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Review Article

Approaches to develop DNA sequence-specific agents

Sanjay K. Sharma, B.S. Narayan Reddy and J. William Lown*

*Department of Chemistry, University of Alberta, Edmonton, Canada T6G 2G2. *Correspondence*

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Introduction

A number of approaches have been employed historically in the development of biologically active drug molecules, ranging from serendipitous discoveries to methods of empirical general and specific pharmacological screening to increasing attempts at rational design on the basis of well-established principles (1, 2). At the frontiers of biological sciences is one of the most significant recent projects being conducted worldwide with the ultimate goal of reading through the human genome by decoding all cellular DNA and some 100,000 human genes (3, 4).

Understanding the specific genes that trigger diseases will undoubtedly help open new avenues to finding cures. Biomacromolecules such as enzymes, other proteins and nucleic acids have been identified as viable targets for mechanistic rational drug design from the present understanding of the biochemical and pharmacological hallmarks of diseases. Nucleic acids such as double-stranded DNA and intramolecularly folded structural sites on single-stranded RNA can also serve as receptor sites for small molecules which can recognize specific regions of base pairs for intercalation or binding to helical grooves by combinations of hydrogen bonding, electrostatic and van der Waals contacts. Such agents are useful in controlling the production or expression of undesirable proteins from the gene segments encoding for them.

Over the past two decades significant advances have been made in understanding the interactions of double helical DNA with small molecules important in antitumor, antibiotic and antitumor chemotherapy. A drug and DNA can have reversible or irreversible interactions. Reversible interactions can be divided into three major classes: electrostatic, intercalation and groove binding (5). Groove binding can be either through major groove or minor groove. Two hollow spiral grooves are formed between the two sugar phosphate chains in such an arrangement of the double helix. These are well-defined channels of information in double helical DNA that can be read from the exterior of the helical structure and are known as minor and major grooves (6). The major groove is 24 A° in width and contains higher informational content than the minor groove. While the minor groove is shallower (10 A° in width), it is generally preferred by small molecules and xenobiotics presumably because it represents a vulnerable site of attack in competing organism's genetic material. Representative examples of this class are netropsin and distamycin, two well-studied members of the pyrrolocarboxamidine class of naturally occurring antibiotic oligopeptides. Early work in this area has been reviewed previously by Zimmer and Wahnert (7). In this review, we will cover the sequence targeting by DNA binding polyamides.

Information reading molecules

Initial structural studies using x-ray crystallography (8, 9) and NMR techniques (10, 11) of netropsin DNA complexes showed how sequence-specific association within the minor groove of adenine-thymine (AT) rich segment is accomplished (Fig. 1). Dickerson (8) and Lown (12) first conceived the idea of "lexitropsin", or information reading molecules, where a modification of *N*-methylpyrrole carboxamide framework of netropsin or distamycin could provide an additional stabilizing interaction thereby permitting binding to GC base pairs. By replacing the pyrrole CHs, which discriminate between adenine-2H and

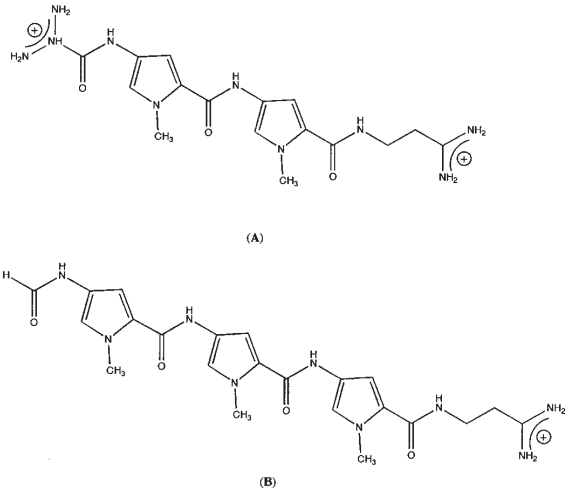


Fig. 1. Structures of the naturally occurring antibiotic oligopeptides netropsin (A) and distamycin (B).

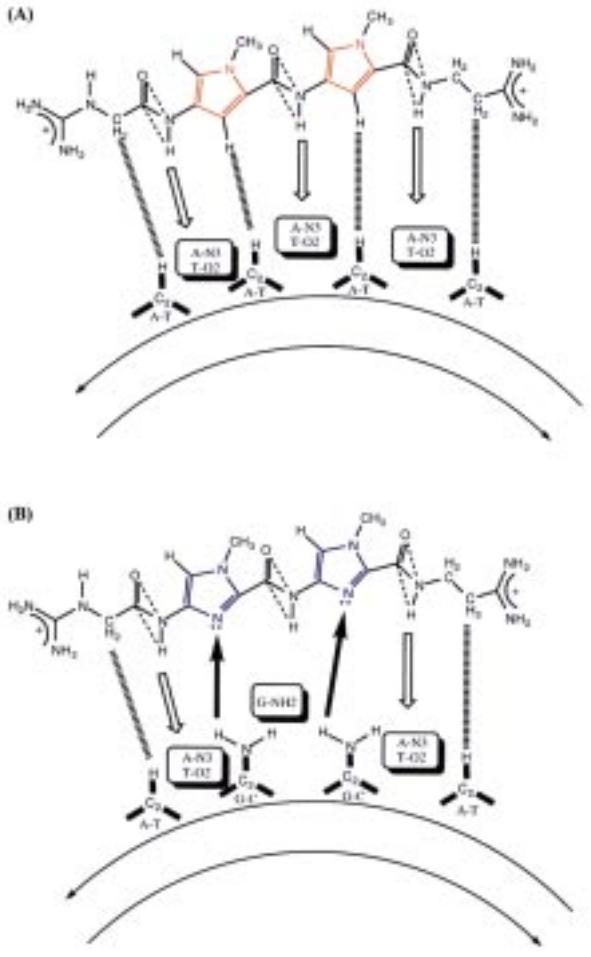


Fig. 2. (A) Interaction of netropsin with AT base pairs through the DNA minor groove. (B) Model for interaction of mixed AT/GC base pairs with an imidazole-containing lexitropsin designed to accept hydrogen bonds from guanine-NH_2 groups.

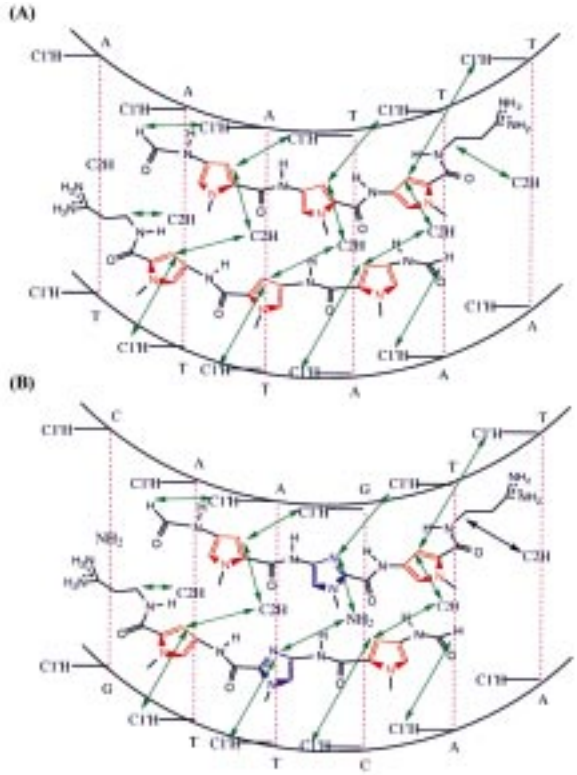


Fig. 3. (A) Anti-parallel side by side 2:1 binding motif and intermolecular NOE contracts (double headed arrows) for two distamycin molecules bound simultaneously and overlapping in the DNA minor groove at A_3T_3 sites, and (B) the same for imidazole-containing compound (f-Py-Im-Py-Am^+) designed to accept GC base pair in (AAGTT).(AACTT) site.

guanine-$2NH_2$ groups on the minor groove facing edges of AT and GC base pairs, with a nitrogen atom to afford *N*-methylimidazole carboxamides, this would offer the opportunity of forming a new hydrogen bond between guanine-2-amino group and the nucleophilic imidazole nitrogen (Fig. 2). At the same time this structural alteration removes the steric clash due to the pyrrole CH groups set deep into the minor groove for netropsin. Monocationic lexitropsins based on distamycin exhibit not only a greater ability to bind to the GC site but also an increased sequence preference when compared with the corresponding dicationic lexitropsin, as shown by the analysis of the binding sites from footprints obtained on plasmid pBR322 DNA restriction fragments (13). Progress made in the synthesis of lexitropsins was reported a few years ago by Singh and Lown (14). The chemistry of the synthetic DNA minor groove binders and DNA interactions of lexitropsin conjugates have been summarized recently by Lown and coworkers (15-17).

Classification of polyamides

NMR studies by Pelton and Wemmer (18) have shown that, at sufficiently high drug to DNA ratios, two

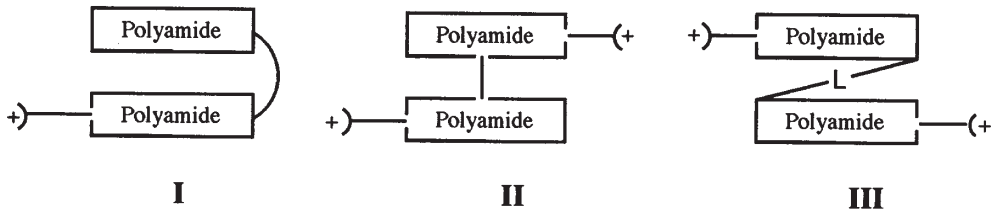


Fig. 4. Modes of linking polyamides: hairpin (I), cross-linked (II) and bis-polyamide (III).

distamycin molecules are simultaneously located in the minor groove in a highly overlapped antiparallel side by side manner (Fig. 3). This observation provided new scope for the information reading capacity of polyamides. The polyamides can be linked in three ways: hairpin (19): a single polyamide with a flexible central linker; cross-linked (20, 21): lateral bridge or staple that connects the centers of the two side by side polyamide units and bis-polyamides (22): two polyamide units tethered by a linker of appropriate dimensions (Fig. 4).

HIV-1 integrase inhibition by bis-polyamides

The phenomenon of the rapid emergence of human immunodeficiency virus (HIV) strains resistant to available drugs implies that effective treatment modalities will require the use of a combination of drugs targeting different sites of the HIV life cycle (23, 24). Integrase is emerging as a novel target for intervention by chemotherapeutics and to date few effective inhibitors of this enzyme have been reported (25, 26). Integrase is responsible for the insertion of the viral DNA into a host chromosome. This process is essential for effective viral replication and can be reproduced *in vitro*. Insertion takes place in two consecutive steps, initially integrase processes the linear viral DNA by removing two nucleotides from each 3' end, leaving the recessed 3'-OH termini. This reaction is followed by trans-esterification of phosphodiester bonds in which a host DNA strand is cut and the 5' end of the cut is joined to a processed viral 3' terminus. These two steps are known as 3' processing and 3' end joining (strand transfer) and can be examined in an *in vitro* assay employing purified recombinant HIV-1 integrase and an oligonucleotide corresponding to the U5 region of HIV-LTR sequence. Inspection of the available HIV-1 viral DNA termini (U5 and U3 LTRs) shows a high degree of conservation and the presence of a stretch of 5-6 consecutive AT positioned 10 nucleotides away from each LTR end. Thirty-five minor groove binders from the family of netropsin and distamycin were tested for anti-HIV-1 integrase activity. The results of this study can be grouped under two different classes, one related to netropsin and distamycin analogs, and the other with the bis-polyamide analogs (bis-lexitropsin).

Netropsin and distamycin analogs

The bis-netropsin (**1-6**) and bis-distamycin (**8-19**) family of compounds composed of two netropsin or distamycin units separated by aliphatic or aromatic linkers with different size and geometry were examined (27, 28) (Table I). The comparison of the potency of distamycin **7** and bis-distamycin **8** suggested the importance of the dimeric structure. The length and the geometry of the linkers is also critical for effective inhibition. The *para*-1,4-disubstituted derivative **8** exhibited markedly higher potency than the *ortho*-1,2-disubstituted **9** or *meta*-1,3-disubstituted **10** derivatives. A similar observation was noted for the pyridinyl derivatives (*para*-substituted **11** with *meta*-substituted **12**). The linear *trans*-1,2-substituted **13** was 30-50 times more potent than the rigid cyclobutyl **14** and norbornyl **15** derivatives. The dimers with the short dimethylene linker **16** or a long aliphatic chain linker **19** exhibited significantly reduced potency compared with **17** or **18** with hexamethylene or octamethylene linkers, respectively.

Bis-polyamide analogs

A series of bis-distamycins with modified side chains, bis-polyamides (bis-lexitropsins) with varying linkers, were also examined for their HIV-1 integrase inhibition activity in *in vitro* assays (28) (Table II). The common structural features required for potency is *para*-substitution, with the 1,4-disubstituted **26** being > 100 times more potent than the 1,3-disubstituted **27**. Similarly, derivative **30** is 10, 30 and > 100 times more potent than the corresponding **31**, **32** and **33** derivatives, respectively. The monosubstituted polyamides **28**, **34** and **35** were practically inactive. The cyclic congener **29** was moderately active. The bis-polyamides have shown a similar trend in potency for HIV-2 integrase.

Inhibition of RNA polymerase II transcription

Sequence-specific DNA-binding small molecules (29) that can permeate human cells could potentially regulate transcription of specific genes. The polyamides (**36-39**) were designed according to the sequences of the HIV-1 enhancer and promoter region (30) (Fig. 5). The

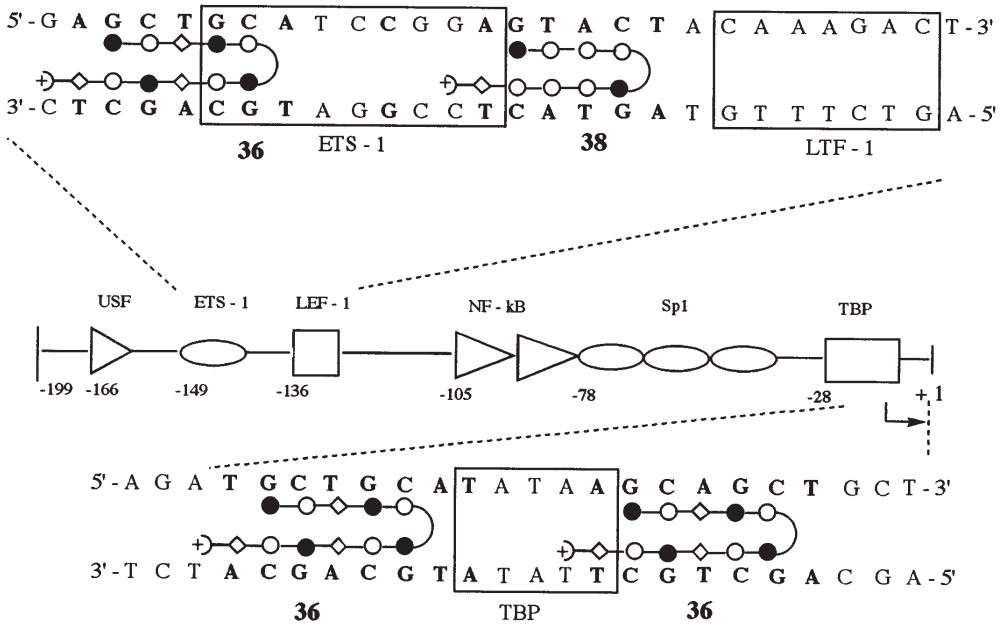


Fig. 5. Schematic of the HIV-1 enhancer and promoter (nucleotide position -199 to +1) showing binding sites for polyamides **36** and **38**.

polyamide **36** inhibits TBP (TATA box binding protein) binding to a double-stranded oligonucleotide corresponding to the HIV-1 TATA box region. Additionally, polyamide **36** does not inhibit TBP binding to the TATA box region of the adenovirus major late promoter which contains a mismatch flanking sequence. The Ets-1 recognition site in the HIV-1 enhancer is flanked by binding sites for polyamides **36** and **38**. The polyamide **38** had no effect on Ets-1 DNA binding; polyamide **36**, however, prevented Ets-1/DNA complex formation. Two mismatch polyamides **37** and **39** did not prevent complex formation. Polyamide **36** inhibits Ets-1 binding by steric interaction with protein contacts in the minor groove, whereas polyamide **38** must occupy the minor groove beyond the Ets-1 contacts. LEF-1 is a member of the high mobility group family of minor groove binding proteins (31) and has been shown to be essential for HIV-1 transcription and replication in lymphoid cells. Polyamide **38**, located in the minor groove immediately adjacent to site L1, inhibits LEF-1 binding to this site and also inhibits LEF-1 binding to sites L2 and L3 at markedly higher concentrations (3 nM and above) as there are no polyamide recognition sites adjacent to sites L2 and L3. Mismatch polyamide **39** failed to inhibit LEF-1 binding.

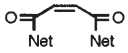
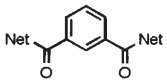
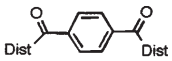
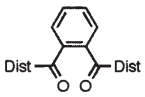
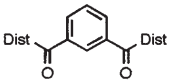
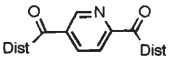
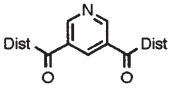
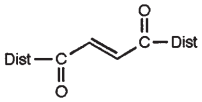
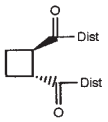
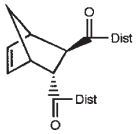
Inhibition of transcription

The effects of polyamides **36** and **37** on HIV-1 transcription were tested in an *in vitro* transcription assay with a HeLa cell nuclear extract. Polyamide **36** inhibited basal transcription from the HIV-1 promoter but not from CMV major immediate early promoter, which contains a mismatched TATA flanking sequence. The polyamide **37** did not inhibit basal transcription, while 50% inhibition of HIV-1 transcription at 60 nM by polyamide **36** was observed which corresponds to a 6- to 10-fold excess of polyamide overbinding sites. Polyamides **38** and **39** were tested on LEF-1 activated HIV-1 transcription. Polyamide **38** inhibited HIV-1 transcription in this system with a 50% reduction of transcription at a concentration of 10-30 nM. Polyamide **38** failed to inhibit HIV-1 transcription in the LEF-1 depleted extract. Polyamide **39** failed to inhibit either HIV-1 or CMV transcription. Polyamide **38** does not inhibit basal transcription, although binding sites for this polyamide are present at the start site for transcription and within the HIV-1 RNA coding sequence. These observations suggest that pol II can transcribe DNA with a polyamide bound in the minor groove and that polyamides are only inhibitory to transcription when these compounds interfere with the DNA binding activity of a required transcription factor (32).

Inhibition of virus replication

HIV-1 transcription was indirectly studied by measuring the levels and kinetics of HIV-1 replication in human peripheral blood mononuclear cells (PBMCs) in culture. PBMCs were infected with a T cell tropic strain WEAU1.6 or with the macrophage tropic strain SF162. It was found that polyamide **36** at 1 μM concentration caused 80% reduction in virus whereas polyamide **38** at 1 mM concentration caused 60% reduction after 6-8 days. The combination of **36**+**38** inhibited HIV-1 replication at 10 nM to 1 μM concentration. The combination of **36**+**38** at 1 μM concentration acted in synergy to reduce viral p24 levels below the threshold of detection after 6-8 days for WEAU

Table I: Inhibition of HIV-1 replication of CEM cells and inhibition of HIV-1 in catalytic activities by a series of bis-netropsin and bis-distamycin analogs.

Compound	Structure	IC_{50} (μM) 3'-Processing	IC_{50} (μM) Integration	Cellular anti-HIV-1 data (μM) IC_{50}	EC_{50}	TI
1	Net-CO-Net	42.8; 78.4[a]	29.0; 45.0	83.5	11.9	7.01
2	Net-CO$(CH_2)_2$CO-Net	05.8 ± 1.6	7.5 ± 2.5 40.3; 10.0	75.3 57	12 3.9	6.3 14.6
3	Net-CI$(CH_2)_9$CO-Net	37.06; 64.8	29.2; 10.0	78	6.6	11.7
4	Net-CO$(CH_2)_{10}$CO-Net	16.4; 33.8				
5		98.9	30.0	199	0.35	566
6		7.5 ± 1.3	6.9 ± 1.4	284	3.35	80
7	Distamycin	56.9	50.2			
8		0.1 ± 0.08	0.09 + 0.001	4.7	0.39	12
9		43.7 ± 4.6	10.0			
10		72 ± 64	10.0	140	21	6.6
11		0.08 ± 0.05	0.1 ± 0.03	69	1.6	43
12		33.5	0.4	69	9.8	7.0
13		0.8	0.3	207	10.4	19.8
14		30.7 ± 8.1	10.0	71	16	4.5
15		36.5 ± 4.9	10.0			
16	Dist-CO$(CH_2)_2$CO-Dist	21 ± 8.5	9.5 ± 1.2		41	1.0
17	Dist-CO$(CH_2)_6$CO-Dist	0.025; 0.09 (4.9)[b]	0.005; 0.009 (2.2) 0.015; 0.09			
18	Dist-CO$(CH_2)_8$CO-Dist	0.032; 0.09 (7.4)	(6.5) 8.0		14	2.0
19	Dist-CO$(CH_2)_{22}$CO-Dist	12.2 ± 3.3				
20	Hoechst-33258	>100	>100			
21	DAPI	>100	>100			
22	Pentamidine	>100	>100			
23	Berenil	>100	>100			
24	Spermine	>100	>100			
25	Spermidine	>100	>100			

[a]Second independent experiment. [b]Numbers in parentheses refer to GC rich DNA duplex.

R =

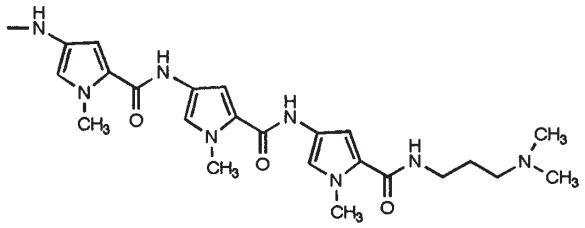
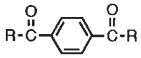
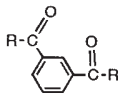
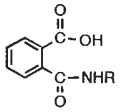
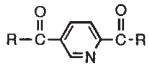
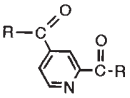
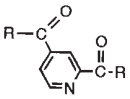
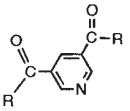
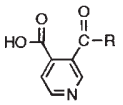
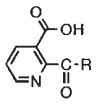


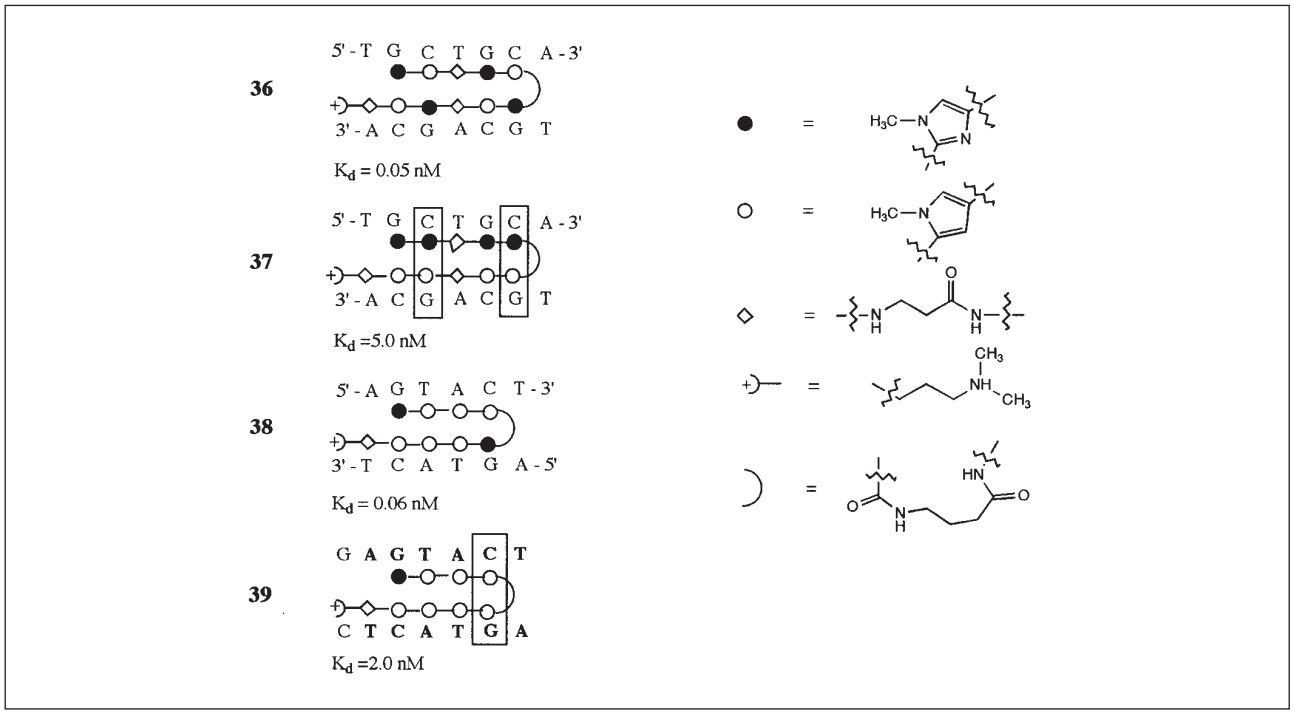
Table II: Inhibition of HIV-1 and HIV-2 integrase catalytic activities by a series of novel bis-polyamides (bis-lexitropsins).

Compound	Structure	HIV-1 IC_{50} (μM) 3'-Processing	Integration	Disintegration	HIV-2 IC_{50} (μM) 3'-Processing	Integration
26		0.19 ± 0.1	0.07 ± 0.002	0.61	0.27 0.28	0.07 0.07
27		22.0	22.0	77 33	66.9	66.9
28		92.5 100	92.5 100	>100 >100	>100	>100
29		8.0 5.5	8.0 5.5	28.5 10.2		
30		0.04	0.01	0.68 0.18	0.37 0.52	0.37 0.05
31		1.2	0.4	0.3		
32		33.3 100	33.4 100	57 14.1	>100	>100
33		>100	>100	>100 86	>100	>100
34		>100	>100	>100	>100	>100
35		>100	>100	>100	>100	>100

(< 10 pg/ml; 99.9% inhibition of viral replication) and were as effective as 1 μM azidothymidine (AZT) in blocking HIV-1 replication. The observed polyamide inhibition of virus replication is likely caused by interference with the DNA binding activities of TBP and Ets-1 by polyamide **36** and the binding activity of LEF-1 by polyamide **38**, but it is possible that inhibition of cellular genes involved in T cell activation could have an indirect effect on HIV-1 replication (32).

RNase activity by di-imidazole polyamides

A number of small molecules with RNA cleavage activity are used for the investigation of RNA structure in solution (33, 34). Two major groups of these small molecules are often termed chemical nucleases and nuclease mimics for their catalytic activity. The chain scission by chemical nucleases often proceeds via hydrogen abstrac-



tion from the ribose, while nuclease mimics induce true hydrolysis of the phosphate diester bone in the RNA backbone by both acid and base hydrolysis (35). Imidazole and compounds containing imidazole residues have been shown to cleave RNA in a RNase A-mimicking manner. Half of the molecules of di-imidazole (**40**) are protonated at neutral pH because the pK_b of imidazole is 7.0 and can act as an acid, while the other half acts as a base in RNA hydrolysis. Di-imidazole (**40**) is a compound derived from the polyamide drugs distamycin and netropsin by the replacement of two pyrrole heterocycles with *N*-methylimidazole residues. This enables **40** to bind to the minor groove of B-DNA in a sequence-specific manner to GC rich sites. The overall cleavage optimum pH of 7.0 is consistent with that of other imidazole containing compounds. The bell-shaped concentration dependence of the cleavage activity of di-imidazole polyamide is known from other synthetic RNA cleavers. Particularly encouraging is the low concentration of 0.3-0.5 mM di-imidazole polyamide at which cleavage is already quite intense. This is about 1 order of magnitude lower than the concentration required of the previous artificial nucleases (36).

From the cleavage patterns obtained by the action of di-imidazole polyamide (**40**) on different RNA substrates, it can be inferred that cleavage is structure-specific to some extent. Cleavage occurs preferentially in the single-stranded regions of the different tRNA substrates, but not exclusively so. In the less structured human mitochondrial tRNA transcript, cleavages are also seen in stem regions. In these RNAs, cleavages occur with a clear preference for pyrimidine-adenine sites (Fig. 6). This sequence specificity is a typical feature of RNase. Another typical feature of RNase is that it cuts after accessible pyrimidines in RNAs where pyrimidine-adenine sites are rare. By analogy, di-imidazole polyamide cleaves after U35 and C36 in the anticodon loop of this tRNA. The cutting mechanism of the di-imidazole polyamide may rely on positioning of an imidazole moiety at the proper place along the RNA strand (37).

DNA gyrase inhibition

DNA gyrase is a prokaryotic type II topoisomerase enzyme that controls the topological state of DNA and catalyzes the negative supercoiling of DNA. The mechanism of DNA supercoiling by gyrase involves cleavage of double-strand DNA, an ATP driven passage of a DNA segment through the cleavage site and a religation step. GyrA is responsible for cleavage and religation, whereas GyrB is involved in the ATP-dependent strand passage step (38). Drugs which are DNA minor groove binders can exhibit inhibition of DNA gyrase *in vitro* (39). Since a sequence specificity is known for several minor groove binders, these agents can be regarded as useful probes in the elucidation of gyrase binding and cleavage sites on DNA (40). Polyamides from two different groups have been evaluated *in vitro* for their anti-gyrase activity – hairpin polyamides and cross-linked polyamides, both of which have shown marked activity against this enzyme.

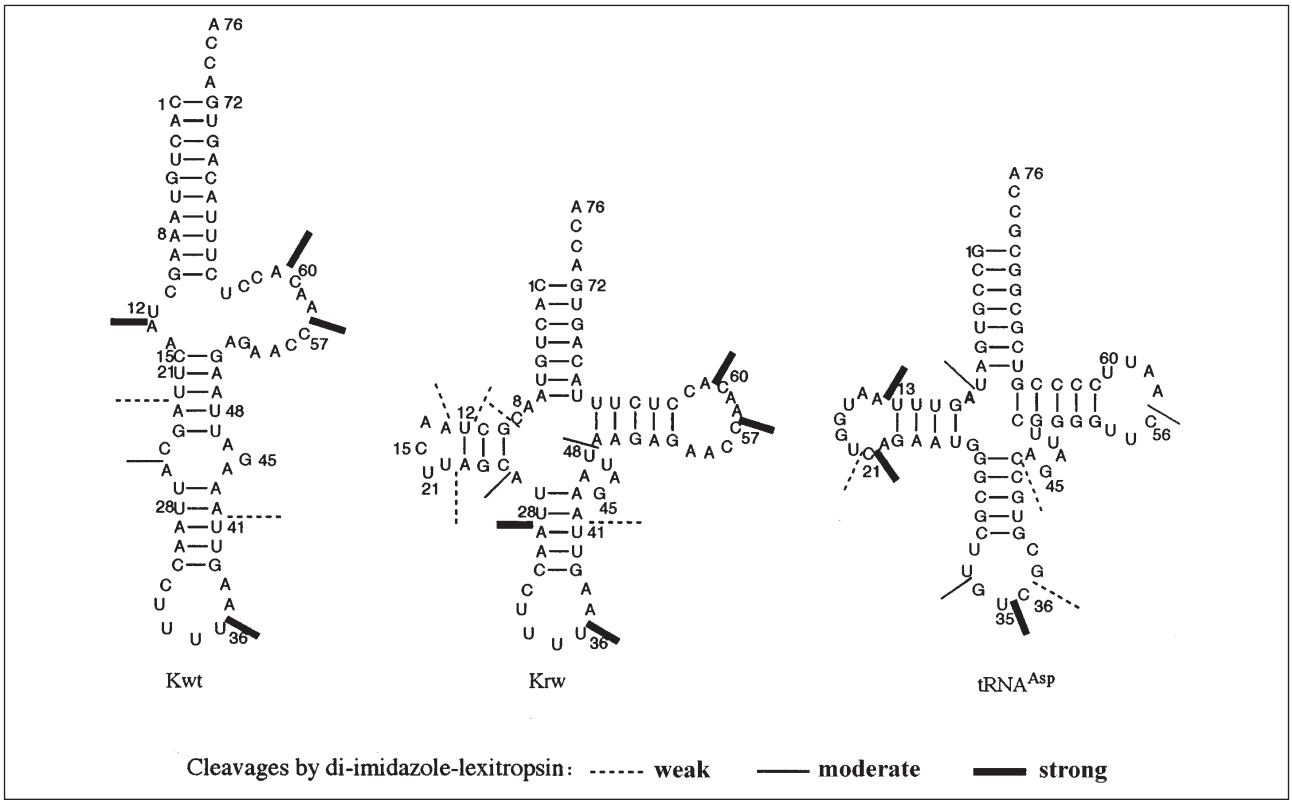
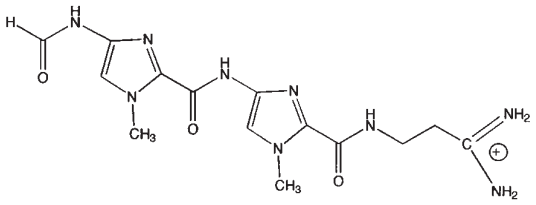


Fig. 6. Cleavage position on the secondary structures of the substrate RNAs.

Inhibition by hairpin polyamides

An 8-ring hairpin polyamide (**41**), which recognizes a GC rich 6-base pair DNA sequence, effectively inhibits the bacterial DNA gyrase activity *in vitro*. This inhibitory potency of polyamide **41** is observed to be most pronounced for the enzyme-mediated cleavage reaction when a 162 bp fragment with a single specific gyrase site is used as a substrate, whereas, in contrast, the supercoiling reaction is much less affected. The inhibition of the cleavage steps occurs in the nanomolar concentration range, which is 3-4 orders of magnitude lower than that



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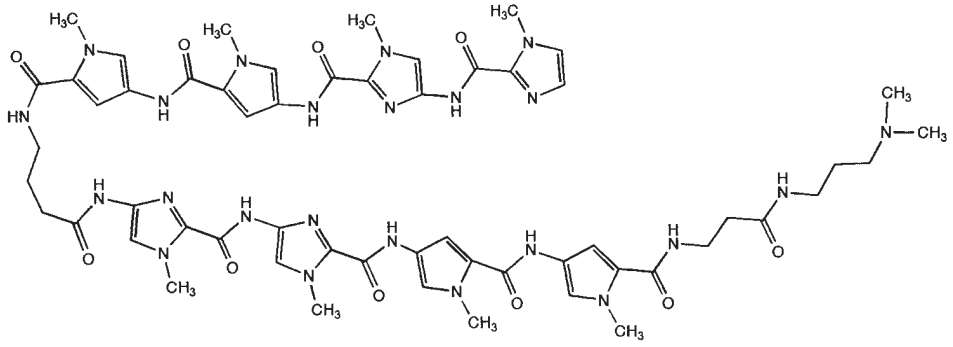

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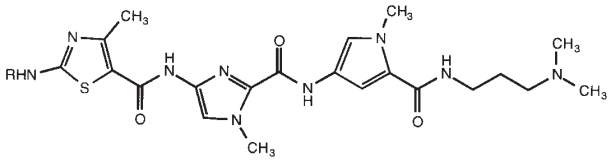


42 R = H
43 R = CHO
44 R = $COCH_3$

observed for supercoiling (41). The hairpin polyamide **41** exhibits the lowest IC_{50} value at 3 nM for *S. noursei* gyrase in the cleavage assay, which is 10-fold less than that of a netropsin analog, 20-fold less than chromomycin A_3 and more than 300-fold less than distamycin. The IC_{50} value is even lower for *E. coli* gyrase (IC_{50} = 0.7 nM). The 8-ring hairpin polyamide **41** has the strongest anti-gyrase effect when compared with the other DNA minor groove binders acting on a specific sequence of DNA substrate. The strong effect of ligand **41** can be best explained by the hairpin formation of the polyamide upon binding to the GGCC enzyme recognition site which directly blocks the double-strand breakage reaction catalyzed by the GyrA subunits of the DNA gyrase.

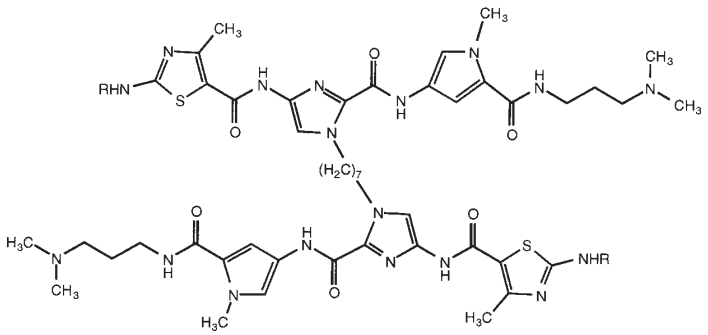
Inhibition by cross-linked polyamides

The monomers (**42-44**) and the cross-linked analogs (42) of these monomers (**45-47**) were evaluated for their gyrase inhibition activity. The dimers (**45-47**) exhibited inhibition of gyrase supercoiling of pBR322 DNA in a low concentration range similar to distamycin, whereas the monomers possesses a significantly weaker inhibitory potency (43). The acetylated dimer (**47**) and the corresponding monomer (**44**) are almost completely inactive. No sequence correlation is possible since pBR322 DNA contains several different cleavage sites for the enzymes. The formation of the 107 bp fragment is inhibited beginning at 0.1 μM and is nearly completely blocked at 1-2 μM of the dimer. The inhibitory effect of the cleavage reaction is most pronounced for the dimer followed by the acetylated analog. The dimer (**45**) completely blocks the cleavage below 1 μM, whereas for distamycin and the monomeric polyamide, concentrations > 1 μM and 10 μM, respectively, are required. The effect can be ascribed to the side by side binding mode on the substrate, which can more effectively prevent the enzyme from cleaving the DNA substrate. Design of appropriate cross-linked polyamide dimers with specific affinity to the enzyme recognition site or to other flanking regions could be a basis for developing a drug of higher inhibitory potency.

Conclusions

The design and synthesis of many compounds and their detailed examination, and hence much time, will be required before we may perfectly control the targeting to definite DNA sequences. Small molecules that specifically bind to any sequence in the human genome would be useful tools in molecular biology and potentially in human medicine. Interactions that stabilize the present 2:1 mode of drug:DNA binding should be useful as a guide in the design of such sequence-specific drugs. A simple code has been developed to rationally alter the sequence specificity of the minor groove binding molecules and has guided the design of polyamides that specifically target a wide variety of DNA sequences. Inhibition of RNA polymerase transcription and HIV-1 integrase represent promising initial steps towards exploring the potential of polyamides for the artificial regulation of eukaryotic gene expression.

It remains to be determined whether a broad panel of genes can be selectively targeted within a variety of cell types with these polyamides. In addition to downregulation of genes, polyamides might be designed specifically to upregulate transcription, thereby expanding the repertoire of synthetic cell-permeable transcription factors with potential clinical application.



45 R = H
46 R = CHO
47 R = $COCH_3$

Acknowledgements

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