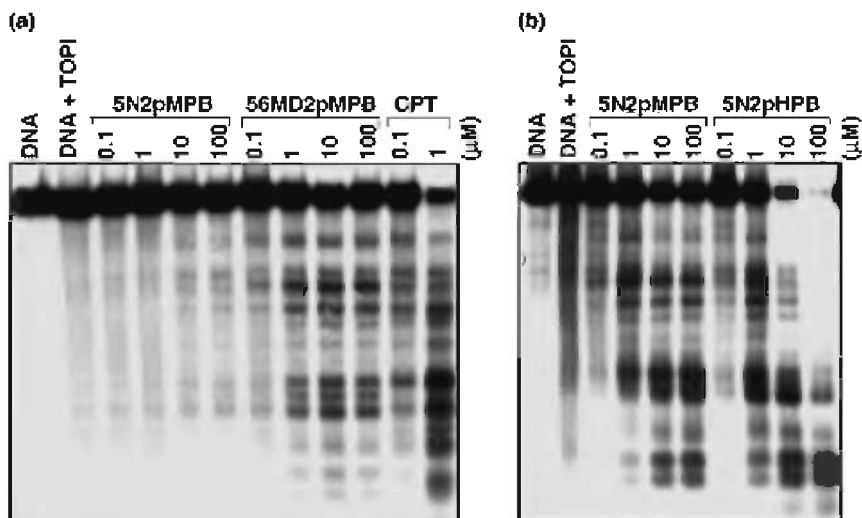


Fig. 21.2. Agarose gels showing hTOP1-mediated DNA cleavage of linearized YEpG DNA induced by various benzimidazole derivatives and CPT, with the ligands and their concentrations (in units of μM) indicated at the tops of the lanes. Following incubation

with both TOP1 and the ligand, the DNA samples were treated with SDS and proteinase K and then alkali-denatured prior to loading on to a 0.8% agarose gel in 0.5X Tris–Phosphate–EDTA buffer (see Refs [54, 87] for details of the experimental methodology).

under the conditions employed in these experiments, CPT is at least 100-fold more effective at poisoning hTOP1 than any of the three BZ analogs.

Table 21.1 summarizes the relative TOP1-poisoning efficacies of the three BZ analogs whose induced DNA cleavage profiles are shown in Fig. 21.2, as well those of two other BZ compounds whose induced DNA cleavage profiles are not shown (see structures of all five compounds in Fig. 21.1). Inspection of these data reveals the following structure–activity relationships: (1) a 5-nitro substituent enhances TOP1-poisoning activity relative to a 5,6-methylenedioxy functionality; (2) TOP1-poisoning activity is enhanced with a hydroxyl relative to a methoxyl substituent at the 2-para-phenyl position; (3) the absence of 2-phenyl substituent essentially abolishes TOP1-poisoning activity. In the sections that follow, we describe DNA binding and computational studies that provide potential molecular interpretations for these observed structure–activity relationships.

21.3.2

Identification of hTOP1 as the Specific Cytotoxic Target of 5N2pMPB

Given the abilities of various BZ derivatives to poison hTOP1, it is of interest to evaluate the role of TOP1 as the cytotoxic target of these compounds in cells. Yeast strains that express hTOP1, but in which the endogenous *top1* gene is deleted, have been used previously to identify hTOP1 as the sole cytotoxic target of CPT in yeast cells [51, 62]. We employed a similar approach to investigate the role of hTOP1 in the cytotoxicity of 5N2pMPB. Figure 21.3 presents the results of our

Tab. 21.1. Relative TOP1-mediated DNA cleavage induced by various 2-, 5-, and/or 6-substituted benzimidazoles.

Ligand	Relative TOP1-mediated DNA cleavage ^a
5N2pMPB	1
56MD2pMPB	>100
5N2pHPB	<0.01
56MDpHPB	10
56MD2TFMB	>1000

^aRelative TOP1-mediated DNA cleavage values are reported as the ratio of the concentrations of a given BZ derivative and 5N2pMPB that induce the same degree of DNA cleavage (i.e. [BZ]/[5N2pMPB], where [5N2pMPB] is arbitrarily assigned a value of 1).

studies. Note that 5N2pMPB is cytotoxic against yeast cells expressing hTOP1 (bottom row) but is not cytotoxic against yeast cells expressing yeast TOP1 (ScTOP1; top row). This result suggests that hTOP1 is the cytotoxic target of 5N2pMPB in the yeast system. BZ derivatives have been shown to exhibit cytotoxicities (expressed as IC₅₀ values) against human cells in the range of 10⁻⁷ to 10⁻⁵ M [55] (D. S. Pilch, E. J. LaVoie, and L. F. Liu, unpublished results), depending on the structure of the derivative. However, the role of TOP1 as the cytotoxic target of BZ derivatives in human cells remains to be established.

21.3.3
Viscometric Measurements Reveal that 56MD2pMPB Binding Unwinds Negative Supercoils in pUC19, Consistent with an Intercalative Mode of Interaction

Viscosity studies in which negatively supercoiled circular DNA duplexes are used as targets for a ligand provide a means of investigating the mode of ligand binding.

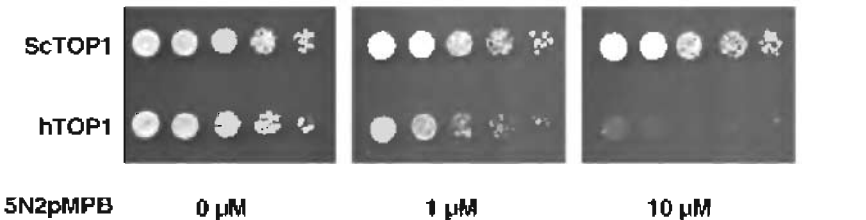


Fig. 21.3. Results of yeast cytotoxicity assay. The yeast strain JN2-134 lacking the chromosomal copy of the TOP1 gene was transformed with either the wild-type yeast TOP1 gene (top row) or the wild-type human TOP1 gene (bottom row). Yeast cells were serially (5-fold) diluted and grown on minimal medium containing 2% galactose and uracil as a selection marker. The medium also contained 0 (left), 1 (middle), or 10 (right) μM 5N2pMPB. The plates were grown for 3 days at 30°C to assess the lethal effect of 5N2pMPB on *S. cerevisiae* expressing either wild-type yeast (ScTOP1) or wild-type human TOP1 (hTOP1) (see Ref. [51] for details of the experimental methodology).

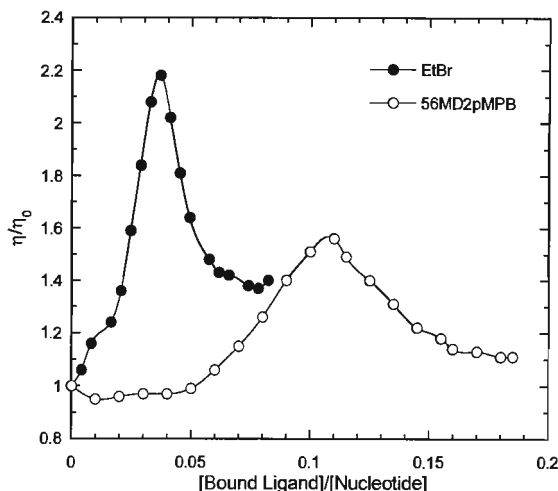


Fig. 21.4. Viscometric titrations of pUC19 with ethidium bromide (EtBr) (solid circles) or 56MD2pMPB (open circles) at 25.5 °C. η_0 is the solution viscosity in the absence of ligand, and η/η_0 is the relative solution viscosity. Solution conditions were 5 mM sodium acetate (pH 5.5) and 0.1mM EDTA (see Ref. [54] for details of the experimental methodology).

In general, ligand intercalation into circular duplex DNA unwinds negative supercoils, ultimately creating a fully relaxed circular molecule, followed by formation of a positively supercoiled molecule [63, 64]. Such ligand-induced changes in tertiary structure are characterized by an increase in solution viscosity upon unwinding of negative supercoils, followed by a decrease in viscosity with the subsequent formation of positive supercoils [65, 66]. Figure 21.4 shows the effect of either 56MD2pMPB or ethidium bromide (EtBr) on the viscosity of a solution containing the negatively supercoiled plasmid pUC19. Note that addition of EtBr, a classic intercalating ligand known to strongly unwind negatively supercoiled DNA [64, 67, 68], yields the expected viscosity profile for intercalation. Significantly, addition of 56MD2pMPB yields a similar viscosity profile to EtBr, although a larger ratio of [bound ligand]/[nucleotide] is required for this compound to achieve complete relaxation of the plasmid than for EtBr. This observation is consistent with 56MD2pMPB binding duplex DNA by intercalation.

Figure 21.5a shows the viscosity profiles resulting from titration of 56MD2pMPB into solutions containing three different concentrations of pUC19 (150, 200, and 250 μ M nucleotide). Figure 21.5b shows the corresponding plot of the DNA-bound 56MD2pMPB concentration at which the curves in Fig. 21.5a reach their maxima (i.e. at equivalence) as a function of nucleotide concentration. The slope of this plot yields the critical saturation ratio, $\bar{\nu}$, which corresponds to the ratio of DNA-bound ligand to nucleotide concentration at which the plasmid DNA molecule is fully relaxed. A linear regression analysis of the data in Fig. 21.5b (depicted as the solid line) yielded a $\bar{\nu}$ value of 0.11. Corresponding control experi-

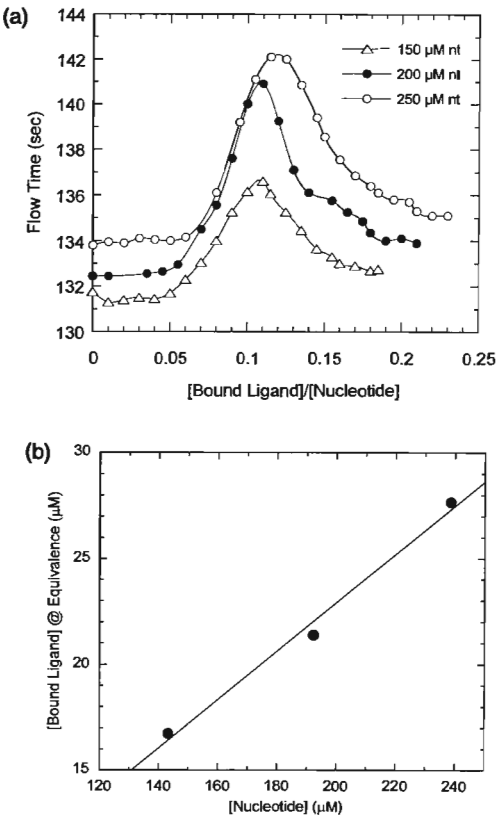


Fig. 21.5. (a) Viscometric titrations of 56MD2pMPB into solutions of pUC19 at the indicated concentrations (where nt denotes nucleotide). (b) Concentrations of DNA-bound 56MD2pMPB at which the curves in (a) are at their maxima (i.e. equivalence concentrations)

plotted as a function of pUC19 concentration. The solid line reflects a linear regression fit of the experimental data. Solution and temperature conditions were as described in the legend to Figure 21.4.

ments (not shown) with EtBr resulted in a $\tilde{\nu}$ value of 0.033, similar to the $\tilde{\nu}$ values previously observed by us as well as others for EtBr binding to pBR322, polyoma virus DNA, and $\Phi\text{X-174 RF I}$ DNA [54, 64, 69]. The DNA helix unwinding angle, ϕ_B , of 56MD2pMPB was determined to be approximately -7° using the following relationship [70]:

$$\phi_B \tilde{\nu}_B = \phi_E \tilde{\nu}_E \tag{1}$$

In this equation, $\tilde{\nu}_B$ and $\tilde{\nu}_E$ are the critical saturation ratios of 56MD2pMPB and EtBr, respectively, and ϕ_E is the helix unwinding angle of EtBr, an angle known to be -26° [67, 71].

21.3.4

The Affinity of Benzimidazoles for Duplex DNA is Modulated by the Structure and Electronic Properties of the Substituents on the Benzimidazole Rings

We used absorption spectroscopy to detect and characterize the binding of selected BZ compounds to the poly(dA-dT)·poly(dT-dA) duplex, which hitherto will be denoted by the abbreviation poly[d(A-T)]₂. Figure 21.6a,b shows the absorption spectra from 410 to 300 nm obtained by incremental titration of poly[d(A-T)]₂ into a solution of either 56MD2pMPB (a) or 56MD2pHPB (b). Inspection of each family of absorption titration spectra reveals the presence of a single isosbestic point at 343 nm, an observation consistent with each ligand exhibiting a single mode of binding to the host DNA. Scatchard plots derived from the absorption titration spectra shown in Fig. 21.6a,b are presented in Fig. 21.6c,d, respectively. Note the downward curvature of both Scatchard plots, an observation suggestive of

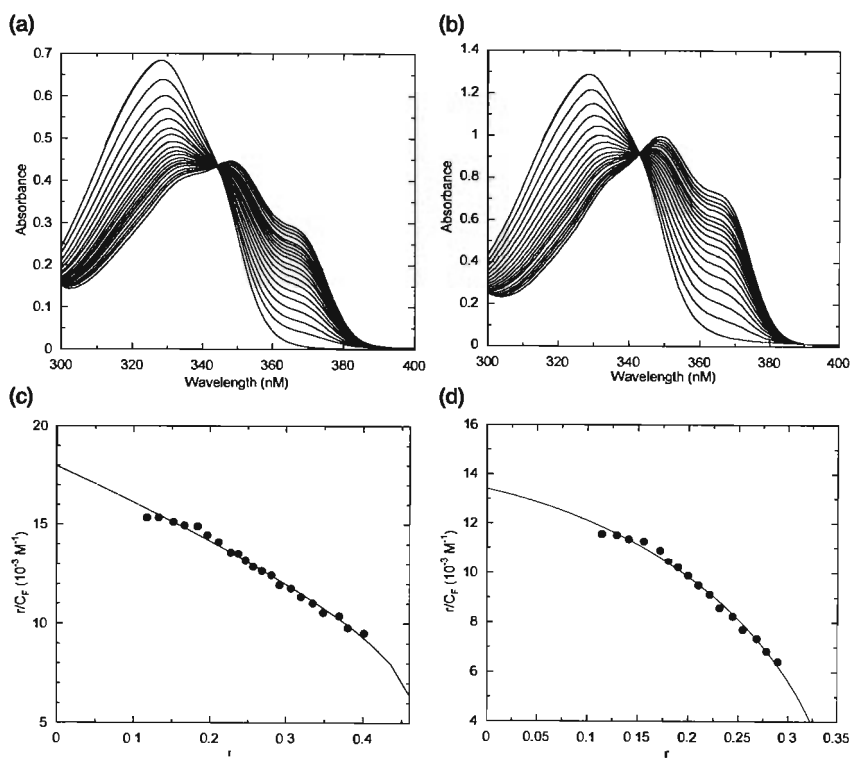


Fig. 21.6. Absorbance titrations of 56MD2pMPB (a) and 56MD2pHPB (b) (both at a concentration of 50 μM) with poly[d(A-T)]₂ at 25°C. From top to bottom at 330 nm, the absorption spectra in (a) and (b) correspond to [base pair]/[ligand] ratios ranging from 0 to 10. Scatchard plots derived from the titrations

shown in (a) and (b) are presented in (c) and (d), respectively. The solid lines reflect non-linear least squares fits of the experimental data with Eq. (2). C_F and r are defined in the text. The solution conditions were 10 mM sodium cacodylate (pH 7.0), 0.1 mM EDTA.

Tab. 21.2. Parameters for the binding of 56MD2pMPB and 56MD2pHPB to the poly[d(A-T)]₂ duplex at pH 7.0 and 25°C.^a

Ligand	<i>K</i> _a (M ⁻¹)	<i>n</i>	ω
56MD2pMPB	17,800 ± 310	2.1 ± 0.1	1.1 ± 0.1
56MD2pHPB	13,400 ± 490	2.8 ± 0.1	2.0 ± 0.2

^aData were derived from fits of the Scatchard plots shown in Fig. 21.6 with Eq. (2). The indicated uncertainties reflect the standard deviations of the experimental data from the fitted curves. The experiments were conducted in 10 mM sodium cacodylate, pH 7.0.

positively cooperative binding. The solid lines in Fig. 21.6c,d represent fits of the experimental points with the neighbor exclusion binding formalism of McGhee and von Hippel [72], which describes a single mode of ligand binding to a homogeneous polymer lattice with the inclusion of cooperative effects.

This binding formalism is given by the following relationship:

$$\frac{r}{C_F} = K(1 - nr) \left[\frac{(2\omega - 1)(1 - nr) + (r - R)}{2(\omega - 1)(1 - nr)} \right]^{n-1} \left[\frac{1 - (n + 1)r + R}{2(1 - nr)} \right]^2 \tag{2}$$
$$R = \sqrt{[1 - (n + 1)r]^2 + 4\omega r(1 - nr)}$$

In this relationship, *n* denotes the number of base pairs covered by the bound ligand, *K* is the association constant for all binding sites, *C_F* is the concentration of free ligand, *r* is the ratio of bound ligand to DNA base pairs (*C_B*/*C_N*), and ω is a cooperativity parameter, which represents the equilibrium constant for transfer of a bound BZ molecule from an isolated to a singly contiguous binding site. The values of *K*, *n*, and ω derived from the fits of the Scatchard plots shown in Fig. 21.6c,d are summarized in Table 21.2. Inspection of these data reveals several significant features: (1) 56MD2pMPB and 56MD2pHPB exhibit apparent binding sites sizes (*n* values) of 2.1 and 2.8, respectively. Recall that our viscometric studies on 56MD2pMPB were consistent with an intercalative mode of DNA binding. Our observed binding site sizes of 2.1 and 2.8 are also consistent with an intercalative mode of interaction, although they are slightly higher than the expected values of 2.0 for classic neighbor exclusion intercalative binding. (2) Under the conditions employed in these measurements (8.4 mM Na⁺, pH 7.0), both 56MD2pMPB and 56MD2pHPB exhibit similar affinities for the poly[d(A-T)]₂ duplex, with their binding constants differing by only 1.3-fold (*K* = 17,980 and 13,400 M⁻¹ for 56MD2pMPB and 56MD2pHPB, respectively). It is of interest to compare these affinities with those exhibited by other intercalating ligands for the poly[d(A-T)]₂ duplex under similar solution conditions to those employed here. Previous studies on the intercalating ligands EtBr, propidium iodide, and the acridine derivatives 9-aminoacridine and m-AMSA have revealed ligand-poly[d(A-T)]₂ association constants that are on the order of 10- to 100-fold greater than those re-

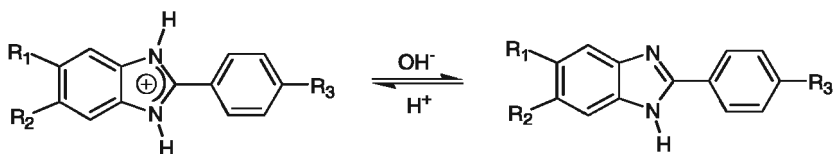
ported here for 56MD2pMPB and 56MD2pHPB [73, 74]. Thus, 56MD2pMPB and 56MD2pHPB are comparatively weak DNA intercalators. (3) 56MD2pMPB and 56MD2pHPB exhibit ω values of 1.1 and 2.0, respectively. Hence, the DNA binding of 56MD2pHPB is more positively cooperative than the binding of 56MD2pMPB. The positively cooperative nature ($\omega > 1$) of 56MD2pMPB and 56MD2pHPB binding to the poly[d(A-T)]₂ duplex differs from the behavior of the classic intercalators EtBr and propidium iodide, which exhibit either non-cooperative ($\omega = 1$) or anticooperative ($\omega < 1$) binding to poly[d(A-T)]₂ [73, 74].

We conducted similar absorption measurements to those described above for 56MD2pMPB and 56MD2pHPB to probe the DNA-binding properties of 5N2pMPB, 5N2pHPB, and 56MD2TFMB. Significantly, under the solution conditions employed (8.4 mM Na⁺, pH 7.0), none of these three compounds exhibited an appreciable degree of DNA binding (data not shown). In the aggregate, our DNA-binding studies reveal the following structure–activity relationships: (1) Substitution of a methoxyl group at the 2-para-phenyl position with a hydroxyl functionality does not significantly alter DNA binding affinity. (2) Substitution of a 2-phenyl functionality with a trifluoromethyl group abolishes DNA binding, an observation suggesting that the presence of a 2-phenyl substituent is critical for binding. (3) Substitution of a 5,6-methylenedioxy functionality with a 5-nitro group abolishes DNA binding. In the section that follows, we describe pH-dependent absorbance studies, suggesting that this observation is due to the reduced p*K*_a of the imidazole ring induced by the presence of the strongly electron withdrawing 5-nitro versus the 5,6-methylenedioxy group.

21.3.5

Benzimidazole Binding to Duplex DNA Requires the Ligand to be in its Fully Protonated Cationic State

We used the pH-dependent changes in the absorbance signals of both DNA-free and DNA-bound BZ compounds to determine the p*K*_a values for the equilibrium between the singly and doubly protonated forms of their imidazole rings, with the former protonation state being non-charged and the latter protonation state being positively charged. Figure 21.7 shows the absorbance of 56MD2pMPB at 355 nm (a) and of 5N2pMPB at 325 nm (b) plotted as a function of pH. Inspection of each curve reveals a single OH[−]-induced transition that corresponds to the conversion of the doubly to the singly protonated form of the benzimidazole, as schematically presented below.



Each pH-dependent transition can be fit with the equation shown below to yield the associated p*K*_a value.

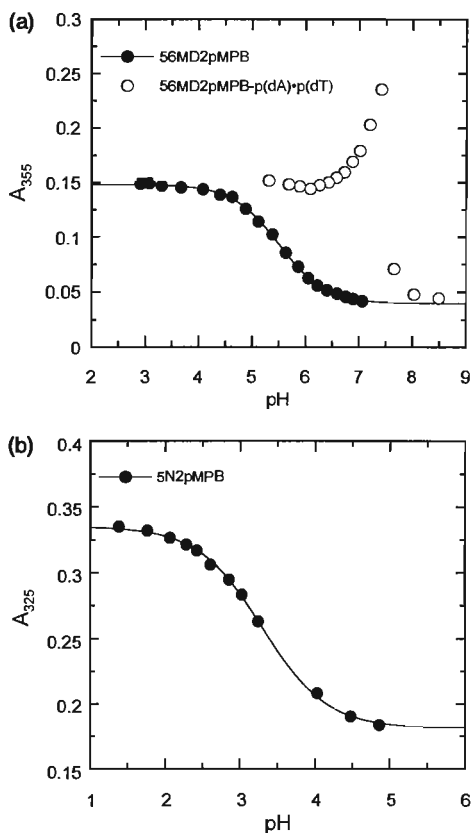


Fig. 21.7. (a) Absorbance of DNA-free (filled circles) and poly(dA)·poly(dT)-bound (open circles) 56MD2pMPB (20 μ M) at 355 nm plotted as a function of pH. (b) Absorbance of DNA-free 5N2pMPB (20 μ M) at 325 nm plotted as a function of pH. The solid lines in (a) and (b) reflect non-linear least squares fits

of the experimental data with Eq. (3). The pH titrations were conducted at 25°C in buffer containing 10 mM sodium phosphate, 10 mM sodium acetate, 10 mM borate, and 0.1 mM EDTA. The pH was adjusted by addition of either NaOH or HCl at concentrations ranging from 0.5 to 5 N.

$$A = \frac{(A_{\max} - A_{\min})(10^{\text{pH}-\text{p}K})}{1 + (10^{\text{pH}-\text{p}K})} + A_{\max} \quad (3)$$

The resulting fits, which are depicted as solid lines in Fig. 21.7a,b, yield $\text{p}K_a$ values of 5.5 ± 0.1 and 3.3 ± 0.1 for 56MD2pMPB (Fig. 21.7a) and 5N2pMPB (Fig. 21.7b), respectively. Thus, substitution of the 5,6-methylenedioxy functionality in 56MD2pMPB with a strongly electron-withdrawing 5-nitro group to form 5N2pMPB reduces the $\text{p}K_a$ of the imidazole ring by 2.2 units.

In addition to the pH-dependent profile of DNA-free 56MD2pMPB, Fig. 21.7a also shows the corresponding profile of 56MD2pMPB in complex with the poly(dA)·poly(dT) duplex. Note that as the pH increases from 5.3 to 7.4,

poly(dA)-poly(dT)-bound 56MD2pMPB undergoes an increase in absorbance corresponding to a partial transition from the doubly to the singly protonated state. However, upon further increase in pH above 7.4, the absorbance drops dramatically to the level of the DNA-free ligand. This observation is consistent with the ligand dissociating from the DNA duplex at pH values above 7.4. Thus, binding to DNA stabilizes the fully protonated cationic form of 56MD2pMPB, increasing the pK_a of the imidazole ring by more than 1.9 units. Moreover, only the fully protonated cationic form of the ligand is able to bind to the host DNA. Recall that BZ compounds containing nitro groups at their 5-positions exhibit essentially no affinity for DNA at pH 7.0. This inability to bind DNA at pH 7.0 is most likely due to the strongly stabilizing effect of the 5-nitro substituent on the non-charged versus the cationic form of the ligand, an effect demonstrated by the substantially lower pK_a of 5N2pMPB ($pK_a = 3.3$) versus 56MD2pMPB ($pK_a = 5.5$).

21.3.6

DNA Binding Alone is not Sufficient to Impart Benzimidazoles with the Ability to Trap and Stabilize the Cleavable TOP1–DNA Complex

A comparison of the DNA binding and TOP1 poisoning results discussed in Sections 21.3.1 and 21.3.4 reveals that the BZ compounds studied here exhibit markedly different hierarchies with respect to their relative DNA-binding affinities and TOP1-poisoning activities. Specifically, the relative TOP1-poisoning activities of the BZ compounds follow the hierarchy 5N2pHPB > 5N2pMPB > 56MD2pHPB > 56MD2pMPB $\gg$ 56MD2TFMB, while the relative DNA-binding affinities of the same five compounds follow the hierarchy 56MD2pHPB $\approx$ 56MD2pMPB $\gg$ 5N2pHPB, 5N2pMPB, 56MD2TFMB. Thus, relative DNA-binding affinity does not correlate one-to-one with relative TOP1-poisoning activity. In other words, DNA binding alone is not sufficient to impart BZ compounds with the ability to trap and stabilize the cleavable TOP1–DNA complex. Clearly, interactions between the bound BZ molecule and TOP1 must also be important. In the section that follows, we describe computational studies on the ternary cleavable complex formed between hTOP1, DNA, and 5N2pHPB that reveal the potential nature of these BZ–TOP1 interactions.

21.3.7

A Structural Model for the Ternary hTOP1–5N2pHPB–DNA Cleavable Complex that is Consistent with the Current Structure–Activity Database

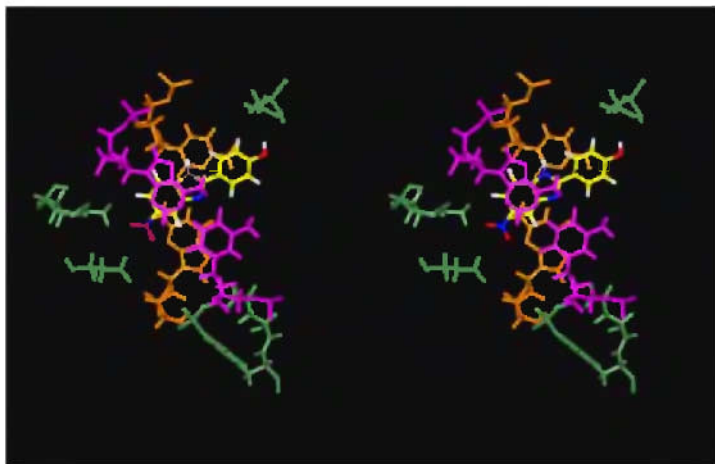
The Hol group has recently reported an X-ray crystal structure of the binary hTOP1–DNA cleavable complex [12]. This crystal structure affords us the opportunity of using computational techniques to derive structural models of the ternary cleavable complexes formed by a broad range of drug families. To this end, we used the published crystal structure of the hTOP1–DNA cleavable complex as a starting point to derive a structural model for the ternary cleavable complex formed between hTOP1, 5N2pHPB, and DNA in the following two main steps: (1) prep-

aration of the TOP1–DNA cleavable complex for ligand docking and (2) docking of the ligand into the TOP1–DNA complex, followed by refinement of the docked complex to generate a final structure. The details of these steps have been previously described [75]. Figure 21.8a shows a portion of the final energy minimized structure for the ternary hTOP1–5N2pHPB–DNA cleavable complex. This structural model has the ligand intercalated between the –1 and +1 base pairs, with the 5- and 6-positions of the benzimidazole moiety facing the DNA minor groove and the 2-phenyl substituent directed toward the major groove. A similar type of intercalative structural model has been proposed for the ternary hTOP1–CPT–DNA cleavable complex [12, 75, 76]. This type of intercalative model for the ternary hTOP1–5N2pHPB–DNA cleavable complex is consistent with our viscometric studies (see Section 21.3.3), which reveal that 56MD2pMPB binds DNA by intercalation, as well as with our TOP1-mediated DNA cleavage studies (see Section 21.3.1), which demonstrate that BZ compounds stimulate similar DNA cleavage profiles to CPT. Further inspection of the model shown in Fig. 21.8a reveals that Arg364, Asp533, Asn722, and Asn352 are the closest amino acids to the intercalated ligand. Arg364 and Asp533 lie in the DNA minor groove, while Asn722 and Asn352 lie on the major groove side of the cleavage site. These amino acid residues, as well as the –1 and +1 base pairs of the DNA, represent potential sources for favorable interactions with the bound ligand.

Figure 21.8b schematically illustrates the hydrogen-bonding interactions suggested by our model of the ternary hTOP1–5N2pHPB–DNA cleavable complex. Note that 56MD2pHPB stabilizes the TOP1–DNA cleavable complex, at least in part, through an array of hydrogen bonds with both the DNA and the enzyme. Specifically, the 3N atom of the benzimidazole moiety makes hydrogen bonding contacts to the 6-NH₂ group of the –1 adenine on the non-scissile strand and the 3-imino proton of the –1 thymine on the scissile strand. In addition, one of the two oxygen atoms on the 5-nitro group forms a hydrogen bond with the side chain of Arg364. A fourth hydrogen bond is formed between the hydroxyl proton at the 2-*para*-phenyl position of the ligand and the carbonyl oxygen atom in the side chain of Asn352. The latter two hydrogen-bonding contacts between the ligand and TOP1 provide potential molecular explanations for our experimental observations (see Section 21.3.1), indicating that a 5-nitro substituent enhances TOP1-poisoning activity relative to a 5,6-methylenedioxy functionality and that a hydroxyl relative to a methoxyl substituent at the 2-*para*-phenyl position also enhances TOP1-poisoning activity.

In addition to the hydrogen-bonding interactions noted above, hydrophobic and NH– π interactions also contribute to the stability of the ternary cleavable complex. Specifically, the benzene component of the benzimidazole moiety in 56MD2pHPB engages in stacking interactions with the –1 adenine base on the non-scissile strand (see Fig. 21.8a). In addition, the 4-NH₂ group of the +1 cytosine on the non-scissile strand forms an interaction with the π -system of the 2-phenyl substituent of the ligand (Fig. 21.8a,b). This interaction may account, at least in part, for our observations (see Sections 21.3.1 and 21.3.4) that the 2-phenyl substituent is required for both TOP1-poisoning and DNA-binding activities.

(a)



(b)

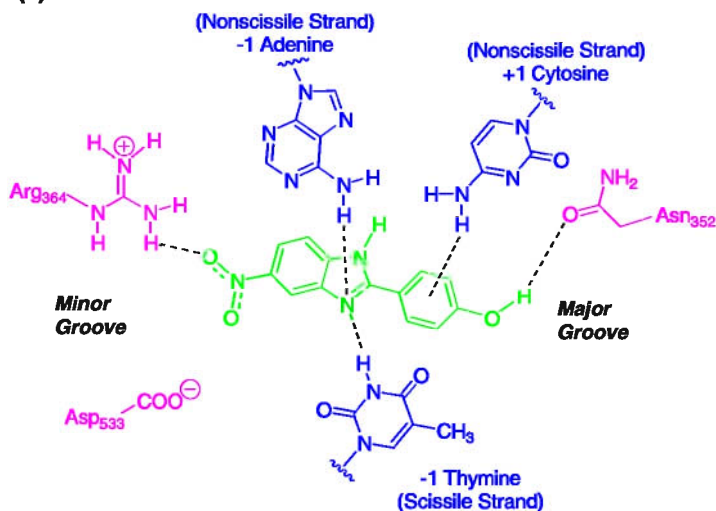


Fig. 21.8. (a) Stereo view looking down the axis of the DNA helix of a portion of the structural model for the ternary hTOP1–5N2pHPB–DNA cleavable complex. The –1 T–A base pair is depicted in magenta, while the +1 G–C base pair is depicted in orange. The hTOP1 amino acid residues are depicted in green, while the 5N2pHPB molecule is depicted according to the following color code: carbon in yellow, nitrogen in blue, oxygen in red, and hydrogen in white. (b) A schematic representation of the potential hydrogen-bonding contacts between the bound

5N2pHPB molecule and both the DNA and TOP1. In this figure, DNA functionalities are depicted in blue, 5N2pHPB is depicted in green, and TOP1 amino acid residues are depicted in magenta. The putative hydrogen bonds are depicted as black dashed lines.

These computational studies were conducted as previously described [75], with the exception that the TABU algorithm [121] was used in the docking calculations instead of a simulated annealing algorithm. In addition, the docking calculations simulated solvation implicitly using the GB/SA method [122].

Note that such NH- π or aromatic “hydrogen-bonding” interactions have been previously observed in interactions between small molecules [77] as well as between macromolecules [78]. In the aggregate, our computational results suggest that 56MD2pHPB stabilizes the TOP1–DNA cleavable complex through an array of hydrogen bonding and hydrophobic interactions with both the enzyme and the DNA. Significantly, our proposed structural model is fully consistent with the current body of experimental DNA binding and structure–activity data.

21.4

Terbenzimidazoles

21.4.1

The Pattern of hTOP1-mediated DNA Cleavage Induced by Terbenzimidazoles is Distinct from that Induced by CPT

We conducted DNA cleavage experiments similar to those discussed above in Section 21.3.1 on four TB derivatives that differ with respect to the substituents on their 5-, 6- and/or 2"-positions (see Fig. 21.9 for structures). Figure 21.10 shows representative gels comparing the DNA-cleavage patterns induced by the different TB analogs as well as by CPT. Note that the DNA cleavage pattern induced by the TB analogs is distinct from the corresponding pattern induced by CPT. This observation suggests that the TB analogs and CPT have different modes of interaction in the ternary drug–DNA–TOP1 cleavable complex. In fact, our spectroscopic studies discussed in a later section suggest that, in contrast to CPT, which is

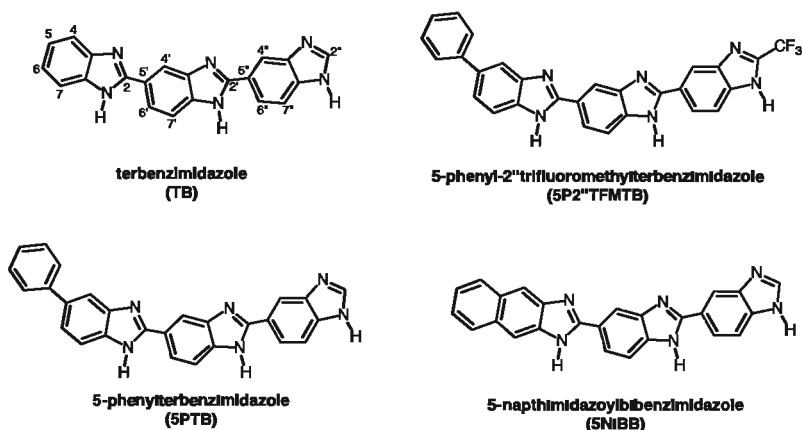


Fig. 21.9. Chemical structures of various terbenzimidazole derivatives. The atomic numbering is indicated in the structure of TB (top left).

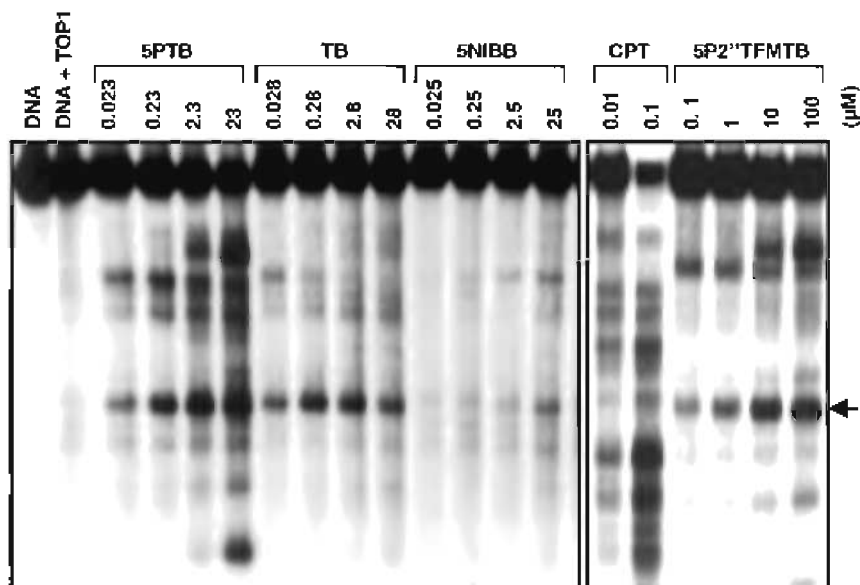


Fig. 21.10. Agarose gels showing hTOP1-mediated DNA cleavage of linearized YEpG DNA induced by various terbenzimidazole derivatives and CPT, with the ligands and their concentrations (micromolar) indicated at the tops of the lanes. The band labeled with the

arrow corresponds to the band C cleavage product induced by Hoechst 33342, as reported previously by the Liu group [86, 87]. Experimental details are as described in the legend to Fig. 21.2.

postulated to bind intercalatively in the ternary cleavable complex, TB derivatives stabilizes the TOP1–DNA cleavable complex by binding in the DNA minor groove. Further inspection of Fig. 21.10 reveals that the overall extents to which the TB derivatives stimulate DNA cleavage differ markedly, with these extents following the hierarchy $5\text{PTB} \approx 5\text{P2''TFMTB} > \text{TB} \gg 5\text{NIBB}$. This hierarchy of ligand-induced cleavage does not apply to all of the observed cleavage products. For example, the extent to which 5PTB, 5P2''TFMTB, and TB stimulate TOP1-mediated cleavage at the site corresponding to the arrow in Fig. 21.10 is similar, with this extent of induced cleavage being substantially greater than that induced by 5NIBB.

In the aggregate, our TB-induced DNA cleavage data reveal the following structure–activity relationships: (1) *Appending* a phenyl ring to the 5-position enhances TOP1-poisoning activity. (2) By contrast, *fusing* the phenyl ring to the 5,6-positions reduces TOP1-poisoning activity. (3) Adding a trifluoromethyl substituent to the 2''-position of a TB analog that already bears a 5-phenyl functionality does not significantly alter TOP1-poisoning activity. In the sections that follow, we describe studies suggesting that these observed structure–activity relationships are correlated with the differential affinities of the TB derivatives for the DNA minor groove.

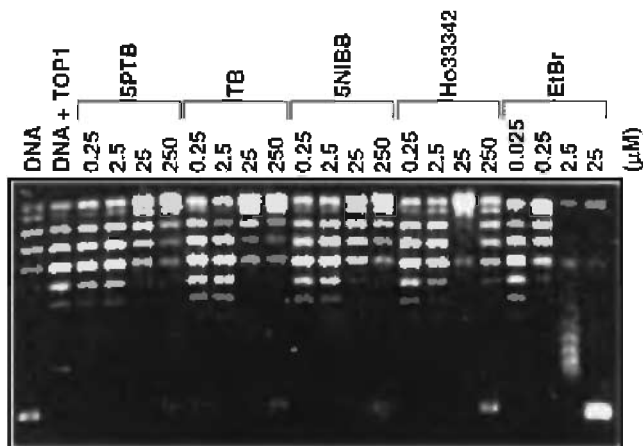


Fig. 21.11. Agarose gel showing the extent of DNA duplex unwinding by increasing concentrations of 5PTB, TB, 5NIBB, ethidium bromide (EtBr), and Hoechst 33342 (Ho33342) in the presence of hTOP1 (see Ref. [58] for details of the experimental methodology). The ligands and their concentrations are indicated at the tops of the lanes.

21.4.2

TOP1 Poisoning by Terbenzimidazoles is not the Result of Ligand-induced DNA Unwinding

We conducted DNA unwinding measurements to assess the impact, if any, of 5PTB, TB, and 5NIBB on the superhelical state of a DNA plasmid (pRS413). The resulting DNA unwinding profiles are shown in Fig. 21.11. In these experiments, ligand-induced DNA unwinding (a characteristic associated with an intercalative mode of DNA binding) is revealed by an upward and subsequent downward shift in the mobilities of the Gaussian distributions of topoisomers as a function of increasing ligand concentration. Inspection of Fig. 21.11 reveals that the three TB analogs behave similarly in inducing very little DNA unwinding relative to the large degree of DNA unwinding induced by the classic intercalator EtBr. This observation suggests that the three TB compounds bind DNA non-intercalatively. In fact, as described in the next section, 5PTB, TB, and 5NIBB exhibit linear dichroism (LD) properties characteristic of minor groove-directed DNA binding. Recall that the overall extents to which the three TB derivatives poison TOP1 follows the hierarchy, 5PTB > TB > 5NIBB (see Fig. 21.10). However, the three ligands are similarly weak DNA unwinders. Thus, it is unlikely that TOP1 poisoning by TB derivatives is related to ligand-induced DNA unwinding.

Further inspection of Fig. 21.11 reveals that the bibenzimidazole derivative Hoechst 33342 (Ho33342) does not induce DNA unwinding at concentrations ≤ 2.5 μM , but induces a substantial degree of DNA unwinding at a concentration of 25 μM . NMR and X-ray crystallographic studies on Hoechst 33258 (also known as pibenzimol (NSC322921)), which differs from Ho33342 only in that it contains a 2'-phenol rather than a 2'-(*p*-ethoxyphenyl) substitution, have demonstrated that

this ligand binds to the minor groove of A·T-tracts in duplex DNA [79–84]. By contrast, pibenzimol exhibits LD properties characteristic of intercalation at G·C-rich tracts of duplex DNA [85]. Given their structural similarities, it is reasonable to propose that Ho33342 and pibenzimol exhibit similar DNA-binding behaviors. Thus, our observed DNA unwinding results for Ho33342 may reflect high-affinity, minor groove-directed DNA binding to A·T-rich sites and lower-affinity, intercalative binding to G·C-rich sites. The Liu group has shown that both pibenzimol and Ho33342 are potent TOP1 poisons [86, 87], with pibenzimol already having been through phase I–II clinical trials against advanced pancreatic carcinomas [88]. The “dual-mode” DNA-binding properties (i.e. minor groove-directed and intercalative binding properties) exhibited by these two bibenzimidazole analogs has raised the question as to which DNA-binding mode is important for TOP1 poisoning by these compounds. Our DNA unwinding results described above for the TB derivatives suggest that the TOP1-poisoning activities of pibenzimol and Ho33342 are not the result of their DNA unwinding activities (i.e. intercalative binding properties). In other words, the TOP1-poisoning properties of these two bibenzimidazole analogs are probably correlated with their minor groove-directed DNA-binding modes.

21.4.3

TB Derivatives Exhibit Linear Dichroism Properties Characteristic of Minor Groove-directed DNA Binding

In addition to the DNA unwinding studies described above, we used flow linear dichroism (LD) methods to characterize the mode by which TBs bind to duplex DNA [89, 90]. Briefly, when the transition dipole moment of a DNA-bound aromatic ligand (e.g. a TB ligand) lies within the plane of the ligand molecule and makes an angle, θ , with the helical axis of the DNA, this angle can be related to the ratio of the LD and absorbance (A) signals (a ratio defined as the reduced LD or LD^r) by the following equation [89–92]:

$$LD^r = \frac{LD}{A} = \frac{3}{2}(3\cos^2\theta - 1)[F(G)] \quad (4)$$

In this equation, $F(G)$ is an orientation factor with values in the range of $0 < F(G) < 1$. Equation 4 implies that a positive LD^r signal is associated with an orientation angle θ of $<55^\circ$, while a negative LD^r signal is associated with an orientation angle θ of $>55^\circ$. In a typical flow LD experiment, the long axis of the nucleic acid helix is aligned along the flow lines [89–92]. Thus, the nucleic acid bases, which are oriented roughly perpendicular to the helix axis (i.e. $\theta_{\text{bases}} \approx 90^\circ$), give rise to a negative LD^r signal. A ligand molecule bound in the minor groove of a DNA duplex gives rise to a positive LD^r signal, since it typically exhibits an orientation angle, θ_{lig} , of $25\text{--}45^\circ$ relative to the helical axis. By contrast, an intercalated ligand molecule gives rise to negative LD^r signal, since it is oriented roughly perpendicular to the helix axis (i.e. $\theta_{\text{lig}} \approx 90^\circ$). An estimate of the average ligand orientation angle (θ_{lig}) in a ligand–nucleic acid complex can be derived from the

LD^r values of the nucleic acid alone $[(LD^r)_{NA}]$ and its ligand complex $[(LD^r)_{lig-NA}]$ using the following relationship [93]:

$$\frac{LD^r_{lig-NA}}{(LD^r)_{NA}} = 1 - 3 \cos^3(\theta_{lig}) \quad (5)$$

Figure 21.12 shows the LD^r spectra of the poly(dA)·poly(dT) duplex alone and in complex with either 5PTB (Fig. 21.12a), TB (Fig. 21.12b), or 5NIBB (Fig. 21.12c). Note that the LD^r spectra of the three ligand–poly(dA)·poly(dT) complexes are positive in the wavelength region (310–370 nm) where the ligands absorb, an observation consistent with each of the three TB derivatives binding in minor groove of the poly(dA)·poly(dT) duplex. Although not shown, we observed similar LD results when using natural DNA duplexes, such as salmon testes DNA, as targets for TB binding, while observing very weak or no TB binding to G·C-rich duplexes, such as poly(dG·dC)·poly(dC·dG), under the solution conditions employed ($[Na^+] = 110$ mM). Analysis of the LD^r data shown in Fig. 21.12 with Eq. (5) yields θ values of 27°, 34°, and 27° for the complexes of poly(dA)·poly(dT) with 5PTB, TB, and 5NIBB, respectively. In the aggregate, our LD results indicate that TB derivatives bind in the minor groove of duplex DNA, with a preference for A·T-rich sequences. These observations are in agreement with a recent X-ray crystallographic study by the Neidle group [94], which revealed that the TB analog, 5-piperazinyl-2''-(*p*-methoxyphenyl)terbenzimidazole, binds in the minor groove of the d(CGCAAATTTGCG)₂ duplex, covering the central 5'-AAATTT-3' sequence. Interestingly, the DNA binding of this TB derivative was accompanied by changes in helical twist suggestive of a small degree of binding-induced helix unwinding [94]. This effect may account for the small amount of TB-induced DNA unwinding we observed in our electrophoretic studies described above in Section 21.4.2, as well as in previous studies probing the interaction between 5PTB and the poly(dA)·poly(dT) duplex [95].

21.4.4

The Relative DNA-binding Affinities of the Terbenzimidazoles is Correlated with their Relative TOP1-poisoning Activities

Having established that 5PTB, TB, and 5NIBB bind in the minor groove of A·T-rich duplex DNA, we sought to determine whether this mode of DNA binding is important for the TOP1-poisoning activities of these ligands. To accomplish this task, we used a combination of spectroscopic and calorimetric techniques, the details of which have been previously described [58], to determine the binding affinities of the three TB analogs to the A·T-rich oligonucleotide duplex d(GA₄T₄C)₂. These studies revealed the affinities of 5PTB, TB, and 5NIBB for the d(GA₄T₄C)₂ duplex to be $(8.9 \pm 2.4) \times 10^9$, $(3.9 \pm 0.7) \times 10^8$, and $(2.6 \pm 0.6) \times 10^7$ M⁻¹, respectively. We observed a similar hierarchy of binding affinities for the poly(dA)·poly(dT) duplex. Note that the relative binding affinities of 5PTB, TB, and 5NIBB for the d(GA₄T₄C)₂ and poly(dA)·poly(dT) duplexes follow the same hierarchy noted above (see Section 21.4.1) for overall TOP1-poisoning activity; namely, 5PTB > TB >

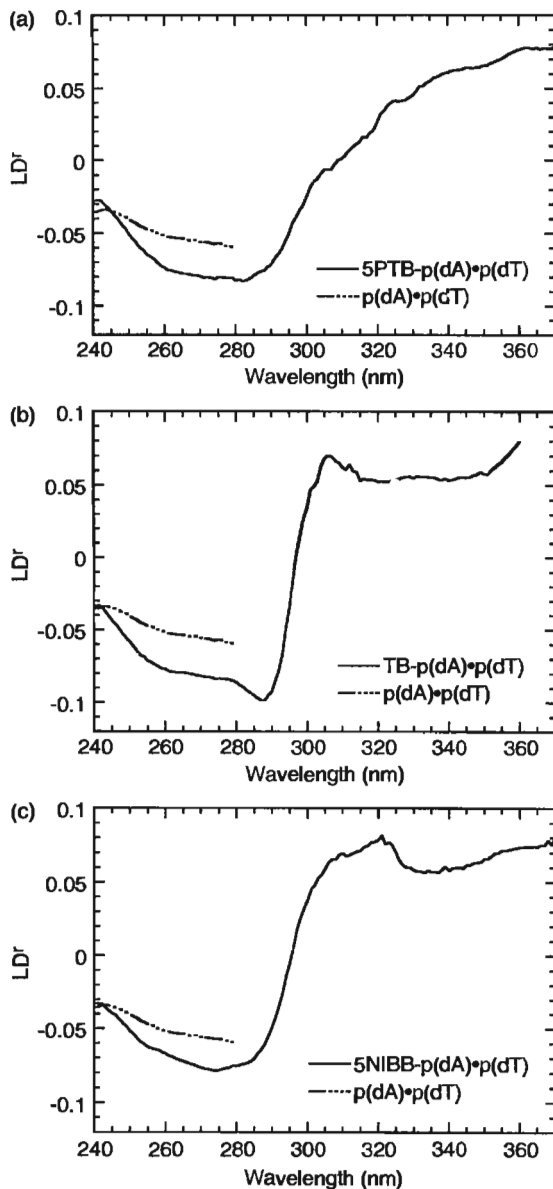


Fig. 21.12. Reduced linear dichroism (LD') spectra at 25°C of poly(dA)·poly(dT) and its complexes with 5PTB (a), TB (b), and 5NIBB (c) at a [ligand]/[base pair] ratio of 0.1 (see

Ref. [58] for details of the experimental methodology). Solution conditions were 10 mM sodium phosphate (pH 6.8), 100 mM NaCl, and 0.1 mM EDTA.

5NIBB. This observation suggests that the relative affinity of TB derivatives for the DNA minor groove is correlated with the relative extents to which these ligands poison TOP1. Recent reports have indicated that minor groove-directed ligand–DNA interactions appear to be involved in the poisoning of TOP1 by protoberberines [53] and nogalamycin [96]. Thus, ligand interactions with the DNA minor groove may be of general importance in the induction of TOP1-mediated DNA cleavage.

21.4.5

A Potential Role for Ligand Interactions with the DNA Minor Groove in Stabilization of the TOP1–DNA Cleavable Complex

As noted above, numerous studies by us as well as others have suggested that the DNA minor groove-binding properties of TB derivatives and Hoechst 33342 play an important role in their TOP1-poisoning capabilities [58, 60, 61, 86, 87, 97]. One of the more challenging questions confronting us is the mechanism by which minor groove-directed ligand–DNA interactions induce TOP1 poisoning. In fact, the role of DNA minor groove binding in TOP1 poisoning has been obscured by the observation that netropsin and distamycin, two antibiotics that have similar affinities for the DNA minor groove as Hoechst 33342, are much weaker TOP1 poisons [86, 87].

One potential role for ligand interactions with the DNA minor groove in stabilization of the TOP1–DNA cleavable complex is to induce a structural perturbation in the host DNA, which, in turn, enhances TOP1-mediated DNA cleavage. A number of recent observations suggest that DNA bending or curvature may be a candidate for such a structural perturbation. (1) Actinomycin D is a dual poison of both TOP1 and TOP2. Its binding to DNA is known to cause a 40° kink (bend) in the DNA helix [98, 99]. This bend is clearly due to the DNA minor groove-directed interactions of the pentapeptide side chains of the drug [98–101]. (2) UV-damaged DNA is known to poison TOP1, probably through formation of cyclobutane pyrimidine dimers (CPDs) [102]. Significantly, CPDs are known to cause DNA bending [103, 104]. In fact, some CPDs have been shown to induce TOP1-mediated DNA cleavage at a distance of up to 10 base pairs away from the site of cleavage [102]. (3) Recent studies have shown that a sequence-directed bend created by a junction between an A·T-tract and normal B-form DNA sequences can stimulate TOP1-mediated DNA cleavage when properly positioned downstream from the site of cleavage [105, 106]. (4) Recent studies have revealed that substitution of a nogalamycin-binding site with a DNA bending sequence (d(A)₅·d(T)₅) results in the stimulation of TOP1-mediated DNA cleavage in the absence of nogalamycin [107]. This observation, coupled with previous NMR-based observations that nogalamycin binding induces a 22° bend in the host DNA [108], suggests that nogalamycin may stimulate TOP1-mediated DNA cleavage by inducing a bend in the DNA that, in turn, stabilizes the TOP1–DNA cleavable complex.

In the aggregate, the observations noted above are consistent with a “DNA curvature” model for stabilization of the TOP1–DNA cleavable complex. In this model

(schematically depicted in Fig. 21.13, see p. 598), we consider two forms of the TOP1–DNA complex, the non-cleavable and cleavable forms, which are in rapid equilibrium. Under normal circumstances, this equilibrium favors the non-cleavable TOP1–DNA complex. However, the presence of a bend in the DNA helix may shift the equilibrium toward the cleavable TOP1–DNA complex. A bent DNA conformation is energetically unfavorable due to the rigidity of normal B-form DNA [109]. Thus, based on a simple thermodynamic consideration, any DNA structural perturbation that can reduce this unfavorable free energy should, in principle, stabilize the bent conformation and, in turn, stabilize the cleavable TOP1–DNA complex. Such DNA structural perturbations might include the following:

1. Sequence-directed bends that are positioned properly relative to the periodicity of the helix (i.e. the helical repeat). Junctions between B-form DNA and A-T tracts are known to exhibit bends in the helical axis of $\approx 22^\circ$ [110–112]. Interestingly, neutralization of the negative charges on the phosphate moieties of the DNA backbone (e.g. by methylation or ethylation) also has been shown to cause significant DNA bending [113].
2. Increased DNA flexibility. DNA is quite rigid. However, gaps, mismatches, bulges, and other forms of altered DNA structure are likely to reduce the rigidity of DNA [114].
3. The writhe of superhelical DNA. In addition to DNA sequence and charge neutralization, superhelical stress can also stabilize DNA curvature. The relaxation activity of TOP1 may be tightly linked to the proposed stabilization of the cleavable complex by DNA curvature [105, 106].
4. Ligand-induced changes in DNA structure. As discussed above, the binding of both actinomycin D and nogalamycin induces a substantial bend in the helical axis of the target duplex [99, 108]. In the sections that follow, we describe studies supporting the notion that TB derivatives may stimulate TOP1-mediated DNA cleavage by the induction and/or stabilization of DNA curvature.

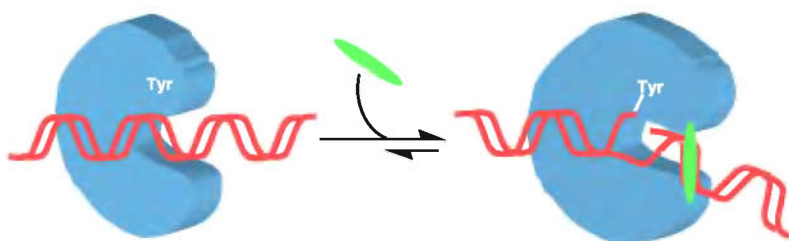
Tab. 21.3. Thermodynamic parameters for the binding of 5PTB, netropsin, and DAPI to the d(GA₄T₄C)₂ and d(GT₄A₄C)₂ duplexes.^a

Duplex	Ligand	ΔH_b^b (kcal mol ⁻¹)	$T\Delta S_b^b$ (kcal mol ⁻¹)	ΔG_{b-20}^c (kcal mol ⁻¹)	K_{20}^c (M ⁻¹)
d(GA ₄ T ₄ C) ₂	5PTB	-4.9 ± 0.6	+8.4 ± 0.8	-13.3 ± 0.2	(8.9 ± 2.4) × 10 ⁹
d(GA ₄ T ₄ C) ₂	Netropsin	-3.4 ± 0.7	+9.0 ± 0.9	-12.4 ± 0.2	(1.7 ± 0.5) × 10 ⁹
d(GA ₄ T ₄ C) ₂	DAPI	-3.6 ± 0.6	+7.4 ± 0.8	-11.0 ± 0.2	(1.5 ± 0.4) × 10 ⁸
d(GT ₄ A ₄ C) ₂	5PTB	-5.9 ± 0.6	+5.1 ± 1.2	-11.0 ± 0.2	(1.7 ± 0.6) × 10 ⁸
d(GT ₄ A ₄ C) ₂	Netropsin	-9.2 ± 0.9	+2.7 ± 1.1	-11.9 ± 0.2	(7.9 ± 2.1) × 10 ⁸
d(GT ₄ A ₄ C) ₂	DAPI	-5.5 ± 0.7	+5.4 ± 0.9	-10.9 ± 0.2	(1.4 ± 0.3) × 10 ⁸

^a Data were derived as detailed previously [61].

^b ΔH_b and ΔS_b are the binding enthalpy and entropy, respectively.

^c ΔG_{b-20} is the binding free energy and K_{20} is the association constant (both at 20°C).



Noncleavable Complex

Fig. 21.13. A “DNA curvature” model for stabilization of the TOP1–DNA cleavable complex. One way the bent state of the DNA (depicted in red) and thus the cleavable complex might be stabilized is through

Cleavable Complex

complexation with a ligand, such as a minor groove-directed TB molecule (depicted as a green oval). In this schematic, the TOP1 enzyme is depicted in blue and the active site tyrosine residue (Tyr) is indicated.

21.4.5.1 5PTB preferentially binds and stabilizes bent versus normal duplex DNA

We used a combination of spectroscopic, calorimetric, and gel electrophoretic techniques (detailed in Ref. [61]) to characterize the binding properties of 5PTB (see structure in Fig. 21.9), to a “bent” decameric DNA duplex, $d(\text{GA}_4\text{T}_4\text{C})_2$, and to a “normal” decameric DNA duplex, $d(\text{GT}_4\text{A}_4\text{C})_2$. For comparative purposes, we also characterized the corresponding binding properties of two other DNA minor groove-binding drugs, netropsin and DAPI (4',6-diamidino-2-phenylindole), which are weak or inactive as human TOP1 poisons [86]. Our spectroscopic and calorimetric data, which are summarized in Tab. 21.3, reveal that, at 20°C, 5PTB exhibits an entropically-driven, 52-fold ($\approx 2.3 \text{ kcal mol}^{-1}$) greater affinity for the bent $d(\text{GA}_4\text{T}_4\text{C})_2$ duplex relative to the sequence-isomeric, normal $d(\text{GT}_4\text{A}_4\text{C})_2$ duplex. By contrast, netropsin and DAPI bind to either host duplex with a similar affinity. In other words, the TOP1 poison (5PTB) preferentially binds and stabilizes bent versus normal duplex DNA, while the weak/inactive TOP1 poisons (DAPI and netropsin) do not distinguish between these two DNA conformational forms.

In addition to the DNA-binding affinity measurements described above, we also conducted PAGE experiments to assess the impact, if any, of 5PTB and netropsin binding on the global conformations (as assessed by electrophoretic mobility) of the $d(\text{GA}_4\text{T}_4\text{C})_2$ and $d(\text{GT}_4\text{A}_4\text{C})_2$ duplexes. The resulting gels are shown in Fig. 21.14. Note that in the absence of ligand, $d(\text{GA}_4\text{T}_4\text{C})_2$ migrates more slowly than $d(\text{GT}_4\text{A}_4\text{C})_2$, an observation consistent with a previous electrophoretic study on the same two decameric duplexes [115]. A reduction in electrophoretic mobility relative to that expected of a DNA molecule on the basis of its molecular weight has been employed as the “gold standard” for scoring DNA bending [111, 116–118]. Thus, the anomalously slow migration of $d(\text{GA}_4\text{T}_4\text{C})_2$ is consistent with this duplex adopting a bent conformation relative to the “normal” conformation adopted by $d(\text{GT}_4\text{A}_4\text{C})_2$. Further inspection of Fig. 21.14 reveals that addition of 5PTB retards the migration of both duplexes, with the extent of this retardation being greater for $d(\text{GA}_4\text{T}_4\text{C})_2$. By contrast, corresponding electrophoretic experiments

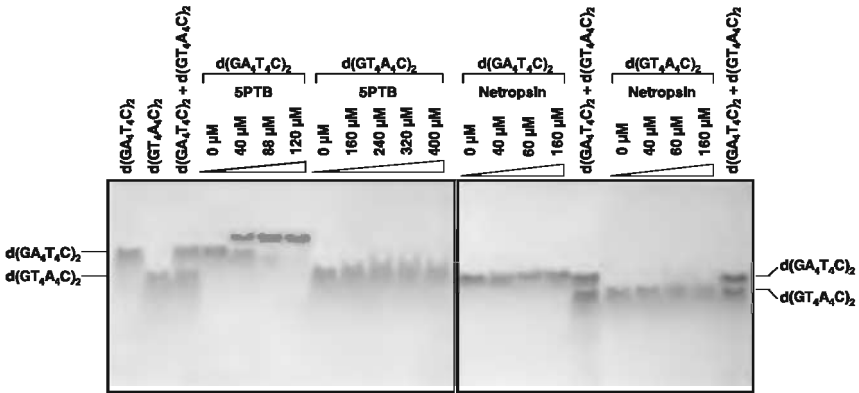


Fig. 21.14. Non-denaturing 28% polyacrylamide gels containing the $d(\text{GA}_4\text{T}_4\text{C})_2$ and $d(\text{GT}_4\text{A}_4\text{C})_2$ duplexes and their complexes with either 5PTB (left gel) or netropsin (right gel) at the indicated concentrations (see Ref. [61] for details of the experimental methodology).

with netropsin (Fig. 21.14) and DAPI (not shown) reveal little or no binding-induced retardation in duplex migration. Hence, it is unlikely that the observed 5PTB-induced retardation is due to differences in size, charge, or stiffness between the ligand-free and ligand-bound duplexes. Instead, this retarded mobility may be due to 5PTB-induced bending of both duplexes, which, for the $d(\text{GA}_4\text{T}_4\text{C})_2$ duplex, is synergistic with the endogenous sequence-directed helical curvature. In the aggregate, these observations are consistent with the hypothesis that minor groove-directed TB–DNA interactions that induce/stabilize bent DNA conformations may be important for TOP1 poisoning.

21.4.5.2 5PTB binding does not remove helical bends from kinetoplast DNA

Restriction fragments from *Leishmania tarentolae* kinetoplast DNA minicircles are known to adopt bent helical structures [118–120]. These globally bent DNA fragments arise from local bends that occur at the junctions between oligo(dA)–oligo(dT) tracts and adjacent segments of B-form DNA [111, 116, 117]. In order to yield a globally bent DNA fragment, the oligo(dA)–oligo(dT) tracts must occur in-phase with the periodicity of the DNA helix (i.e. every 10–11 base pairs) [111, 116, 117]. As noted in the previous section, a hallmark of bent DNA fragments is their anomalously slow migration in polyacrylamide gels [111, 116–118]. We investigated the impact of 5PTB binding on the gel mobility of a 264-bp kinetoplast DNA (k-DNA) fragment. For comparative purposes, we also analyzed the corresponding impact of the DNA minor groove-binding drug distamycin A (Dist A), which, unlike 5PTB, is inactive as a TOP1 poison [86]. The resulting electrophoretic analysis is shown in Fig. 21.15. Note that, in the absence of drug, the 264-bp k-DNA fragment migrates as if it has an apparent length of ≈ 600 bp. Further note that this anomalously slow migration is minimally altered by addition

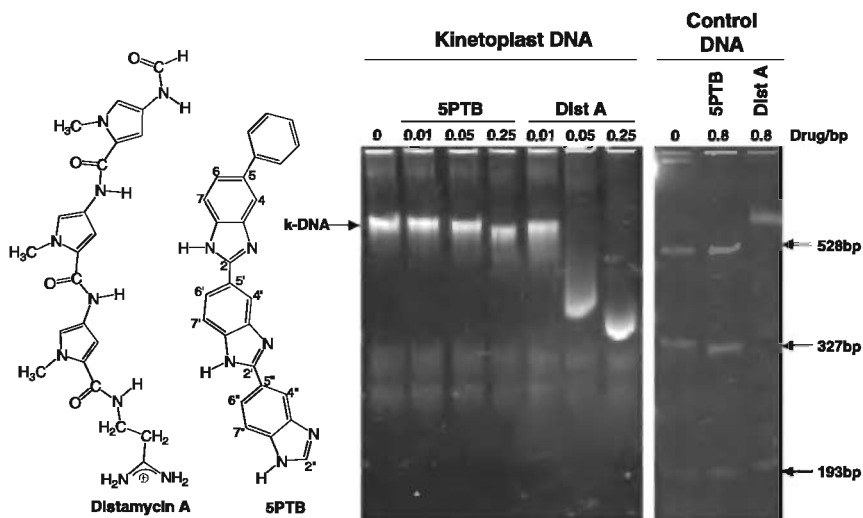


Fig. 21.15. Non-denaturing 6% polyacrylamide gel showing the impact of 5PTB and distamycin A (Dist A) binding on the mobility of a 264 base pair kinetoplast DNA (k-DNA) fragment (left gel). As a control, the gel on the right shows the corresponding impact of the

two DNA minor groove binding ligands on the mobilities of non-k-DNA fragments (resulting from a *Hind*III digestion of the *E. coli mh1* gene). The structures of 5PTB and Dist A are shown on the left.

of 5PTB. By contrast, addition of Dist A, which binds in the minor groove of A-T-tracts with a similar affinity to 5PTB, enhances the mobility of the k-DNA fragment to a near normal level, an observation consistent with the normalizing impact of Dist A on the electrophoretic mobilities of K-DNA fragments previously reported by the Crothers group [110].

Our observations suggest that 5PTB binding does not alter the sequence-directed bent state of the k-DNA fragment, while Dist A binding straightens the DNA fragment. In other words, the TOP1 poison (5PTB) does not remove pre-existing helical bends from DNA; in marked contrast to the inactive TOP1 poison (Dist A), which does. This result is consistent with our studies described in Section 21.4.5.1 probing the effects of 5PTB binding to a bent versus a normal decameric duplex. In the aggregate, both the k-DNA and the decamer studies are consistent with the notion that the induction and/or stabilization of DNA curvature may play a role in the poisoning of TOP1 by DNA minor groove-binding drugs, such as the TBs and Hoechst 33342.

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22

Binding and Reaction of Calicheamicin and Other Eneidyne Antibiotics with DNA

Joseph P. Cosgrove and Peter C. Dedon

22.1

Introduction

The US Food and Drug Administration recently approved the use of gemtuzumab ozogamicin for the treatment of relapsing acute myeloid leukemia [1, 2]. This antibody-targeted chemotherapeutic agent consists of a DNA-cleaving enediyne cytotoxin, calicheamicin, covalently attached to a humanized monoclonal antibody directed against the CD33 cell surface glycoprotein antigen expressed by blast cells in 90% of patients with acute myeloid leukemia [3]. While the selectivity of gemtuzumab ozogamicin is due to targeting of the antibody to leukemia cells, its potent cytotoxicity arises from the attached calicheamicin molecule. This potent cytotoxin is one of over a dozen members of the enediyne family of bacterially produced antibiotics, including esperamicins A1 and C, C-1027, kedarcidin, maduropeptin, dynemycin, and neocarzinostatin, the first enediyne discovered. Since enediyne chemistry has been given thorough coverage [4–9], this review represents an update of a 1992 review written by Dedon and Goldberg [5], with a focus on some of the most recent work on the mechanisms by which enediynes select their DNA targets and how cells respond when exposed to the drugs.

22.2

Sources, Biosynthesis, and Structural Conservation

All of the enediynes isolated to date are produced by eubacteria of the order Actinomycetales (Table 22.1). One of the most intriguing features of enediynes, a feature relevant to any family of bacterially produced antibiotics, is the remarkable conservation of the antibiotic structure. In addition to the conserved core structures of the several enediyne classes (Fig. 22.1), the various appendages are also highly conserved in both structure and function. This is not surprising in light of the emerging picture of enediyne biosynthesis, most notably for the biosynthetic pathways of calicheamicin and C-1027. The Thorson group has cloned many of the calicheamicin biosynthetic genes from *Micromonospora echinospora* [18].

Tab. 22.1. Bacterial species producing enediyne antibiotics.

<i>Eneidyne</i>	<i>Producing species</i>	<i>Ref.</i>
Calicheamicin γ_1^I	<i>Micromonospora echinospora</i>	10
Esperamicin A1	<i>Actinomadura verrucosospota</i>	11
Neocarzinostatin	<i>Streptomyces carzinostaticus</i>	12
C-1027	<i>Streptomyces globisporus</i>	13
Kedarcidin	Unidentified actinomycete	14
Namenamicin	Unidentified actinomycete	15
Dynemicin	<i>Micromonospora chersina</i>	16
Maduropeptin	<i>Actinomadura madurae</i>	17

They have proposed a plausible biosynthetic model involving synthesis of the alkycone followed by attachment of the carbohydrate side chain by a series of glycosyltransferases with flexible substrate selectivity, a flexibility that likely extends to other actinomycete species. The conservation of the structure and function of the decorations of the enediyne core is illustrated with several examples. First, the deoxyfucose-anthranilate of esperamicin A1 is analogous to the terminal carbohydrate-aromatic group of the side chain of calicheamicin γ_1^I (Fig. 22.1), though located on the opposite side of the enediyne core. The trisaccharide of esperamicin A, is highly conserved in calicheamicin γ , namenamicin, and esperamicin C (Fig. 22.1). Another feature of structural conservation, though of unknown function, is the presence of ansa bridges in maduropeptin, C-1027, and kedarcidin (Fig. 22.1).

This conservation of structure also extends to function. For example, neocarzinostatin, C-1027, and esperamicin A1 all possess functional groups that confer an intercalative binding mode: the naphthoate of neocarzinostatin [19], the benzoxazolate of C-1027 [20] and the anthranilate of esperamicin A1 [21, 22]. The structure and physical properties of the anthranilate of esperamicin A1 make it unusual among intercalating agents. The deoxyfucose-anthranilate of esperamicin A1 does not by itself interact with DNA, yet it contributes 1–2 kcal mol⁻¹ to the DNA-binding energetics of the drug molecule [22]. This is similar to the situation with the naphthoate of neocarzinostatin [19]. The basis for intercalation of the *N*-(2-methoxyacrylyl) anthranilate group is its ability to adopt a planar structure capable of insertion between base pairs. Indeed, the benzoxazolate groups present in the enediyne C-1027 [23–25] and in the chromophore of auromomycin [26] represent bicyclic analogs of the anthranilate of esperamicin A1, in which the terminal oxygen of the methoxyacrylyl group is bonded to the aromatic ring. On the basis of this observation, the Dedon group demonstrated that the benzoxazolate of C-1027 confers an intercalative binding mode [20]. It is thus a reasonable and easily verified prediction that the naphthoate moieties of kedarcidin and N1999A1 serve as DNA intercalators.

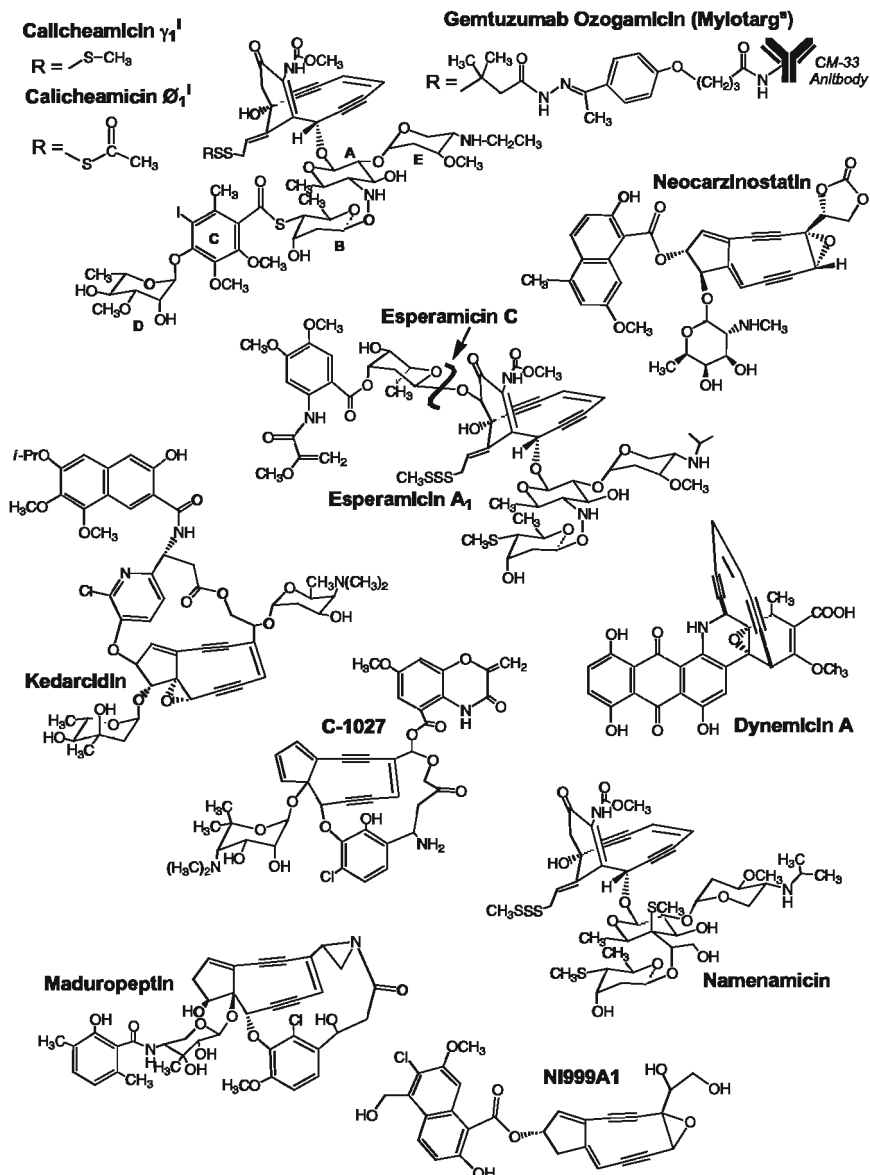


Fig. 22.1. Structures of natural and synthetic enediynes and gemtuzumab ozogamicin (Mylotarg).

22.3

Mechanisms of Target Recognition

The earliest studies of enediynes pointed to a minor groove location for drug binding and DNA damage. This conclusion was based on competitive binding studies and the 3'-stagger of cleavage sites on opposing strands (27, 28). In the intervening years, however, numerous structural, biophysical, and biochemical studies have revealed a more complicated set of determinants for the recognition of DNA targets by enediynes, with the bulk of the studies focusing on esperamicin and calicheamicin.

22.3.1

Overall Scheme for Binding and Activation

A likely scenario for a target search by calicheamicin starts with non-specific binding to DNA, possibly by superficial association in the hydrophobic minor groove, followed by tracking along the groove or along the outer surface of the helix in search of a target site. This is a reasonable model given the established one-dimensional diffusion or sliding of proteins along the DNA helix [29, 30]. The positive charge of calicheamicin might be expected to facilitate an approach to the negatively charged sugar-phosphate backbone, while the hydrophobic properties of the drug molecule [31] and its structural complementarity to the minor groove [32–34] would provide further stability to a superficial association of the drug with DNA. Binding to a specific site then occurs with micromolar to nanomolar affinity [22, 35–37] determined by the physical and chemical properties of both the drug and the DNA-binding site, as discussed shortly. For example, a compatible site for calicheamicin binding optimizes local structural flexibility to accommodate minor groove widening [32, 33, 38] and DNA bending [39], as well as hydrogen bonding and van der Waals contacts in the minor groove [39].

Whether it occurs before or after target site binding, DNA damage by calicheamicin at any site requires reduction of the methyltrisulfide, most likely by cellular glutathione, to produce the diradical intermediate (Fig. 22.2). However, it remains a matter of some controversy as to whether the reduction of the methyltrisulfide of calicheamicin and esperamicin takes place with the drug bound at the target site, in a loosely bound configuration, or in solution. The recent studies of Townsend and co-workers demonstrate that the rate of methyltrisulfide reduction by glutathione is slowed by a factor of 1.5–4 when calicheamicin is bound to DNA compared with reactions in solution [40]. This modest slowing is consistent with other observations of glutathione interactions with DNA, which lead to the conclusion that repulsion of negative charge of the tripeptide and the negatively charged sugar-phosphate backbone limits access of glutathione to reactions in the minor groove of DNA [41–44]. However, Townsend has also shown that the relative stability of the post-reduction, dihydrothiophene intermediate (Fig. 22.2; $t_{1/2} \sim 3$ s) allows it to bind to DNA and equilibrate with the target site prior to formation of the diradical [35]. Indeed, the relative stability of the dihydrothiophene suggests

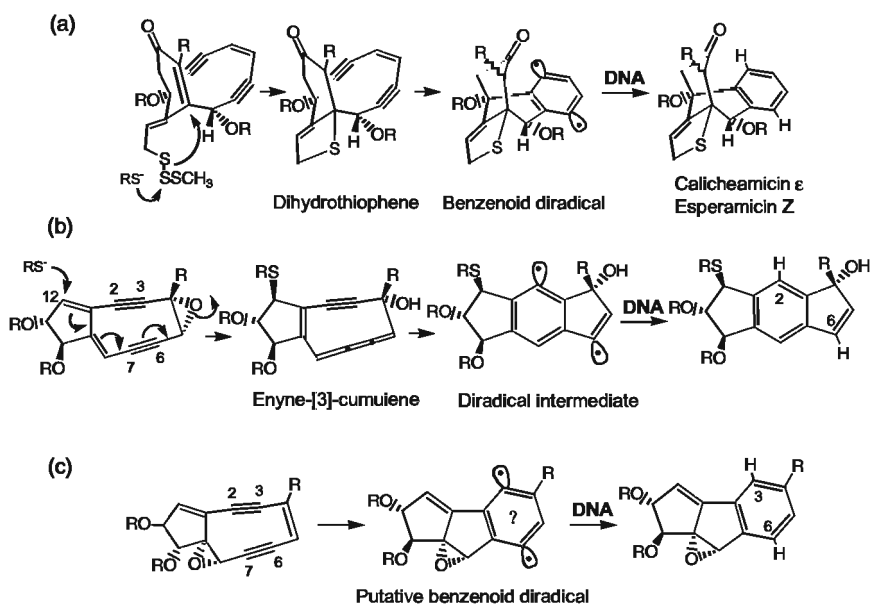


Fig. 22.2. Mechanisms of formation of the diradical intermediates by various enediyne. (a) Reductively-induced Bergman cyclization of the bicyclo[7.3.1]tridecadiynene core of calicheamicins, esperamicins, and probably namenamycin. (b) Nucleophilic attack at the C12 of neocarzinostatin and related enediyne chromophores results in the formation of an enyne-[3]-cumulene intermediate that undergoes cyclization between the C3 and

C7 positions to form the C2/C6 diradical intermediate capable of hydrogen atom abstraction. (c) The enyne-[3]-cumulene can also cyclize by an alternative pathway involving ring formation between the C2 and C7 radical centers to form a benzenoid diradical [203], a pathway consistent with the final structures of the aromatized forms of kedarcidin [204] and C-1027 [25].

that glutathione-mediated reduction of calicheamicin could occur virtually anywhere in a cell with diffusion of the molecule to mitochondrial or nuclear DNA for subsequent formation of the diradical. Given these points and the identical sequence selectivities of the parent calicheamicin [12], the dihydrothiophene [35], and the aromatized calicheamicin ϵ (Fig. 22.2) [33, 37], it seems reasonable to conclude that the question of the location of the calicheamicin activation event is moot from the standpoint of biological consequence and that there is no requirement for activation of a DNA-bound drug molecule.

22.3.2

Structure of the Calicheamicin–DNA Complex

A series of NMR studies over the past decade has produced a nearly complete picture of the structure of the DNA complexes of calicheamicin [32–34, 38, 45–47] and esperamicin [21, 48]. Dinshaw Patel's group has provided what are arguably

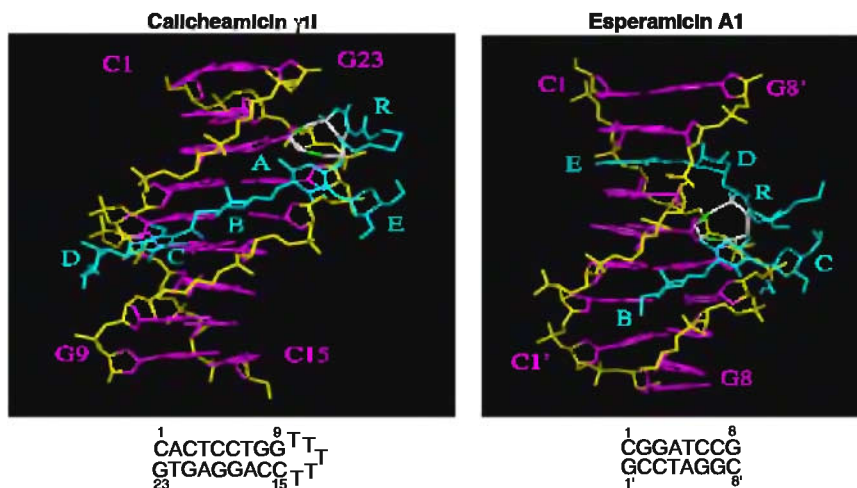


Fig. 22.3. NMR structural models for the DNA complexes of calicheamicin γ_1 (left, Ref. [38]) and esperamicin A1 (right, Ref. [48]). The calicheamicin complex was prepared with a hairpin deoxyoligonucleotide shown beneath the image, while the esperamicin complex

was prepared with a self-complementary deoxyoligonucleotide. The letters correspond to the various components of the side chains, as shown in Fig. 22.1. These images were kindly provided by Dr Dinshaw Patel, Memorial Sloan Kettering Cancer Center.

the most comprehensive studies of the structures of the calicheamicin and esperamicin DNA complexes, as shown in Fig. 22.3.

In all sequences studied (AGGA·TCCT, AGGT·ACCT, AAAA·TTTT), calicheamicin binds to DNA in the minor groove with the aglycone positioned at the 3' end of the purine tract and the carbohydrate tail extending in the 5' direction [32–34, 38, 45]. The drug adopts a right-handed screw conformation that complements the shape of the B-DNA minor groove, with numerous hydrogen bonding and van der Waals contacts. In the AGGA sequence, there are hydrogen bonds between the iodine and sulfur atoms of calicheamicin and the amino protons of the 5'- and 3'-guanines [32, 38]. In spite of the significance proposed for the iodine–dG interaction, which was predicted in modeling studies (49), it is clearly not a critical feature of calicheamicin target recognition given the high-affinity binding and efficient cleavage of (A)_n·(T)_n sequences [50] and other purine runs not containing the appropriately placed dG targets [28, 35, 37, 39, 51–54].

A prominent feature of the NMR studies is the critical role of the aryl tetrasaccharide in defining the sequence selectivity of calicheamicin, an observation that is entirely consistent with the earlier biochemical studies [51]. The aryl tetrasaccharide binds in essentially the same orientation with or without the aglycone and orients the C3 and C6 pro-radical centers near the cleavage sites (Fig. 22.3) [32, 33, 46, 47]. Kahne and co-workers demonstrated that the aryl tetrasaccharide is a relatively rigid structure that does not undergo major conformational changes

upon binding of calicheamicin to DNA [34, 55]. On the basis of studies in AGGT·ACCT and AAAA·TTTT sequences, they argue that DNA conformational changes in the vicinity of the aryl tetrasaccharide, which were observed to be larger in the pyrimidine strand than the purine strand, could arise as a result of an asymmetrically positioned and rigid aryl tetrasaccharide exerting steric pressure on the pyrimidine strand [34].

Another drug-induced change in DNA structure occurs in the vicinity of the calicheamicin aglycone. Patel and co-workers observed that a tilted aglycone spans a widened minor groove while the cycloaromatized aglycone lies parallel to the helix axis [32, 38]. The NMR studies of Nicolaou and co-workers suggest a degree of motion of the aglycone, with the observation of two slowly exchanging binding modes [33]. A widening of the minor groove upon drug binding is consistent with inhibition of calicheamicin-induced cleavage in the presence of a protein that binds in the major groove and widens it opposite the cleavage site [56], and with the observation of DNA bending by calicheamicin [39].

22.3.3

Esperamicin Structure and Function

Esperamicin A1 has not received the attention paid to calicheamicin in spite of similar structures and cytotoxic potencies, possibly due to its weak sequence selectivity [27] and less efficient production of double-strand DNA lesions [22, 57, 58]. Beyond the early observation of a minor groove binding mode for esperamicin A1, it was recognized that the deoxyfucose anthranilate side chain (Fig. 22.1) caused a reduction in the frequency of double-stranded DNA lesions [27]. The first indication of a role in DNA binding for the sugar-aromatic group came from the Dedon group's studies of esperamicin-induced DNA damage in chromatin [59]. The drug was found to cleave DNA in chromatin only in the linker region between adjacent nucleosome cores [59], a behavior reminiscent of other DNA-intercalating agents such as bleomycin [60, 61]. Removal of the deoxyfucose-anthranilate to form esperamicin C (Fig. 22.1) caused the enediyne to cleave DNA in both the core and linker regions of the nucleosome, as observed with calicheamicin [59].

Formal confirmation of the intercalative properties of the anthranilate of esperamicin A1 was provided by the Dedon group's subsequent biophysical studies [22]. The structure and physical properties of the methoxyacrylyl-anthranilate make it unusual among intercalating agents. It had no apparent affinity for DNA yet contributed 1–2 kcal mol⁻¹ to the DNA-binding energetics of esperamicin A1 [22]. As noted earlier, the benzoxazolate groups present in the enediyne C-1027 [23–25] and in the chromophore of the antitumor antibiotic auromomycin [26] represent planar analogs of the anthranilate in which the terminal oxygen of the methoxyacrylyl group is bonded to the aromatic ring (Fig. 22.1). As a demonstration of conserved structure and function in the enediynes, the Dedon group subsequently proved that the benzoxazolate of C-1027 also functioned as a DNA intercalator [20].

The intercalative function of the anthranilate was also apparent in the only

NMR study of the esperamicin A1–DNA complex, again the work of Patel and co-workers [21, 48]. Though esperamicin produces DNA damage with weaker sequence selectivity than calicheamicin, the self-complementary CGGATCCG chosen for the esperamicin study produced only one complex. Consistent with the biophysical studies [22, 59], esperamicin was found to bind to DNA by intercalation of the methoxyacrylyl-anthranilate moiety between the GG·CC step. Additional bonding interactions are provided by the trisaccharide moiety lying in the minor groove. The pro-radical centers of the enediyne core were positioned near the deoxyribose 5'-hydrogen atom (pro-S) on one strand and the 1'-hydrogen atom on the other strand. Again, this is consistent with the known deoxyribose oxidation chemistry associated with esperamicin A1-induced double-stranded lesions [22]. The only major DNA structural changes associated with esperamicin binding are unwinding of the helix, due to the anthranilate intercalation, and widening of the minor groove in the vicinity of the enediyne core, as observed with calicheamicin.

22.3.4

Structure and Dynamics of Calicheamicin Binding Sites

Calicheamicin target selection was first proposed to involve G·C-containing tetrapurine sequences such as AGGA·TGGT [62, 63]. This led to the proposal of a critical interaction between the aryl iodide atom and the exocyclic N2 of dG [49], which, as discussed earlier, is clearly not critical for calicheamicin target recognition. The contribution of hydrophobic interactions to the stability of the calicheamicin–DNA complex [31] suggests another mechanism for targeting of tetrapurine sequences by the drug, which involves an increase in entropy due to the displacement of water molecules. The basis for this model is the observation that homopurine sequences are more hydrated than mixed sequence DNA [64], which leads to the expectation that entropic effects should contribute substantially to the thermodynamics of calicheamicin binding to oligopurine tracts. However, recent studies have revealed a more complicated picture of the structure and dynamics of calicheamicin-binding sites.

Over the past decade, the concepts of shape selection, as opposed to reading of the DNA sequence *per se*, and induced-fit target selection have been invoked to account for the complexity of small molecule–DNA interactions. These two principles can also be applied to the mechanisms by which enediynes select their DNA targets. Kahne and co-workers proposed that calicheamicin target selection involves DNA sequences that can most easily undergo conformational changes to accommodate binding by a relatively rigid drug molecule [45, 55]. However, the strongest support for shape recognition and induced-fit binding has come from recent biophysical and biological studies of calicheamicin [39, 54, 56, 65].

With regard to shape selection, several observations beyond the broad range of sequences bound by calicheamicin [50, 53, 54, 63, 66] suggest a direct role for DNA conformation in the target recognition process. The first is that the quantity of calicheamicin-induced DNA damage is affected by sequences outside the binding site. For example, damage at the C of AGGGTC is 40% greater than at the G of AGGGTG [36, 54, 66] even though the bulk of the bonding contacts occur in the

AGGG sequence. A second important observation involves the enhancement of calicheamicin-induced DNA damage in DNA incorporated into a nucleosome [54, 65]. Using the 5S rDNA model nucleosome system, the Tullius and Dedon groups demonstrated that the quantity of DNA cleavage at the 3' end of a purine tract in the 5S rDNA sequence increased 4- to 10-fold when the DNA was wrapped around the histone octamer of the nucleosome core [52, 54, 65]. The damage site was situated such that the bending of the DNA created a widened minor groove facing away from the histone core. Of several mechanisms that could account for this phenomenon, it is likely that a change in the topology of this calicheamicin-binding site caused an increase in its reactivity. A recent study by Sissi *et al.* lends support to this model [56]. They observed that transcription factor-induced changes in DNA conformation affect calicheamicin binding and damage, with protein-mediated narrowing of the minor groove causing a reduction in drug-induced cleavage [56]. However, on the basis of calicheamicin-induced cleavage frequency in poly(A) tracts, Tullius and co-workers proposed that calicheamicin prefers sequences with a narrow minor groove [50]. These apparently conflicting interpretations of cleavage data rule out minor groove width as a major determinant of calicheamicin target recognition. Instead, several groups have argued that high-affinity binding of calicheamicin occurs with DNA sequences that readily undergo conformational changes to accommodate the shape of the drug [45, 50, 54].

This induced-fit model of calicheamicin binding can be viewed as an active or passive form of shape selection. There are two scenarios here: (1) trapping a DNA conformation that exists in a population of conformers; and (2) binding-induced changes in DNA structure. Either mechanism is consistent with the results of circular dichroism [67], DNA sedimentation [68], and NMR studies [33, 34, 69] that point to calicheamicin-associated changes in DNA conformation. The key to understanding the relationship between calicheamicin binding and the malleability of DNA structure may lie in the observation made by Tullius and co-workers that calicheamicin prefers the 3' end of an A-tract and more generally by the Dedon group that calicheamicin targets the 3' ends of purine tracts, not simply tetrapurine sequences [50, 54]. In the case of A-tracts, there is a helical deformation at the 3' end of the tract, most probably a kink or bend [70–72]. So, it is possible that calicheamicin prefers to bind to bent DNA, a model supported by the nucleosome studies discussed earlier [52, 54, 65]. However, preference for bent DNA, which may be viewed as simple shape selection, does not explain binding at mixed-sequence targets such as AGGA and ACAA. These sequences do not possess intrinsic curvature, as revealed by gel-mobility studies performed by the Dedon group, as discussed later [39].

These observations led Dedon and co-workers to propose a model in which calicheamicin targets DNA sequences that are bent or that can induce or trap a bent conformation. That a bent or flexible sequence element should occur in calicheamicin-binding sites is wholly consistent with observations of A- and G-tract DNA that suggest the existence of a structural discontinuity at the 3' end of homopurine sequences [73, 74]. To test this hypothesis, two different DNA cyclization techniques were used to assess the ability of calicheamicin ϵ , the aromatized form of calicheamicin γ_1^1 (Fig. 22.2), to bend DNA upon binding [39]. Using oli-

gonucleotide constructs containing two known binding sites (AGGA·TCCT and ACAA·TTGT) in phase with the helical repeat, it was observed that calicheamicin binding caused a shift to smaller circle sizes in T4 ligase-mediated circle formation assays. A substantially smaller shift was observed with oligodeoxynucleotide constructs containing binding sites positioned out of phase with the helical repeat. It was also observed that binding of calicheamicin ϵ to a 273-bp construct with phased binding sites caused an increase in the molar cyclization factor, J , from 8×10^{-8} to 9×10^{-6} M. Both sets of results prove that drug-induced DNA bending contributes to the overall changes in DNA conformation that occur upon binding of calicheamicin [33, 34, 67–69].

The observation of calicheamicin-induced DNA bending is entirely consistent with an induced-fit model for calicheamicin binding to DNA. Kahne and co-workers proposed that a rigid aryl tetrasaccharide [55] exerts steric pressure on the pyrimidine strand of the binding site, which leads to widening of the minor groove [34, 45]. It is not surprising that DNA bending is not grossly apparent in the NMR studies given the limited ability to detect all but the sharpest deflections of the helix axis. Though the bending experiments do not permit an assessment of directionality, minor groove widening suggests bending toward the major groove. This is consistent with the reduction of calicheamicin damage that occurs as a result of protein-induced bending and subsequent narrowing of the minor groove [56] and with the enhancement of calicheamicin-induced damage when the minor groove is widened by bending of DNA around a histone octamer [52, 54, 65].

These observations permit some refinement of the calicheamicin target search model. Sampling of sites in the minor groove involves optimization of the various hydrogen-bonding and van der Waals interactions with the bases and sugar-phosphate backbone [33, 34, 51, 69, 75]. The maximum number of bonding contacts can only be achieved when the helix is bent or flexible enough to accommodate bending and widening of the minor groove. In cases where the DNA is bent, such as A_4 sequences, the energetic cost of drug binding is reduced by external forces that either bend or move the DNA. However, for “straight” DNA targets, a balance must be achieved between the intrinsic resistance of DNA to movement and those forces (e.g. electrostatic and hydrophobic) involved in the binding of calicheamicin to DNA. In addition, since the binding characteristics of individual sequences are heterogeneous, we should expect not one, but several, optimal binding scenarios, each unique to a particular sequence. As a result, the amount of bending induced need not be uniform across all calicheamicin-binding sites. Indeed, factors such as bonding interactions between the aryl iodide and the N2 of a local dG likely serve to modulate the binding interaction.

22.3.5

Neocarzinostatin Recognition of Bulged DNA Structures

While most of the studies with enediynes have focused on B-DNA targets, the Goldberg group has made major inroads into defining the role of non-B-DNA sec-

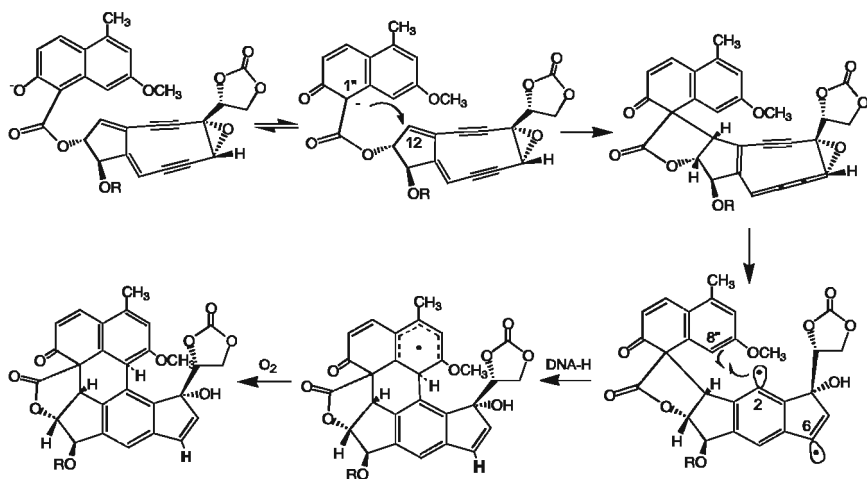


Fig. 22.4. Thiol-independent base-catalyzed activation of neocarcinostatin that occurs selectively at DNA bulges as described by Goldberg and co-workers [80]. An enolate anion at the C1'' position of the naphthoate adds to the C12 position of the enediyne core

and causes formation of the cumulene intermediate and subsequent diradical. While the C2 radical center attacks the naphthoate moiety, the C6 radical is free to abstract a hydrogen atom from the deoxyribose in the nucleotide 3' to the bulge.

ondary structure, specifically bulges, in small molecule–DNA interactions. They observed that necarcinostatin not only binds with micromolar affinity to unpaired loops of two to five nucleotides [76], but that the local DNA conformation in a two-base bulge causes the drug to undergo a thiol-independent activation to produce DNA cleavage and adduct formation [77]. A similar phenomenon occurred with RNA–DNA hybrids, but to a lesser extent [78]. Goldberg and co-workers have presented strong chemical [76–81] and structural [82] evidence in support of a highly atypical and elegant mechanism for this DNA conformation-specific enediyne activation.

As shown in Fig. 22.4, the naphthoate moiety, normally an intercalator, undergoes a general base-catalyzed formation of an enolate anion at the C1'' position that adds by Michael addition to the C12 position of the enediyne core, the site at which thiol normally adds to initiate formation of the diradical intermediate [80, 81]. Following formation of the diradical, the C2 radical center attacks the C8'' in the naphthoate to form a cyclospironolactone species [80, 81] and the C6 radical is free to abstract the 5'-hydrogen atom of deoxyribose in the nucleotide 3' to the bulge [77, 79]. The basis for recognition of the DNA bulge appears to involve the complementary fit of the wedge-shaped active cyclospironolactone intermediate in the major groove of the pocket formed by the looped out bases [82]. The biological relevance of this bulge-specific targeting and drug activation is highlighted by the established formation of bulges in repetitive sequences [83] and as a consequence of alignment slippage during DNA replication [84].

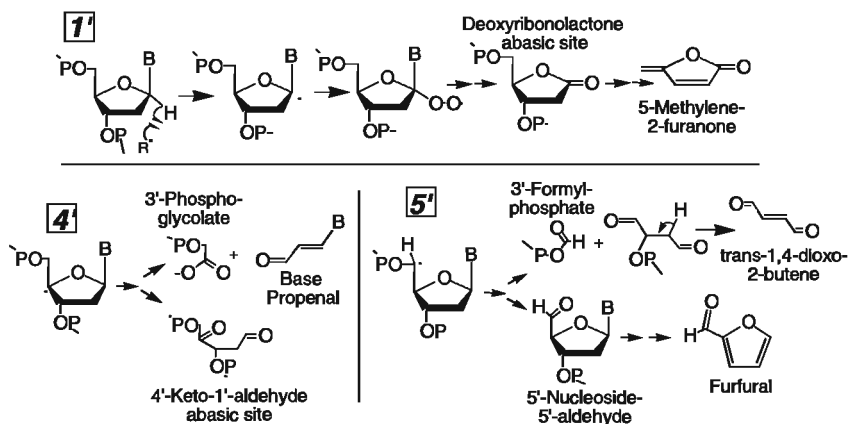


Fig. 22.5. Products of enediyne-induced deoxyribose oxidation in DNA.

22.4

Products of Enediyne-induced DNA Damage

As discussed earlier, enediynes cause DNA damage by forming a diradical species that is capable of abstracting hydrogen atoms from deoxyribose in DNA. The minor groove specificity of the enediynes limits the radical-mediated hydrogen atom abstractions to the 1'-, 4'-, and 5'-positions of deoxyribose, with a spectrum of products shown in Fig. 22.5. Given the numerous reviews of the deoxyribose oxidation chemistry associated with enediynes and other radical-mediated agents such as bleomycin and ionizing radiation [12, 16, 85, 86], this section will address only the recent studies of the chemistry and biology of deoxyribose oxidation relevant to the enediynes.

22.4.1

Proportions of Single- and Double-stranded DNA Lesions Produced by Eneidiynes

One of the unique features of enediynes is their ability to produce double-stranded DNA lesions by the action of a single drug molecule [41, 87]. Like restriction endonucleases with relatively weak sequence selectivity, the diradical form of all of the enediynes can abstract deoxyribose hydrogen atoms from both strands of DNA to produce lethal double-stranded DNA lesions. While esperamicin A1 [22, 57, 58, 88], C-1027 [23, 89], kedarcidin [90], and neocarzinostatin [41, 87] all produce more single-stranded lesions than bistranded damage, the Dedon group found that calicheamicin and esperamicin C produce double-stranded DNA lesions almost exclusively [22, 91]. This might lead one to conclude that calicheamicin should be the most potent enediyne given the strong correlation between double-stranded lesions and cytotoxicity [92]. However, there has been no systematic comparison of the various enediynes with regard to relative cytotoxic potency.

The presence of the anthranilate of esperamicin A1 causes important changes in the chemistry of esperamicin A1-induced DNA damage. The Stubbe and Kozarich groups had previously demonstrated that esperamicin C produces double-stranded lesions involving both 4'- and 5'-oxidation of deoxyribose [58]. They and others concluded that esperamicin A1 causes only single-stranded lesions involving 5'-oxidation of deoxyribose [57, 58]. However, the Dedon group found that 25% of the DNA damage produced by esperamicin A1 consists of double-stranded DNA lesions composed of a strand break, generated by 5'-oxidation of deoxyribose, positioned opposite an abasic site derived from [1'-oxidation] of deoxyribose [22]. The latter is likely to be the deoxyribonolactone abasic site observed with neocarzinostatin (Fig. 22.5) [93]. Abasic sites are often overlooked in experiments employing gel-mobility shifts to identify deoxyribose oxidation products, especially when the abasic site is present as a minor component of the deoxyribose oxidation products.

The basis for the different proportions of single- and double-stranded lesions produced by esperamicins A1 and C was addressed by the Stubbe and Kozarich groups [88]. They demonstrated that DNA is the source of hydrogen atoms abstracted by both radical centers in activated esperamicin A1 [58], which raised the questions about the basis for the paucity of esperamicin A1-induced double-stranded lesions. The answer involved chemical repair of the deoxyribose radicals by the reductant used to activate the enediyne (Fig. 22.2). With esperamicin C, though surprisingly not esperamicin A1, glutathione was less efficient at chemical repair of the deoxyribose radical and thus caused more double-stranded lesions than neutral thiols such as dithiothreitol, which were used in the original studies of Stubbe and Kozarich [58]. The likely basis for the inefficient repair of deoxyribose radicals by the negatively charged glutathione is its exclusion from the minor groove by charge repulsion by the sugar-phosphate backbone, as observed in studies of radiation-induced DNA damage (e.g. Refs [94, 95]) and in the Dedon group's studies of DNA damage produced by calicheamicin [44].

The switch from 1'/5' to 4'/5' oxidation chemistry for esperamicin C is analogous to the different chemistries produced by neocarzinostatin and C-1027 at different binding sites and, for C-1027, within the same binding site. Neocarzinostatin was long ago shown to produce bistranded DNA lesions at AGC-GCT sites by a combination of 1'- and 5'-oxidation, respectively, while oxidation of AGT-ACT sites involved 4'- and 5'-chemistry, respectively (reviewed in Ref. [12]). More recently, the Goldberg group has shown that C-1027 produces double-stranded lesions at a variety of sequences but most prominently at the GTTAT-ATAAC sequence [23]. Within this single binding site, there are two different bistranded lesions produced. One involves 4'- and 1'-oxidation of the A₁ and A₂ positions, respectively, in the GTTA₁T-ATA₂A₃C sequence, while the other bistranded lesion involves 4'- and 5'-oxidation of the A₁ and A₃ sites [96]. A similar shuttling between different deoxyribose hydrogen atoms at a single binding site has also been observed with neocarzinostatin [97]. This pronounced heterogeneity of deoxyribose oxidation chemistry suggests a high degree of conformational flexibility for the C-1027 and neocarzinostatin enediyne cores. However, similar effects have not

been demonstrated with calicheamicin and esperamicin A1. Townsend and co-workers demonstrated a strong specificity for 5'- and 4'-oxidation of deoxyribose in calicheamicin-induced bistranded lesions, though this was in a single DNA sequence [98]. It is quite likely that there is some variation in the cleavage chemistry as a function of the binding site conformation to greater or lesser extent for all enediynes, but more sensitive and direct chemical analysis of the deoxyribose oxidation products will need to be applied to test this hypothesis.

22.4.2

Oxidation of the 1'-Position of Deoxyribose and the Biochemistry of the Deoxyribonolactone Abasic Site

The deoxyribonolactone abasic site (Fig. 22.5) is the product of 1'-hydrogen atom abstraction by neocarzinostatin [93], copper-phenanthroline [99], and radiation [100, 101]. This repertoire of 1'-oxidants has now been expanded to include esperamicin A1 [22] and C-1027 [96]. While there is no direct chemical evidence for the formation of the deoxyribonolactone by these agents, it is highly likely given the precedent with neocarzinostatin and the production of the abasic site as a general feature of 1'-oxidation of deoxyribose.

The deoxyribonolactone abasic site has now been successfully synthesized by several independent routes [102–104] and the abasic site has been the subject of several important studies, including an NMR study of the effect of the abasic site on DNA structure by the Lhomme group [105]. In terms of biological consequences, Greenberg and co-workers have collaborated with the Cunningham and Demple groups to study the repair of the abasic site by endonuclease III [106] and the effect of the abasic site on the DNA repair polymerase beta [107]. The striking feature of both studies is the formation of covalent protein–DNA crosslinks between the deoxyribonolactone and the repair enzymes. In the case of endonuclease III, an active site lysine, that normally forms a Schiff base with the C1-aldehyde of a native abasic site, reacts with the C1 of the deoxyribonolactone to form a stable amide linkage (Fig. 22.6). The crosslink forms with an amazingly high frequency of 20%, at least *in vitro*, which suggests that the futile repair of this lesion in cells will result in a high level of protein–DNA crosslinks. With regard to polymerase beta, preliminary processing of a normal abasic site by the human abasic endonuclease, APE1, leaves a 5'-terminal abasic site residue that is subsequently removed

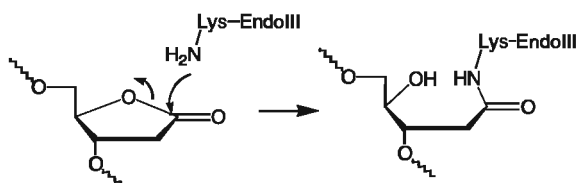


Fig. 22.6. Formation of a protein–DNA crosslink during processing of the deoxyribonolactone abasic site, derived from 1'-oxidation of deoxyribose, by endonuclease III, as proposed by the Greenberg and Cunningham groups [106].

by polymerase beta [107]. However, the presence of the deoxyribonolactone, while efficiently incised by APE1, again leads to the formation of an amide linkage between the C1 position of the abasic site and an active site lysine of the polymerase [107]. These protein–DNA crosslinks are likely to pose a serious impediment to further DNA repair.

22.4.3

Oxidation of the 4'-Position of Deoxyribose and the Chemistry of Base Propenal

The chemistry of 4'-oxidation of deoxyribose partitions along two pathways to form a 4'-keto-1'-aldehyde abasic site or a strand break composed of a 3'-phosphoglycolate residue, a base propenal, and a 5'-phosphate-ended fragment (Fig. 22.5). This pathway has been very well defined for enediynes and bleomycin [12]. However, the results of recent studies with gamma-radiation stand in contrast to earlier findings and suggest that the base propenal is not a constant feature radical-induced 4'-oxidation of deoxyribose. Henner and co-workers had tentatively identified thymidine propenal as a product of gamma-irradiation of thymidine deoxynucleoside and proposed that base propenals account for the thiobarbituric acid-reactive species generated by gamma-irradiation of DNA [108]. However, in a recent chromatographic study, von Sonntag and co-workers failed to find base propenals in irradiated DNA [109]. This suggests that the mechanism of degradation of the 4'-radical center in deoxyribose does not follow the same pathways for small oxidants and gamma-radiation. Further confirmatory studies are needed here, as are cellular studies to define the role of the *in vivo* biochemical environment on deoxyribose oxidation chemistry.

The biological consequences of base propenals, including those generated by enediynes, have also been the subject of study by several groups. In terms of cellular defense mechanisms against these electrophiles, Mannervick and co-workers observed that base propenals are highly efficient substrates for several classes of glutathione transferase, especially glutathione transferase P1-1 [110]. Given the nuclear location of several glutathione transferases [111], it is possible that these enzymes serve to protect the cell against electrophilic deoxyribose oxidation products generated by endogenous oxidative processes. On the other hand, Grollman and co-workers observed that base propenals possess cytotoxic properties [112], possibly by virtue of inhibition of thymidylate synthesis [113]. Dedon and co-workers have recently discovered that base propenals react with dG to form the mutagenic pyrimidopurinone adduct, M₁G (Fig. 22.7) [114], an adduct originally attributed to the lipid peroxidation product malondialdehyde [115]. Calicheamicin and bleomycin, both of which cause formation of base propenals [12], were shown to react with DNA to form M₁G [114]. Interestingly, gamma-radiation did not cause formation of M₁G [116], with is consistent with von Sonntag's observation of a lack of base propenal in irradiated DNA [109]. The ability of base propenal, and possibly other electrophilic products of deoxyribose oxidation, to react with nucleobases to form DNA adducts raises the possibility that a single activated enediyne molecule can cause three or more DNA lesions: two from the immediate reaction

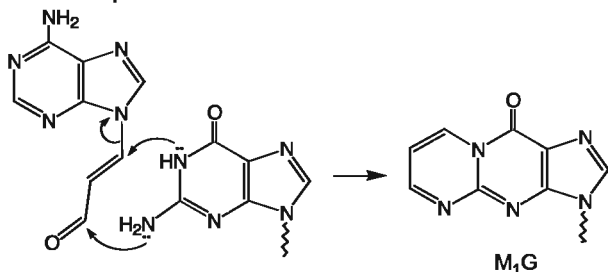
Adenine Propenal

Fig. 22.7. Reaction of dG with adenine propenal, derived from 4'-oxidation of deoxyribose, to form M₁G, the pyrimidopuranone adduct of dG [114].

of the radical centers with deoxyribose on each strand and one or more adducts derived from reaction of neighboring bases with the deoxyribose oxidation products. This would represent a form of “complex lesion” that has been argued to be a hallmark of ionizing radiation [117]. Such a lesion poses an obvious impediment to DNA repair and could contribute to the potent cytotoxicity of enediynes antibiotics.

22.4.4

Oxidation of the 5'-Position of Deoxyribose and the Chemistry of Butenedialdehyde

The Dedon group recently discovered that 5'-oxidation of deoxyribose results in the formation of a [*trans*]-1,4-dioxo-2-butene product that is likely derived by β -elimination of a [2 phosphoryl-1,4-dioxobutane] residue, as shown in Fig. 22.5 (Chatterji, Venkitachalam, and Dedon, submitted for publication). This four-carbon fragment completes the picture of deoxyribose 5'-oxidation chemistry [12], with partitioning of the reaction pathway to form an accompanying 3'-formylphosphate residue or a nucleoside-5'-aldehyde residue (Fig. 22.5). Again, it was demonstrated that this potent electrophile reacts with dC in a highly efficient manner to form a novel bicyclic oxadiazabicyclooctamine adduct, as shown in Fig. 22.8 [118], which in preliminary studies was observed in DNA oxidized by ionizing radiation (Chatterji and Dedon, unpublished observation). This second example of base adduct formation from deoxyribose oxidation products suggests an important role for this phenomenon in the spectrum of DNA lesions produced by enediynes and other oxidizing agents.

22.4.5

Covalent Adducts of Eneidiynes with Deoxyribose

In addition to DNA cleavage, enediynes have also been shown to form covalent adducts with deoxyribose in DNA. This phenomenon was first described by Goldberg and Povirk for neocarzinostatin [119, 120]. They observed the formation of a

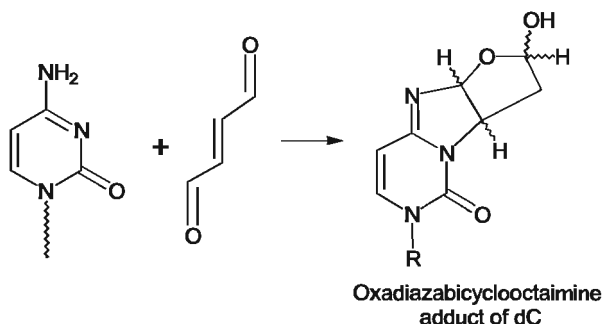


Fig. 22.8. Reaction of dC with butenedialdehyde, derived from 5'-oxidation of deoxyribose, to form a novel bicyclic adduct [118].

neocarzinostatin adduct at the 5'-position of deoxyribose, one of the major sites of drug-induced oxidation, in native DNA with a frequency of about 5% [119, 120]. While the site of adduction in the enediyne was not identified in the studies with B-DNA, a similar 5'-adduct in deoxyribose was observed in the reaction of neocarzinostatin at a DNA bulge. It was argued, on the basis of known site specificity, that the C6 radical center in neocarzinostatin is the site of adduction [121]. The proposed mechanism for the formation of a drug-deoxyribose adduct involves back reaction of one of the radicals of the activated enediyne with a deoxyribose radical generated by the other drug radical center. In support of this mechanism, it was observed that adduct formation increased significantly under anaerobic conditions, as expected if the deoxyribose radical cannot be fixed by reaction with molecular oxygen [122]. Indeed, the adduct represents the major product formed in a reaction of neocarzinostatin with a DNA bulge under anaerobic conditions [121].

The Goldberg group extended these studies to the enediyne C-1027 with the discovery of novel interstrand drug-DNA crosslinks [123, 124]. Under anaerobic conditions, virtually all of the strand cleavage reactions mediated by C-1027 (*vide supra*) resulted in the formation of drug-deoxyribose monoadducts and interstrand crosslinks [123, 124]. In the oligodeoxynucleotide sequence GTTA₁T·ATA₂A₃C, crosslinks occurred between A1 and A2 or A1 and A3 while monoadducts were limited to A2 and A3 [124]. As argued for neocarzinostatin, the mechanism was proposed to involve back-reaction of the drug-induced deoxyribose radicals with the aromatic ring system of the aromatized C-1027 enediyne core.

There are several potential biological consequences of these enediyne-deoxyribose adducts. The first involves resistance to DNA replication and repair. Goldberg and co-workers demonstrated that the neocarzinostatin adducts are resistant to exonuclease activity [125]. Furthermore, the adducts represent a blocking lesion for a variety of DNA polymerases [126]. Prokaryotic DNA polymerases (Klenow fragment, T4, herpes simplex virus, and cytomegalovirus DNA polymerases) all stop at the base immediately 3' to the adduct, while those lacking 3' to 5' exonuclease proof-reading activity (e.g. exo-minus Klenow and exo-minus T7 DNA

polymerase) insert a nucleotide opposite the lesion, as does human DNA repair polymerase beta. Interestingly, there was no impairment of polymerase fidelity as observed with base adducts [126]. The C-1027 interstrand crosslinks also pose a block to DNA replication, but one of a more serious nature. One might expect that the recently discovered bypass polymerases in human cells will extend DNA synthesis past enediynes monoadducts [127], possibly in an error-free manner if Goldberg's polymerase studies are representative. However, the interstrand crosslink poses a unique problem for DNA repair and may thus represent a more toxic lesion than a double-strand break [128].

22.5

Enediynes-induced DNA Damage in Cells

To this point, we have considered enediynes target recognition and DNA damage in the context of isolated and purified DNA. However, as a logical consequence of the systematic search for antitumor natural products beginning in the 1960s and 1970s, the cellular effects of enediynes have been the subject of studies too numerous to review here. The reader is referred to the very comprehensive review of the biological and clinical aspects of the enediynes edited by Doyle and Borders [129]. Here, we will discuss the most recent studies in chromatin and cells.

22.5.1

Enediynes Target Recognition in Chromatin and Cells

Several studies have addressed the selection of enediynes targets in cellular and isolated chromatin, with the unsurprising result that studies with purified DNA are good predictors of target selection in cells. Beerman and co-workers demonstrated almost two decades ago that neocarzinostatin caused the release of nucleosome-sized DNA fragments from HeLa nuclei exposed to the drug [130]. Though the connection was not made at the time, the preferential cleavage of the DNA joining adjacent nucleosome cores (i.e. histone octamer with approximately 150 base pairs of DNA) was, in retrospect, likely a consequence of the intercalative DNA-binding mode of neocarzinostatin [19]. This conclusion is based on the Dedon group's studies of esperamicin-induced DNA cleavage in HeLa cells and nuclei [59]. As shown schematically in Fig. 22.9, these studies revealed that nucleosome-sized DNA fragments were released from treated nuclei and cells treated with esperamicin A1, while esperamicin C, which lacks the deoxyfucose-anthranilate moiety of esperamicin A1, and calicheamicin γ_1^I produced essentially random DNA cleavage [59]. On the basis of earlier studies with bleomycin and other intercalating agents [131–133], the linker-selective cleavage apparent with esperamicin A1, but not esperamicin C, led us to propose that the anthranilate of esperamicin A1 functioned as an intercalating moiety. This later proved to be correct, as discussed earlier in Section 22.3.3. The basis for linker-selective binding by esperamicin A1 and other intercalating agents likely arises as a result of exclusion of the drug from the dy-

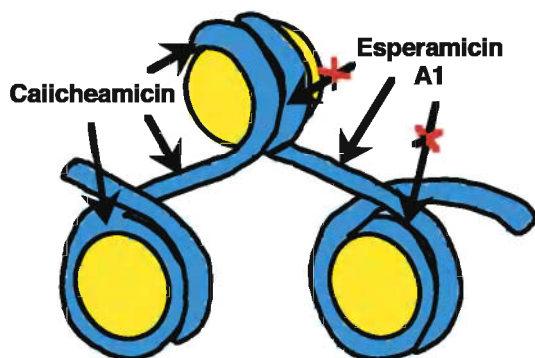


Fig. 22.9. Stylized representation of the promiscuous selection of nucleosomal DNA targets by calicheamicin (and esperamicin C) and the intercalation-limited targeting of nucleosome linker DNA by esperamicin A1 (and other intercalating enediynes).

namically constrained DNA of the nucleosome core (Fig. 22.9). Essentially the same conclusion was reached by Povirk and co-workers in their studies of bleomycin- and neocarzinostatin-induced DNA cleavage in a reconstituted nucleosome model [134].

These studies of enediynes-induced DNA cleavage in nuclei also revealed an interesting feature of calicheamicin target recognition. The drug was observed to cleave the DNA wrapped around the nucleosome core with a 10-nucleotide periodicity consistent with binding of the drug to regions of the core DNA where the minor groove faced away from the histones [59]. In subsequent studies by the Tullius and Dedon groups [54, 65], this model was borne out by calicheamicin- and esperamicin C-induced cleavage in reconstituted nucleosomes, as described earlier in Section 22.3.4.

At the next level of complexity, several groups have studied enediyne target selection in whole cells as a function of transcriptional activity. Beerman and co-workers employed a glucocorticoid-inducible gene expression model to demonstrate that bleomycin and neocarzinostatin preferentially cleaved transcriptionally active DNA sequences [135]. The location of the DNA targets for these drugs coincided with the deoxyribonuclease-sensitive glucocorticoid receptor binding sites in the regulatory sequences of the expressed gene. Interestingly, neocarzinostatin showed a significantly higher selectivity for these sites than did bleomycin, though the basis for this difference is unclear. These results are entirely consistent with the general observation of selective DNA damage in transcriptionally active genes by a variety of DNA-damaging agents. For example, Wogan and co-workers found that aflatoxin forms 4- to 5-fold more guanine adducts in the multicopy, transcriptionally active ribosomal RNA genes than in the bulk nuclear DNA of rat liver cells [136, 137]. The basis for this preference is generally regarded as a matter of accessibility of DNA-binding sites in the less compacted chromatin structure of active genes [137].

The advent of ligation-mediated PCR technology provided the means to define the location and quantity of DNA damage at single-nucleotide resolution in individual DNA sequences within the native setting of the cell [138]. Using this technology, the Dedon group carried the observations of enediyne target recognition into whole cells using the human *p53* and phosphoglycerate kinase (*pgk1*) genes as models [139, 140]. One set of studies in the upstream region of the *pgk1* gene addressed the effects of a positioned nucleosome on enediyne target recognition [139]. As anticipated by the studies in nuclei and nucleosomes, esperamicin A1 preferentially targeted the linker regions surrounding a defined position nucleosome, while calicheamicin and esperamicin C cleaved throughout the nucleosome core and linker DNA [139]. Loss of a detectable nucleosome structure in this region of the transcriptionally active *pgk1* gene removed the impediment to esperamicin A1-induced DNA cleavage [139].

Looking further upstream in the *pgk1* regulatory sequence, it was observed that esperamicin-induced DNA cleavage was modulated at sites within and surrounding the binding sites for several transcription factors. Again consistent with predictions from *in vitro* studies, esperamicin A1-induced cleavage was inhibited at the binding sites for GC and CCAAT box binding proteins as well as the NF1 protein [140]. The basis for these results may lie in dynamic constraints on DNA imposed by the GC and CCAAT box proteins and the circumferential binding of NF1 to DNA [140]. An unexpected result of these studies was the observation of enhanced cleavage by both esperamicins A1 and C in a 10-nucleotide stretch lying between the NF1 and CCAAT transcription factor-binding sites [140]. The basis for this enhancement is unclear but may involve DNA conformational effects related to binding site interactions.

Another surprising result arose from studies of esperamicin-induced DNA cleavage in exon 5 of the human *p53* gene in HeLa cells [140]. On the basis of photo-footprinting and deoxyribonuclease-mapping techniques, two groups had proposed the presence of a nucleosome-like structure at the 3' end of the *p53* exon 5 [141, 142]. However, cleavage with esperamicin A1 failed to reveal the presence of a nucleosome at this site [140], in spite of the strong selectivity of this enediyne for nucleosome linker DNA. One possible explanation for this apparent discrepancy is the unusual structure of nucleosomes in transcriptionally active genes (see references in [140]).

22.5.2

Molecular and Genomics Approaches to Understanding Cellular Responses to Eneidyne

Ultimately, the goal of developing more effective cancer therapies is best served by understanding the response of cells to exposure to DNA-directed chemotherapeutic agents, from the standpoint of resistance mechanisms as well as defining the link between DNA damage and cell death or mutation. In this regard, there have been numerous studies of the cellular responses to enedynes. The bulk of the studies

have focused on one or a few molecular pathways in cultured cells, while a recent report from the Myers group has attempted to quantify the response of the entire genome to neocarzinostatin.

The molecular studies can be very broadly divided into four categories: genetic toxicology, inhibition of DNA transcription and replication, cell cycle arrest, and apoptosis. It was recognized very early that enediynes caused DNA damage [143, 144]. Though it is still unproven that DNA is the cytotoxic target of enediynes, or any other DNA-damaging agent for that matter, there is strong evidence in support of this hypothesis. Perhaps most notable is the hypersensitivity of ataxia telangiectasia cells, which are defective in double-strand break repair, to enediynes, bleomycin, and ionizing radiation [145–150]. Not surprisingly, low concentrations of neocarzinostatin cause a block in the G₂ phase of the cell cycle in CHO cells [151–153]. Among the expected consequences of enediyne-induced double- and single-stranded DNA lesions are mutations [154–157], induction of DNA-repair activities [158, 159], and chromosome rearrangements [157, 160–167], an especially important outcome in light of the G₂ arrest noted earlier. Regarding DNA repair, Strauss and co-workers observed that neocarzinostatin induces repair in a xeroderma pigmentosum cell line deficient in the ability to repair bulky DNA adducts (i.e. nucleotide excision repair) [168]. The repair activity consisted of short patches of DNA synthesis accompanying disappearance of drug-induced single-strand breaks and oxidized abasic sites [168]. Following on numerous earlier studies demonstrating inhibition of DNA and RNA synthesis by neocarzinostatin (e.g. Refs [143, 169–171]), Beerman and co-workers have made extensive study of the effects of enediynes on the molecular events associated with inhibition of DNA replication [172–177]. Another possible pathway for the cytotoxic effects of enediynes, and other DNA-cleaving agents, involves activation of poly(ADP-ribose) polymerase (PARP) and consequent depletion of cellular pools of NAD [153, 178–182]. The proposed mechanism for PARP involvement in enediyne-induced DNA-damage response involves binding of PARP to the damage sites followed by auto-modification of the enzyme by ADP-ribose polymerization. Lindahl and co-workers have demonstrated that PARP activity inhibits DNA-repair processes by blocking access of the repair enzymes to the site of a strand break [182].

By whatever pathway, the enediynes all appear to cause apoptosis as the ultimate mechanism of cytotoxicity [183–191]. This is an expected result given the ubiquity of apoptotic pathways in the cytotoxic mechanisms of most, if not all, anticancer drugs [192]. The link between enediyne-induced DNA damage and apoptosis has been challenged by the Wrasildo group with the observation that synthetic analogs of dynemicin A induce apoptosis in the absence of DNA binding or DNA cleavage [190]. On the other hand, the Schor group has made the interesting observation that treatment of cells with the anti-apoptotic protein, Bcl-2, paradoxically enhances neocarzinostatin-induced apoptosis [184, 187] mediated in part by caspase 3 activation of Bcl-2 [193]. The basis for this phenomenon appears to lie in the Bcl-2-induced increase in cellular glutathione levels, since there is no similar enhancement for thiol-independent enediynes [184, 187]. The Schor group observes that

the cytotoxicity of enediynes that require reductive activation could be enhanced in tumors in which resistance to chemotherapeutic agents is manifest as upregulation of Bcl-2, such as such as estrogen-responsive breast cancers [187].

Finally, a recent gene expression array study by Myers and co-workers revealed several novel features of the response of *Saccharomyces cerevisiae* to neocarzinostatin holoantibiotic [194]. Their results show that, with a 4-h treatment of neocarzinostatin at $50 \mu\text{g mL}^{-1}$, the levels of 18% of the transcripts were altered by a factor of 2 or more. Again not surprisingly, a number of DNA-damage repair genes such as RNR2/3/4 and RAD50/51/52 were observed to increase more than two-fold. The expression of these genes, in particular RAD52, has been shown to be essential for the survival of yeast in which a lone double-strand break has been induced by the HO endonuclease [195, 196].

An especially novel finding in the Myers study was the upregulation of NCE103, a gene involved in the non-classical protein export pathway. When the yeast cells were exposed to the apo-protein component of neocarzinostatin holoantibiotic, NCE103 was the only gene to show a substantially modified expression [194]. The expression of this gene may thus represent a defensive mechanism for removal of the neocarzinostatin apo-protein. Indeed, Myers and co-workers demonstrated that the apo-protein was internalized into the yeast cells, an observation consistent with the demonstration of neocarzinostatin holoantibiotic uptake into human epithelial cells [197] and C-1027 holoantibiotic into Ehrlich carcinoma cells [198]. The importance of this finding lies in the proposal that the apo-protein components of neocarzinostatin, C-1027, kedarcidin, and maduropeptin have been claimed to possess proteolytic activity [199–201]. Though they did not perform a survey of different protein substrates, Zein and co-workers argue that the apo-proteins selectively cleave histone proteins, especially H1, and that the proteolytic activity contributes to the toxicity of enediynes by directing the chromophore to linker regions between nucleosomes, the location of histone H1 [200]. As tempting as this scenario might be, the proteolytic activity of the enediyne apo-proteins has recently been called into question by Heyd *et al.* [202]. A rigorous investigation of the purity of neocarzinostatin apo-protein preparations derived from an expression vector revealed that the proteolytic activity arose from a contaminating protease and not from the purified apo-protein [202]. It thus appears unlikely that an apo-protein-mediated proteolysis contributes to enediyne toxicity or entry into a cell, though the apo-protein may indeed serve to deliver the enediyne chromophore into the cell.

22.6

Summary

Given the clinical success of the Mylotarg calicheamicin–antibody conjugate, there is now considerable impetus for biological studies aimed at understanding the mechanisms by which enediynes cause cell death. The structural and mechanistic diversity of the enediyne family suggests that, in spite of any commonality of

radical-based DNA cleavage, there are likely to be many important differences in the cellular responses to individual enediynes. The success of calicheamicin has also served as motivation for the synthesis and study of novel enediynes, an area that unfortunately could not be covered in this review.

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23

Devising a Structural Basis for the Potent Cytotoxic Effects of Ecteinascidin 743

Federico Gago and Laurence H. Hurley

23.1

Introduction

Ecteinascidia turbinata is a tunicate species from the Caribbean and the Mediterranean that belongs to the class Ascidiacea within the subphylum Tunicata (also called Urochordata). Ascidians, or sea squirts, are small bottom-dwelling soft-bodied marine animals that form colonies comprising many individuals, called zooids. The term “tunicate” derives from their characteristic protective covering, or tunic, which functions to a certain extent as an external skeleton and consists of some cells, blood vessels, and a secretion of a variety of proteins and carbohydrates, including cellulose, an unusual finding in animals. Within the tunic is the muscular body wall, which controls the opening of the siphons used for feeding. Colonies are formed by asexual reproduction through budding; that is, outgrowths on the parent break off as new individuals. Sexual reproduction, on the other hand, leads to a fertilized egg that develops into a free-swimming tadpole larva. The larval stage is brief and is used to find an appropriate place for the adult to live. Larvae have notochords and nerve cords, as well as muscular tails twice as long as their bodies. Following attachment to a surface, the body resorbs the tail, using it as a food supply during metamorphosis into its sessile adult form. Degeneration of the dorsal nerve cord and sensory organs leaves in the adult a single ganglion between the oral and atrial openings from which nerves grow to the various organs of the body.

23.2

Biological Activity and Characterization of the Active Compounds

Crude aqueous ethanol extracts of *Ecteinascidia turbinata* were shown to have powerful immunomodulating and antiproliferative properties as early as 1969 [1]. Many unsuccessful attempts at isolation of the active compounds were followed by concerted efforts that led to the characterization of six alkaloids called ecteinascidins 729, 743, 745, 759A, 759B, and 770 (Fig. 23.1), of which Et 743 was the most

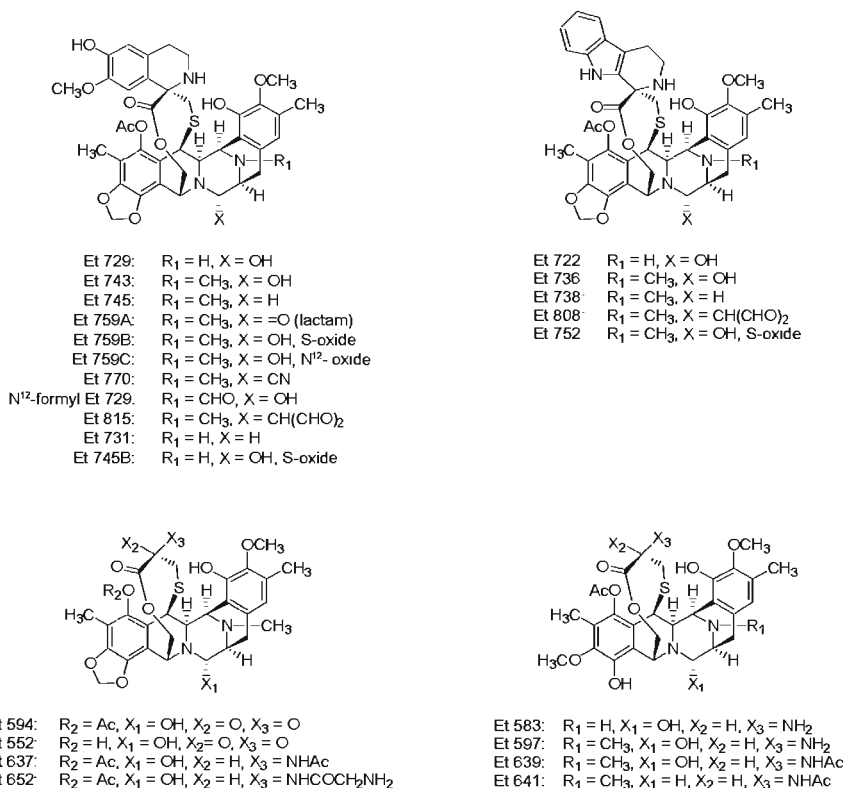


Fig. 23.1. Chemical structures of selected ecteinascidins.

abundant (0.0001% yield) [2]. The numbers after the abbreviation “Et” refer to the masses (M) deduced from the highest mass ions (M + H) observed in positive-ion fast atom bombardment mass spectrometry (FABMS) spectra. Tandem FABMS/MS was particularly useful in defining structural units in the compounds, as discussed in the recent review by Kenneth L. Rinehart [3].

The novel and unique chemical structure of the ecteinascidins (Fig. 23.2) is formed by a monobridged pentacyclic skeleton composed of two fused tetrahydroisoquinoline rings (subunits A and B) linked to a 10-member lactone bridge through a benzylic sulfide linkage. Most ecteinascidins have an additional tetrahydroisoquinoline or tetrahydro β -carboline ring (subunit C) attached to the rest of the structure through a spiro ring. The existence of these two families reveals different biosynthetic pathways very likely involving condensation of carbonyl groups with either dopamine or tryptamine equivalents.

A clear structural similarity is apparent with microbially derived safracins [4] and saframycins [5], both less potent anticancer agents than ecteinascidins, and also with sponge-derived renieramycins, for which no antitumor activity has been reported (Fig. 23.3). The antiproliferative action was early thought to be largely due

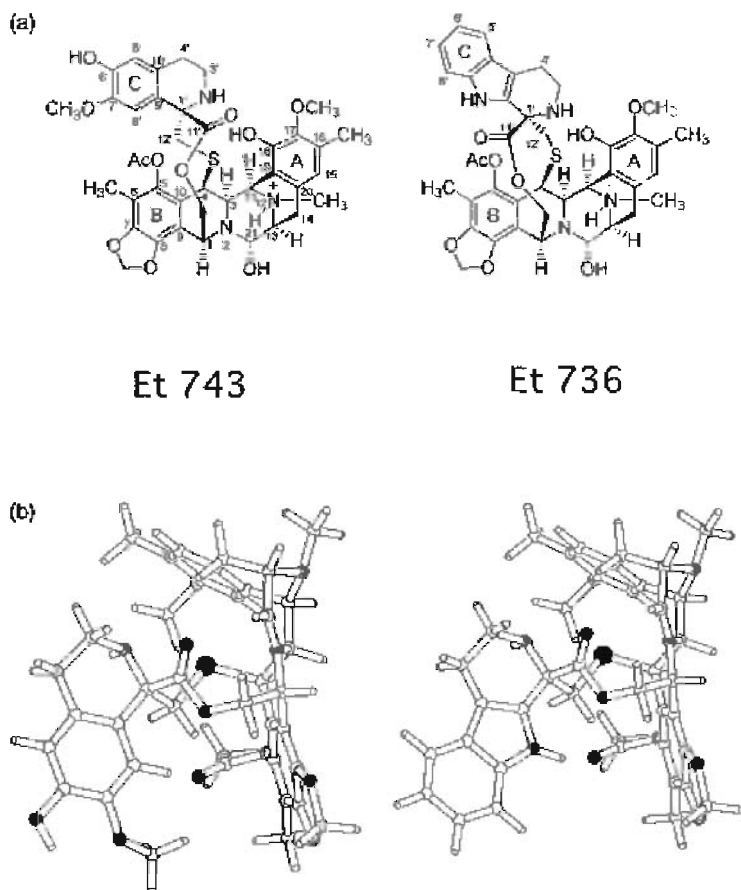


Fig. 23.2. (a) Chemical structures and numbering of Et 743 and Et 736. (b) Three-dimensional structures of the calculated iminium forms of Et 743 and Et 736.

to the presence of an active α -carbinolamine (N-C-OH) group [6], which is also present in naphthyridinomycins, quinocarcins, and pyrrolo(1,4)benzodiazepine antibiotics such as anthramycin, sibiromycin, and tomaymycin.

Reduction of the quinone skeleton of saframycin A to hydroquinone results in the concomitant elimination of the nitrile group and enhanced covalent binding to DNA [7], in a reaction reminiscent of that between anthramycin and DNA [8]. An α -carbinolamine or an iminium were the species suggested to be involved in the interaction, and the amino group of guanine in the minor groove was later identified as the site of covalent attachment [9]. By analogy to these related antibiotics, the potent biological activity of the ecteinascidins was then rapidly associated with their ability to form a covalent adduct to DNA using the reactive carbinolamine group [10].

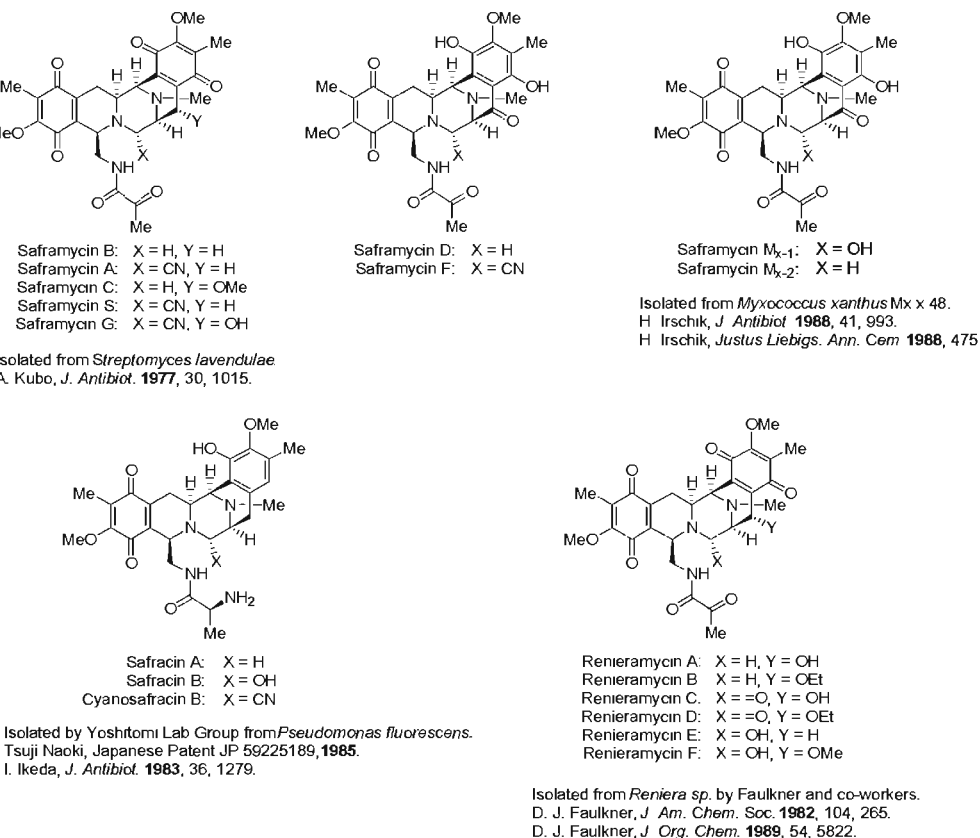


Fig. 23.3. Chemical structures of selected saframycins, safracins, and renieramycins.

The chemical structures of the ecteinascidins were assigned by correlation NMR techniques and FABMS spectra, whereas the unequivocal assignment of the relative stereochemistries at C4 and C1' (=C22) was achieved only when the X-ray crystal structures of the natural N^{12} -oxide of Et 743 and the 21-O-methyl- N^{12} -formyl derivative of Et 729 were solved [11].

23.3

Structural Characterization and Synthesis of Ecteinascidins

The crystal structures of N^{12} -formyl C^{21} -methoxy Et 729 formic acid methanol solvate dihydrate and Et 743 N^{12} -oxide acetonitrile solvate octahydrate (deposited in the Cambridge Structural Data Base under accession codes WAMBAN and WAMBER, respectively) revealed two independent ecteinascidin molecules per asymmetric unit plus several solvent molecules. The molecular shape of both

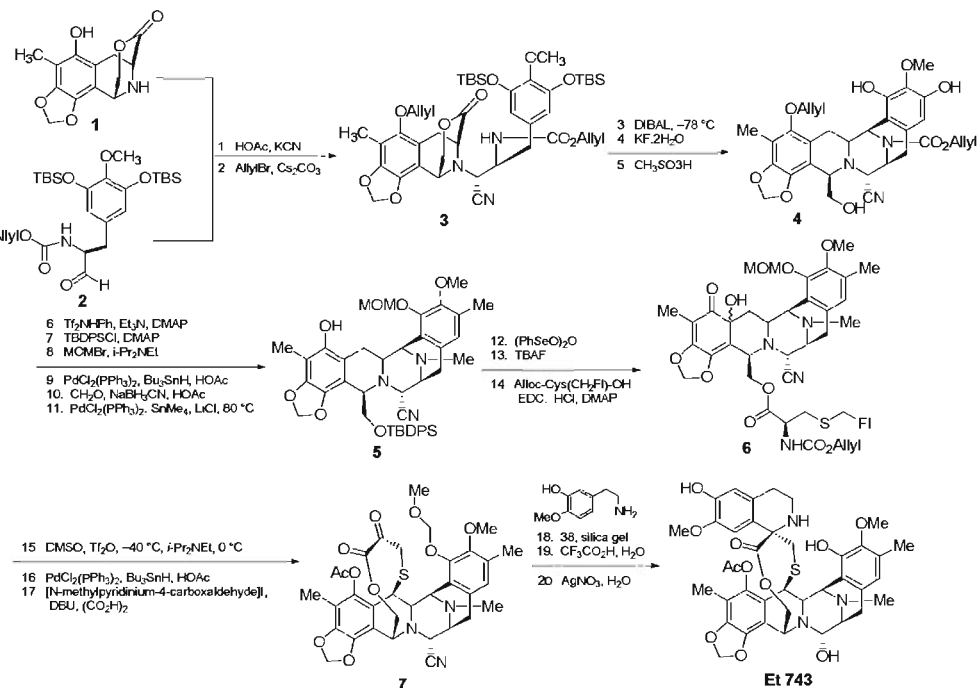
ecteinascidins is highly compact, with much of the surface occupied by hydrophobic methyl, methylene, and aryl-CH hydrogens, and only the carbinol and the hydroxyl groups at C18 of subunit A and C6' (=C27) of subunit C, which point away from the molecule, are available for hydrogen bonding.

The ecteinascidin molecule was first described as a platform formed by the B-subunit ring system on which both subunits A and C stand using their cross-bridging skeletons (Fig. 23.2). In the crystals, the two molecules are associated into dimers of ellipsoidal shape through the stacking of their ring B systems and formation of an intermolecular hydrogen bond involving the hydroxyl group on C18 of one molecule and either the formyl or the *N*-oxide oxygen on N12 of the other molecule. This hydrogen bond appears to provide a swivel point for the association. The presence of a formate ion in the WAMBAN crystal structure suggested protonation of one of the nitrogen atoms. Since N2 is almost totally shielded from the solvent, N12 was proposed as the most likely candidate, in analogy to saframycin, which becomes selectively protonated at the N12 position at pH levels below 5.5. Later, proof was obtained by NMR spectroscopy that N12 is indeed protonated in the covalent DNA adduct as well [12, 13].

Ecteinascidia turbinata has been successfully grown by Pharma Mar in aquaculture facilities in Spain (near Formentera island, in the Mediterranean sea) in what represents a more practical and environmentally sound practice than harvesting the creature from the wild. Nevertheless, in recent years several synthetic schemes have been developed that make it possible to produce Et 743 in the kilogram quantities required for further clinical studies worldwide.

The precedents to the first total synthesis of Et 743 (for a review, see Ref. [14]) are to be found in synthetic work leading to related molecules isolated from bacterial sources (e.g. saframycins and safracins) or marine sponges (e.g. renieramycins). These compounds (Fig. 23.3) have antimicrobial properties and possess the same pentacyclic skeleton as the ecteinascidins, but one or two of the aromatic rings contain a different oxidation pattern, resulting in dimeric mono- or bisquinone structures. The first enantioselective total synthesis of Et 743 (Scheme 23.1) was achieved in 1996 by using a multistep enantiocontrolled process [15]. This procedure was subsequently improved by the more efficient preparation of an analog of the key intermediate 3 in which the CN group was replaced by a carbonyl moiety [16]. The complete semisynthesis starting from cyanosafracin B (Scheme 23.2) was reported a few years later [17].

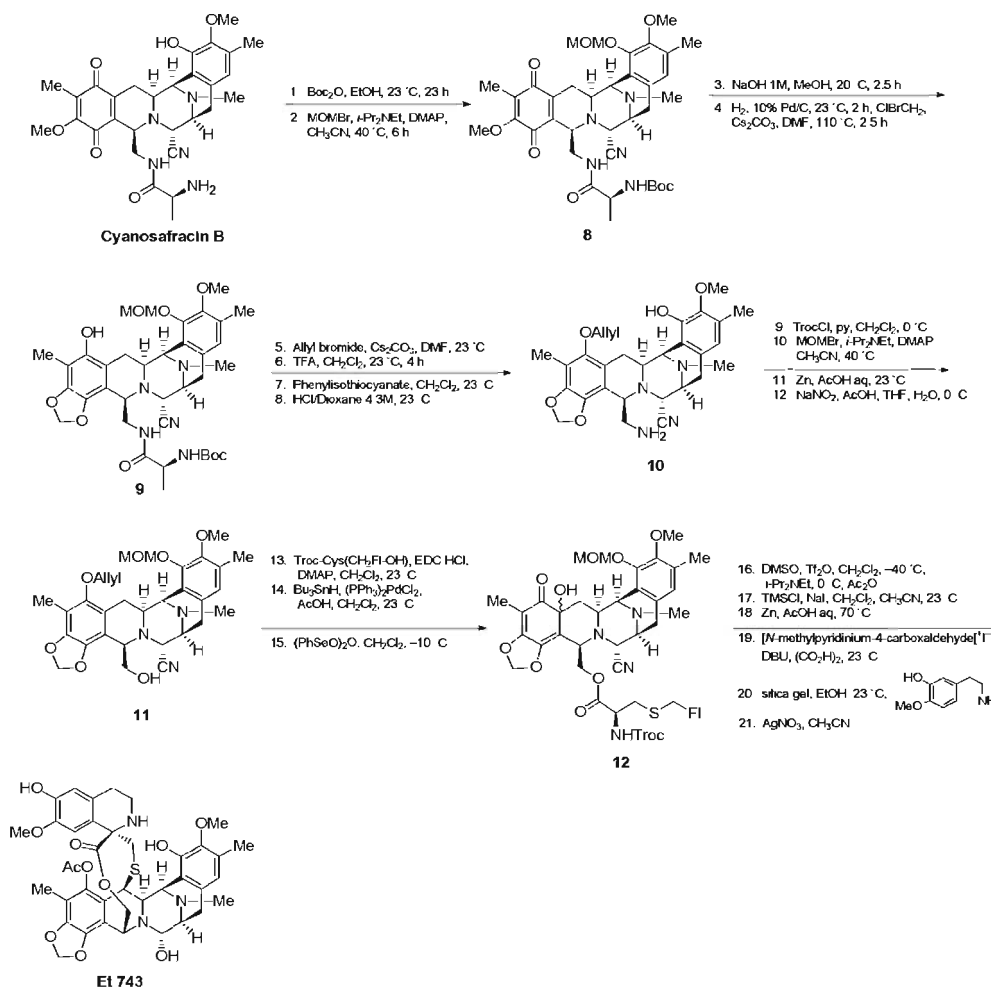
Two important intermediates to build the piperazine-bridged bis(tetrahydroisoquinoline) framework were provided by enantioselective syntheses of bridge lactone intermediate 1 and *N*-protected α -amino aldehyde 2 using catalytic asymmetric hydrogenation (Scheme 23.1). Coupling of building blocks 1 and 2, reduction of intermediate lactone 3 to the corresponding lactol, and internal Mannich bisannulation gave the monobridged pentacyclic intermediate 4. After protection, *N*-methylation and replacement of CF₃SO₃ by CH₃ led to 5, which has the required substitution pattern in the pentacyclic framework. The following key transformations completed the synthesis of Et 743: (1) oxidation of phenol 5 permitted the selective hydroxylation of the angular position of the aromatic ring; (2) after ester-



Scheme 23.1

ification of the primary hydroxyl function with a protected cysteine derivative, compound **6** was transformed in a one-pot reaction into the 10-membered lactone bridge by creation of exo quinone methide followed by nucleophilic addition of deprotected cysteine and further acetylation of the resulting phenoxide ion; (3) deprotection of the allyloxycarbonyl (Alloc) group and subsequent transamination of the α -amino lactone gave the α -keto lactone **7**; (4) Pictet-Spengler cyclization with phenethylamine generated the spiro tetrahydroisoquinoline stereospecifically.

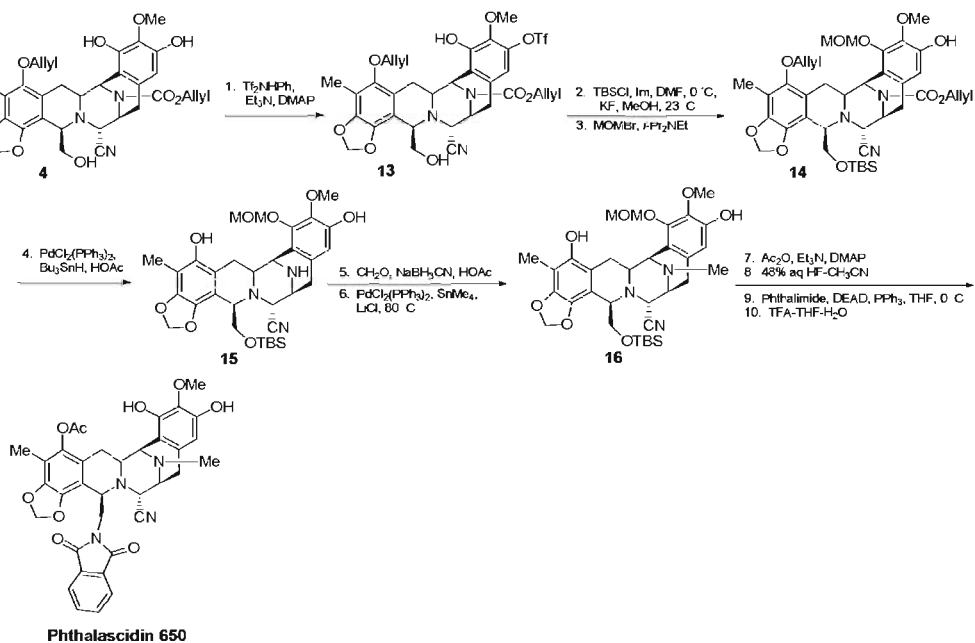
Optimization of fermentation reaction conditions for preparation of cyanosacarin B by *Pseudomonas fluorescens* has resulted in the availability of this material in kilogram quantities. From it the synthesis of Et 743 can be accomplished in a very short and straightforward way, as follows (Scheme 23.2). Selective hydrolyzation of protected methoxy-*p*-quinone **8**, reduction of the quinone, and reaction of the reduced compound with bromochloromethane affords the methylenedioxy intermediate **9**. Protection of **9** with allyl bromide, deprotection of the methoxymethyl (MOM) and *t*-butoxycarbonyl (Boc) groups, and subsequent cleavage of the amide bond by Edman's degradation gives the amine **10**. Protection of the phenolic function in **10** and conversion of the primary amino group into an alcohol affords intermediate **11**. Esterification of compound **11**, allyl deprotection, and angular oxidation provides intermediate **12**, and the synthesis is completed in six additional steps. Salient features of this end-game operation are the early removal of the MOM group with trimethylsilyl iodide (TMSI) and the use of β , β , β ,



Scheme 23.2

trichloroethoxy-carbonyl (Troc) protection for the amino function of the (*S*)-cysteine derivative.

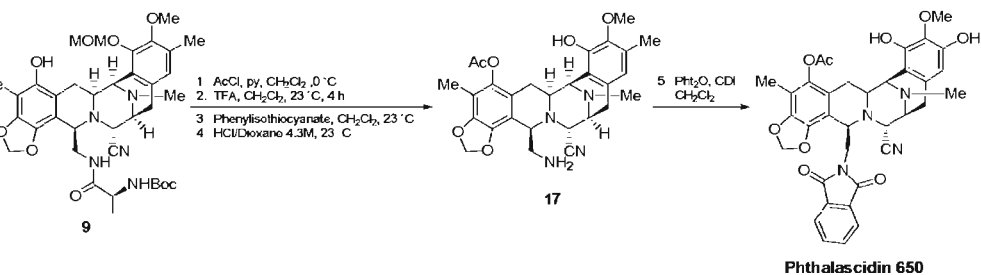
This latter methodology has resulted in both a much cheaper process and a substantial improvement in overall yield with respect to the original total synthesis. Moreover, the availability of some of the intermediates bearing reactive primary amino or alcohol functionalities (e.g. **10** and **11**) allows for the preparation of a wide range of Et 743 analogs in multigram amounts. One of these analogs is phthalascidin (Pt 650), a compound with comparable *in vitro* activity to Et 743 [18]. Pt 650 was originally synthesized (Scheme 23.3) using the common pentacyclic **4** obtained in the synthetic route to Et 743 (Scheme 23.1). The pentacyclic triol **4** was converted to the phenolic monotriflate **13**. Protection of the alcohol and phenol



Scheme 23.3

groups gave compound **14**. Cleavage of the *N*-alloc and *O*-allyl group in **14** afforded the secondary amine **15**, which was *N*-methylated and *C*-methylated to **16**. Acetylation of phenol **16**, deprotection of the silyl group, and Mitsunobu displacement of the primary alcohol produced the phthalimide, which upon acid catalyzed cleavage of the methoxymethyl ether provided pure Pt 650.

Using the new semisynthetic procedure, acetylation of intermediate **9**, previously utilized in the synthesis of Et 743 from cyanosafrafrin B, followed by deprotection of the *N*-Boc and MOM groups and by Edman's degradation of the alanine side chain affords **17**. Reaction of **17** with phthalic anhydride and carbonyldiimidazole gives Pt 650 (Scheme 23.4).



Scheme 23.4

23.4

Structure–Activity Relationships

Initial structure–activity relationships among natural members of the ecteinascidin class were based on results from *in vitro* cytotoxic screening assays against murine leukemia cells L1210 [19]. The importance of the reactive carbinolamine for optimal activity was highlighted by the 175-fold decrease in activity in going from Et 743 to its C21-deoxy counterpart Et 745, and the 17-fold reduction in potency upon oxidation of the carbinolamine to the lactam (Et 759A). Likewise, replacement of the C21 hydroxyl group by a malonaldehyde (Et 815) or a cyano (Et 770) group reduced the cytotoxic potency relative to Et 743, most likely due to slower formation of the reactive iminium intermediate. Other structural modifications were also shown to influence cytotoxicity. Thus, deacetylation of Et 743 to give Et 701 was seen to reduce the activity 40 times, whereas the *N*-12 demethylated analog Et 729 was 10 times more active than Et 743. Acetylation or methylation of the free phenol group in subunit C, on the other hand, did not significantly alter the biological properties of Et 743, but the activity was reduced 40 times when the phenol group at position C-18 (subunit A) was methylated. In general, the presence of the aromatic C-subunit was found to be important for potency, as exemplified by comparing Et 743 and Et 729 with Et 583, Et 594, and Et 597, but little difference in activity was found between ecteinascidins containing a tetrahydroisoquinoline ring system as subunit C (e.g. Et 729 or Et 743), and the corresponding analogs containing a tetrahydro- β -carboline ring (e.g. Et 722 or Et 736).

In vitro cytotoxicity studies with L1210 mouse leukemia cells were then extended to other cell lines, and subnanomolar potencies were also established against P388 mouse leukemia, A549 lung cancer, HT29 colon cancer, MEL-28 melanoma cells, and human tumors explanted from patients. *In vivo* activity was then evaluated in mouse tumor models and a variety of human tumors xenografted in nude mice. Complete regressions were observed in MEXF989 melanoma, MX-1 breast carcinoma, LXFL529 non-small cell lung carcinoma, and HOC22 ovarian carcinoma xenografts, and partial regressions were seen in renal MRIH121 and PC2 prostate carcinoma xenografts. Interestingly, changes in the C-subunit were seen to modulate the biological activity of both Et 736 and its *N*-12 demethylated analog Et 722, relative to Et 743, suggesting that this part of the molecule plays an important role in cytotoxicity. For example, Et 722 and Et 736 displayed a higher level of activity *in vivo* against P388 leukemia in mice, whereas Et 729 and Et 743 showed higher activity against B16 melanoma, Lewis lung carcinoma, M5076 ovarian sarcoma, and MX1 human mammary carcinoma xenografts. Et 743 was then selected for clinical development because of its greater relative abundance in the tunicate and entered clinical trials in early 1996 in both Europe and the United States. Et 743 is now in phase II clinical trials, and toxicity so far has been shown to follow a transient-reversible pattern and to be predictable, dose-related, and mostly limited to bone marrow and liver [20, 21]. Following a favorable opinion adopted by the Committee for Orphan Medicinal Products (COMP) of the European Agency for the Evaluation of Medicinal Products (EMA), Et 743 was granted, in April 2001,

orphan medicinal product designation for the treatment of soft tissue sarcoma by the European Commission [21].

The observation that Et 743 analogs lacking subunit C and possessing various substituents on the nitrogen atom of the α -amino lactone still retained good anti-tumor activity led to the design and synthesis of a number of ecteinascidin-like compounds. Replacement of the 10-membered lactone bearing the C-subunit with benzamide, *cis*-cyclohexane-1,2-dicarboxyimide, *cis*-cyclohexene-1,2-dicarboxyimide, or phthalimide moieties, together with protection of the phenolic hydroxyl group on the B-subunit by acetyl, propionyl, methoxyacetyl, methansulfonyl, methyl, or ethyl groups, yielded a number of derivatives [16]. Their primary antiproliferative activities were tested *in vitro* using different human solid cancer cell lines. After 3-day continuous exposure to the drugs, a metabolic assay was used in which the cellular reduction of a tetrazolium salt afforded a readily detectable formazan in proportion to viable cell number. These studies confirmed and expanded the initial structure–activity relationships and showed that an acetoxyl group in the B-subunit afforded optimum activity, whereas protection of the other phenolic hydroxyl group in the A-subunit resulted in diminished antitumor activity. The most potent compound turned out to be Pt 650, which displayed antiproliferative activities comparable to those of Et 743, and in both cases independent of cellular growth rate and *p53* tumor suppressor gene status. The dose–response curves were also similar, including the intriguing observation that cell viability after 24 h of drug exposure appeared to be 10–20% greater at 10–100 nM than at 1–2 nM, a finding suggestive of a relatively narrow concentration range for optimum bioactivity.

23.5

Structural Characterization of Et 743–DNA Adducts

Following determination of the crystal structure of the ecteinascidin derivatives, a molecular modeling study addressed the question of sequence specificity and the mode of interaction of Et 729 and Et 743 with DNA. Given the distinctive pattern of hydrogen bond acceptors and donors, and the fact that guanine is alkylated on the exocyclic amino group, critical spatial positioning between the active carbinolamine functional group (N2-C21-OH) of the drug and the N2 of guanine appeared crucial for the crosslinking reaction in the DNA minor groove. As a result of this study, both an orientation for bound ecteinascidin and a role for hydrogen bonding in the sequence recognition of DNA by these drugs were suggested: subunits B and C would stack on the sugar–phosphate backbone, each on one side of the DNA minor groove, following the right-handed curvature of the double helix, and a number of intermolecular hydrogen bonds involving N2 of guanines would stabilize the complex. Accordingly, GGG was proposed to be the best sequence for complex formation. It is remarkable that, despite the bulkiness of the ecteinascidin molecule, the minor groove was reported to be minimally disturbed, but this was probably a consequence of using only a steepest descent energy minimization algorithm in model refinement.

Direct evidence that Et 743 alkylates duplex DNA at guanines was provided by a series of gel electrophoresis experiments [22] that showed (1) no detectable effect on single-stranded DNA; (2) retarded migration of both supercoiled and nicked simian virus 40 (SV40) DNA following incubation with increasing concentrations of Et 743; (3) strong retardation with a homopolymeric duplex decanucleotide containing dG:dC but no discernible effects with decamers containing either dA:dT or dI:dC (dI standing for deoxyinosine, the nucleoside containing hypoxanthine in place of guanine and therefore lacking the purine 2-amino group); (4) progressive covalent adduct formation following noncovalent binding of the drug; and (5) reversion of Et 743-induced DNA mobility effects upon DNA denaturation. DNA footprinting experiments using DNase I and 1,10-phenantroline-copper plus hydrogen peroxide (both of them reactives that attack the DNA from the minor groove), and also exonuclease III (which digests DNA from its 3' ends and is stopped by DNA adducts), revealed a protection by Et 743 on the minor groove of three to five bases and a sequence preference different from that of anthramycin. As expected, no effects were detectable when guanine was replaced by inosine in the *Escherichia coli* tyrosine tRNA promoter (*tyrT* DNA) used in the experiments.

To gain further insight into the sequence selectivity, a series of duplex 14-mer oligonucleotides containing a central guanine flanked on each side by different combinations of bases (5'-XGY) were studied in a band shift assay. Outside the central triplet, all guanines were replaced with inosine to eliminate possible ambiguities. Et 743 was shown to retard the DNA in a concentration-dependent manner and to produce a more pronounced shift when compared with anthramycin. When all central triplets were compared with 5'-GGG (run as a standard for calculation), alkylation by 10 and 100 μM Et 743 was shown to be approximately equal in 5'-TGG and $\sim 50\%$ more efficient in 5'-CGG and 5'-GGC. In general, Et 743 better alkylated those oligonucleotides in which Y was G or C, whereas those possessing A or T on both sides of the central guanine were not detectably recognized by the drug, even at the higher concentration tested. A drawback of this study, later addressed by Seaman and Hurley [13], was that several of the 16 sample sequences studied actually contained more than one potential binding site for Et 743. This was due to either overlapped triplet target sites or the simultaneous presence of one or more alternative binding sites on the complementary strand of the duplex. When the previous experimental results were reinterpreted on a structural basis, a much clearer picture arose, and a set of reactivity rules were proposed that also took into account the hydrogen bonding possibilities. Thus, it was established that in the target sequence, 5'-XGY, the favored base for Y is either G or C, in agreement with the previous proposal. In addition, a pyrimidine base (T or C) is preferred for X when Y is G, whereas a purine (A or G) is needed if Y is C. If both 3' and 5' requirements are met, the sequence is highly reactive (e.g. 5'-AGC, 5'-GGC, 5'-CGG, and 5'-TGG) (Fig. 23.4). Failure to satisfy the first requirement (that Y be G or C) results in low reactivity sequences, while fulfillment of only this condition is characteristic of moderately reactive sequences (e.g. 5'-GGG and 5'-CGC). The success of this relatively simple set of rules further supports the idea that reactivity to Et 743 (and ecteinascidins in general) is governed by a combined direct

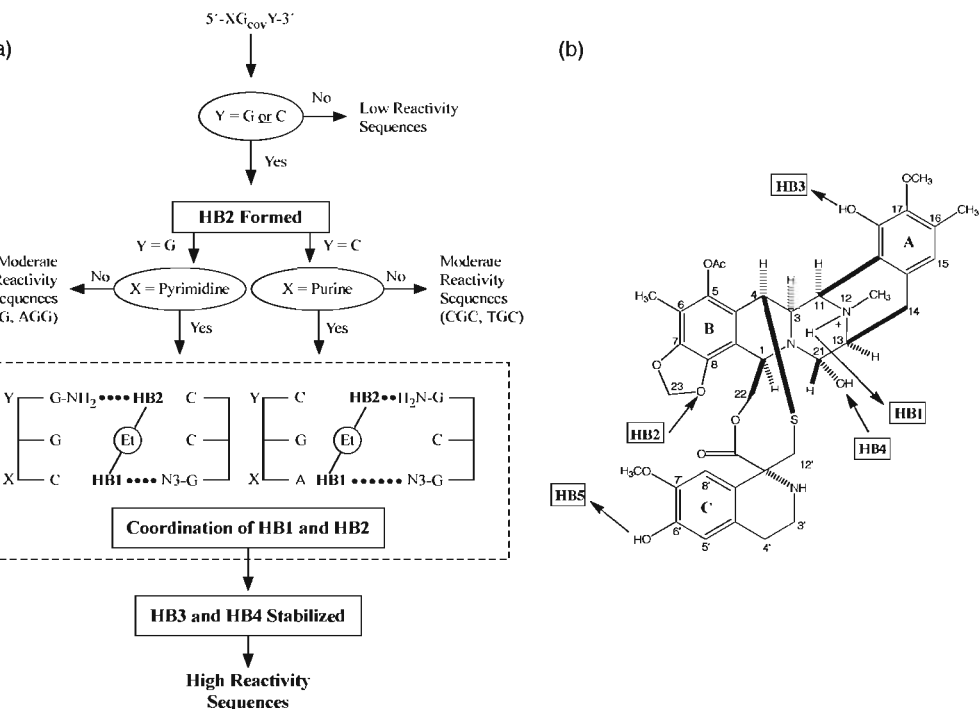


Fig. 23.4. (a) Flow chart analysis of the sequence specificity of the three-base sequence $5'\text{-XG}_{\text{cov}}\text{Y-3'}$ shows that Y must be either G or C, and depending on which base occupies Y, the X choice must be of the opposite ring system type (e.g. $\text{Y} = \text{G}$ and $\text{X} = \text{pyrimidine}$). The

relationship between these sequence rules and DNA–drug hydrogen bonding is displayed in the flow chart, which ultimately leads to the goal of high reactivity. (b) The structure of Et 743, showing the donor-acceptor sites HB1, HB2, HB3, HB4, and HB5.

read-out of a central guanine plus two additional flanking bases. This view is amply substantiated by experimental and modeling structural work, demonstrating that the extent and spatial orientation of a hydrogen-bonding network involving specific DNA base donors/acceptors does indeed direct the course of sequence recognition.

23.6

Structural Studies of Ecteinascidin–DNA Complexes

Knowledge of the above rules and potential problems is important in order to design a suitable duplex oligonucleotide for structural studies. If the sequence contains, for example, a $5'\text{-AGC}$ target triplet site on one strand followed by a cytosine, there will be an alternative binding site, $5'\text{-GGC}$, on the complementary strand, and this may compromise the success of the experiment. Although inosine could be used to replace guanine at some key positions, as in the band shift experiments

reported above, to our knowledge this strategy has not yet been used in NMR studies of ecteinascidin–DNA complexes.

The first report of a 1:1 Et 743:DNA adduct involved the self-complementary dodecanucleotide $d(\text{CGTAA}\underline{\text{G}}\text{CTTACG})_2$ [12]. The two-dimensional NMR spectra exhibited well-resolved cross-peaks that could be assigned to a single species and allowed the identification of a number of intermolecular contacts. Several critical connectivities from the nuclear Overhauser effect spectroscopy (NOESY) spectrum supported the proposed covalent bond between C21 and N2(G6) and clearly defined the precise orientation of the drug in the minor groove, with the A-subunit to the 5'-side of the alkylated guanine (G6) and the B- and C-subunits to the 3'-side. Evidence for the interaction of Et 743 with the alkylated DNA strand was provided by nuclear Overhauser effects (NOEs) of the H21 proton into H1'(G6), H1'(C7), H1'(T8), H2'/H2''(C7), H6(C7), and H6'(T8) and of the H22 protons into H1'(C7) and H3'(T8). Association of subunits A and B with the opposite strand was shown by NOE connectivities between the 6Me, 5OAc, H23, H4, and H11 protons of Et 743 to the G18, C19, T20, and T21 protons, as well as by aromatic shielding of subunit B over the deoxyriboses of G18 and C19. In contrast, the broad intramolecular NOEs of subunit C suggested interconversion between different conformers and lack of specific association with a site on the DNA, which was supported by the presence of just a single weak Et 743–DNA NOE involving this subunit (H3'(T8)–7'OMe). Taken together, these observations provided evidence that this part of the molecule is perpendicularly projected above the minor groove, in accordance with earlier proposals. One of the major differences between the resulting NMR-based model and the previous theoretical model was that the protonated N12(Me) hydrogen bonds to O2(T20) rather than to the G6:C19 base pair at the alkylation site. Since another hydrogen bond exists between the amino group of G18 and one of the dioxymethylene oxygens, subunit B penetrates more deeply into the minor groove and gets into closer contact with the wall of the non-alkylated strand. Subunit A remains perpendicular to the minor groove, and a hydrogen bond is proposed between 18OH and O1'(T20).

On the basis of this first NMR-based model, the importance of suitably placed DNA hydrogen bond acceptors to the 5'-side and a hydrogen bond donor to the 3'-side of the alkylation site was highlighted. Perhaps more importantly, the role of subunits A and B in DNA recognition and bonding was rationalized, as well as the paucity of subunit C–DNA interactions.

Further insight into the protonation state of ecteinascidin–DNA adducts was gained when the same dodecamer was isotopically labeled with ^{13}C and ^{15}N [23]. The NMR spectra confirmed lack of protonation on either the exocyclic nitrogen of G6 or N2 of the drug, whereas N12 appeared protonated. A mechanism for activation (Fig. 23.5) was then proposed [23] that takes advantage of the increased strength of the hydrogen bond between the proton on N12 and the hydroxyl group on C21 as the Et 743 molecule approaches the minor groove and is desolvated. This proton, which is essential for both sequence recognition and adduct stabilization, would then catalyze the dehydration of the carbinolamine, yielding the reactive iminium intermediate that undergoes nucleophilic attack at C21 by the exo-

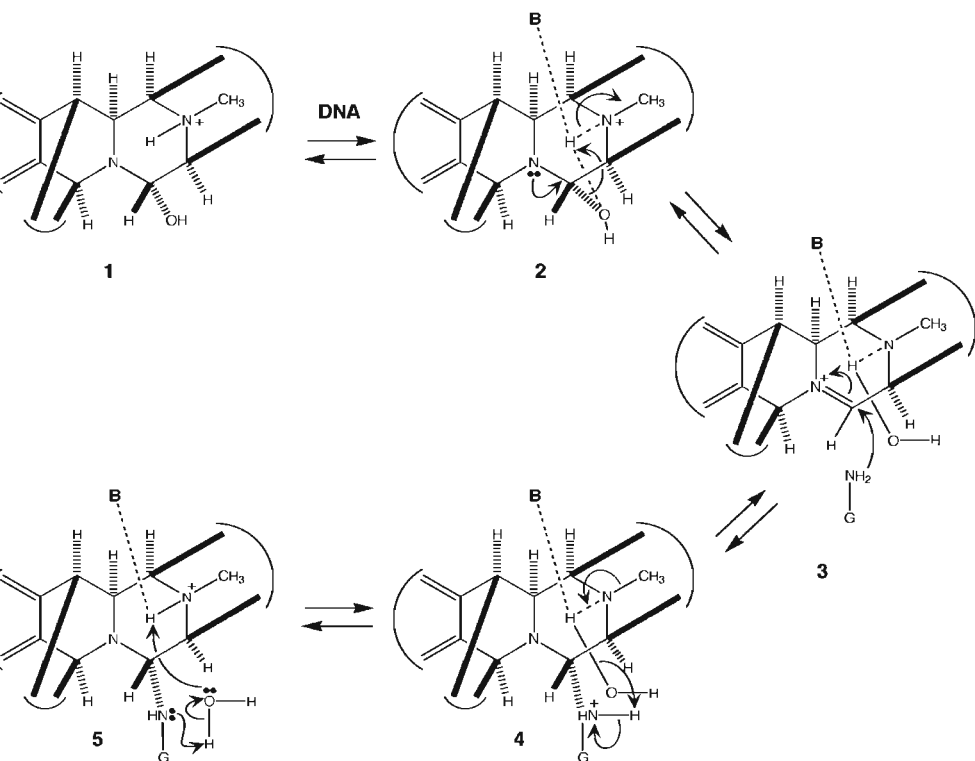


Fig. 23.5. Reversible reaction of Et 743 with DNA to form the Et 743-(N2-guanine)-DNA adduct. “B” is a DNA base hydrogen bond acceptor. Adapted from Ref. [30].

cyclic amino group of G6. The resulting alkylated guanine would be initially protonated, but it would regain neutrality by transferring the proton either to N12 of Et 743 or to the transiently bound water molecule that originated in the previous step. This proton shuttle would restore the original protonation state of the drug and would expel the water molecule as the transition state collapses to the final covalent adduct. Since a similar mechanism operates in the activation of pyrrolo(1,4)benzodiazepine antibiotics [24], it appears that nature ensures the reactivity of these carbinolamine-containing molecules by the inclusion of an internal catalytic proton adjacent to the leaving hydroxyl group.

The two-dimensional ^1H NMR study with the Et 743- $d(\text{CGTAAGCTTACG})_2$ adduct was expanded to include a similar complex with the closely related Et 736, which allowed a better characterization of the hydrogen-bonding network associating the covalently bonded drug with the DNA duplex than in the corresponding adduct with Et 743 [13]. Nevertheless, similar hydrogen-bonding interactions for the common A/B-subunit scaffold were deduced from comparison of exchangeable and nonexchangeable ^1H NMR data. In both cases, unbroken ^1H - ^1H NOESY

cross-connectivity patterns did not reveal any radical structural perturbation in the DNA molecule as a consequence of drug binding. The single set of NMR signals of the unbound self-complementary duplex was of course split into two distinct sets of signals corresponding to the unmodified (C13–G24) and covalently modified (C1–G12) strands. A model for the structure of the Et 736–DNA adduct based on the one- and two-dimensional NMR studies is shown in Fig. 23.6 (see p. 654).

The limited set of intermolecular distance restraints obtained for this covalent complex was then used to model the precovalent complex and the covalent adduct between Et 743 and a double-stranded non-self-complementary nonamer containing d(TAAAGCTTA) in one strand. The modeling was done by means of unrestrained molecular dynamics (MD) simulations in explicit solvent and in the presence of neutralizing sodium ions, and a parallel simulation of the free oligo was also run for comparison [25]. The prealkylation binding complex showed that the protonated N12 hydrogen bonded to the O2 acceptor atom of T15 and the carbinol OH, acting both as a hydrogen bond donor to O2 of C14 and as a hydrogen bond acceptor from N2 of G5. A distance (which cannot be measured experimentally) of about 4.0 Å between C21 and N2(G5) suggested that, in this complex, C21 is a suitable target for nucleophilic attack by G5. Further anchoring to the DNA minor groove was provided by a hydrogen bond between the methylenedioxy oxygen and N2 of G13, as suggested. The OH on subunit C was found to hydrogen bond alternatively to its neighboring methoxy group and to O1P of the phosphate linking T7 and T8, whereas the OH on subunit A was not involved in intermolecular hydrogen bonding. As a consequence of these interactions, the inter-strand O6(G5)–N4(C14) and N1(G5)–N3(C14) hydrogen bonds were broken, and both the opening and the negative propeller twist of the G5–C14 base pair increased relative to the free oligonucleotide. This distortion, however, could be easily accommodated by the neighboring base pairs so that the double helical structure was only minimally perturbed, except for widening of the minor groove and a net bending toward the major groove. Interestingly, when the precovalent complex of Et 743 with the duplex d(TAACGATTA)–d(TAATCGTTA) was simulated under the same conditions, the MD trajectory was unstable and no suitable geometry for adduct formation was found, which is in agreement with the fact that a CGA triplet does not represent a good binding site for the drug. Therefore, the simultaneous occurrence of three hydrogen bonds seems to be critical for stabilization of ecteinascidin–DNA prealkylation complexes, and there are two optimal ways to achieve this, as shown in Fig. 23.4.

Replacement of the central AGC triplet with CGG in the oligonucleotide studied provided another example of an optimal binding site for Et 743. This CGG-containing sequence was also simulated in complex with the related Pt 650. All the covalent adducts possessing a drug-modified guanine in one strand revealed a considerably enlarged DNA minor groove and significant bending toward the major groove (Fig. 23.7), mostly due to an increase in positive roll at the base pair step involved in covalent bond formation. Interestingly, the central CGG triplet appeared to accommodate both Et 743 and Pt 650 with less distortion than AGC, suggesting that the intrinsic bendability of a particular DNA sequence and its overall preorganization

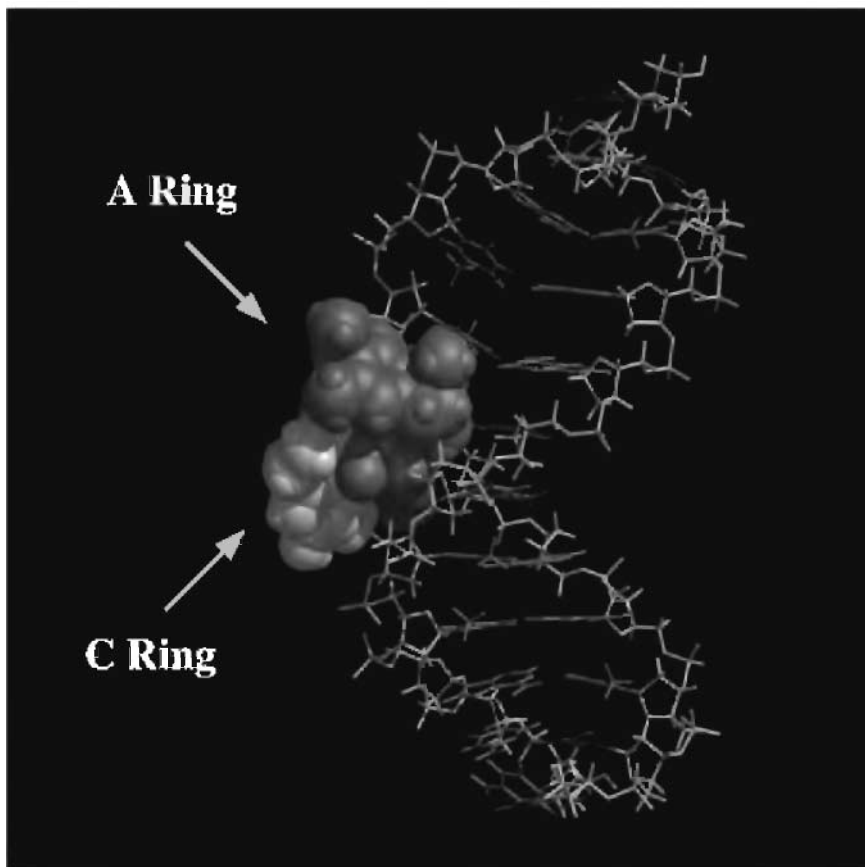


Fig. 23.6. A molecular dynamics-generated structure of Et 736 at the preferred bonding site. The three Et 736 subunits A (yellow), B (blue), and C (white) are positioned in the minor groove of DNA. Guanine is shown in red.

could facilitate specific recognition by Et 743. In relation to this, in the band shift experiments designed to evaluate the influence of the bases flanking a centrally located guanine, the CCG triplet indeed appeared as the most reactive of all the two-base permutations [22].

The validity of the results from these MD simulations was supported by evidence obtained previously from gel migration and cyclization kinetics experiments carried out with a 21-base-pair oligonucleotide containing a single guanine in the middle of a favored (5'-AGC) triplet [26]. The 3' cytosine was paired to an inosine on the non-covalently modified strand, and a 3-base overhang was included to allow for head-to-tail self-ligation in the presence of T4 DNA ligase. The ligated 21-mers containing the Et 743 adduct showed a retardation in electrophoretic mobility relative to the unmodified linear multimers and a greater tendency to form closed

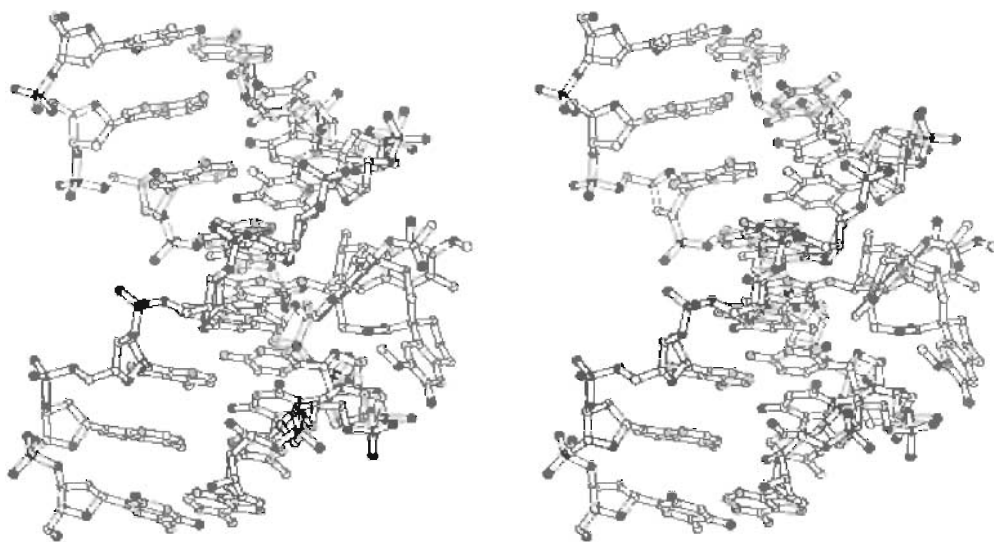


Fig. 23.7. Stereoview of a representative covalent adduct formed between Et 743 and a dodecanucleotide containing a central AGC triplet. Coordinates for representative refined average structures from the MD simulations of the Et 743–d(TAAAGCTTA)–d(TAAGCTTAA)

and Et 743–d(TAACCGTTA)–d(TAACCGTTA) complexes are publicly available from the Research Collaboratory for Structural Bioinformatics with PDB identification codes 1EZ5 and 1EZH, respectively. Adapted from Ref. [14].

circular DNA. Both observations were suggestive of induced bending, and the angle of absolute curvature was estimated to be $17 \pm 3^\circ$, in good agreement with the independent estimates from the MD simulations.

To determine the directionality of the induced bend, a series of four oligonucleotides was designed in which the same single alkylation site as before was positioned 5, 7, 11, and 13 base pairs away from the center of a tract containing five consecutive adenines. Despite the fact that X-ray crystallography has consistently shown them as straight [27], these A-tracts are known to bend DNA toward the minor groove, and this bend can be used as a reference point for studying curvature by placing the A-tract “in phase” or “out of phase” with another bending element [28]. Since the anomalous mobility was at a maximum when the Et 743 alkylation site was positioned five base pairs (one-half helical turn) away from the center of the A-tract and at a minimum when the separation was 11 base pairs (one complete helical turn), these observations were taken as an indication that the bend caused by Et 743 was in a direction opposite to that of the A-tract, that is, toward the major groove, again in good agreement with the findings from the unrestrained MD simulations. This feature was novel among minor groove DNA monoalkylating agents, since covalent modification of N3 of adenine in AT-rich regions by (+)-CC-1065 and related compounds is accompanied by bending of the DNA into the minor groove [29].

23.7

Molecular Basis for Covalent Reactivity and Sequence Selectivity

As described in the previous section, the site selectivity of Et 743 is believed to be governed by a three-base-pair recognition sequence where the flanking base pairs immediately 5' and 3' to the modified guanine are involved in stabilizing the Et 743–DNA adduct. These previously reported studies provided a possible molecular basis for the recognition of, and reactivity toward, different DNA sequences by Et 743. Experiments that indicated differential stability of the high and low reactivity sequences prompted us to further explore the premise behind the apparent reactivity of Et 743.

23.8

The Rate of Reversal of Et 743 from Drug-Modified 5'-AGT Is Faster Than That from Drug-Modified 5'-AGC Sequences

The first indication that the Et 743 alkylation reaction is reversible came from initial endeavors to create a 60-mer oligonucleotide containing an Et 743 site-directed adduct at either a 5'-AGT or 5'-AGC sequence. The substrates were constructed by ligating a duplex oligonucleotide modified with Et 743 at a single guanine to flanking duplex oligonucleotides on both the 5'- and 3'-sides. Although we succeeded at creating a site-directed adduct at a 5'-AGC sequence, attempts at generating a site-directed adduct at the 5'-AGT sequence failed [30]. After several ligation and purification steps, it was observed that the ligated 60-mer no longer contained Et 743 covalently bound at the 5'-AGT sequence. This result prompted us to compare the stability of the Et 743–DNA adduct at the 5'-AGC versus 5'-AGT sequences.

A band shift assay was used to monitor the amount of drug-modified duplex in order to determine if the covalent adduct is stable over time [30]. A 22-mer oligonucleotide containing a single modification site, either 5'-AGT or 5'-AGC, surrounded by a common sequence (Fig. 23.8a), was modified completely with Et 743. The level of Et 743-modified DNA decreased over time for both Et 743 target sequences. At the end of the 8-h time period, the amount of the Et 743–DNA adduct at the 5'-AGC sequence was reduced by approximately 20%, whereas the amount of the Et 743–DNA adduct at the 5'-AGT sequence decreased by over 60% (Fig. 23.8b). This three-fold difference indicates that the Et 743 adduct at the 5'-AGC sequence is more stable than the Et 743 adduct at the 5'-AGT sequence. Furthermore, the results suggest that Et 743 can reverse from DNA and that the rate of reversal depends on the base pair immediately 3' to the site of covalent attachment of Et 743 (5'-AGC versus 5'-AGT). The observed reduction in the amount of Et 743–DNA adducts with time prompted us to look at the possibility that the released Et 743 might be able to “walk” the DNA, migrating from one sequence to another.

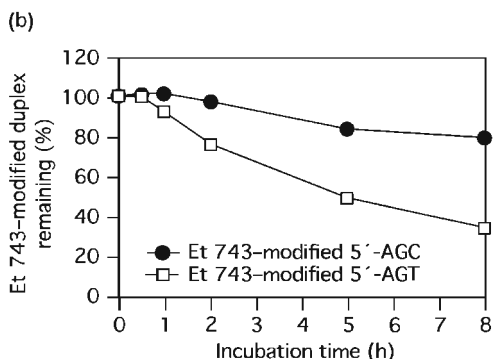
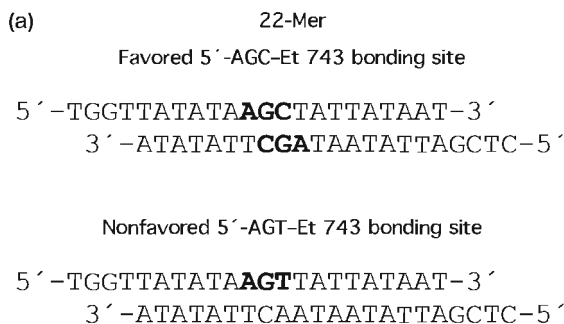


Fig. 23.8. Time dependency of the reduction of Et 743 adducts at different target sequences.

(a) Sequence of the 22-mer duplex DNA containing either the favored 5'-AGC or the non-favored 5'-AGT Et 743 target sequence. These oligonucleotides, containing either a single 5'-AGC or 5'-AGT, were incubated with 20 μM Et 743. The resulting covalent adduct

was incubated for the indicated amount of time at room temperature and loaded on a native gel. (b) Plot of the amount of each drug-modified duplex, expressed as a percentage, over time. The intensity of the band corresponding to Et 743-modified DNA at the designated times is normalized to the intensity at time zero, which is expressed as 100%.

23.9

Et 743 Can Reverse from Its Initial Covalent Adduct Site and Bond to an Unmodified Target Sequence

In principle, once Et 743 is released from the covalent modification sites, it could alkylate other available target sites. To determine if Et 743 can rebond at other recognition sequences, Et 743-modified oligonucleotides (22-mer) containing the site-directed adducts at either the 5'-AGC or 5'-AGT target sequence were incubated with a different size oligonucleotide (30-mer) (Fig. 23.9a) containing either the favored or the non-favored Et 743 target sequences, respectively [30]. Figure 23.9b shows that as the level of Et 743-modified 5'-AGT adduct decreases over time, the other duplex (30-mer) containing the favored drug target sites becomes modified with Et 743. After 10 h, approximately 20% of the 30-mer is modified by Et 743, suggesting that as Et 743 reverses from the non-favored 5'-AGT target sequence, it

(a)

30-Mer

Favored 5'-NGC-Et 743 bonding site

5'-TTTTTT**AGCAAGCTAGCT**ATCTCTCTCTCT-3'
 3'-AAAAAAT**CGTTCGATCGATA**-5'

Nonfavored 5'-NG(A/T)-Et 743 bonding site

5'-TTTTTTACAA**CGTCGTGAC**TCTCTCTCTCT-3'
 3'-AAAAAAT**TGTTGCAGCACTGA**-5'

(b)

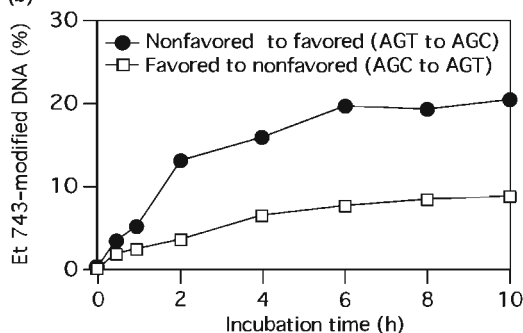


Fig. 23.9. Translocation of covalently bound Et 743 from its DNA adduct to another, unmodified target sequence. (a) Sequence of the 30-mer containing either the favored or non-favored Et 743 target sites. (b) A 30-mer containing only favored Et 743 target sequences was incubated for the indicated

amount of time with an Et 743-modified 5'-AGC site-directed adduct (22-mer). The resulting mixture was run on a 16% native gel. The amount of drug-modified duplex was quantified and plotted as a function of incubation time.

can rebind another DNA strand containing the favored target sites (Fig. 23.9b). The same profile can be seen when the 5'-AGC sequence is modified, but to a much smaller extent (Fig. 23.9b). Here, less than 10% of the 30-mer becomes modified with Et 743 after 10 h (Fig. 23.9b). These results support the notion that the reaction of Et 743 with duplex DNA is reversible and that, in the presence of competitor DNA, free drug released from the covalent DNA adducts can alkylate other available DNA sites, particularly when the initially alkylated sequence is a non-favored sequence.

23.10

The Kinetics of the Covalent Modification of 5'-AGC and 5'-AGT Sequences by Et 743 Are Similar

In addition to differences in the rate of the reverse reaction, there could also be significant differences in the rate of DNA alkylation at different bonding se-

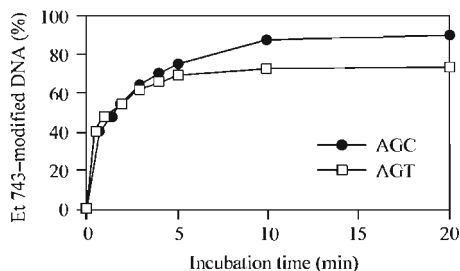
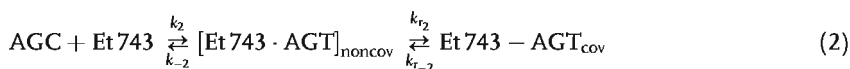
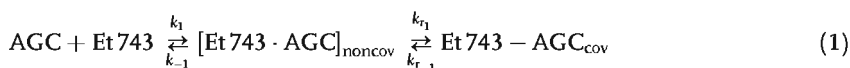


Fig. 23.10. Time-course experiment showing the reaction of Et 743 with DNA. An oligonucleotide containing either the 5'-AGC or 5'-AGT sequence was reacted with 20 μ M of Et 743. The amount of Et 743-modified duplex DNA was quantified and plotted as a function of time.

quences. In order to measure the relative kinetics of the forward reaction in different Et 743 target sequences, the 22-mer duplex DNA containing either the 5'-AGC or 5'-AGT sequence (Fig. 23.8a) was used to monitor the formation of Et 743-modified DNA as a function of time [30]. Although the reaction of Et 743 with both target sequences is equally fast up to 3 min, the final extent of modification is slightly less for the 5'-AGT sequence (Fig. 23.10). In the reaction of Et 743 with the 5'-AGC sequence, 75% of the duplex DNA becomes modified after 5 min, whereas with the 5'-AGT sequence, about 65% of the DNA is modified in the same time period. This small variation probably results from differences in equilibrium between covalently bound and unbound Et 743 at favored and non-favored sequences.

On the basis of these studies, two insights into the chemical reaction of Et 743 with DNA were proposed [30]. The first insight is an important modification of the previously reported catalytic mechanism for the reaction of Et 743 with DNA [23], which shows the reversible reaction (Fig. 23.5). The second insight provides an explanation for the apparent kinetics involved in the reaction of Et 743 with DNA. Assuming that the reaction takes place in two stages – a non-covalent association with DNA (noncov) followed by the formation of a covalent adduct (cov), as suggested previously [13] – the following kinetic model is proposed for the two different sequences:



The forward reaction is equally fast for both DNA sequences (i.e. $k_{r1} \cong k_{r2}$) and appears to be independent of the DNA target sequence. Indeed, the catalytic mechanism involving a proton shuttle does not involve the sequence-specific hydrogen bonding unique to either sequence, and it is therefore not surprising that the initial rate is independent of sequence. In contrast, the rates of the reverse

reaction for 5'-AGC and 5'-AGT (k_{r-1} and k_{r-2}) do depend on the triplet target sequence and show an approximate first-order rate constant of $2.8 \times 10^{-4} \text{ s}^{-1}$ for 5'-AGC and $1.2 \times 10^{-3} \text{ s}^{-1}$ for 5'-AGT (Fig. 23.8B), with $k_{r-2} > k_{r-1}$.

23.11

The Differences in the Rate of the Reverse Reaction May Be Derived from Structural Differences between Et 743–DNA Adducts at the 5'-AGC and 5'-AGT Sequences

The observed differences in the rate of dealkylation between the different sequences can be explained in terms of differential hydrogen bonding and the resulting stability of the covalent adducts. The results of previous studies from Texas led us to postulate that the ability to form the hydrogen-bonding network between Et 743 and the recognition sequence determines the relative drug-binding stability and reactivity. In the Et 743–AGC adduct, Et 743 is positioned within the minor groove such that the coordination of hydrogen bonds is maximized, which results in maximum stabilization of the covalent adduct. On the basis of NMR data [30], it has been demonstrated that changing the target sequence from 5'-AGC to 5'-AGT yields an Et 743 adduct in which Watson–Crick base pairing is disrupted at least to the 5'-side of the target guanine. As a result, the thermal stability of the covalent adduct at the 5'-AGT sequence is reduced. These results demonstrate the importance of the hydrogen bond between the 2-amino group of guanine on the 3'-side of the covalently modified guanine and C8–C23 oxygen of Et 743 as a major contributor to the stability of Et 743–DNA adducts.

This loss of structural integrity of the Et 743–AGT adduct could account for the differential rate of reversibility that is observed between the two target sequences. For example, this could lead to a change in the microenvironment surrounding the covalent linkage between DNA and Et 743. At the 5'-AGC target sequence, where Et 743 forms a stable, tight complex, the linkage is less accessible to attack by a water molecule ($5 \rightarrow 4$ in Fig. 23.5). This is in direct contrast to the Et 743–AGT adduct, where the minor groove site of covalent attachment is more accessible to solvation due to the dynamic nature of the DNA adduct forming an open complex. Hence, this increased solvent accessibility could increase the rate of the reverse reaction of Et 743 with DNA, whereas stable adducts (e.g. AGC) that are excluded from attack by the water molecule would have a lower rate of reversibility.

23.12

Molecular Targets for Et 743

While it is clear that Et 743 can covalently modify DNA with resulting structural effects on DNA, how this mediates the cytotoxicity, and indeed whether this is the only mechanism for producing other biologically relevant events (e.g. reversal of MDR resistance), is still not completely clear. A wide range of studies have been carried out to elucidate the mechanism of action of Et 743 [16, 18, 32–40]. Perhaps

one of the more important early observations was that in the NCI 60 cell panel line, the COMPARE analysis demonstrated that Et 743 had a unique mechanism of action that encouraged further development.

A drug such as Et 743, which covalently reacts with DNA to occupy the minor groove and bend DNA, is likely to affect a wide range of processes that require protein binding to DNA (transcription, replication, repair, and recombination) as well as nucleosomal assembly and helicase unwinding. It is perhaps not surprising that many of these processes have indeed been observed to be inhibited [3, 11, 19, 31]. It is therefore important to differentiate processes that are inhibited at physiologically relevant concentrations of Et 743 (1–100 nM) from those that only occur at much higher concentrations (i.e. 10 μ M). Thus inhibition of topoisomerase I, which occurs at 10 μ M [16, 33], binding of nuclear transcription factor Y (NF-Y) to DNA, which occurs at micromolar concentrations [35], and disorganization of microtubule networks, which also occurs at micromolar concentrations [32], are unlikely to be relevant to the cellular mechanism of action of Et 743.

There are at least two processes that are disrupted by Et 743 that appear to be biologically relevant. The first is the hijacking of the nuclear excision repair (NER) mechanism [38], which gives rise to a lethal lesion on DNA, and the second is the suppression of MDR1 transcription [34, 40], which leads to avoidance of a drug-resistance mechanism. Only the first has been directly related to the covalent modification of DNA, while the second may be related to Et 743 modification of DNA or protein.

23.12.1

Involvement of Transcription-Coupled Nucleotide Excision Repair in Mediating the Cytotoxic Effects of Et 743

D'Incalci and colleagues [35, 36] first demonstrated that hamster cells deficient in XPB, ERCC1, or CSB, and human XPA cells had reduced sensitivity to Et 743. Pommier and colleagues selected a human colon carcinoma cell line (MCT 116/ER5) derived from HCT 116 cells that was resistant to Et 743 through exposure to increasing concentrations of this drug for one year until they were 23-fold more resistant than the parent cell line [38]. These cells were used to characterize the genomic change resulting in the resistance and the effects of exposure to Et 743. The chromosome alteration occurred in a region that included the gene (XPG) implicated in xeroderma pigmentosum. Analysis of the XPGc DNA showed insertion of an adenine in codon 240, resulting in frameshift mutation and a stop codon at position 243 in the HCT 116/ER5 cells. Complementation with wild-type XPG restored drug sensitivity. Significantly, studies in cells deficient in XPC demonstrated that drug sensitivity is specifically dependent on the transcription-coupled pathway of NER. Finally, it was determined that interaction of Et 743 with transcriptionally coupled (TC)-NER machinery results in DNA strand breaks, presumably associated with an aberrant repair process, which is blocked in the NER-deficient cell lines. The formation of Et 743-associated DNA strand breaks was not found in a second study [36].

In parallel studies carried out in Texas and Arizona [39] using the bacterial UvrABC proteins, it was demonstrated that Et 743–DNA lesions at the favored bonding sites (i.e. 5'-CGG or 5'-AGC) were inefficiently incised by the bacterial NER system, resulting in aberrant strand breaks on the 5'-side of the lesion and some evidence of uncoupled 3' and 5' incisions. These results, taken in conjunction with the unique characteristics of Et 743, were used to propose a mechanism for how the NER processing of the Et 743–DNA adduct would lead to aberrant repair. In this proposal stable Et 743–DNA duplex adducts must have structural features that somehow mimic the bent and open form of the normal UvrA₂B heterotrimeric complex with DNA. Indeed, Et 743–DNA adducts are bent into the major groove through minor groove expansion due to occupancy by the wedge-shaped Et 743. The extrahelical protrusion of the C-subunit of Et 743 may also play a role in promoting the dissociation of UvrA and, perhaps, stabilizing the UvrB preincision complex. However, in the absence of defined structural interactions among the various protein–DNA components of the UvrA₂B–, UvrB–, and UvrBC–DNA complexes, it is impossible to be more specific than just suggesting possible structural mimics (bending) and steric interactions (extrahelical protrusion) that could lead to aberrant DNA repair outcomes. One could envisage a situation similar to that of a drug-poisoned topoisomerase I or II complex where a drug molecule intercepts an intermediate in which neither a 3' nor a 5' incision is made, but the UvrBC, or even the UvrABC, is trapped as a non-covalent protein–Et 743–DNA complex, which is more detrimental to cells than the original Et 743–DNA adduct. Just as with the drug-poisoned topoisomerase I or II complexes, where elevated levels of topoisomerases would lead to populations of more susceptible cells, a similar situation exists for elevated levels of NER proteins.

23.12.2

Suppression of MDR1 Transcription by Et 743

In addition to the mechanism involving subversion of the TC-NER system to produce lethal lesions on DNA, Et 743 has been shown to inhibit MDR1 transcription activation [34]. This is an important attribute of a cytotoxic compound because MDR1-mediated drug resistance is a prime way that cancer cells avoid the toxic effects of anticancer agents. The underlying mechanism for this resistance is still unclear and may involve Et 743 alkylation of DNA or direct interaction at the protein level (see later). It has been proposed that the mechanism occurs at the DNA level by blocking of transcriptional factor NF-Y [35]. However, this only occurs at 10³-fold higher levels of Et 743 than required to produce the inhibition of MDR1. In a recent publication [40], Forman and collaborators have demonstrated that nanomolar concentrations of Et 743 potentially inhibit SR12813-induced activation of CYP3A4 and MDR1 expression by the pregnane X receptor and broad-specificity xenobiotic sensor PXR/SXR. This antagonism of SXR activation by Et 743, leading to downregulation of the P-glycoprotein pathway of drug efflux, could be advantageously used to prevent development in cancer cells of a multidrug resistance phenotype.

23.13

Relationship of Structural Consequences of DNA Modification by Et 743 to Biological Effects

The reported Et 743-induced DNA bending and DNA minor groove widening could be important for either impairing the binding of sequence-specific transcription factors, as reported for nogalamycin, chromomycin A₃ [41], and the bisanthracycline WP631 [42], or for enhancing recognition by DNA-binding proteins, such as those that recognize cis-platinated DNA [43]. Disruption of protein–DNA association could be brought about by occupancy of the minor groove or by drug-induced bends that are incompatible with the direction of bending required by the DNA-binding protein at its recognition site. Alternatively, the existing bend could be advantageous for binding of some other proteins to the major groove, but no such proteins have been identified so far. On the other hand, if multiple potential binding sites for Et 743 are properly phased in a relatively short stretch of DNA, the imposed cumulative curvature could bring closer together specified fragments not contiguous in primary sequence [44], and the drug could be serving a surrogate protein function. This has been demonstrated for (+)-CC-1065, which binds to the DNA minor groove and can partially substitute for the transcription factor Sp1 in upregulating the level of basal gene expression [45].

Since there are many occurrences of the triplet sequences favored by Et 743 and related compounds in genomic DNA, one may wonder whether the cytotoxic effect caused at very low concentrations (between picomolar and nanomolar, depending on cell type) relies on binding to a specific DNA sequence or to a particular DNA architectural motif. A sequence that has elicited some interest is the TGG triplet contained in the complementary strand of the so-called CCAAT box, a common element in eukaryotic promoters that can be found in both the forward and the reverse orientation (i.e. ATTGG). These five nucleotides are absolutely required for binding of the evolutionary conserved activator NF-Y (CBF, HAP2/3/4/5) [46] to the DNA minor groove, and in fact, binding of NF-Y to this sequence has been shown to be inhibited by Et 743 [47]. While it seems clear that occupancy of the minor groove by the covalently bonded drug will preclude association of NF-Y with its cognate DNA element, the micromolar concentrations necessary to achieve this effect, together with the fact that stably bound NF-Y is not displaced from the CCAAT box by the low concentrations of Et 743 used both in cell cultures and in patients [33], point to a different DNA site as the likely target for Et 743.

From the structural standpoint, a remarkable parallel has been found in the manner Et 743 and the DNA-binding domain of the early growth response protein 1 (EGR1) and other zinc finger-containing transcription factors (e.g. Sp1) induce DNA distortions [48]. Each Cys₂His₂ zinc finger domain in EGR1 and Sp1 typically binds three successive base pairs of DNA sequence and the α -helical portion of each finger is capable of fitting in the major groove of the DNA in such a way that binding of successive fingers causes the protein to wrap around the DNA [49]. Base recognition capitalizes on three key residues in this α -helical “reading head,”

and the relatively simple set of contacts they make resembles a well-studied protein–DNA recognition code [50].

Coincidentally, some of the G-rich triplets recognized by individual zinc fingers of these proteins (i.e. TGG), as well as some overlapping combinations (i.e. CGG and GGC), contain some of the favored target sites for Et 743 binding. Thus, in a protein–DNA binary complex these triplets still provide the hydrogen-bonding functionalities in the minor groove associated with high-affinity Et 743-binding sites, with the added bonus that the DNA is already locally bent and unwound and presents a widened minor groove relative to the canonical B-conformation [51]. As a consequence, the exocyclic amino group of the central guanine and the carbonyl oxygen of the pairing cytosine in any of these triplets are more exposed to the solvent and thereby more readily accessible for interaction with Et 743-like molecules. The possibility then exists that Et 743 will prefer to bind with higher preference to this distinctive DNA conformation than to “naked” DNA. In other words, it is conceivable that the demonstrated effect of Et 743 on DNA curvature, which reflects an influence on the energetics of DNA conformation, can be accompanied by a reciprocal effect of DNA conformation on drug binding. Therefore, if Et 743 widens the minor groove and bends DNA toward the major groove, Et 743 can be expected to bind more tightly to locally bent DNA–protein complexes displaying these structural characteristics than to linear B-DNA. Such thermodynamic linkage between cyclization and binding (Fig. 23.11) has been demonstrated for catabolite activator protein binding to its DNA target site, which also has been shown to be intrinsically slightly bent [52]. Likewise, the association of TATA-binding protein to the minor groove of the TATA element has been reported to be enhanced when the DNA is slightly pre-bent in the direction of the major groove [53].

Interestingly, EGR1 and the ubiquitously expressed Sp1 recognize different, but related, nucleotide sequences that can be partially overlapped [54], in which case their binding is mutually exclusive. Binding of one or the other protein will depend on their relative concentrations and their intrinsic affinities, which can be finely tuned by phosphorylation [55, 56]. The hypothesis that the resulting balance in such competition can be influenced by the presence of G-bonded Et 743 to one or more sites has not been experimentally tested yet. Some DNA triplet subsites, such as TGG or CGG, can theoretically provide simultaneous binding sites for a zinc finger (in the major groove) and Et 743 (in the minor groove). In other instances, the Et 743 binding site may overlap two adjacent zinc finger subsites, and the possibility also exists that, given the right sequence, two or more Et 743 molecules are bonded consecutively. An example of several triplet sites contiguous in sequence that are favorable for Et 743 binding is found in the variable D6 region of the human 28S ribosomal RNA gene [57]: the CGG triplet is tandemly repeated between 5 and 8 times. This gene is transcribed by RNA polymerase I in the nucleolus, and since elevated rRNA synthesis is a hallmark of rapidly proliferating cells, it may be worth testing whether expression of this gene is affected by Et 743.

Another example of such a composite site, TGGCGGCGG, is found in the 5'-upstream promoter region of the human *p73* gene, which encodes a protein with structural and functional homologies with the p53 tumor suppressor protein. To

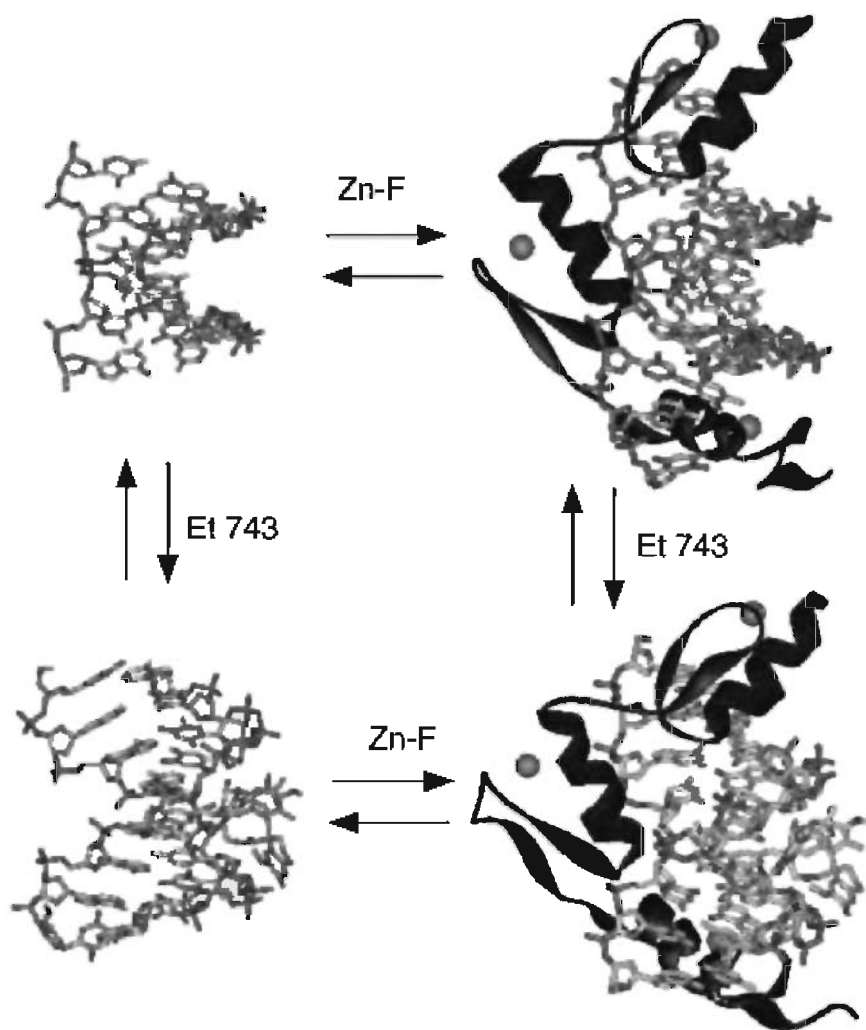


Fig. 23.11. Quasi-thermodynamic cycle for binding of Et 743 and EGR1/Sp1 (Zn-F) to either a naked double-stranded DNA molecule (top left) or to a binary drug-DNA (down left) or protein-DNA complex (top right).

assess the feasibility of having more than one Et 743 molecule bonded to adjacent target sites, molecular dynamics simulations in aqueous solution of the complex between the double-stranded dodecanucleotide d(GTGGCGCGCGCC) and three tandemly bound Et 743 molecules (ecteinascidin-bonded guanines underlined) were recently undertaken [58]. This study showed that head-to-tail binding of multiple Et 743 molecules to adjacent optimal sites is indeed structurally and energetically possible (PDB code 1KML) and is probably favored over binding to individual sites, since it brings about the possibility of cooperativity. The DNA in

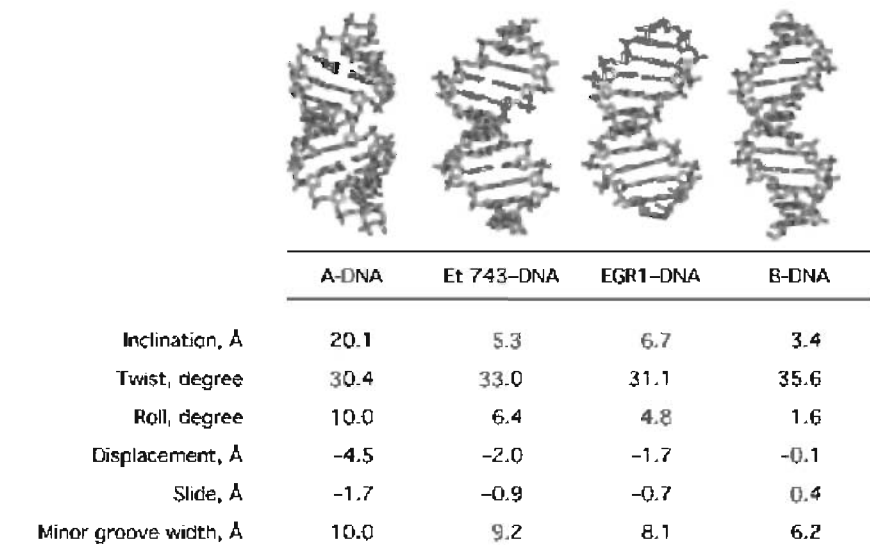


Fig. 23.12. Comparison of typical structural parameters for (from left to right) canonical A-DNA, DNA in a complex with three tandemly bound Et 743 molecules (Et 743-DNA, PDB code 1KML) [58], DNA in a complex with the three zinc fingers of transcription factor EGR-1 (EGR1-DNA, PDB code 1AAY) [49], and canonical B-DNA.

this complex showed a very good superimposition onto the DNA recognized by the three zinc fingers present in the DNA-binding domain of EGR1. In fact, the conformation of the DNA in both complexes was shown to be intermediate between those of canonical A- and B-DNA (Fig. 23.12).

These structural features are also reminiscent of those found in DNA-RNA hybrids, which suggests that Et 743 could also preferably target such conformations, as found in the double helix of template DNA paired to nascent RNA, that could be found in the active site of RNA polymerase II [59, 60]. Inhibition of RNA polymerase activity and decreased RNA synthesis were indeed demonstrated in early bioassays using purified ecteinascidins. In a previous section we proposed how the unique structural features of the Et 743-DNA adduct can trap the TC-NER system.

Notwithstanding the relatively well-studied interaction of Et 743 with DNA, a possible interaction with other targets cannot be neglected. The intriguing observation that inhibition of NF- κ B binding to DNA was achieved at lower concentrations when the protein was preincubated with Et 743 than when the drug was allowed to react with DNA suggests some sort of drug-protein interaction. In this respect, the recent observation that Et 743 can inhibit ligand-induced transcriptional activation of genes regulated by the nuclear receptor PXR/SXR [40] is of particular interest. Preliminary docking experiments (E. Marco and F. Gago, unpublished) suggest that Et 743 could be targeting the co-activator binding site that is formed upon agonist binding to this receptor (Fig. 23.13). This site, which is mostly hydrophobic, appears to be a suitable target for Et 743 binding and subse-

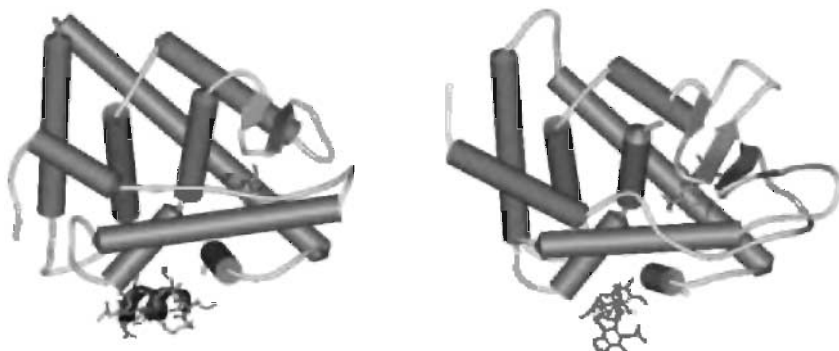


Fig. 23.13. (a) Schematic representation of the agonist-bound conformation of the human estrogen receptor ligand binding domain (LBD) co-crystallized with a short fragment of bound co-activator (green ribbon) (PDB entry

3ERD). (b) Similar view of the related human SXR nuclear receptor LBD in complex with the agonist SR12813 (PDB entry 1ILH) showing Et 743 tentatively docked in the co-activator binding cleft.

quent nucleophilic attack by a neighboring lysine residue. Therefore, the possibility that protein-binding sites for Et 743 exist, in addition to the better studied DNA-binding sites, needs to be seriously considered.

23.14 Conclusions

The high expectations raised in 1969 by the potent antiproliferative activities of natural ecteinascidins have been realized by the optimistic results obtained from phase I and II clinical trials with Et 743 and the demonstration of both a high potency and a favorable therapeutic index. Limitations on testing initially imposed by the low amounts of drug present in the natural source have been overcome by current availability in kilogram quantities from chemical modification of microbially produced cyanosafrafrin B. Et 743 alkylates DNA on the exocyclic amino group of guanines following non-covalent binding to the minor groove of some preferred triplet sequences in a reaction that can be reversed on DNA denaturation. Sequence selectivity has been rationalized on the basis of a direct read-out through a set of well-defined hydrogen-bonding rules dictating that the preferred targets are $R\underline{C}C$ and $Y\underline{C}G$, where R and Y stand for purine and pyrimidine, respectively, and the underlined guanine is the base that undergoes alkylation by Et 743. Intriguingly, it is the differential rates of reversibility from preferred ($R\underline{C}C$ or $Y\underline{C}G$) versus non-preferred (XGA or $X\underline{C}T$), where X is any base, that is the main determinant of sequence preference rather than reaction rate.

Et 743 interferes with at least two biological processes important to cancer cells. First, Et 743 appears to subvert the NER system to yield a poisoned complex in which the Et 743–DNA adduct traps the repair complex, possibly leading to single-

strand breaks in DNA. Second, Et 743 causes MDR1 reversal by a mechanism that is as yet undefined. At the transcriptional level Et 743 blocks NF- κ B binding to its cognate sequence in the transcriptional region of MDR1, but only at concentrations much higher than those required to inhibit MDR1 expression.

Binding of an Et 743 molecule to a DNA target site brings about widening of the minor groove and curvature toward the major groove. The resulting DNA deformation strongly resembles that induced by zinc finger-containing transcription factors, such as EGR1 and Sp1, which bind to GC-rich regulatory sequences in many gene promoters. Since the transcription factor and Et 743 can bind simultaneously on opposite sides of the double helix, binding of Et 743 to the fully accessible minor groove in well-defined steps of Sp1-DNA or EGR1-DNA complexes may be facilitated by protein-induced structural changes and could lead to increased complex stabilization. The resulting enhanced specificity could account for the unusually high potency and the remarkable effects on gene transcription of Et 743 and other ecteinascidin-like molecules.

The unique effects of Et 743 on DNA structure, and the corresponding effects on NER and perhaps MDR1, provide clinical oncologists with an agent quite different from existing drugs. Et 743 has already shown excellent clinical activity as a single agent in soft tissue sarcomas, and because of its ability to trap NER complexes and reverse MDR1, its use in combination with other alkylating agents should give excellent responses.

Acknowledgments

Federico Gago would like to thank his past and present collaborators. Laurence Hurley is grateful to David M. Bishop for preparing, proofreading, and editing the final version of the manuscript and figures.

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24

The Azinomycins. Discovery, Synthesis, and DNA-binding Studies

Maxwell Casely-Hayford and Mark Searcey

24.1

Introduction

Nature is a rich source of potential antitumor agents that can act as a paradigm for the development of synthetic agents that retain the biological activity of the natural product but are targeted to the tumor, have increased bioavailability or are simply prodrug forms that are inactive until administered. Examples of potent antitumor antibiotics that interact with DNA include the clinically used anthracyclines [1] and bleomycins [2], while agents under investigation include CC-1065 and the duocarmycins [3], ecteinascidin 743 [4], and rebeccamycin [5]. The effective exploitation of the biological activity of natural products requires a combination of synthesis, computational studies, an array of DNA-binding studies and both *in vitro* and *in vivo* biological studies. Such a combination has led to the development of agents such as mitoxantrone [6] and, more recently, the pro-drug AQ4N [7], based upon extensive knowledge of the biological activity of the anthracycline antibiotics.

Synthetic alkylating agents have a long history in cancer chemotherapy [8]. With their origins as the sulfur mustards developed for chemical attacks during the First World War, they were first used clinically in the fourth decade of the twentieth century. Typically, synthetic alkylating agents derive their biological activity from an ability to alkylate and crosslink DNA, and extensive studies have been made of relationships between structure, sequence selectivity, and antitumor activity. Nature has also developed agents that can bind covalently to nucleic acids and that have potential therapeutic value, particularly in the treatment of cancer. Currently used clinically in the treatment of gastrointestinal, breast and bladder cancers, mitomycin C is an example of an antitumor antibiotic that can bind to and crosslink DNA [9]. CC-1065 and the duocarmycins are minor groove binding alkylating agents that are amongst the most potent antitumor antibiotics discovered to date. Their mode of action, through alkylation of DNA in the minor groove, has been extensively studied and reviewed [3].

The azinomycins A (1) and B (2) (Fig. 24.1) [10, 11] and the truncated analog 3 represent novel structures that have been extensively explored from a synthetic

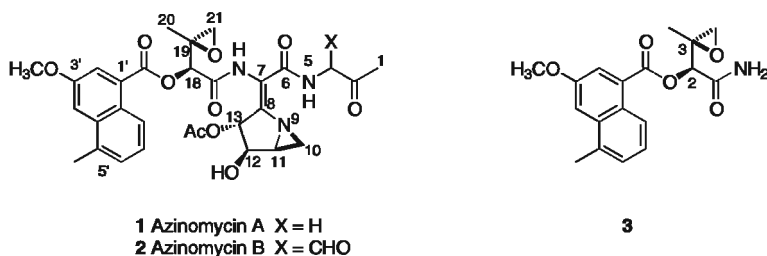


Fig. 24.1. Structure of the azinomycins.

viewpoint [12], but for which studies of structure–activity relationships from a DNA-binding and biological viewpoint have been few and widely spaced chronologically [12]. To date, little advance has been made in progressing these agents into a clinical setting. In this chapter, we aim to bring together the details of the isolation, synthesis, structural, and DNA-binding studies of the azinomycins, to generate a starting point from which to begin to understand the properties of these natural products that could lead to new, therapeutically relevant compounds.

24.2

Isolation of the Azinomycins 1–3

The azinomycins A (1) and B (2), along with the truncated analog 3, which consists of the naphthalene and the epoxide fragments, were isolated from *Streptomyces griseofuscus* S42227 by researchers at the SS Pharmaceutical company in Japan [10]. The structures and the *in vivo* biological activities of the compounds in a number of murine tumors were described [13]. Interestingly, this group reported no cytotoxicity for 3, although more recently this compound has been reported by a number of other groups to have potent antitumor activity (see below, Section 24.3.2). The structures of the three compounds were ascertained by inspection of the mass, ^1H and ^{13}C NMR spectral data and these workers noted that the spectral data were similar to that for carzinophilin, an antitumor antibiotic that was isolated in 1954 from *Streptomyces sahachiroi* [14]. Armstrong later disclosed that the ^1H and ^{13}C NMR of carzinophilin and azinomycin B were superimposable [15] and since that date, the two compounds have been regarded as being the same.

Carzinophilin had an interesting history, as attempts to assign a structure to the compound had been dogged by revisions until the discovery of the azinomycins. The structure originally proposed by Lown and co-workers to be a bisintercalating bisalkylator with the structure 4 [16], was later revised by Onda [17] to an *N*-acetylaziridine intercalator possessing a highly oxygenated structure 5 (Fig. 24.2). These early studies led to Shibuya's synthesis of the naphthalene ring structure, at the beginning of a synthetic route to Lown's compound [18], which has been used with small variations, by all workers in the area (see Section 24.3).

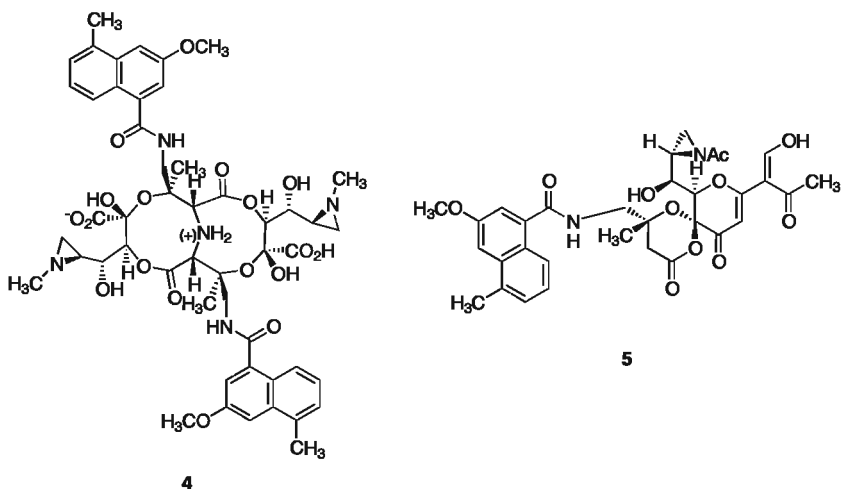


Fig. 24.2. Reported structures of carzinophilin.

24.3

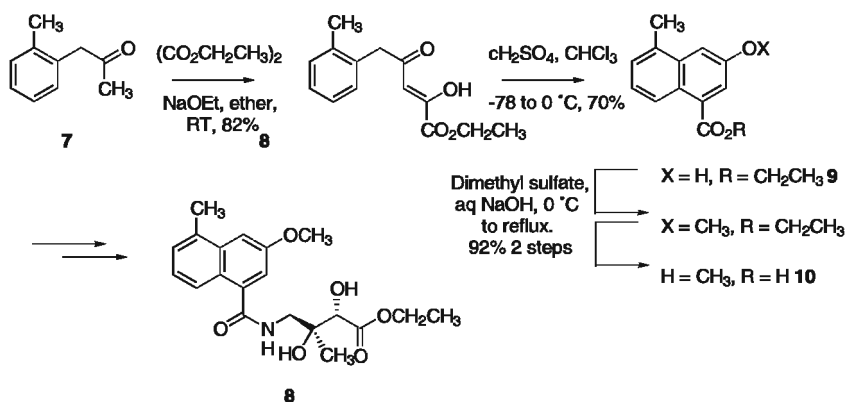
Studies of the Truncated Analog 3

24.3.1

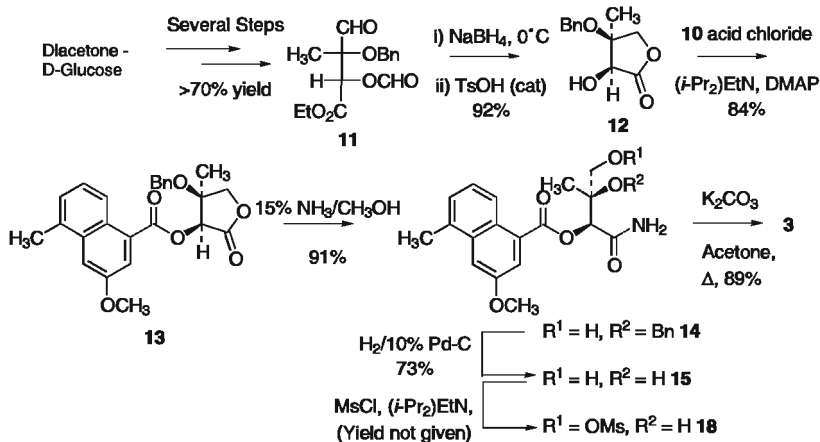
Stereoselective Synthesis of the Truncated Azinomycin 3

The first synthetic studies of the azinomycins [18] were based upon the erroneous structure of carzinophilin suggested by Lown and co-workers [16]. Although it has some historical interest, it also forms the basis of the synthesis of the azinomycin chromophore that has been utilized by all workers since this time. In essence, Shibuya synthesized one of the degradation products of azinomycin A (compound 6), which formed the C1–C18 segment of the structure 4 [18]. 3-Methoxy-5-methylnaphthalene-1-carboxylic acid 10 was synthesized via a condensation reaction of 1-(2-methylphenyl)-2-propanone 7 with diethyl oxalate to give the enol 8, which was cyclized to the naphthol 9 under acidic conditions. The naphthol was methylated and hydrolyzed in high yield, under basic conditions to give 10 (Scheme 24.1).

The side chain synthesis noted in this work is of interest in that Shibuya went on to use a similar approach in the synthesis of the truncated azinomycin analog 3, the first description of the synthesis of this compound [19]. To achieve this aim, Shibuya used the “chiral pool” and began from commercially available diacetone D-glucose, using a five-step synthesis to generate the aldehyde 11, which was reduced and refluxed with catalytic acid to generate the γ -lactone 12 (Scheme 24.2). This lactone could be coupled directly with the acid chloride of naphthalene 10 and ring-opened with ammonia to give the amide 14 in excellent yield and without cleavage of the ester bond. Removal of the benzyl group, mesylation of the primary alcohol and base-induced cyclization generated 3.



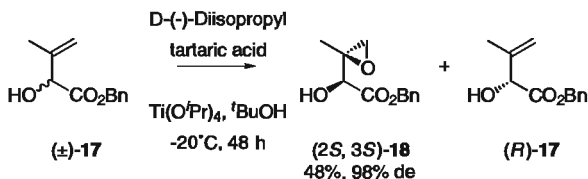
Scheme 24.1. Synthesis of the naphthalene ring system [18].

Scheme 24.2. Shibuya synthesis of **3** [19].

This stereospecific synthesis of **3**, while somewhat lengthy compared with later efforts, established the stereochemistry of the epoxide in the natural products, as the (18*S*, 19*S*) diastereoisomer (azinomycin numbering) matched exactly that of the isolated natural product of **3** and, crucially, had the same optical rotation. Shibuya and co-workers also used a second route to the epoxide via the chiral pool, beginning from fructose [20].

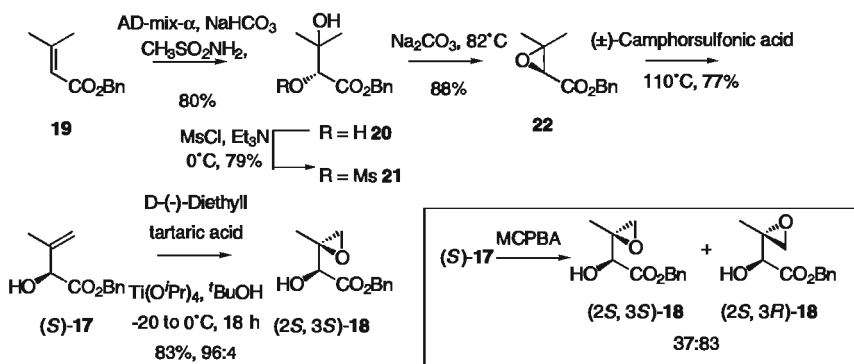
The majority of other studies on the synthesis of the epoxide fragment of the azinomycins and on the synthesis of **3** have focused upon the use of the Sharpless asymmetric epoxidation, for which the fragment is ideally suited. First reports of the use of this reaction were by Armstrong and co-workers [21] and Shishido *et al.* [22], but more direct efforts on the precursor **17** were reported by Konda [23]. Kinetic resolution of racemic **17** under Sharpless AE conditions using *D*-(−)-diisopropyl tartrate gave a 48% isolated yield and 98% ee of (2*S*, 3*S*)-**18**, along with

recovered (*R*)-**17** (Scheme 24.3). Due to errors in the reporting of optical rotation in Shibuya's earlier work [20], Konda mis-assigned the stereochemistry in his original paper and Shipman and co-workers [24] later revised his results to show that the above was correct. Konda believed that he had obtained the opposite to the expected stereochemistry.



Scheme 24.3. Konda synthesis of the epoxide fragment [23].

The use of the Sharpless AE in the synthesis of the epoxide, which has been further investigated by Coleman [25], is a rapid route into the epoxide fragment and to the synthesis of **3**. However, in an elegant approach, Shipman and co-workers used stereocontrol throughout the synthesis by the development of a Sharpless asymmetric dihydroxylation/asymmetric epoxidation methodology [24]. Using the readily available benzyl ester of dimethyl acrylic acid **19**, they performed an asymmetric dihydroxylation using AD-mix- α to generate **20**. Mesylation, epoxide formation and acid catalyzed epoxide opening gave the intermediate (*S*)-**17**, which was now correctly functionalized for Sharpless AE. Use of D-(-)-diethyl tartrate generated the required (2*S*, 3*S*)-**18** with minor separable amounts of the (2*S*, 3*R*)-**18** (Scheme 24.4). Reaction with the acid chloride of **10**, deprotection and reaction with ammonia under coupling conditions gave a high yielding route to **3**. Single crystal X-ray structures of intermediates firmly established the stereochemistry of **18**. One of the strengths of this approach is the ability to generate all four stereoisomers of **18**. Shipman reported a 37:63 mixture of diastereoisomers of **18** when MCPBA was used as oxidizing agent and in the authors'



Scheme 24.4. Shipman synthesis of the epoxide fragment [24].

laboratory, we have used this method to generate all four diastereoisomers of **3**, including the (2*S*, 3*R*) and (2*R*, 3*S*) isomers of **18**, which are easily separated by chromatography and which have not been previously studied. Such investigations may aid in our understanding of factors that contribute to the biological activity of the natural products (M. Casely-Hayford and M. Searcey, unpublished observations).

24.3.2

DNA-binding and Biological Activity Studies of the Truncated Fragment and Synthetic Analogs

The synthesis of the left-hand fragment of the azinomycins, in itself a natural product with some demonstrated, although controversial, antitumor activity, allowed the study of its interactions with DNA and further assessment of its antitumor activity *in vitro*. Zang and Gates have disclosed the most comprehensive study of this compound and its interactions with its nucleic acid target (Fig. 24.3) [26]. Using a 5'-³²P-labeled 145-base-pair restriction fragment, they showed that **3** alkylated DNA at guanine residues with little, if any, sequence selectivity but at much lower concentrations than a simple epoxide alkylating agent **23**. This strongly supports the hypothesis that **3** may bind non-covalently to DNA. They also demonstrated that addition of ethidium bromide inhibits DNA alkylation, that DNA binding is accompanied by bathochromic and hypochromic shifts in the UV-visible spectrum and that in a T4 ligase DNA-winding assay **3** requires a concentration 200–500 times that of daunomycin to induce similar effects.

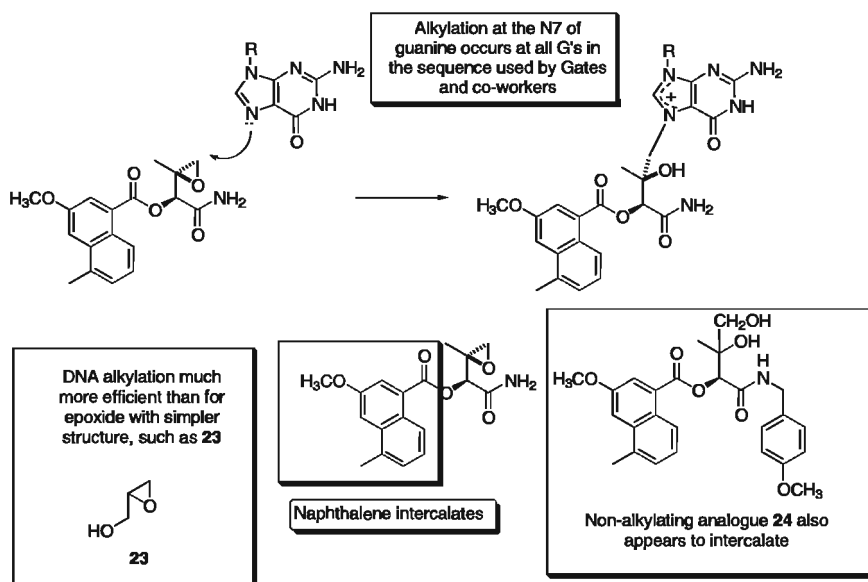
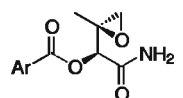


Fig. 24.3. Zang and Gates results confirm intercalation and guanine alkylation by **3** [26].



Cytotoxicity IC₅₀ Values (μM)

	A2780	A2780cisR	CH1	SKOV-3	HT29
3	<0.05	0.076	<0.05	1.25	0.33
25	1.8	3.5	1.4	12.0	4.3
26	0.44	1.1	0.55	5.1	2.15
27	0.39	1.35	0.17	13.0	2.8
28	>25	>25	>25	>25	>25
29	>25	>25	>25	>25	>25

Chemical Structures:

3: R = CH₃ (3-methoxy-5-methylnaphthalene derivative)

26: R = CH₂CH₃ (3-ethoxy-5-methylnaphthalene derivative)

28: Phenyl group (1-phenyl-2-epoxynaphthalene derivative)

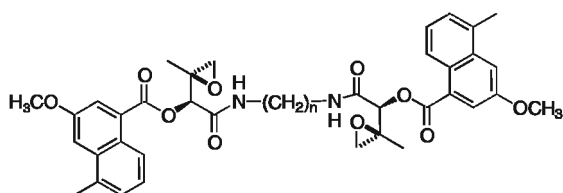
29: Quinoxaline chromophore (2-epoxy-3-methylquinoxaline derivative)

Fig. 24.4. Effect of intercalator structure on antitumor activity [27].

All of these experiments point to an intercalative mode of binding for the naphthalene chromophore, a mode that has been suggested for azinomycin but that is somewhat surprising in view of the generally held belief that naphthalene derivatives are poor DNA intercalators. Crucially, these authors used viscometric experiments that showed increases in viscosity of double-stranded DNA-containing solutions upon the addition of both **3** and the non-alkylating analog **24**, strongly indicative of an intercalative mode of binding for these compounds.

These findings correlate with, and help to explain, the observations of other groups with regard to the biological activity of analog structures of the natural product. Upon completion of the stereoselective synthesis of **3**, Shipman and co-workers began a synthetic effort to identify analogs of the natural product that may be useful antitumor agents (Fig. 24.4) [27]. They began a small structure–activity survey of the substituents to the left (the naphthalene) and to the right (the amide) of the epoxide. Replacement of the 3-methoxy-5-methylnaphthalene with a phenyl group (compound **28**, Fig. 24.4), which would be expected to show little affinity for DNA through intercalation, effectively removes the biological potency of the epoxide in a variety of cell lines. Intriguingly, both 1- and 2-naphthalene-carboxylate analogs, while losing significant (up to 20-fold) activity, remain relatively potent with biological activities as low as 100–200 nM in some cell lines. Introduction of the quinoxaline chromophore, the intercalating moiety of the cyclic depsipeptide antibiotics such as triostin A, led to a complete loss of biological activity confirming, perhaps, that the chromophore alone, even in this well-studied family of bisintercalators, is not sufficient for DNA intercalation.

In a further effort to generate more potent analogs of **3**, Shipman has also designed, synthesized, and studied a crosslinking dimer of the epoxide that can form interstrand crosslinks in DNA (Fig. 24.5, compounds **30–32**) [28]. Using an alkyl linker through the right-hand carboxylic acid group, they demonstrated that an optimum linker length appeared to be four methylene groups and that all of these



Cytotoxicity IC₅₀ Values (μM)

	n	A2780	CH1	CH1cisR	SKOV-3	HT29	K562
30	2	<0.05	<0.05	<0.05	1.4	0.42	0.067
31	4	0.065	<0.05	<0.05	0.82	0.55	0.027
32	6	0.11	0.08	0.08	1.2	0.50	0.062
3	-	<0.05	<0.05	-	1.25	0.33	-

Fig. 24.5. DNA crosslinking agents [28]. Comparison of antitumor activity with **3**. From Ref. [27].

agents ($n = 2, 4, 6$ in Fig. 24.5) can crosslink DNA and have potent cytotoxic activity, although none of the compounds had significantly greater activity than the non-crosslinking **3** (see Section 24.6). An interesting study in this context would be the effect of stereochemistry on the biological activity of these compounds. Presumably a dimer formed from the (*S,S*) and (*R,R*) diastereoisomers would be able to form intrastrand crosslinks more easily than interstrand ones.

24.4

Studies on the Total Synthesis of the Azinomycin A

24.4.1

Synthesis of the 1-Azabicyclo[3.1.0]hex-2-ylidene Dehydroamino Acid Subunit

Most of the synthetic work on the azinomycins has been focused upon the densely functionalized 1-azabicyclo[3.1.0]hex-2-ylidene dehydroamino acid subunit, chiefly as the research was driven by the enquiries of synthetic organic chemists for whom the chemistry was a significant and attractive challenge. It is outside the scope of this review to comprehensively cover all of the efforts on this unit and the reader is referred to reviews by Shipman [12] (who also covers Tereshima's work in more detail than here) and Coleman [29] (covering his own work) for a more synthetic approach. Here, we will briefly review efforts that ultimately led to the total synthesis of the azinomycins.

Both Armstrong [30] and Coleman [31] have, independently and concurrently, described a route to the aziridine core unit that involves disconnection of the intact aziridine from the alkene, and of the alkene from the backbone (Fig. 24.6). In the forward direction, this requires the reaction of a glycine phosphonate with a functionalized aldehyde, carrying the intact aziridine, a stereoselective bromination and a Michael addition–elimination reaction to complete the synthesis. Drawbacks to this approach became apparent as the reactivity of the 12-OH meant that selection

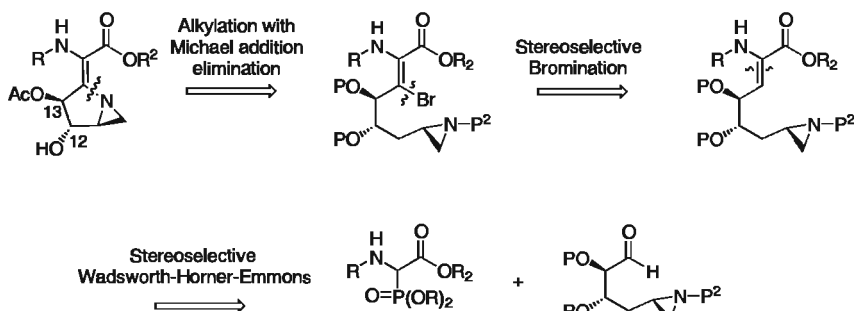


Fig. 24.6. Retrosynthetic analysis of the aziridine segment by Armstrong [30] and Coleman [31].

of the protecting groups was a key issue and must be contemplated early in the synthesis [29].

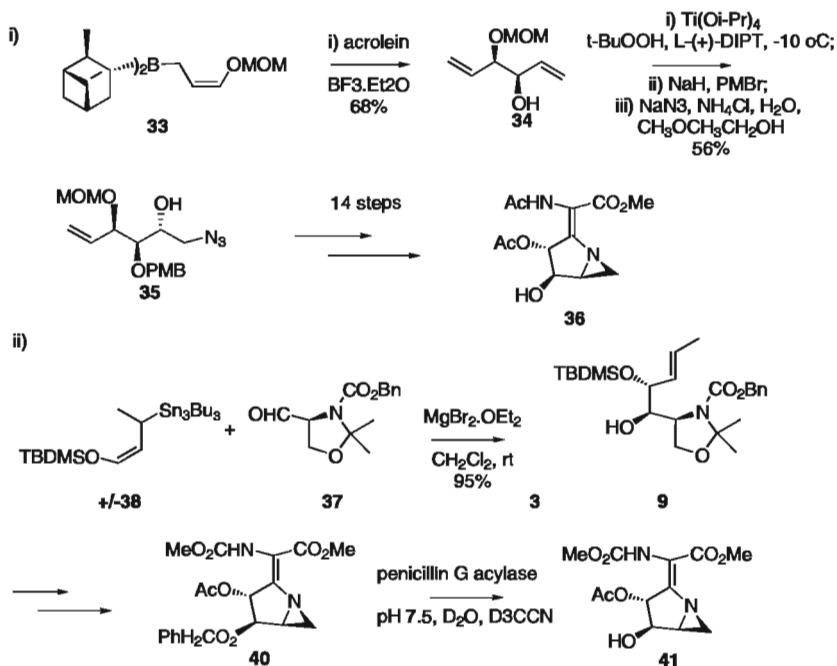
24.4.1.1 Synthesis of the aldehyde unit

In initial studies, Armstrong utilized both L-serine and D-arabinose as starting points for the synthesis of the aldehyde [32]. Coleman, in a similar fashion to Armstrong, initially utilized the natural “chiral pool” to make the aldehyde side chain, beginning his synthesis from D-glucosamine [33]. However, it is clearly of benefit to differentiate between the 12- and 13-oxygens early in the synthesis and while Coleman achieved this end using glucosamine, the protecting group strategy proved eventually to be unworkable. This group has more recently described two elegant routes to the differentiated aldehyde, one involving asymmetric allylation of an organoborane **33** followed by Sharpless AE and ring opening of the resulting epoxide **34** with azide to put in place all the required functionality [34]. Similarly, chiral aldehyde **37** underwent a Lewis acid-promoted addition reaction with allyl stannane **38**, thus differentiating the two alcohols and allowing the introduction of an enzyme cleavable protecting group at position 12 in the final compound [35].

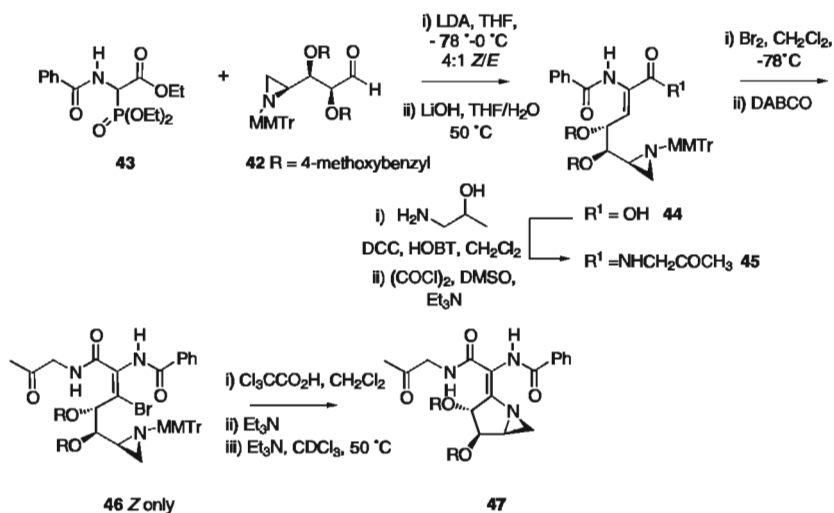
24.4.1.2 Wadsworth–Horner–Emmons reaction, bromination, and ring closure

Armstrong’s initial work utilized the reaction of the aldehyde **42** with a glycine phosphonate **43** into which he also introduced the left-hand ketone group of azinomycin A (Scheme 24.6) [30]. Reaction of the phosphonate **43** with the aldehyde **42** gave a mixture of (*Z*)/(*E*)-stereoisomers (4:1) which was subsequently hydrolyzed to give only the (*Z*)-isomer **44**, the (*E*)-isomer being destroyed under the reaction conditions. Following coupling to the ketoamine, bromination with bromine at low temperatures, followed by addition of DABCO gave only the (*Z*)-isomer **46**, which upon deprotection and ring closure under basic conditions gave the unnatural (*Z*)-isomer **47** (Scheme 24.6).

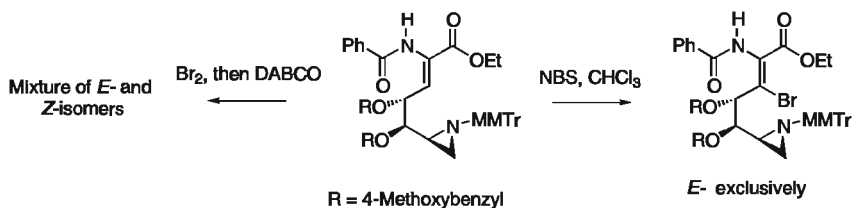
Armstrong and co-workers later discovered that the conditions of the bromination were key to the successful production of the compound with the correct (natural) stereochemistry [36]. Thus, bromination of the (*Z*)-isomer at -78°C with bromine, as noted, gave the (*Z*)-isomer, while treatment with NBS in dichloromethane gave exclusively the (*E*)-isomer (Scheme 24.7).



Scheme 24.5. Coleman's syntheses of the aldehyde with differentiated 12- and 13-OH groups [34, 35].

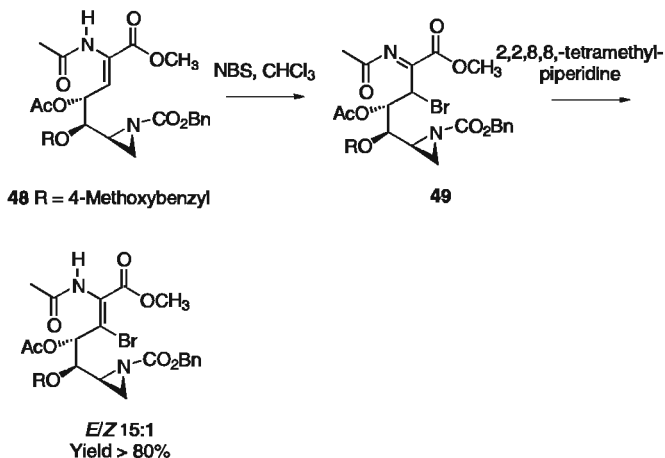


Scheme 24.6. Armstrong synthesis of the Z-isomer of the aziridine subunit [30].



Scheme 24.7. Bromination with NBS or bromine [36].

Early in their studies, Coleman and co-workers noted the isolable nature of the bromoimine intermediate **49** in the bromination of the double bond in **48** by NBS [31]. They later showed that treatment of this compound with hindered bases led to, at best, a stereocontrol of only 1.5:1 (*E*)/(*Z*)-product when the 12-OH was protected as a phenylacetyl group, but as high as 10:1 (*E*)/(*Z*) with a *p*-methoxybenzyl 12-OH protecting group [37] (Scheme 24.8).



Scheme 24.8. Bromination with NBS, isolation of the bromoimine and base-induced tautomerism [37].

Deprotection and final ring closure of the aziridine has been shown by both Armstrong and Colemans groups to occur with complete stereocontrol [30, 31], emphasizing that introduction of the bromine with correct stereochemistry is key to the production of the natural product stereochemistry.

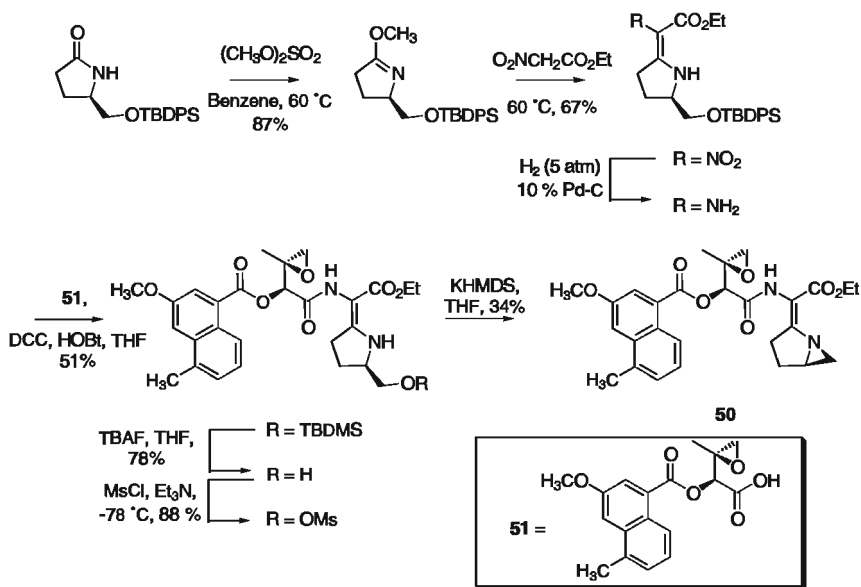
24.4.2

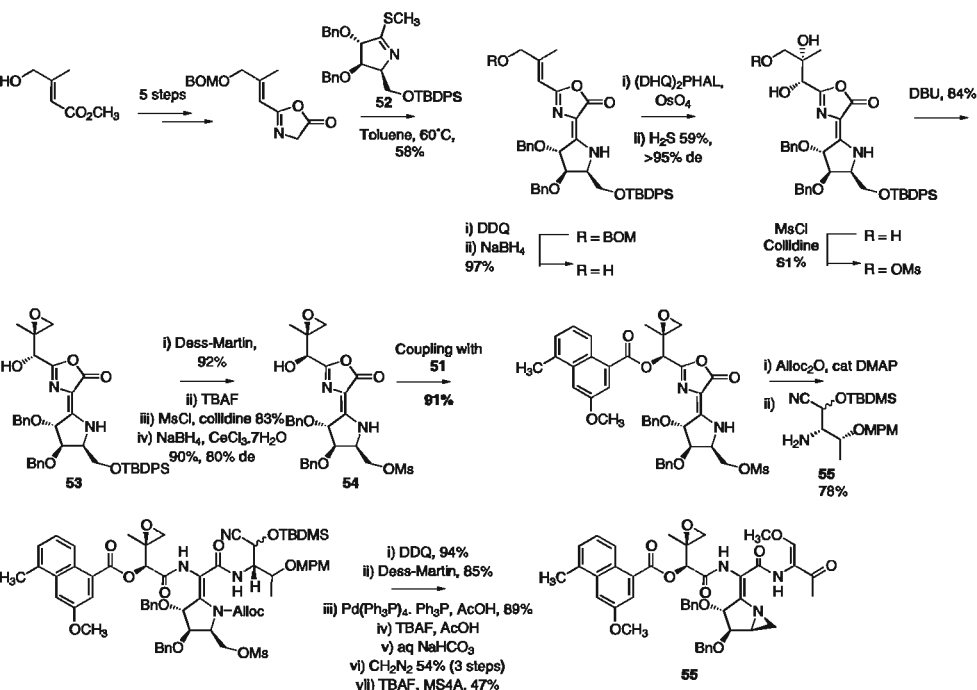
The Total Synthesis of Azinomycin A

The total synthesis of one of the azinomycins proved elusive, in spite of extensive studies of both **3** and the densely functionalized aziridine moiety, until the recent report of the synthesis of azinomycin A by Coleman and co-workers [38]. To finally

achieve their aim, this group had to rethink the segment-based synthesis approach originally identified and turn to a slightly less convergent, but elegant and, eventually, successful approach.

Studies of the aziridine fragment of the azinomycins had suggested that the dense functionality of this group, and in particular the 12-OH group of the full structure, leads to marked instability in the natural products and in synthetic analogs [29]. In early studies of carzinophilin (azinomycin B), Terashima and co-workers synthesized the skeleton of the azinomycins up to the aziridine (compound 50, Scheme 24.9) but without the problematic substituents on the pyrrolidine ring [39]. In a similar, recent synthesis, Shipman and co-workers made the same ester by a different route in order to demonstrate its DNA-crosslinking ability [40].

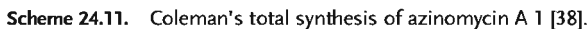




Scheme 24.10. Tereshima synthesis of fully protected azinomycin B analog **56** [42].

by reaction of the 2-oxazolin-5-one with the protected compound **55** derived from methyl L-threonine. A series of manipulations gave the fully protected precursor to azinomycin B **56**. At this stage, the authors discovered that it was not possible, under a variety of conditions, to remove the benzyl protecting groups from the aziridine fragment, presumably due once more to the instability of the unprotected compound at the 12-OH position.

To complete the total synthesis of an azinomycin and to access sufficient material for biological studies, Coleman and co-workers realized that an alternative approach to the construction of the natural product would be required [38]. Most synthetic efforts had concentrated on synthesizing the parts of the azinomycins separately and then bringing the final pieces together. This was justified, as each piece represented a different synthetic challenge, both for the stereochemically defined epoxide and the stereochemically challenging and functionally dense aziridine. However, it is clear that in a demanding natural product synthesis, introduction of sensitive functional groups should lie as close to the end of the synthesis as possible and with the observation in hand that the 12-OH caused extensive problems, Coleman and co-workers adjusted their approach to a late stage introduction of this group. Key to the total synthesis was the assembly of the backbone of the natural product, including the epoxide moiety, followed by the late stage introduction of the azabicyclic system through a Wadsworth–Horner–Emmons reaction (Scheme 24.11).



24.5

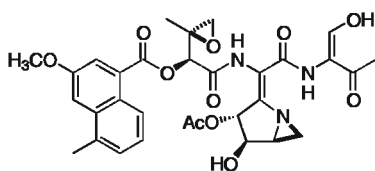
As considerable amounts of the azinomycins remained elusive and DNA-binding studies were therefore difficult, Coleman and co-workers also carried out a number of computational studies of the binding of the natural product to DNA. Having

developed force-field parameters for the natural products [43], a model was developed for DNA crosslinking by azinomycin B [44]. Two approaches were considered. The first involved generating a DNA duplex containing an intercalation site, based upon the 9-aminoacridine-4-carboxamide crystal structure [45], whilst the second used a non-intercalative model. Both models suggested that initial alkylation should occur at the N7 of adenine through alkylation by the aziridine followed by N7-G alkylation on the opposite strand by the epoxide group. This model has been supported by experimental observations by Fujiwara and Saito [48] and is discussed in more detail in the following section.

24.6

Experimental DNA-binding Studies and Antitumor Activities of the Full Azinomycin Structures – Is Crosslinking Required for Biological Activity?

In early studies on the biological activity of azinomycin B, Lown and co-workers noted the ability of the natural product to form covalent links between complementary strands of DNA [46] and this was confirmed by Armstrong's group who used ^{32}P -labeled synthetic oligonucleotides to demonstrate crosslinking [47]. Essentially, they took synthetic DNA strands containing a 5'-GCT and modified only this sequence, suggested as an excellent crosslinking sequence for azinomycin B, to try to understand the requirements for DNA alkylation. The results are summarized in Fig. 24.7. Azinomycin B formed crosslinks most efficiently in the



Azinomycin B 2

Most efficient crosslinking	5'-GCT 3'-CGA	5'-GCT 3'-C I A	5'-GCC 3'-CGG
Less efficient crosslinking	5'-GCT 3'-CZA	5'-I CT 3'-CGA	5'-GAC 3'-CTG
No crosslinking	5'-ZCT 3'-CGA	5'-GAT 3'-CTA	5'-GTT 3'-CAA
	5'-GCA 3'-CGT		

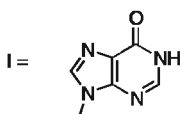
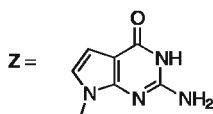


Fig. 24.7. Crosslinking of DNA by azinomycin B demonstrated by Armstrong and co-workers [47].

5'-GCT sequence, as well as 5'-GCC, whereas crosslinking decreased to trace amounts with a central A or T in the sequence. Crosslinking of 5'-GC was not observed. These authors also noted that piperidine-sensitive base-induced lesions were seen, to some extent, at all G's within the fragments they were analyzing and suggested a model in which initial G-alkylation is followed by a crosslinking reaction at suitably positioned A and G two bases to the 3'-side of the original lesion and on the opposite strand.

More recently, Fujiwara and Saito [48] have suggested an alternative crosslinking reaction, which supports the hypothesis of Coleman's modeling studies [44]. Using a self-complementary short oligonucleotide containing a 5'-GCT sequence, HPLC analysis revealed that incubation with azinomycin B at 0°C gave two products, one corresponding (by MS) to the crosslinked adduct and the other to the mono-alkylated product. The latter compound was found to convert to the crosslinked product on further incubation. Heating the monoadduct to 90°C for 5min induced depurination of the oligonucleotide at the 5'-A (sequence 5'-(TAGCTA)₂), whereas heating of the diadduct under the same conditions also gave cleavage at the central G. Analysis of the same reaction using the methylated analog **60** of azinomycin B allowed the authors to suggest that epoxide alkylation occurred at the N7 of guanine and thus to propose that initial alkylation occurred through aziridine reaction with the N7 of adenine followed by efficient crosslinking through a second reaction of the N7 of guanine with the epoxide (Fig. 24.8).

In a recent review, Hodgkinson and Shipman gave a comprehensive listing of the tissue culture-based antitumor activities of the azinomycins and their various derivatives [12]. The principal question to arise from this is of the requirement for interstrand crosslinking for biological activity. As Lown [46], Armstrong [47], and Saito [48] have demonstrated crosslinking for the natural products, whatever the order of adduct formation, it has been implied that this is required for biological activity. However, Shipman has shown that the truncated azinomycin analog **3** has potent antitumor activity in a variety of cell lines that matches and in some cases surpasses the activity of a crosslinking derivative synthesized and studied by the same group [12]. In fact, both the natural (*S,S*)- and unnatural (*R,R*)-diastereoisomers have potent antitumor activity (see Tab. 24.1 for comparison of the natural agent with a crosslinking analog **50**). Compound **3** has been demonstrated to be an effective DNA alkylator at the N7 of guanine in the major groove [26] and it is of interest that Armstrong and co-workers noted during their crosslinking studies that azinomycin B alkylated at all Gs within the sequences under study, in spite of forming crosslinks only where the guanine or adenine was correctly position two base pairs to the 3'-side and on the opposite strand [47].

24.7

Conclusions

The azinomycins represent a starting point for the design of new antitumor agents that alkylate DNA, as the instability and relative synthetic inaccessibility of the

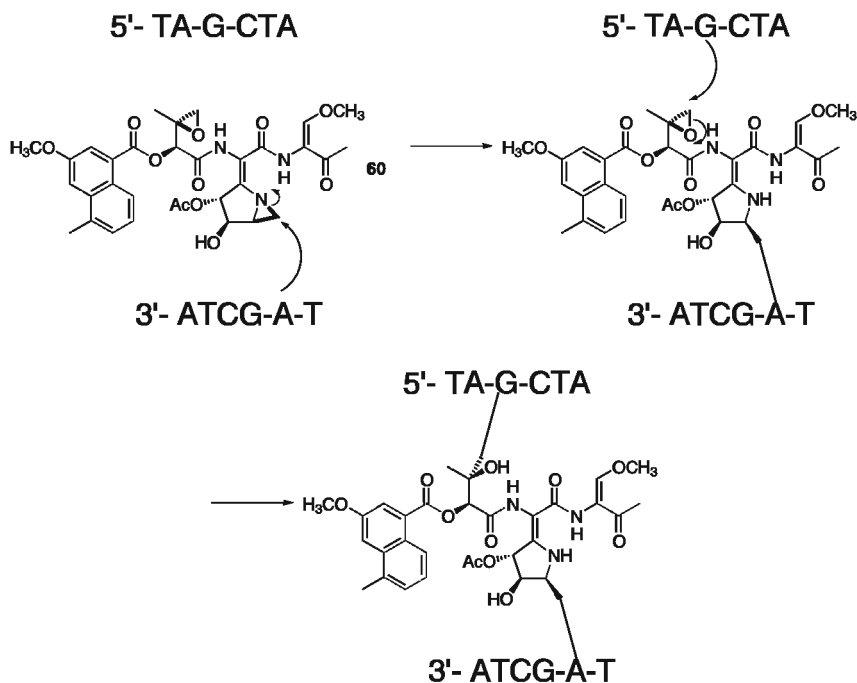


Fig. 24.8. Crosslinking of DNA by azinomycin B as shown by Fujiwara and Saito suggests a different mechanism to Armstrong (Fig. 24.7) [48].

natural products, notwithstanding Coleman's elegant total synthesis, deem them to be unlikely agents for use in the clinic. Synthetic efforts to date have identified a number of routes both to the truncated analog 3 and to the densely functionalized aziridine and at least one of these routes has yielded a total synthesis along with a number that have given very late stage agents or crosslinking analogs. Target

Tab. 24.1. Cytotoxicities for crosslinkers and non-crosslinkers.

No.	Crosslinker?	Cytotoxicity, IC ₅₀ (μM)							
		L5178	P388	A2780	A2780cisR	CH1	CH1cisR	SKOV-3	HT29
1	Yes	0.12	—	—	—	—	—	—	—
2	Yes	0.18	—	—	—	—	—	—	—
3	No		0.011	<0.05	0.076	<0.05	—	1.25	0.33
26	No		—	0.44	1.1	0.55	—	5.1	2.15
27	No		—	0.39	1.35	0.17	—	13	2.6
50	Yes		0.047	0.076	0.28	0.078	0.087	2.1	0.52
56	Yes		0.50	—	—	—	—	—	—

From Ref. [12].

binding and biological activity studies have lagged behind the synthesis effort with only a small number of disclosures of DNA alkylation, crosslinking, and antitumor activity. This lack of fundamental studies stems in part from the lack of natural product to study and the synthetic efforts will contribute extensively to our future understanding of the activity of this class of compound. To date, there would appear to be several conflicting observations with regard to the biological activity and these problems need to be addressed before analog development can lead to useful agents:

1. Is intercalation required for biological activity? Coleman's computational studies have suggested that intercalation of the naphthalene chromophore and DNA crosslinking are unlikely to be compatible due to constraints on the extended structure of the azinomycins [44]. Gates and co-workers have presented strong evidence for intercalation of the truncated agent 3 [26] and the lower activity of both the epoxide and aziridine moieties alone suggests a role in DNA binding for the chromophore that is difficult to explain in any other fashion. More attention to the structure of the chromophore, including the introduction of classical intercalating functions such as acridines, may build on the initial studies of Shipman and co-workers [27] and define the requirement, if any, for intercalation.
2. Is crosslinking required for activation? Any controversy within the study of azinomycins has focused upon the different crosslinking mechanisms suggested by Armstrong [47] and Saito [48]. A more telling consideration is the requirement for crosslinking for biological activity. As noted above, the truncated analog 3 retains high antitumor activity in cell culture in spite of an inability to form crosslinks and Armstrong's observation of non-crosslinking alkylation by azinomycin B [47] suggests that extensive monoalkylation occurs even with the natural products. Although classical antitumor alkylating agents exert their effects through crosslinking DNA, the minor groove binding alkylating agents such as CC-1065 and the duocarmycins form only monoalkylated products [3]. It is possible that major groove alkylation by the azinomycins combined with intercalation of the chromophore is sufficiently disruptive to initiate cell death.

The azinomycins are natural products with potent antitumor activity and yet have not progressed to a stage where they are clinically useful. Considerable synthetic efforts have been successful in synthesizing the truncated analog, advanced intermediates in the total synthesis and, in at least one case, the full natural product. DNA-binding and biological studies have established that the azinomycins can both monoalkylate and crosslink DNA and that they, and some of their analogs, have potent antitumor activity in various cell lines. However, the questions of whether the activity of the azinomycins stems from DNA crosslinking and of how these agents can be made into clinically useful agents remain unanswered. Further key studies of the natural products, analog structures, including potential pro-drug forms, and their effects on biological systems remain to be performed before these questions can be answered.

Acknowledgments

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25

The Generation and DNA-Interaction of PBD and CBI Libraries

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25.1

Introduction

The pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) [1] and cyclopropylbenzoindequinones (CBIs) [2] are natural product-based antibiotic and antitumor agents from *Streptomyces* species that can recognize and bond to guanine and adenine bases, respectively, in specific sequences of DNA. When PBDs and CBIs are attached to solid supports, their ability to bind to DNA in a sequence-selective manner is retained. This property allows the on-bead screening of PBD- or CBI-based combinatorial libraries. This methodology is being developed as part of a gene targeting strategy.

There is interest in developing low molecular weight ligands capable of recognizing and then binding or bonding to specific DNA sequences in order to down-regulate the expression of single genes or sets of genes [3]. This can be achieved either by targeting the coding region of genes and blocking RNA polymerase, or by targeting ligands to the promoter region and blocking the interaction of regulatory proteins and/or transcription factors. This so called “antigene” approach to gene regulation has distinct advantages in comparison to the “antisense” approach. These include the drug-like properties of small molecules (e.g. favorable cellular penetration and pharmacokinetics compared to oligonucleotides) and the lower concentration of agent required for a pharmacological effect given the larger number of RNA transcripts required to be “neutralised” compared to copies of the corresponding DNA template.

We have investigated a number of approaches to gene targeting using low molecular weight ligands, including the solid-phase/combinatorial approach described here. This involves the attachment of PBD and CBI molecules to solid supports in a combinatorial fashion using rationally designed linkers. As these units bind covalently to guanine and adenine DNA bases respectively, the objective is to build-up extended molecules on solid support capable of recognizing and interacting with 10 or more base pairs of DNA.

A wide array of PBD molecules [1] are known, with members containing a vari-

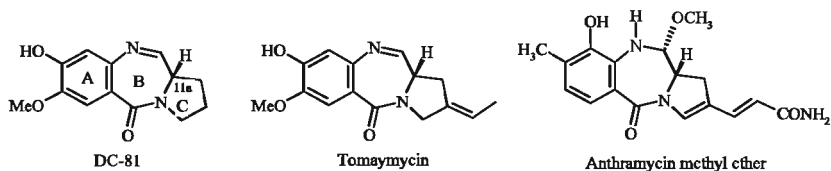


Fig. 25.1. Examples of Structures of PBDs with Varying A-Ring Substitution Patterns and C-Ring Substitution and Unsaturation Patterns.

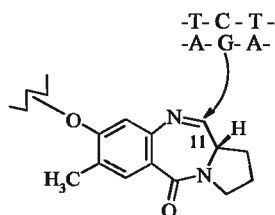


Fig. 25.2. Covalent Interaction of a PBD with the N2 of Guanine in the Minor Groove of DNA.

ety of A- and C-ring substitution patterns and different degrees of C-ring saturation ranging from saturated (e.g. DC-81) to C2-*exo* (e.g. tomaymycin) or C2-*endo* unsaturation (e.g. anthramycin) (Fig. 25.1). It has been established that these agents bind selectively and covalently in the minor groove of DNA via an aminal bond between the electrophilic C-11 position of the PBD and the nucleophilic C2-amino group of a guanine base (Fig. 25.2). The (*S*)-configuration at the C11a position allows the PBD to fit snugly within the minor groove of DNA, spanning three base pairs with a preference for 5'-Pu-G-Pu sequences. These aspects of PBDs have been extensively reviewed in the literature [1, 2, 4]. In contrast, the CBI moiety has been shown to bind covalently to adenine within the minor groove of DNA, with a preference for sequences containing runs of AT base pairs [2, 4] (Fig. 25.3).

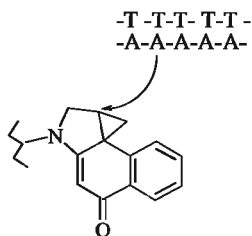


Fig. 25.3. Covalent Interaction of a CBI Moiety with the N3 of Adenine in the Minor Groove of DNA.

We have now shown that these sequence-specific interactions are retained while both the PBD and CBI moieties are tethered to a solid support. This represents a step towards the goal of screening large combinatorial libraries for gene targeting molecules. The initial stages of the development of this approach are described below.

25.2

Synthesis of Tethered PBD and CBI Constructs

25.2.1

Synthesis of Capping Units

The PBD capping unit shown in Fig. 25.4, was designed so that it could be connected through the C8-position either directly to the solid support or to peptidic

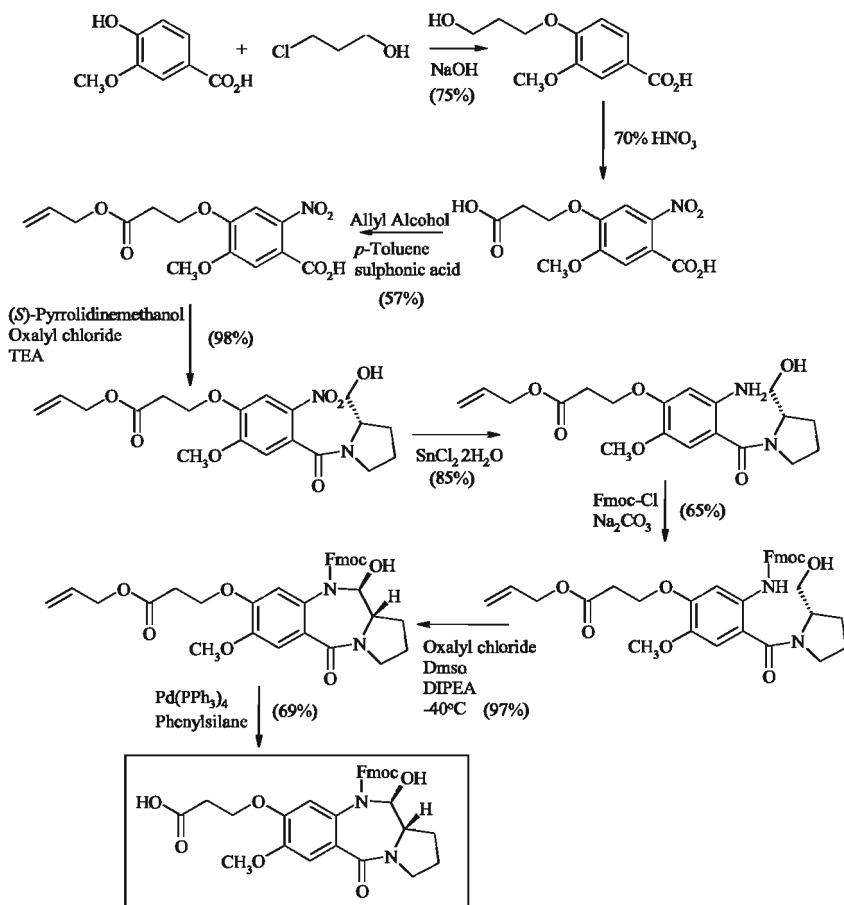


Fig. 25.4. Synthesis of the PBD Building Block Acid.

library members. It has been demonstrated in previous investigations that PBD C8-substituents do not hinder DNA interaction as they point along the minor groove after binding [5]. Also, coupling through this position allows the N10–C11 imine to remain intact for covalent interaction with DNA.

The synthetic route utilized previously developed PBD chemistry [6]. The orthogonally protected intermediate was prepared from a vanillic acid and 2(*S*)-pyrrolidinemethanol. Ultimately, removal of the allyl-protecting group afforded the free acid capping unit. When the N10 position is protected with Fmoc the molecule can be used as a control for on-bead screening, as the Fmoc group sterically hinders DNA binding and prevents formation of the electrophilic N10–C11 imine moiety. The CBI capping unit was synthesized using literature procedures and was a gift from Professor Denny (University of Auckland).

25.2.2

Attachment of PBD and CBI Capping Units to Solid Supports

The capping units were coupled to resin under standard coupling conditions appropriate for the particular resin being used (see Fig. 25.5). The beads were subjected to three coupling cycles to ensure that all available binding sites had been reacted. The efficiency of coupling was assessed using the qualitative Kaiser test [7]. PBD coupling was also assessed by measuring the amount of Fmoc protecting group released during the deprotection reaction using UV spectroscopy. This involved suspending a small weighted sample of resin in a freshly prepared solution of 20% piperidine in dimethylformamide. After 3 h, a sample was removed and subjected to UV analysis at 290 nm [8]. Loading data for typical PBD couplings are shown in Tab. 25.1. Loading could also be monitored by measuring the weight

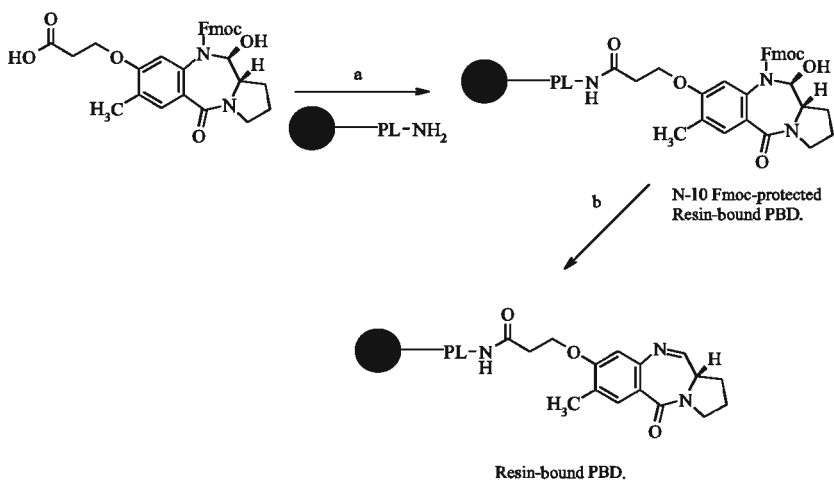


Fig. 25.5. Attachment of the PBD to the Solid Support.

Reagents:–

a = Resin-PL-NH₂/TBTU/CH₂Cl₂ (PL = photocleavable linker)

b = Piperidine/DMF.

Tab. 25.1. Incorporation Levels of the PBD Unit onto Solid Phase.

Sample	Absorbance (average of 3 readings)	Loading (mmol g⁻¹)	Level of incorporation onto the bead (%)
Sample A	0.552	0.279	93
Sample B	0.526	0.266	88.7

Note: Tentagel resin, initial loading 0.3 mmol g⁻¹.

increase of resin and these results were found to compare well (incorporation level 84.5%) with the other methods. The attachment of the CBI unit was performed in an analogous manner (e.g. Fig. 25.5) from the appropriate CBI capping unit [9].

25.3

On-bead DNA Interaction

25.3.1

Interaction of Double-Stranded DNA PBDs and CBIs On-bead

On-bead DNA interaction studies were carried out using double-stranded oligonucleotides which were end-labeled with either ³²P, fluorescein, or rhodamine [10]. The fluorescein- and rhodamine-labeled oligonucleotides were purchased from commercial sources. The ³²P-labeled oligonucleotides were 5'-endcapped with γ-[³²P]-ATP by incubating with T4 polynucleotide kinase followed by purification using mini-prep Biorad Spin columns containing P6 Bio-gel. The beads (1.0 mg) were allowed to swell in DMF for approximately 1h prior to incubating with the labeled oligonucleotides (5 pmol μL⁻¹) for 24 h at 37°C [11]. They were subjected to four cycles of re-suspension in TE buffer (10 mM Tris, 1 mM EDTA), followed by centrifugation and supernatant removal to remove all unbound oligonucleotide. Fluorescence was measured using a Tecan SpectraFluor. For the radiolabeled DNA, after the final wash the pellet was re-suspended in 1 mL of scintillation fluid and counted on a Wallac 1400 scintillation counter.

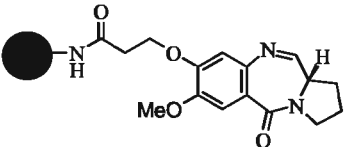
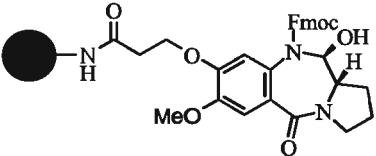
Preliminary studies were carried out on the PBD-resin using a radiolabeled oligonucleotide containing the preferred AGA-binding sequence (Tab. 25.2). The sets of resin evaluated were derived from the same initial batches of Fmoc-protected PBD-resin. After the coupling cycles were complete, each batch was split into two, and then one half was deprotected. This ensured that the loading of the PBD species on the bead was identical, whether the beads were N10-protected or not. This allowed the extent of binding to be directly compared. A significant difference in binding affinity between the protected and unprotected resins was observed (Tab. 25.3). This demonstrated that PBDs attached to the bead surface can interact with DNA efficiently only after the removal of the N10-Fmoc protecting group. Similar results were obtained when the CBI-resin was screened against labeled DNA containing the relevant sequence (i.e. AAAAA), as shown in Tab. 25.4 and Fig. 25.6.

Tab. 25.2. Preferred PBD Binding Sequences.

Oligonucleotide-binding sequence	Full sequence	Counts (CPM)
Pu <u>G</u> Pu	5'-ACA CCT AIA GAT IAA ITC TI-3' 3'-TTT IIA TCT CTA CTT CAI AC-5'	50,394
Py <u>G</u> Py	5'-ACA CCT AIT GTT IAA ITCTI-3' 3'-TTT IIA TCA CAA CTT CAI AC-5'	3,321
Pu <u>I</u> Pu	5'-ACA CCT AIA IAT IAA ITC TI-3' 3'-TTT IIA TCT CTA CTT CAI AC-5'	1,820

CPM = counts per minute. I = Inosine

Tab. 25.3. Comparison of Binding N10-protected and non-protected Resin.

Resin	³² P	Fluorescein	Rhodamine
	50,304	42,355	16,539
	0	1.217	1.122

The experiments with alternative labels (Tab. 25.3) confirmed that significant binding differentials between the N10-protected and -deprotected PBD–resins are achievable, thus indicating the potential to develop sensitive screening procedures based on this strategy.

The covalent interaction between the tethered PBD and DNA is validated by the experiment shown in Fig. 25.6 in which it can be seen that labeled DNA remains associated with the PBD–resin after four washes with buffer. The covalent nature of the interaction was further confirmed by complete removal of labeled DNA after

Tab. 25.4. Selectivity Comparisons between Resin-Bound PBD and CBI Units.

Oligo	Rhodamine RFU	
	PBD-resin	CBI-resin
Pu <u>G</u> Pu	53705	2288
AAAAA	3832	54849

RFU = relative fluorescence units.

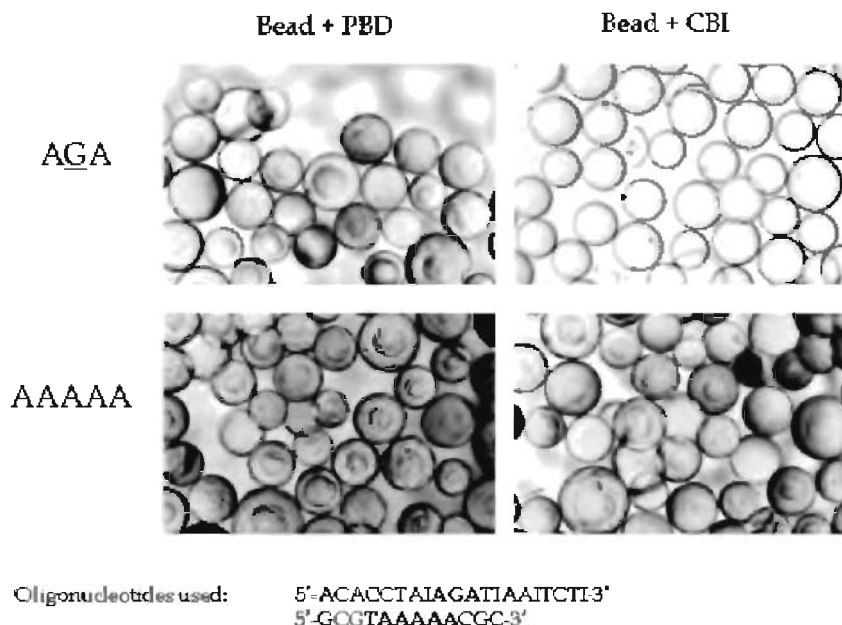


Fig. 25.6. Sequence Selectivity of Resin-Bound PBD and CBI Moieties.

incubation in buffer at pH 3.5, conditions which are known to cleave the aminal bond formed between PBDs and DNA [1].

25.3.2

On-bead DNA Sequence Selectivity

Studies were next carried out to establish whether differences in sequence-selectivity could be observed during DNA binding to a PBD-resin (Tab. 25.2). Oligonucleotides containing AGA (most preferred), TGT (least preferred) and AIA (where I = inosine, which is incapable of covalent interaction with a PBD) sequences were ^{32}P end-labeled. Following incubation with PBD-resin, a greater binding affinity was observed with the preferred AGA-containing sequence compared to the least preferred TGT sequence. For the sequence containing AIA, only residual binding was observed which was presumably due to non-covalent interactions [12].

Figure 25.6 shows the results of challenging both resin-bound PBD and CBI constructs with rhodamine-labeled oligonucleotides which contain either a PBD-preferred or CBI-preferred binding site. The beads were extensively washed with buffer to remove non-covalently bound oligonucleotides and were then distributed in soft agar on a microscope slide and inspected under a light microscope. The selective binding of the PBD and CBI units to their corresponding preferred binding sites is evident from the pink coloration of the appropriate beads. The slight uptake of color by the resin-bound PBD upon treatment with the CBI target se-

quence is due to the presence of two lesser-preferred PBD-binding sites (5'-CGT and 5'-CGC) present on either side of the CBI target sequence (i.e. AAAAA). Quantitative data for this experiment (Tab. 25.4) indicates a 24:1 discrimination between sequences for the CBI-resin and a 14:1 discrimination for the PBD-resin despite the presence of two least-preferred PBD-binding sites.

25.4

Library Synthesis

Peptides were chosen for the initial combinatorial library experiments due to their ease of synthesis and identification. Libraries were synthesized using traditional methods employing the "split and mix" technology pioneered by Furka and co-workers [13]. The first library was constructed using an automated technique employing an ACT-357 synthesizer. Amino Tentagel resin (5 g) was split equally into 17 wells and coupled with one of 17 Fmoc-protected amino acids (Fig. 25.7).

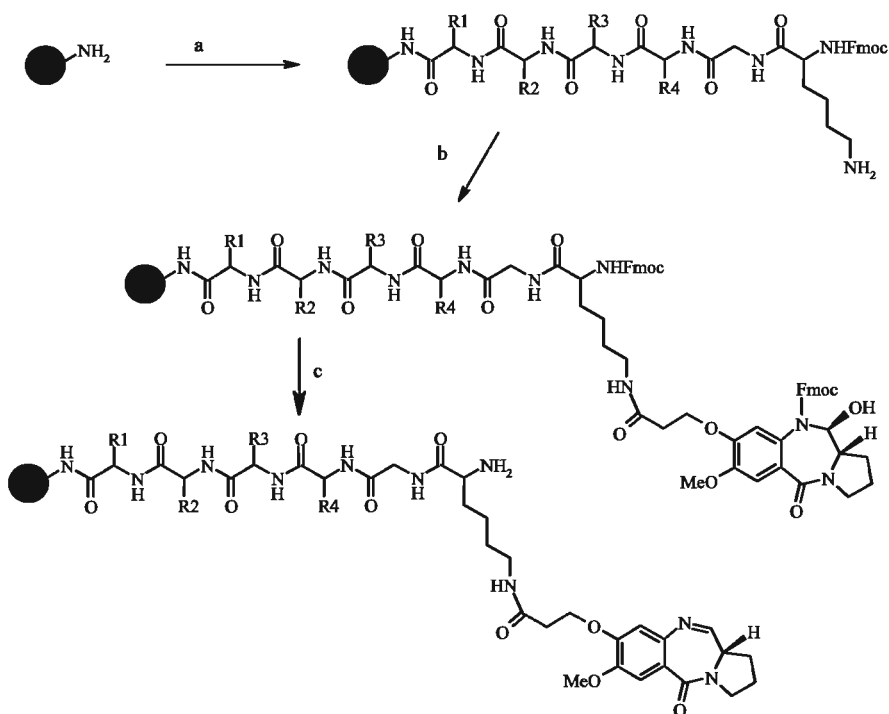


Fig. 25.7. PBD-Peptide Library Synthesis. Reagents: (a) (i) Fmoc amino acid, TBTU, DIPEA, DMF; (ii) 30% Ac_2O /DMF; (iii) 20% piperidine/DMF; (iv) Fmoc glycine, TBTU, DIPEA, DMF; (v) 20% Ac_2O /DMF; (vi) 20% piperidine/DMF; (vii) *N*- α -Fmoc-*N*- ϵ -

Alloc-lysine, TBTU, DIPEA, DMF; (viii) 20% Ac_2O /DMF; (ix) $\text{Pd}(\text{PPh}_3)_4$, phenylsilane, CH_2Cl_2 ; (x) Fmoc PBD acid, TBTU, DIPEA, DMF. (b) (i) 50% TFA/ CH_2Cl_2 ; (ii) 20% piperidine/DMF.

Arginine, cysteine, and histidine were omitted for technical reasons. Following the coupling reaction the resin was recombined, acetylated, and deprotected. Three further re-splitting and mixing cycles were performed, followed by successive additions of the fifth (Fmoc-glycine-OH) and sixth amino acids (Fmoc-lysine(Alloc)-OH) to give 17 sublibraries, where the final two amino acids were known. Following the removal of the side chain protecting group from the terminal lysine derivative, the PBD building block acid was coupled to the resulting amine under standard coupling conditions. Subsequent side chain deprotection of the peptide sequences and deprotection of the N10 position of the PBD afforded sublibraries containing 1.4×10^6 potentially DNA-interactive constructs (Fig. 25.7). The CBI library was made in an analogous fashion (see Fig. 25.8).

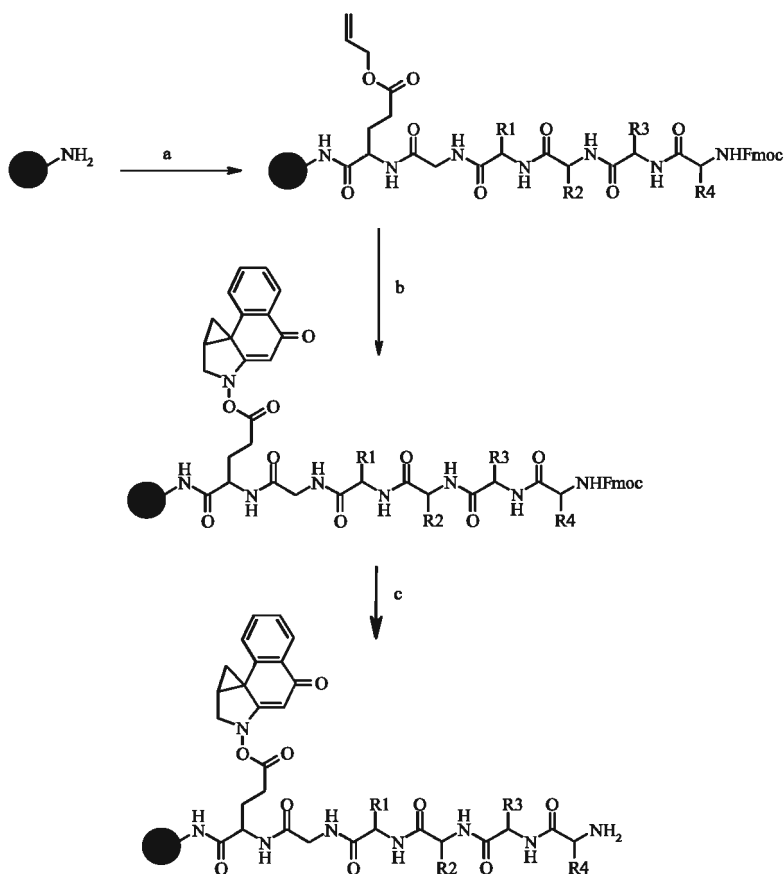


Fig. 25.8. CBI-Peptide Library Synthesis. Reagents: (a) (i) *N*- α -Fmoc-glutamic acid α -allyl ester, TBTU, DIPEA, DMF; (ii) 30% Ac_2O /DMF; (iii) 20% piperidine/DMF; (iv) Fmoc glycine, TBTU, DIPEA, DMF; (v) 20% Ac_2O /DMF; (vi) 20% piperidine/DMF;

(vii) Fmoc amino acid, TBTU, DIPEA, DMF; (viii) 20% Ac_2O /DMF; (ix) $\text{Pd}(\text{PPh}_3)_4$, phenylsilane, CH_2Cl_2 . (b) (i) Dhbt-OH, DIC, DMF; (ii) *seco*-CBI, DMF; (iii) 2% TIS/TFA; (iv) 20% piperidine/DMF.

25.4.1

Library Screening

The libraries were screened against rhodamine labelled target oligonucleotide sequences. Following incubation with the labeled DNA, the beads were extensively washed to remove non-covalently bound oligonucleotide and then distributed in agar and inspected under a light microscope. Active compounds were stained red and could be removed with a pipette for processing. Figure 25.9 shows a selection of beads from a such a library containing an example of a “hit”. The reddest beads from the incubation were isolated and subjected to an Edman Degradation procedure (see table 25.5 for an example Tab. 25.5). Comparison of the sequences showed that a 3,4-lysine motif was found to be present in 25% of the hits.

Constructs of interest could then be resynthesized for off-bead solution studies. For example, of the “hits” obtained, in table 25.5 YPKKGK-PBD, KKKKGK-PBD, NKKKGK-PBD, YYNKGK-PBD, EEGTGK-PBD constructs were resynthesized using Rink Amide resin which enabled cleavage from their support using trifluoroacetic acid. LC-MS was used to confirm the identities of the molecules produced (Tab. 25.6). The most suitable HPLC conditions for the compounds were found to be a methanol/water (65:35) mobile phase containing 0.05% ammonium acetate in conjunction with a Phenomenex Jupiter C4 column (250 mm × 4.6 mm, 5 μm). Figure 25.10 shows an HPLC trace of YYNKGK-PBD before purification showing the main component with three minor but significant deletion product impurities. Figure 25.11 shows the mass spectrum of the major component.

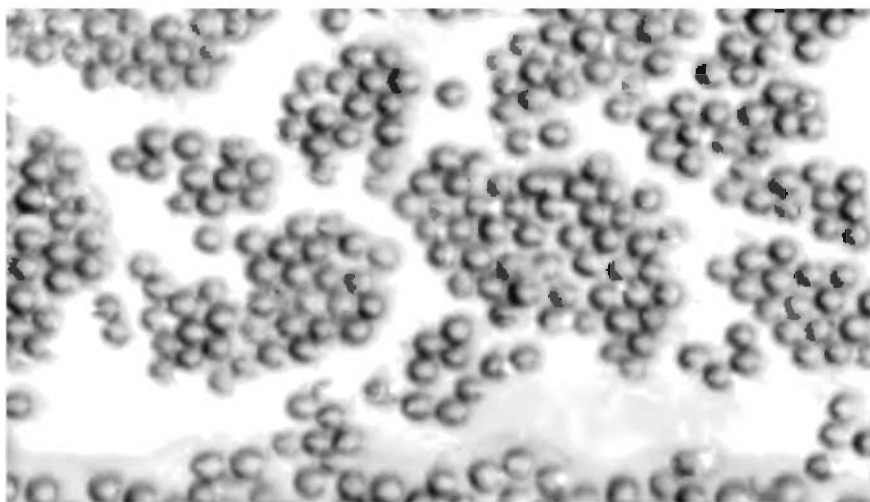


Fig. 25.9. Selection of Beads taken from a CBI-Library screened against 5'-GCCGAAAAACGC. The “Hit” can be seen in red.

Tab. 25.5. Peptide Sequencing Results for PBD library screened against 5'-GCACTTTTGAAGATCATTTT.

<i>Bead number</i>	<i>Sequence</i>
1	KGNTLG
2	KGKKPY
3	Bead lost.
4	KGKKA
5	KGFAQK
6	KGKYN
7	KGKKY
8	KGKKP
9	KGWKFG
10	KGK -
11	KGKYKA
12	KGKPP
13	KGKKF
14	KGFMLK
15	KGKKK
16	KGPKQK
17	KGPTK or KGTKK
18	KGVFMK
19	KGLATK
20	KGKKMK
21	KGKIKT
22	No sequence recorded.
23	KGNNMYK or KGNNPKK
24	KGNNMK
25	KGSKKT
26	KGFWK
27	KGKNYY
28	KGKK -
29	KGAKAK
30	KGIPKK; A
31	KGKIKL
32	KGTSKG
33	KGKY -
34	KGKKKN
35	KG -
36	KGKMYT
37	KGKMF
38	No bead.
39	KGKKKT
40	KGKKP
41	KGAKP
42	No sequence recorded.
43	KGKKK
44	KGKYTK
45	KGIWTK
46	KGTEEE

Tab. 25.6. Analytical Data for PBD-Peptides.

Sequence	Formula	Retention time (min)	Mass expected	Mass found
KKKKGK-PBD	C ₄₈ H ₈₃ N ₁₄ O ₁₀	1.7	1015.28	1017.7
NKKKGK-PBD	C ₄₆ H ₇₇ N ₁₄ O ₁₁	4.3	1001.21	1003.7
YYNKGK-PBD	C ₅₂ H ₇₁ N ₁₂ O ₁₃	4.2	1071.21	1073.6
EEGTGK-PBD	C ₄₀ H ₅₉ N ₁₀ O ₁₅	3.5	918.97	921.5
Boc-PBD building block acid	C ₂₁ H ₃₀ N ₂ O ₇	2.6	436.47	437.2

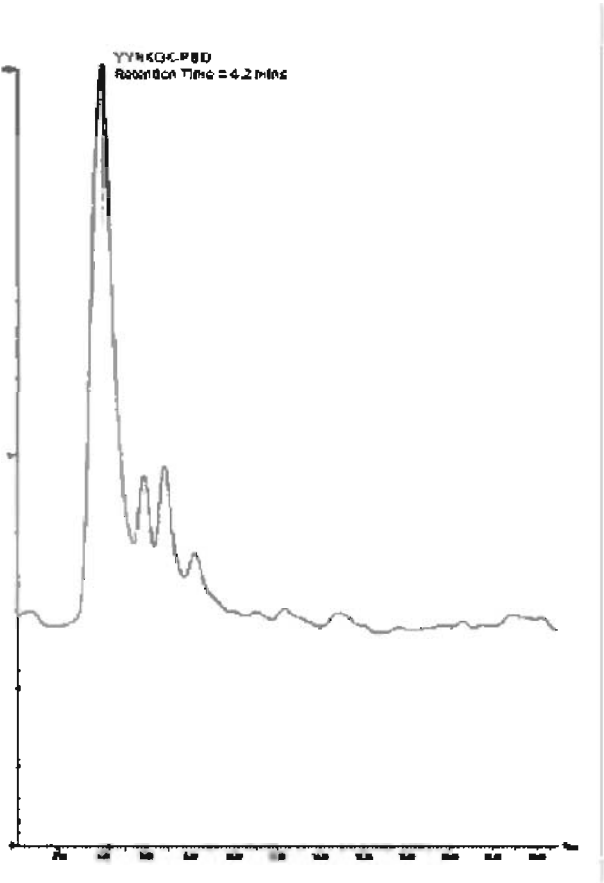


Fig. 25.10. LC-MS Profile of PBD-Peptide YYNKGK-PBD.

YYNKGK-PBD

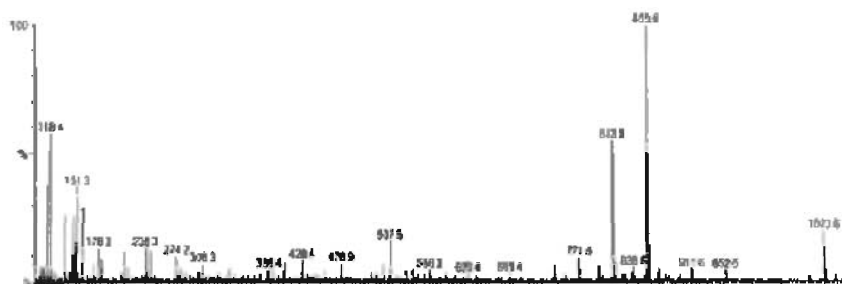


Fig. 25.11. MS Profile of PBD-Peptide YYNKGK-PBD.

25.5

Conclusion

In summary, we have demonstrated that PBD and CBI moieties retain their ability to interact covalently with DNA in a sequence-selective manner when tethered to a solid support. Furthermore, solid-phase combinatorial libraries containing PBD and CBI moieties can be prepared and screened against specific DNA sequences. “Hits” can be identified, sequenced, and the molecules resynthesized off-bead for use in further experiments.

Acknowledgments

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