

Sequence-selective binding to DNA of bis(amidinophenoxy)alkanes related to propamidine and pentamidine

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The DNA sequences targeted by a complete homologous series of aromatic diamidines have been determined at single-nucleotide resolution via protection from cutting by the endonucleases DNase I, DNase II and micrococcal nuclease. Propamidine, pentamidine and to a lesser extent hexamidine bind selectively to nucleotide sequences composed of at least four consecutive A-T base pairs. In contrast, the binding to DNA of butamidine, heptamidine, octamidine and nonamidine is poorly sequence-selective. Sequences composed of only three consecutive A-T base pairs do not afford a potential binding site for propamidine or the longer homologues, and none of the drugs tolerate the presence of a G-C base pair within the binding site. Experiments with DNA molecules containing inosine in place of guanosine

and 2,6-diaminopurine in place of adenine reveal that the lack of binding of propamidine to GC-containing sites is attributable to an obstructive effect of the exocyclic 2-amino group of guanosine. The present data support the view that the local conformation of the double helix (in particular the width of the minor groove) plays a dominant role in the binding reaction and that the capacity of diamidines to recognize AT-rich sequences selectively varies considerably depending on the length of the alkyl chain. The evidence indicates that binding to AT-tracts in DNA must play a role in the biological activity of these diamidines, but there is no simple correlation between binding and pharmacological efficacy.

INTRODUCTION

Aromatic diamidines, and in particular bis(amidinophenoxy)-alkanes (Figure 1), have a long history in the treatment of human diseases. Propamidine (**3**) is an antimicrobial agent used for the treatment of *Acanthamoeba* keratitis and other corneal infections [1,2]. Pentamidine (**5**) is active against *Pneumocystis carinii*, the pathogen responsible for opportunistic infection which occurs in the majority of patients with AIDS [3–6]. Hexamidine (**6**) is a well-known antimicrobial drug used to treat amoebic corneal infection and various bacterial diseases. These diamidines also display a useful spectrum of anti-trypanosomal and antiviral activity. For example, pentamidine and its analogues show significant activity against diverse, widespread parasites that cause serious diseases such as *Giardia lamblia*, *Plasmodium falciparum* and *Leishmania mexicana amazonensis* [7,8]. In fact pentamidine was originally introduced for human clinical use over 40 years ago for the treatment of African trypanosomiasis. Among these various therapeutic properties, the anti-*Pneumocystis carinii* pneumonia (PCP) activity of pentamidine has proved to be particularly useful, especially since the pandemic development of AIDS. But because pentamidine has adverse side effects a large number of analogues have been synthesized [9–14]. Yet, so far as we are aware, pentamidine remains the only diamidine used in the clinic for treating PCP. The quest for improved pentamidine analogues remains more pressing than ever because of the growing prevalence of HIV, but our limited knowledge as to the molecular mechanism of action of pentamidine has confined the search for new anti-PCP drugs mainly to an empirical level.

There is considerable evidence that direct interaction with the

genome of the pathogen contributes to the pharmacological effect of bis(amidinophenoxy)alkanes [15]. Morphological changes at sites containing extranuclear DNA occur when protozoa are treated with pentamidine [16]. It has been known since the early 1970s that pentamidine and structurally related compounds such as berenil (**1**) and stilbamidine (**2**) bind to DNA in a non-intercalative manner [17–19]. Later it was shown that pentamidine fits into the minor groove of double-helical DNA with a strong preference for AT-rich sequences [20–22]. In recent years, Neidle and co-workers have solved the structure of several crystalline complexes between short oligonucleotides and propamidine, pentamidine and analogues [23–29]. These structural studies have allowed us to discern intimate details of the pentamidine–DNA interaction and have contributed to our understanding of the molecular rules that dictate sequence-specific recognition.

These considerations prompted us to investigate the binding to DNA of a complete homologous series of bis(amidinophenoxy)-alkanes that differ from each other only by the length of the alkyl chain. They include the three aforementioned drugs together with butamidine and three longer homologues, heptamidine, octamidine and nonamidine (compounds **3–9** in Figure 1). A recent study showed that the amoebicidal efficiency of these bis(amidinophenoxy)alkanes varies significantly from one congener to another, with propamidine, hexamidine and octamidine being the most active compounds against both trophozoites and cysts of two strains of *Acanthamoeba* [2]. We wondered whether the biological activity of these drugs might be directly related to interaction with DNA. Support for this hypothesis can be found in scattered reports that propamidine, which binds strongly to DNA, is biologically active, whereas butamidine is inactive (as an

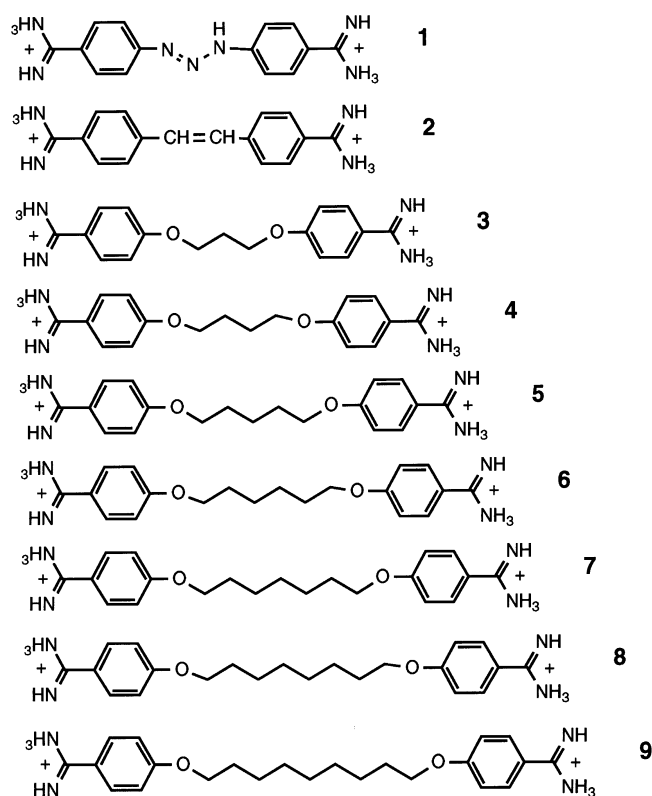


Figure 1 Structures of aromatic diamidines

Berenil (1), stilbamidine (2); the bis(amidinophenoxy)alkanes: propamidine (3), butamidine (4), pentamidine (5), hexamidine (6), heptamidine (7), octamidine (8), nonamidine (9).

amoebicide and also as an anti-PCP and antiparasitic agent) and has been described previously as possibly being a poor DNA binder [11].

To investigate the selectivity of binding of the bis(amidinophenoxy)alkanes (3–9) to DNA, we resorted to the tried and tested methodology of footprinting using three well-characterized endonucleases [30,31]. The results provide clear experimental evidence that the capacity of these drugs to recognize AT-rich sequences selectively varies considerably depending on the length of the alkyl chain between the two amidinophenoxy terminal groups. The results are discussed in relation to previous experimental findings and theoretical DNA-binding studies.

MATERIALS AND METHODS

Drugs

Propamidine and pentamidine were obtained from May and Baker (Dagenham, U.K.) and Sigma Chemical Co. (La Verpillière, France) respectively. The synthesis of the other diamidines has been reported [9,32]. Heptamidine, octamidine and nonamidine are only moderately soluble in water but in the concentration range used (5–50 μ M) purely aqueous solution could be obtained. In the dry state drugs were stored in a desiccator in the dark at 4 °C. Ligand concentrations were determined by direct weighing.

Chemicals and biochemicals

Ammonium persulphate, Tris base, acrylamide, bis-acrylamide,

ultrapure urea, boric acid, tetramethylethylenediamine and dimethyl sulphate were from BDH. Formic acid, piperidine, hydrazine and formamide were from Aldrich. Photographic requisites were from Kodak. Bromophenol Blue and xylene cyanol were from Serva. Nucleoside triphosphates labelled with 32 P (α -dATP and γ -ATP) were obtained from NEN DuPont. Unlabelled dATP was ultrapure grade from Pharmacia. Restriction endonucleases *EcoRI*, *AvaI* and *PvuII* (Boehringer Mannheim) were used according to the supplier's recommended protocol in the activity buffer provided. Alkaline phosphatase, T4 polynucleotide kinase and avian myeloblastosis virus reverse transcriptase were from Pharmacia. Bovine pancreatic DNase I (Sigma Chemical Co.) was stored as a 7200 units/ml solution in 20 mM NaCl/2 mM MgCl₂/2 mM MnCl₂, pH 8.0. Micrococcal nuclease (MNase; Boehringer Mannheim) was stored as a 2500 units/ml stock solution in 50 mM Tris buffer, pH 7.6, containing 2 mM CaCl₂. DNase II (Sigma Chemical Co.) was prepared as a 200 units/ml stock solution in 10 mM ammonium acetate, pH 5.6, containing 0.2 mM EDTA. The stock solutions of enzymes were kept at –20 °C and freshly diluted to the desired concentration immediately before use. All other chemicals were analytical-grade reagents, and all solutions were prepared using doubly deionized Millipore-filtered water.

DNA purification and labelling

Plasmids pKMp27 [33], pUC12 and pBS (Stratagene, La Jolla, CA, U.S.A.) were isolated from *Escherichia coli* by a standard SDS/sodium hydroxide lysis procedure and purified by banding in CsCl/ethidium bromide gradients. Ethidium was removed by several isopropanol extractions followed by exhaustive dialysis against Tris/EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer before digestion by the restriction enzymes. The 178-mer fragment from pUC12 and the 117-mer and 265-mer fragments from plasmid pBS were rendered radioactive by 3'-[32 P]-end labelling of the *EcoRI*–*PvuII* double digest of the plasmid using [α - 32 P]dATP (6000 Ci/mmol) and avian myeloblastosis virus reverse transcriptase or by 5'-[32 P]-end labelling of the *EcoRI*/alkaline phosphatase-treated pBS plasmid using [γ - 32 P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase followed by treatment with *PvuII*. The 160 bp *tyrT*(A93) DNA was prepared by digestion of the plasmid pKMp27 with *EcoRI* and *AvaI* in order to generate sticky ends of unique sequence. It was labelled (i) at the *EcoRI* site on the lower strand (the Crick strand) with [α - 32 P]dATP and (ii) at the *AvaI* site on the upper strand (the Watson strand) with [α - 32 P]dCTP and reverse transcriptase, so as to give specific 3'-end labelling. Finally the labelled digestion products were separated on a 6% (w/v) polyacrylamide gel under non-denaturing conditions in TBE buffer (89 mM Tris/borate, pH 8.3/1 mM EDTA). After autoradiography, the requisite band of DNA was excised, crushed and soaked in elution buffer (500 mM ammonium acetate/10 mM magnesium acetate) overnight at 37 °C. This suspension was filtered through a Millipore 0.22 μ m filter and the DNA was precipitated with ethanol. Following washing with 70% (v/v) ethanol and vacuum drying of the precipitate, the labelled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

Preparation and labelling of DNA fragments containing modified nucleotides

The protocol used to synthesize DNA containing inosine or 2,6-diaminopurine (DAP) in place of guanosine or thymine respect-

ively has been previously described [34]. Briefly, molecules containing normal or modified bases were synthesized by PCR using the primers 5'-AATTCCGGTTACCTTAATC and 5'-TCGGGAACCCACCACGGG bearing a 5'-OH or 5'-NH₂ terminal group so as to permit 5'-phosphorylation of one strand only. The *tyrT*(A93) fragment used as a template bears a 5'-phosphate due to the action of *EcoRI* and thus only the newly synthesized DNA (with normal or modified nucleotides) could be labelled by the kinase. Twenty amplification cycles were performed, each cycle consisting of the following segments: (a) for normal DNA, 94 °C for 1 min, 37 °C for 2 min, and 72 °C for 10 min; (b) for inosine plus 2,6-diaminopurine-containing (I+DAP)-DNA, 84 °C for 1 min, 30 °C for 2 min, and 62 °C for 10 min. The purified PCR products were 5'-end labelled with [γ -³²P]ATP in the presence of T4 polynucleotide kinase and the labelled DNA was isolated by 6% polyacrylamide gel electrophoresis.

Footprinting experiments

Cleavage reactions by DNase I, DNase II and MNase were performed essentially according to the original protocols [35–38]. Reactions were conducted in a total volume of 10 μ l. Samples (3 μ l) of the labelled DNA fragment were incubated with 5 μ l of the buffer solution supplemented with the diamidine under test. After 30 min incubation at 37 °C to ensure equilibration of the binding reaction, digestion was initiated by the addition of 2 μ l of the endonuclease solution whose concentration had been adjusted to limit the enzyme attack to less than 30% of the starting material so as to minimize the incidence of multiple cuts in any strand ('single-hit' kinetic conditions). Optimal enzyme dilutions were established in preliminary calibration experiments. Typically, DNase I experiments included 0.01 unit/ml enzyme, 20 mM NaCl, 2 mM MgCl₂, 2 mM MnCl₂, pH 7.3. DNase II cuts included 40 units/ml enzyme, 10 mM ammonium acetate, 1 mM EDTA, pH 5.6. MNase cuts included 1 unit/ml enzyme, 50 mM Tris/HCl, 2 mM CaCl₂, pH 7.6. At the end of the reaction time (routinely 3 min at room temperature), the digestion was stopped by freeze-drying. After freeze-drying each sample was washed once with deionized water then freeze-dried again before resuspending in 3 μ l of an 80% (v/v) formamide solution containing tracking dyes (purchased from Sigma).

Electrophoresis and autoradiography

DNA cleavage products were resolved by PAGE under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. Electrophoresis was continued until the Bromophenol Blue marker had run out of the gel (approx. 2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer model S2). Gels were soaked in 10% (v/v) acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and subjected to autoradiography at -70 °C with an intensifying screen. Exposure times of the X-ray films (Fuji R-X) were adjusted according to the number of counts per lane loaded on each individual gel (usually 24 h).

Quantification by storage phosphor imaging

A Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature [39]. Baseline-corrected scans

were analysed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with dimethyl sulphate (G) or formic acid (G+A) followed by piperidine-induced cleavage at the modified bases.

RESULTS

Footprinting experiments were performed with four different DNA fragments to provide an assessment of the sequence selectivity of the diamidines with respect to a wide variety of potential binding sites. The 178-mer, 117-mer and 265-mer *EcoRI*-*PvuII* restriction fragments obtained from plasmids pUC12 and pBS were examined together with the 160 bp fragment from *E. coli* containing the *tyrT* promoter sequence that has been used extensively over many years to probe the sequence-selectivity of a large number of DNA-binding drugs [31].

To begin with, experiments were performed using DNase I as cleaving agent. A typical autoradiogram of footprinting gels run with the seven bis(amidinophenoxy)alkanes (25 μ M each) bound to the 117, 178 and 165 bp fragments is shown in Figure 2. With all three DNA species, addition of propamidine led to several areas of decreased intensity (i.e. footprints) that reflect cleavage inhibition due to the ligand bound to particular nucleotide sequences. Similar experiments were performed with each DNA fragment labelled on one or the other of the complementary strands. Selected lanes from each gel were scanned to compare the protection patterns afforded by the various diamidines. Figure 3 reports differential cleavage plots for the 3'-labelled 265-residue, 117-residue and 178-residue fragments in which data for five of the diamidines are superimposed. Plots for octamidine and nonamidine have been omitted for clarity. Negative probabilities of cleavage indicate protection against cutting by DNase I and, conversely, positive values indicate drug-induced enhanced DNA cleavage. It is immediately apparent that all five compounds hinder the cutting reaction to varying degrees. At one extreme, propamidine is the most potent inhibitor, whereas, on the other hand, butamidine has practically no effect on the nuclease cleavage. Although less effective than propamidine, the next two diamidines with an odd number of methylene groups in their alkyl chain, i.e. pentamidine and to a much lesser extent heptamidine, inhibit the access of the enzymic probe. The two analogues having an even number of CH₂ units behave quite differently since butamidine completely fails to inhibit the enzyme, whereas hexamidine does reduce the DNase I cleavage at defined sequences. Close examination of the cleavage plots reveals that hexamidine gives stronger footprints than heptamidine, although its effect is rather modest compared with what can be achieved with propamidine.

For a more accurate quantitative comparison we measured the variation of band intensity as a function of diamidine concentration at a selected binding site and a flanking GC-rich sequence within the target 265 bp DNA fragment from pBS (Figure 4). The results illustrate very clearly how the diamidines protect the AT-tract from cleavage to varying extents. The intensity of the footprint at the TTAATTT site is more or less proportional to the extent of enhanced cleavage at the adjacent GGG sequence. With propamidine and pentamidine the footprinting at the AT-tract occurs with half-maximal effect at concentrations (C₅₀) of approx. 4 μ M and 9 μ M respectively. The C₅₀ values for hexamidine and heptamidine cannot be accurately determined but are clearly higher: the protection by the

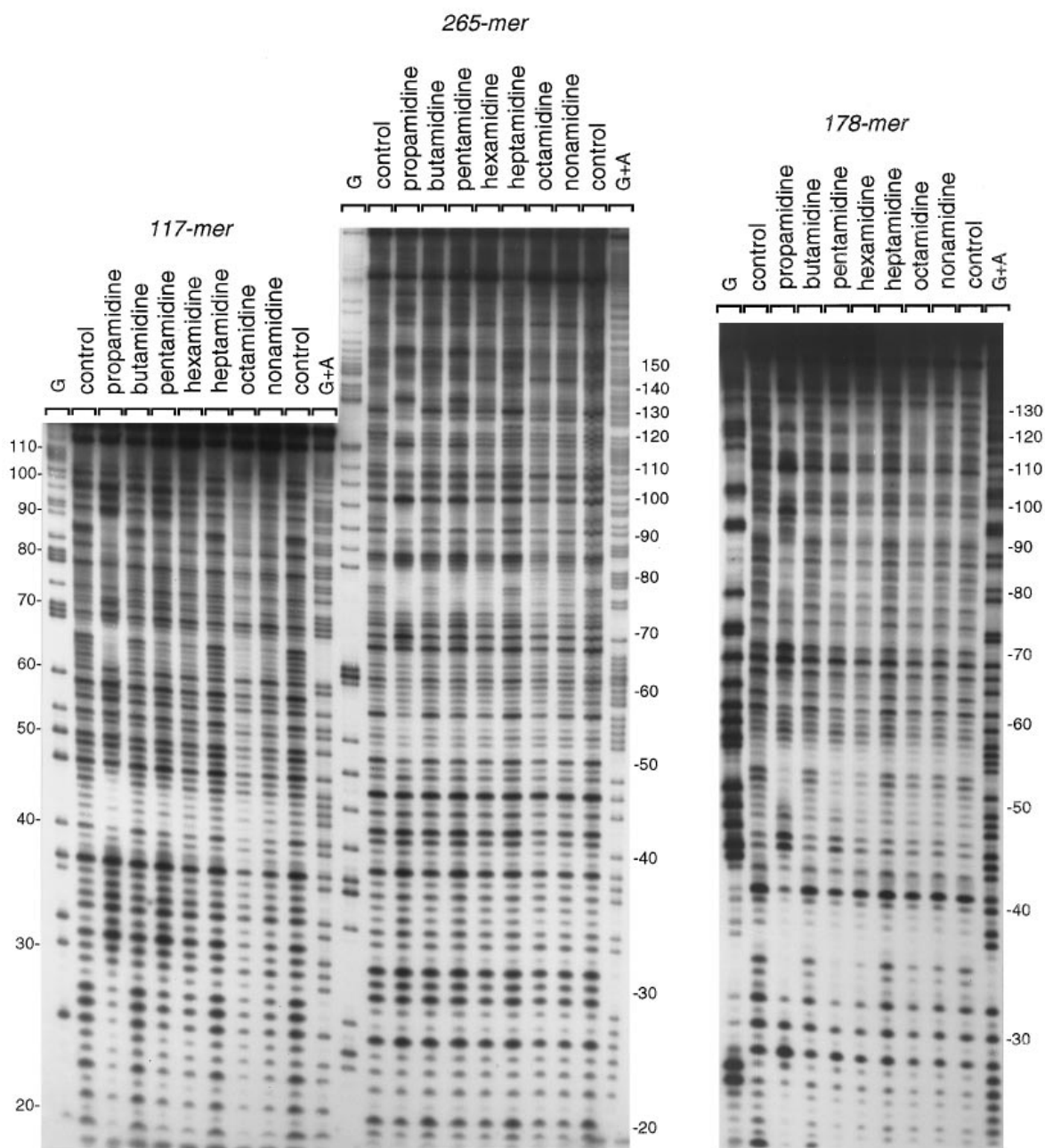


Figure 2 DNase I footprinting of the diamidines on the 117, 265 and 178 bp *EcoRI/PvuII* restriction fragments cut out from plasmids pBS and pUC12

In each case the duplex DNA was 3'-end labelled at the *EcoRI* site with [α - 32 P]dATP in the presence of avian-myeloblastoma-virus (AMV) reverse transcriptase. The products of the DNase I digestion were resolved on an 8% polyacrylamide gel containing 8 M urea. Each drug was used at 25 μ M. Tracks labelled 'control' contained no drug and tracks labelled 'G' and 'G+A' represent dimethyl sulphate- and formic acid-piperidine markers specific for guanines and purines respectively. Numbers at the side of the gels refer to the numbering scheme used in Figure 3.

heptamide is so weak that no sensible estimate of the C_{50} could be determined (see [40] for discussion of this problem). In all probability, the binding of hexamide to the AT-rich site is at least 4–5 times tighter than that of heptamide. Even at high concentrations (50–100 μ M) butamide only marginally protects the cleavage of AT-tracts such as the one analysed here, whereas both octamide and nonamide have absolutely no effect. From the gels in Figure 2 and the plots in Figures 3 and 4, we can conclude that the compounds rank in the order **3** > **5** > **6** > **7** as

regards potency of footprinting, and that **8** and **9** are generally less selective in terms of DNA recognition.

The sequences protected from cleavage by the most potent diamidine, propamide, are listed in Table 1 and clearly all are situated in AT-rich sequences of the DNA. The 3'-offset of the footprints across the two strands of the duplex is in accord with the model for asymmetric cleavage by DNAase I across the minor groove of the B-form helix [41]. It is interesting to note that all protected sequences (presumptive drug-binding sites)

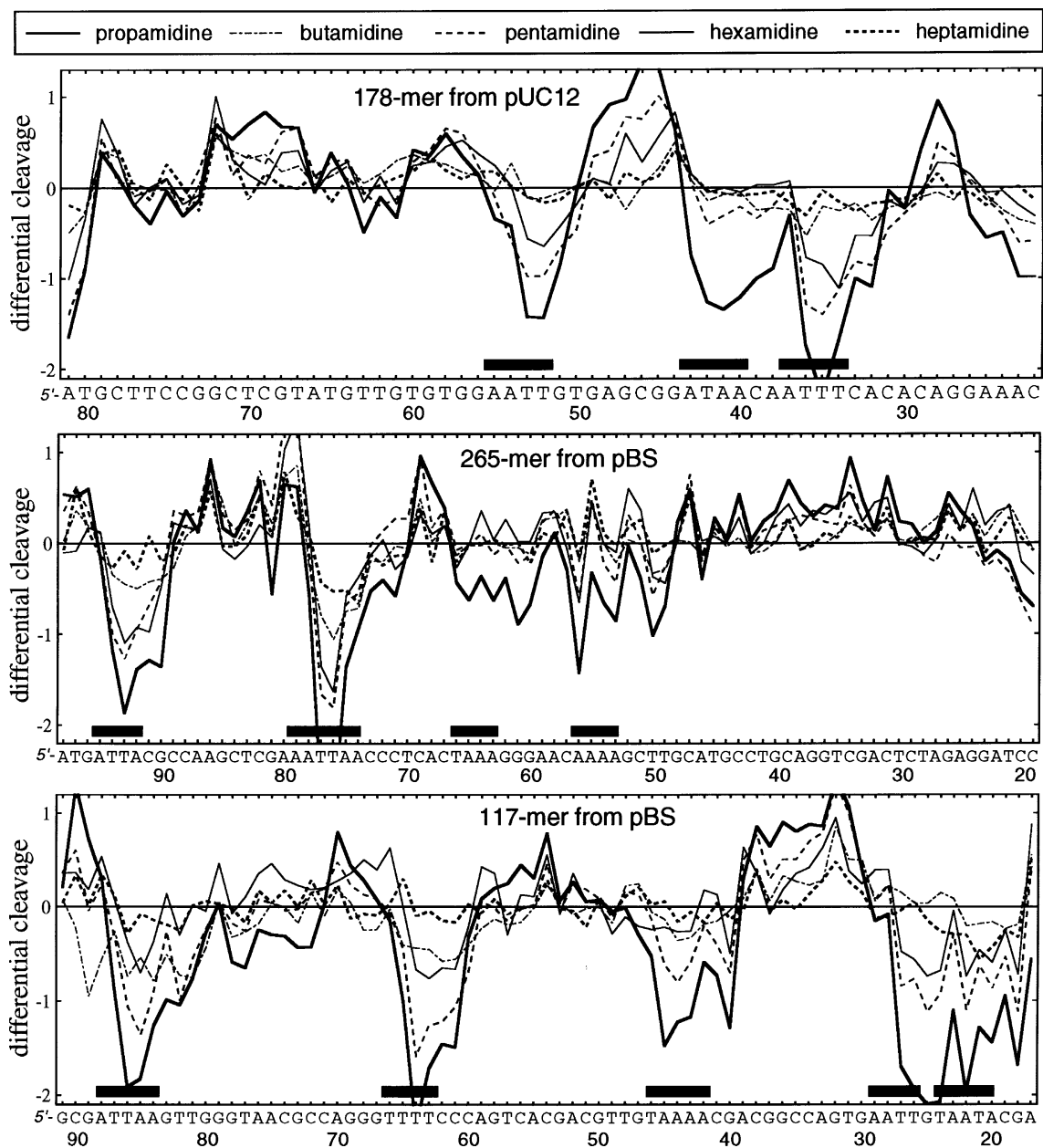


Figure 3 Differential cleavage plots comparing the susceptibility of the 265-mer, 117-mer and 178-mer DNA fragments to DNase I cutting in the presence of the diamidines

For clarity, data for octamidine and nonamidine are not included; they were much the same as those shown for butamidine or heptamidine. Negative values correspond to a ligand-protected site and positive values represent enhanced cleavage. Vertical scales are in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control, given closely similar extents of overall digestion.

contain a minimum of four consecutive A-T base pairs. AT trinucleotides such as the sequences 5'-TAA (75–77) on the 117-mer and 5'-TAT (65–67) on the 178-mer apparently are not recognized as binding sites for propamidine or its homologues. Most footprints encompass more than four base pairs and can occasionally cover up to 10 bp, such as at the sequence 5'-ATAACAATTT (32–43) on the 178-mer, but given the bimodal shape of the footprinting plot at this sequence (Figure 3) it is obvious that the observed footprint really corresponds to two adjacent binding sites each encompassing four or five A-T pairs. The sequences protected from cleavage listed in Table 1 frequently

include a G-C base pair on the 3' and/or 5' side, but if we take into account the 3'-bias introduced by the enzyme we can define more precisely the true drug-binding site (see [42,43] for details). It then appears that all sites coincide with a run of four or more A-T base pairs and G-C pairs are always excluded. Adjoining the protected sites are a few regions where the DNase I cutting rate is substantially enhanced relative to the control. These cleavage enhancements occur in GC-rich regions such as the sequences 5'-GGCCG (34–38) on the 117-mer, 5'-GCAGGC (39–44) on the 265-mer, 5'-GAGCGG (44–49) on the 178-mer and 5'-CGCGCCGC (72–79) on the *tyrT* fragment. There are also a few

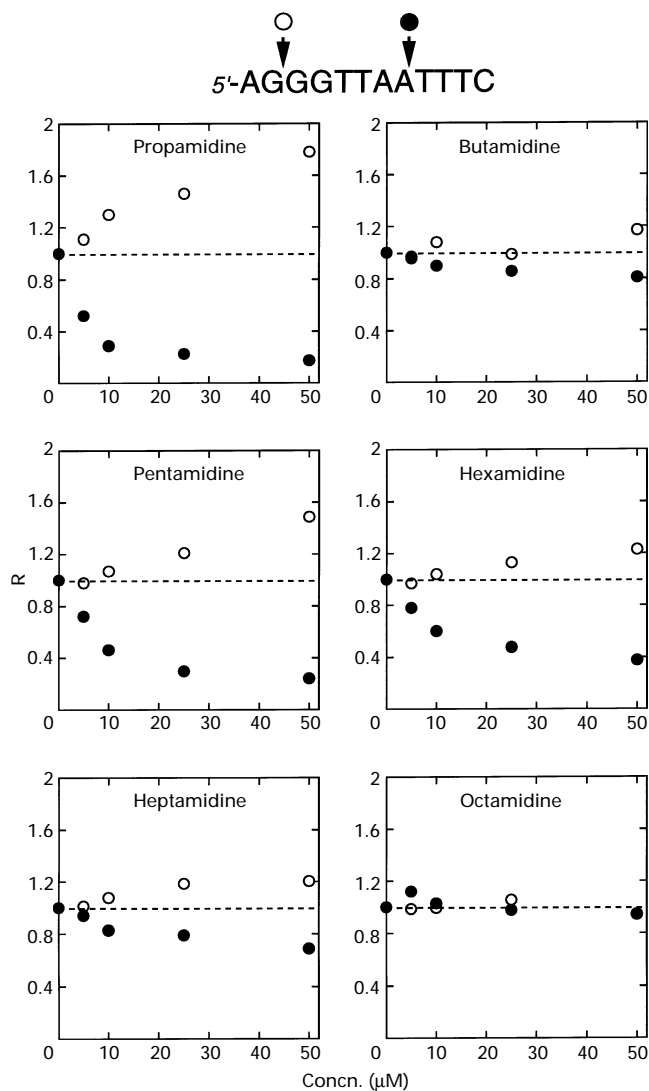


Figure 4 Footprinting plots for bonds GpG 71 (○) and ApT 77 (●) in the 265-mer fragment

The relative band intensity R is plotted as a function of ligand concentration.

regions of mildly enhanced cleavage at sequences remote from identified sites of drug binding. These are unlikely to be explicable by propagated structural changes because these ligands are thought to cause little or no distortion of the double helix [23–29]; they are more likely to reflect the redistribution of enzyme molecules (the ‘mass-action’ effect [44]).

Complementary experiments were performed with DNase II and MNase in an effort to confirm the nature of the various binding sites. Cutting patterns produced by these two nucleases on the 3′- or 5′-end labelled strands of the 117-mer fragment in the presence of the diamidines are shown in Figure 5. The cleavage of DNA by DNase II and MNase is much more sequence selective than cleavage by DNase I and as a result often gives weaker definition of each binding site. However, the gels do provide auxiliary information. With DNase II, footprints can easily be distinguished around nucleotide positions 26 and 45 in the presence of propamidine, pentamidine and hexamidine, whereas little, if any, effect can be seen with butamidine and heptamidine. With the MNase, regions of

Table 1 Sequences in the four DNA fragments protected from cleavage by DNase I in the presence of propamidine inferred from differential cleavage plots

The location of each sequence, with reference to the numbering schemes in Figure 3 and 7, is indicated in parentheses

DNA	Protected sequence	DNA	Protected sequence
117-mer		178-mer	
5′- ATTACAATT (21-29)		5′- AAATTGTTATCC (34-45)	
3′- CATAATGTTA (19-28)		3′- ACTTTAACAAATA (32-43)	
5′- TTTTAC (41-47)		5′- AATTC (52-57)	
3′- GCAAAAT (40-46)		3′- GTTAA (51-55)	
5′- AAAACC (63-68)			
3′- CCTTT (61-66)			
5′- CTTAATC (83-89)			
3′- TTGAATT (81-87)			
265-mer		160-mer (<i>tyrT</i> fragment)	
5′- TTTTGT (53-58)		5′- AAATTAC (29-35)	
3′- CGAAAAC (51-57)		3′- CTTTA (27-32)	
5′- TTTAGT (63-68)		5′- TTTTC (49-53)	
3′- GGAAA (61-65)		3′- GTA AAA (46-51)	
5′- TAATTC (75-81)		5′- ATATGA (87-92)	
3′- CAATTA (73-78)		3′- AACTATA (84-90)	
5′- TAATCA (92-97)		5′- AAAAGC (127-133)	
3′- GCATT (90-94)		3′- CATT TTTTC (125-131)	

attenuated cleavage are less marked but can be identified around nucleotide positions 26, 45, 63 and 86 at least with propamidine. Therefore zones of protection against attack by DNase II and MNase can be observed in many of the same places as for DNase I. The footprinting results with these two nucleases support the conclusions drawn from the DNase I data that (i) propamidine produces more pronounced footprints than the other diamidines, (ii) butamidine has little effect on the cutting of DNA by either nuclease, (iii) the extent of protection against cleavage induced by hexamidine is consistently higher than that produced by heptamidine at all binding sites amenable to examination.

The experiments with the three nucleases concur that all propamidine-induced footprints correspond to AT-rich sequences. Apparently GC base pairs cannot be accommodated within the drug-binding site. The reason for this remains to be determined but, by analogy with what is known for other minor groove binders such as netropsin and distamycin, there is good reason to believe that it is the 2-amino group of guanine exposed in the minor groove of the double helix that is presumed to impede the access of the ligand to the floor of the minor groove at GC sequences, hence reinforcing the preference for AT-rich tracts [34]. We have set out to verify this notion using DNA in which the cognate group has been removed from guanine and added to adenine. The purine 2-amino group can be shifted from G to A nucleotides by replacing them with inosine and 2,6-diaminopurine-containing nucleotides respectively [34,45]. Samples of *tyrT*(A93) DNA containing A-T and G-C pairs (normal DNA) or 2,6-diaminopurine–thymine (DAP–T) and inosine–cytidine (I–C) were prepared using PCR and used as substrates to investigate the sequence-selective binding of propamidine.

The resulting DNase I footprinting patterns (Figure 6) revealed that the effect of shifting the purine 2-amino group from guanines to adenines is to provoke a significant redistribution of

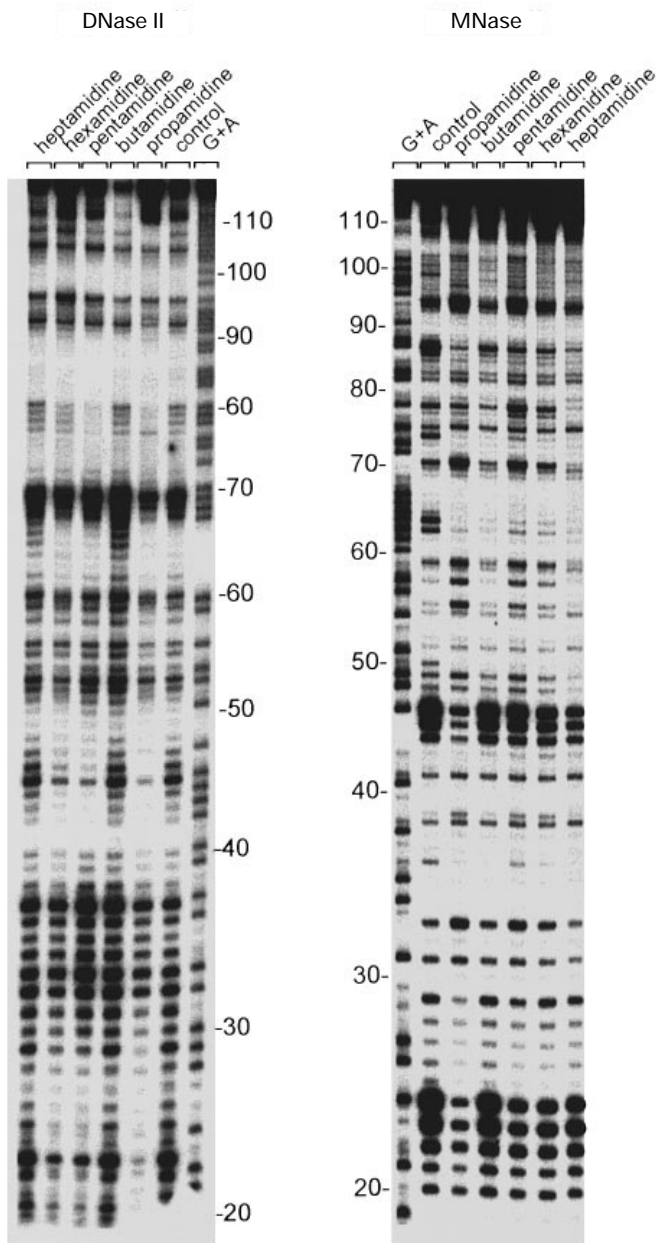


Figure 5 DNase II and MNase digestion patterns of the 117-bp fragment in the absence (control) and presence of diamidines (25 μ M each)

The DNA was labelled at the 3'- and 5'-end for experiments with DNase II and MNase respectively. Presentation as described for Figure 2.

binding sites for propamidine, such that the newly created sites containing I-C pairs are substantially preferred over DAP-T-containing sites. The altered sequence selectivity is emphasized in the differential cleavage plots (Figure 7). The canonical recognition sites in natural DNA, now modified by the inclusion of a purine 2-amino group, frequently appear in regions where the I+DAP-substituted DNA is hypersensitive to cutting by DNase I. These regions of enhanced cleavage commonly appear flanking strong binding sites or clusters of sites in footprinting experiments. Evidently propamidine is excluded from its former canonical sites containing DAP-T base pairs but it finds new

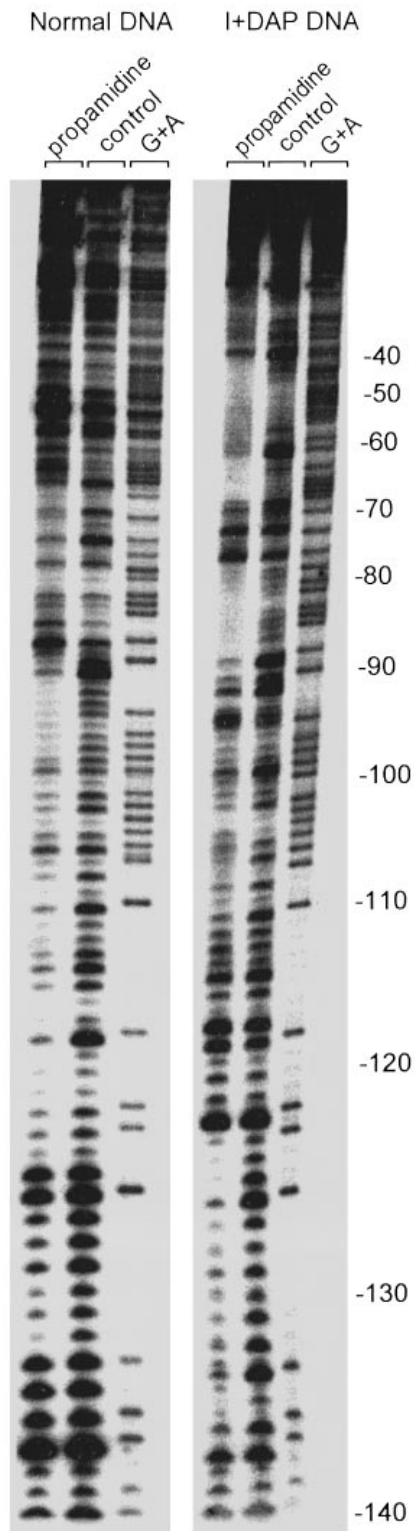


Figure 6 DNase I footprinting of propamidine on normal *tyrT(A93)* DNA and its homologue substituted with inosine and DAP nucleotides

The scale at the side of the gel corresponds to the numbering of the *tyrT(A93)* sequence as represented in Figure 7.

binding sites associated with I-C-rich sequences. Each of the new binding sites can be correlated with a run of I and C nucleotides.

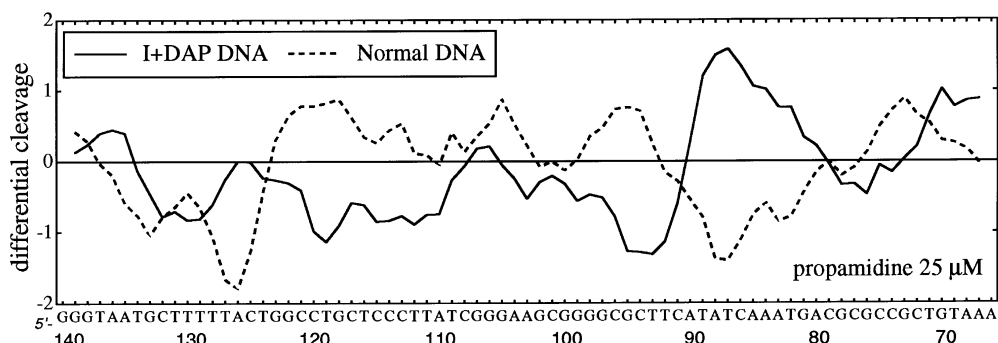


Figure 7 Differential cleavage plots comparing the DNase I-mediated cleavage of normal and I + DAP-containing DNA in the presence of 25 μ M propamidine

The plot drawn as a continuous line refers to the modified DNA fragment containing inosine and DAP residues and the plot indicated by a broken line refers to normal DNA. Other details as for Figures 2 and 3.

DISCUSSION

Before discussing the results, it must be recorded that the footprinting data reported here corroborate the findings of two previous reports by Fox et al. [21] and ourselves [22] that propamidine binds selectively to AT-rich sequences in DNA. The binding of propamidine to DNA has previously been investigated by indirect means but not by footprinting [9,11]. More recently, the structure of propamidine bound to short oligonucleotides has been solved by NMR and X-ray crystallography [26–29]. The interaction of propamidine and analogues (including propamidine) with the dodecanucleotide d(CGCGAATTCGCG)₂ has also been examined by computer modelling [25]. Overall, our footprinting data are consistent with both theoretical and experimental studies suggesting that binding of propamidine to AT-rich sequences is tighter than that of pentamidine. The better anchorage of propamidine in the double helix may be related to a decreased minor groove width at the binding site coupled with a reduced mobility of the bound propamidine molecule [26]. The results also agree with the idea that butamidine binds considerably less tightly to DNA than propamidine and pentamidine. This has been interpreted in terms of drug–DNA shape complementarity: the radius of curvature of the minor groove-bound propamidine and pentamidine molecules is 14 Å and 77 Å respectively, i.e. close to the radius of 19 Å measured with DNA, whereas that of butamidine is much larger [11]. Computer and experimental studies on the binding to DNA of several diamidine derivatives with an alkyl chain of variable length have led to the conclusion that for *para*-substituted bis(amidine) compounds, those with an odd number of methylene units in the linker are more isohelical with the DNA minor groove, thus allowing more effective penetration into that receptor. This explanation accounts for the results obtained with diamidines containing three, four and five methylene groups, but is less satisfactory for the longer analogues with six and seven methylene units. The radius of curvature of hexamidine is considerably larger than that of DNA [11], but the footprinting data reveal that this almost linear molecule does bind selectively to AT-rich sequences. Both our footprinting data and the published T_m measurements [9] indicate that the interaction of hexamidine with DNA is only slightly weaker than that of pentamidine. The poorly sequence-selective binding of octamidine and nonamidine is not surprising because these two molecules are excessively hydrophobic compared with propamidine and behave essentially as detergents.

A structural study on the complexes formed between propamidine and the asymmetric decamer d(CGCTCATTGC-

GCAATGAGCG) has shown that the drug binds preferentially to the minor groove of the 5'-AATG sequence but they also identified a secondary binding site involving a spanned 5'-TGA sequence [29]. Surprisingly, in our experiments no such sequences proved to afford a potential binding site for propamidine. All footprints contain a minimum of four consecutive A-T base pairs; no footprints were detected even at the TAA and TAT triplets (Figure 3). Footprinting studies with other diamidines such as berenil have indicated that a G-C base pair can occasionally be tolerated in the drug-binding site [46,47]. Perhaps propamidine strictly requires a narrow minor groove for optimal fitting whereas other diamidines such as berenil can tolerate sequences having a slightly wider minor groove.

The experiments with the modified DNA are in accord with previous observations on other minor groove binders such as netropsin, distamycin, berenil, DAPI and Hoechst 33258 [34,48,49]. The lack of binding of propamidine to GC-containing sites is attributable to an obstructive effect of the exocyclic 2-amino group of guanosine. This substituent can act directly to limit the access of the drug to the floor of the minor groove and/or indirectly by increasing the minor groove width. When the 2-amino group is shifted to adenine residues, by virtue of the combined G → I and A → DAP substitutions, the width of the minor groove is substantially modified [45] so that drugs can penetrate snugly to the floor of the narrow groove at I-C-rich sequences, whereas their ability to fit within the wider groove at DAP-T-rich sequences becomes impaired.

Finally, an important question to address is whether the DNA sequence selectivity of the diamidines can be related to their biological activity. Recently the compounds studied here have been evaluated for amoebicidal activity against two strains of *Acanthamoeba polyphaga*. The long alkanes proved to be more effective than the shorter analogues in killing both trophozoites and cysts of the parasite [2]. In terms of amoebicidal efficiency, the test molecules rank in the order 8 > 9 > 6, 7 > 5 > 3 > 4 whereas in terms of AT-selective DNA binding the ranking order is 3 > 5 > 6 ≥ 7 with 4, 8 and 9 being poorly able to recognize AT-rich sequences. Therefore, so far as the present data go, AT-selective DNA recognition by this series of diamidines cannot be simply correlated with their potency against *Acanthamoeba keratitis*. In terms of activity against *P. carinii*, it has been reported that pentamidine, propamidine and hexamidine are more effective than butamidine in treating experimental PCP [9] so at first sight it could be envisaged that DNA contributes to the activity. However, studies have failed to find a correlation

between the DNA-binding properties of pentamidine analogues and their effectiveness against PCP *in vivo* [9,12,22]. Similarly, it has been reported that the efficacy of diamidines as agents to treat infection by African parasites such as *Plasmodium* and *Leishmania* has little to do with their capacity to bind to DNA [8]. Therefore we must conclude that if binding to DNA plays a part in the biological activity of these diamidines, then their pharmacological effects must demand more than specific complex formation between the drugs and AT-rich sequences in DNA. Perhaps their mechanism of action involves interaction with a protein complexed to an AT-rich sequence rather than direct interaction with DNA. The minor-groove-binding agent distamycin has proved to compete efficiently with various classes of DNA-binding proteins [50–53]. Other scenarios can be envisaged: for instance that the positively charged diamidines compete for polyamine-binding sites on DNA, mimicking the effects of polyamine deprivation, and/or that they interact selectively with mitochondrial DNA as previously reported with bis-cationic minor-groove binders including the diamidines berenil (**1**) and stilbamidine (**2**) [54]. Once bound to DNA, the drug may establish contact with a second macromolecular species such as a protein (transcription factors, polymerases, topoisomerases, etc.) to initiate the biological response [55].

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