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Large, sequence-dependent effects on DNA conformation by minor groove binding compounds

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ABSTRACT

To determine what topological changes antiparasitic heterocyclic dications can have on kinetoplast DNA, we have constructed ligation ladders, with phased A5 and ATATA sequences in the same flanking sequence context, as models. Bending by the A5 tract is observed, as expected, while the ATATA sequence bends DNA very little. Complexes of these DNAs with three diamidines containing either furan, thiophene or selenophene groups flanked by phenylamidines were investigated along with netropsin. With the bent A5 ladder the compounds caused either a slight increase or decrease in the bending angle. Surprisingly, however, with ATATA all of the compounds caused significant bending, to values close to or even greater than the A5 bend angle. Results with a mixed *cis* sequence, which has one A5 and one ATATA, show that the compounds bend ATATA in the same direction as a reference A5 tract, that is, into the minor groove. These results are interpreted in terms of a groove structure for A5 which is largely pre-organized for a fit to the heterocyclic amidines. With ATATA the groove is intrinsically wider and must close to bind the compounds tightly. The conformational change at the binding site then leads to significant bending of the alternating DNA sequence.

INTRODUCTION

Compounds, such as the diamidines in Figure 1, bind strongly to AT minor groove sites and target AT rich sequences in kinetoplast minicircle DNA (kDNA), with subsequent destruction of the kinetoplast and cell death (1–3). The complete mechanism by which this occurs has not been determined. However, given the AT rich composition of minicircles, their A-tract sequences and the tight curvature of a minicircle of ~1000 bp, it is likely that topological changes in the DNA minicircles induced by minor groove binding have a part in antiparasitic activity. Intrinsically bent DNA is also a common feature of key DNA control elements such as promoters (4). Such bent sequences can be selectively recognized by control proteins or complexes and serve a functional role in gene expression. Changes in DNA topology are clearly important in regulation of transcription of many genes and modulation of bending can enhance or reduce transcription. Designed small molecules that could selectively affect DNA bending would, thus, be valuable for control of expression of some genes.

Intrinsic bending by specific DNA sequences was first discovered in kDNA of the mitochondrial kinetoplast of some parasitic microorganisms (5,6). The kDNA minicircles have short A-tract sequences that are in phase with the helix repeat such that they always appear on the same side of the double helix and their curvature is roughly additive. In order to test the hypothesis that compound induced topological changes in kDNA are important for their antiparasitic activity, it is first necessary to determine whether the compounds can actually cause topological changes in DNA and if so, how the changes depend on compound structure and DNA sequence. The development of therapeutics against neglected and deadly parasitic diseases, for which adequate drugs are not available, is critically important since resistance to current drugs, which are quite toxic, has been reported (7). It is essential to better understand topological effects in DNA complexes of diamidines if their activity profile is to be enhanced in a rational manner.

DNA sequence and compound selection

Our goal is to systematically evaluate the effects of diamidines on straight and bent AT kDNA model sequences. The rationale for selecting DNA sequences for evaluation was to have a validated, bent A-tract sequence and a closely related, relatively unbent, control sequence, both with the same number of AT base pairs, sequence composition, and number of compound binding sites. Appropriate ligation of such sequences can then yield models for similar sequences in parasite kDNA. Having repeated identical binding sites simplifies the analysis of compound effects at a specific sequence and different binding sites can be evaluated in the same manner.

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With phasing of the binding sites and DNA of sufficient length, even small topological effects can be evaluated with high accuracy. DNA sequences with A-tracts have been used extensively to evaluate intrinsic DNA bending (8–14). When phased at a repeat of 10.5 bp, the sequence induces a local bend in the helix axis of ~18° and a dramatic global curvature can be detected by polyacrylamide gel electrophoresis (PAGE). Our A-tract sequence (Figure 2) is identical to the one used by Maher and coworkers to evaluate DNA bending (8,9) and a similar sequence has been used effectively by Kool and coworkers to evaluate the effects of modified base incorporation on DNA topology (11,12). As a comparison, the A5 tract was replaced by an ATATA sequence that binds many minor groove agents quite well. The sequence has not previously been reported in a ligated duplex but it is not expected to have significant curvature. Gel results reported here confirm the findings of Maher with the A5 ligation ladder and show that the ATATA sequence has very little intrinsic curvature. The '*cis*' duplexes have A-tracts that are ideally phased, while the '*trans*' duplexes are out-of-phase. *Trans* duplexes were included to determine if differences in mobility (if any) were due to general, non-specific electrostatic effects, or discrete changes at the binding sites.

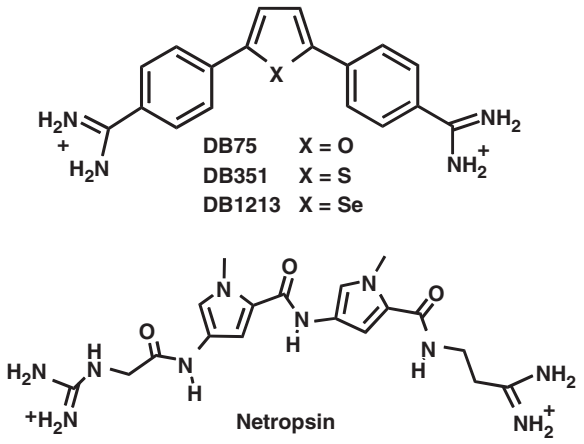

Figure 1. Compounds used in these experiments.

As an initial antiparasitic diamidine to use in development of the method and evaluation of topological effects on AT sequence DNAs, DB75 (Figure 1) was selected. A prodrug of this compound has reached phase III clinical trials against *Trypanosoma brucei* induced human African sleeping sickness and has also served as an important model system in biophysical studies of DNA minor groove complexes (15). To evaluate subtle effects of compound structure on AT sequence topology, compounds with single atom changes in DB75 were also evaluated by replacing the furan with thiophene (DB351) and selenophene (DB1213) groups (Figure 1). We have recently shown that these compounds bind quite well to both A-tract and alternating AT sequences of the type incorporated into the ligation ladder (16). We have also previously shown, using footprinting studies, that these compounds bind well to both A-tracts and alternating AT sequences (17).

PAGE as a probe of topology

PAGE is a very sensitive method for detecting DNA helical topology and small molecule induced changes (18–22). Ligation ladder PAGE assays provide an opportunity for extensive quantitative analysis of DNA bending, including estimating the degree of bend from the relative curvature

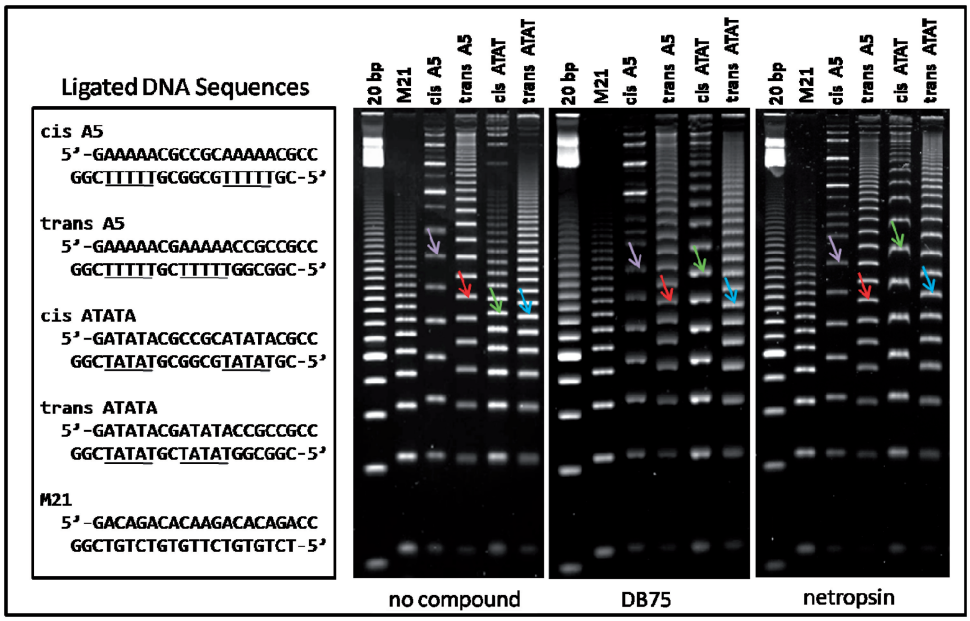

Figure 2. Sequence of duplexes used in this study (left). Typical gels are shown for ligation ladder experiments for gels containing no compound (left), DB75 (middle) and netropsin (right). Lanes for the ligated *cis* and *trans*, 20-bp marker, and M21 duplexes are indicated at the top of each gel. Colored arrows point to the 147-bp ligated linear multimers for each sequence. The double intensity band in the 20-bp ladder is 100 bp.

(C_r), which Crothers defined as the number of A-tracts per helical turn (10). The distance between A-tracts is designed to alternate between 5 and 6 bp. Including the 5-bp length of the A-tract, this gives an average distance between tracts of 10.5 bp, or the length of one helical turn. The electrophoresis of small molecule-DNA complexes, however, can be problematic. The heterocyclic diamidines investigated here are positively charged at the typical buffer pH of 8.3, and thus migrate in a direction opposite that of the DNA strands during electrophoresis.

Surprisingly, small molecules with fairly high binding constants have been shown to co-migrate with DNA to some extent. Barcelo *et al.* (21) proposed several theories to explain this observation; the gel matrix favors the reassociation process, perhaps by forming a 'cage' which restricts ligand diffusion, or the gel structure increases local concentrations of DNA and ligand by volume exclusion effects. They also noted that when multiple bands are migrating in the gel the fastest band loads the gel with compound such that slower bands will maintain a relatively constant free ligand concentration. Small molecule–DNA complexes have previously been investigated by placing the ligand of interest in the gel. Fox and coworkers (23) cast compound in the gel matrix in an analysis of minor groove and intercalating agent effects on DNA restriction fragment topology. Persil *et al.* (24) investigated the ability of the intercalator coralyne to produce homoadenine assemblies by putting ligand in the gel. We circumvented possible problems with differential DNA–complex disassociation by casting compounds in the gel matrix and loading them into the lower buffer chamber. This requires relatively large amounts of compound and means that only one compound can be evaluated per gel. However, the amount of compound bound is kept constant which allows their effects on DNA topology to be analyzed quantitatively.

MATERIALS AND METHODS

DNA-binding compounds

Preparation of the diamidines in Figure 1 has been previously described (16). Netropsin was purchased from Sigma–Aldrich (St Louis, MO). Compounds were prepared as 1 mM stock solutions in water and kept frozen at −4°C away from light. All three diamidines and netropsin have binding constants to A-tract and alternating AT DNA that are greater than $1 \times 10^7\,M^{-1}$ under gel electrophoresis conditions (16,25). In quantitative gel experiments, sufficient compound was added to the gel and lower buffer to maintain compound binding to the AT target sites during the gel experiment.

Oligonucleotides

Two duplexes already investigated by Maher *et al.* (9) (*cis* and *trans* A5 in Figure 2) and three new duplexes (*cis* and *trans* ATATA in Figure 2 and a *cis* mixed sequence in Supplementary Figure 2) were investigated with the compounds in Figure 1. An additional 21-bp oligomer with the same base composition but lacking an AT tract was designed for use as a mobility marker (M21 in Figure 2). Lyophilized DNA oligomers were purchased from Integrated DNA Technologies, Inc. (IDT, Coraville, IA) with HPLC purification. Water was added to the solid DNAs to bring the concentration to ~1.0 mM, based on the reported amount of DNA from IDT. The concentration of these single stranded DNA samples was then determined with the extinction coefficient provided by the manufacturer. For ligation, complementary strands (100 μM) were then combined in a 1:1 ratio (based on their calculated concentration) and annealed in NEB 1× ligation buffer (New England BioLabs, Ipswich, MA) containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 μg/mL bovine serum albumin.

Ligation ladders

Duplexes were ligated using a procedure similar to that previously described (2,8,9). Annealed duplexes were 5′ phosphorylated using T4 polynucleotide kinase (New England Biolabs) and were subsequently ligated with T4 DNA ligase (New England BioLabs) using buffer provided. Ligation reactions typically were 200 μL of 2 μM DNA (in 21-bp duplex) with 1200 U ligase. Room temperature ligation time was 20 min followed by an enzyme inactivation time of 30 min at 65°C. In order to distinguish circular multimers from linear products, a portion of ligated duplex was digested with Bal-31 nuclease (New England BioLabs) according to manufacturer's instructions.

Gel electrophoresis

Ligation ladder products were separated on 8% native polyacrylamide gels (1.5 mM thick, 20 cm long) prepared from a 40% acrylamide solution (29:1, bis–acrylamide: acrylamide, EMD, Gibbstown, NJ) in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, 2.0 mM EDTA, pH 8.3). Gels were conditioned with a 60 min, 25°C, 100 V pre-run prior to sample loading. Electrophoresis was 200 V (10 V/cm) at 25 ± 0.1°C typically for 145 min in 1× TBE buffer. Electrophoretic apparati were connected to circulating temperature control systems to ensure temperature remained constant. Samples of 2 μM ligation ladder (in units of 21-bp duplex, typically 10 μL) were incubated with test compounds at a concentration giving a ratio of 2:1 compound to AT-binding sites. A commercially available 20-bp marker (Bayou Biolabs, Harahan, LA) was also loaded onto the gel. For quantitative experiments, the test compound was also placed in the buffer in the lower chamber and cast in the gel.

Gels were stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA) at the concentration and time recommended by the manufacturer. Stained gels were imaged using an Omega10gD Molecular Imaging System (UltraLum, Claremont, CA).

Calculation of C_r

Molecular weight assignments for ligation ladder products were obtained using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) and were based on the mobility of M21. The relative mobility (R_L) for each

multimer was calculated, where $R_L = L_{apparent}/L_{actual}$, and plotted as a function of L_{actual}. L_{actual} is determined by adding 21 bp to each sequential band, starting with the bottom most bands while skipping bands that are due to circular ligation products.

R_L values for multimers 105 bp through 189 bp were averaged from three gel experiments for the *cis* A5 duplex containing no compound. Data were fitted to the following relationship as described by Crothers (10) and extended by Maher (8);

$$R_L - 1 = (aL^2 - b)C_r^2 \quad \mathbf{1}$$

where L is the length of the multimer and C_r is the relative curvature, or the number of phased A tracts per helical turn. For the *cis* A5 duplex, $C_r = 1$ by definition. Values of a and b vary with electrophoretic conditions such as temperature, gel percentage and acrylamide:bisacrylamide ratios. Values were obtained for a and b under our conditions by using non-linear least squares fitting in KaleidaGraph (Synergy Software, Reading, PA). These values were then substituted into Equation (1) and C_r values were obtained by fitting the data for each duplex without compound as well as in the presence of the four minor groove binders.

RESULTS

Ligation ladders for A5 and ATATA: free DNA

Ladders of the *cis* and *trans* A5 and ATATA sequences are compared in Figure 2. The ligated DNAs exhibited differences in mobility, which are greater than experimental error, from 63 bp or three multimers and higher. The *cis* A5 results are in agreement with previous findings and show that A-tracts migrate much more slowly than their true molecular weight (8–10). Of the four duplexes tested, *cis* A5 tracts had the most anomalous migration, followed by *trans* A_5, *cis* ATATA and *trans* ATATA. Such differences in mobility are especially interesting since the composition of all four duplexes contain AT tracts that differ only in their sequence order and relative positions.

Circular DNAs formed via intramolecular ligation of duplexes can be seen as intense bands at higher multimer numbers and are identified via Bal-31 exonuclease digestion (Supplementary Figure 1B). Due to its high intrinsic curvature, the *cis* A5 sequence produced circular products with the fewest multimers. The relatively linear *trans* ATATA duplex was able to form circular products only at a higher molecular weight and in lower yield. The shuffled sequence (M21), which has the same sequence composition as the other duplexes, does not form circles that are detectable under these experimental conditions.

Ligation ladders as a small molecule–DNA complex topological analysis tool

The compounds in Figure 1 have dissociation rates under the experimental conditions that are relatively rapid; typical half-life is less than one min for these compounds (26), even though they have binding constants in the $10^7 \times 10^8\ M^{-1}$ range. Since the gel experiment is much longer than the dissociation rates of the compounds, they can be separated from the DNA during a typical gel electrophoresis experiment when compound is not present in the gel. This is especially true for the fastest moving, lowest molecular weight duplexes where depletion of the cationic compounds first occurs. The bands at the top of the gel will migrate with compound fully bound, while the fastest moving bands are rapidly depleted of compound during gel electrophoresis. The intermediate bands will have a variable level of compound bound that prevents any quantitative analysis of topological effects on DNA. We have therefore included the compounds in the gel and lower buffer chamber such that the DNAs in all bands migrate in a constant compound concentration throughout the experiment. As we will show below, however, experiments without compound in the gel can be used as a qualitative screening tool.

Ligation ladders as a quantitative topological tool

Changing the ratio of compound to DNA in the sample prior to electrophoresis had little effect on the final band mobility as long as compound was present in the gel (results not shown). This suggests that when binding sites are saturated at the beginning of electrophoresis they stayed that way through the duration of the experiment, as long as ligand was also present in the gel matrix.

Quantitative analysis of bending angle. In order to calculate relative curvature of the DNAs and their complexes and quantitatively evaluate the compound's effects on DNA topology, results from R_L versus L_{act} plots (Figure 3) were transformed into plots of $R_L - 1$ versus L_{act}^2 (Figure 4), as originally suggested by Crothers (10). The plot for ligation ladders in absence of compound is shown in Figure 4. Values for a and b in Equation (1) were found from the linear fit of the line for *cis* A_5, assuming a C_r value of 1.0 for *cis* A5 with no compound, giving Equation (2).

$$R_L - 1 = \left(-0.074 + 4.552 \times 10^{-5} L_{act}^2\right)(C_r)^2 \quad \mathbf{2}$$

Thus, values for C_r that are greater than 1.0 are more curved relative to the *cis* A5 duplex and values of less than one represent duplexes that are less bent than *cis* A5.

Using Equation 2 with these values, the relative curvature for each duplex can be determined from the slopes in Figure 4 when no compound is present and compared to the curvature in the presence of compound (Figure 4). The relative curvature for *trans* A_5 was found to be 0.56. Previously reported values for this sequence were 0.47 and 0.58 in the presence of 0 mM and 10 mM Mg^{2+}, respectively (9). The C_r values for *cis* ATATA, 0.29, and *trans* ATATA, 0.20, which have not been previously reported, are significantly less than for *cis* A5 (Table 1). Once the fit for the four duplexes in the absence of compound was obtained, these values were used as standards by which the effects of various compounds on DNA topology were quantitatively evaluated. A typical plot for netropsin is shown in Figure 4 as an illustration of the approach. Assuming C_r of 1.0 is equivalent to $\sim 18^\circ$ of curvature (the proposed value for the curvature of an A-tract)

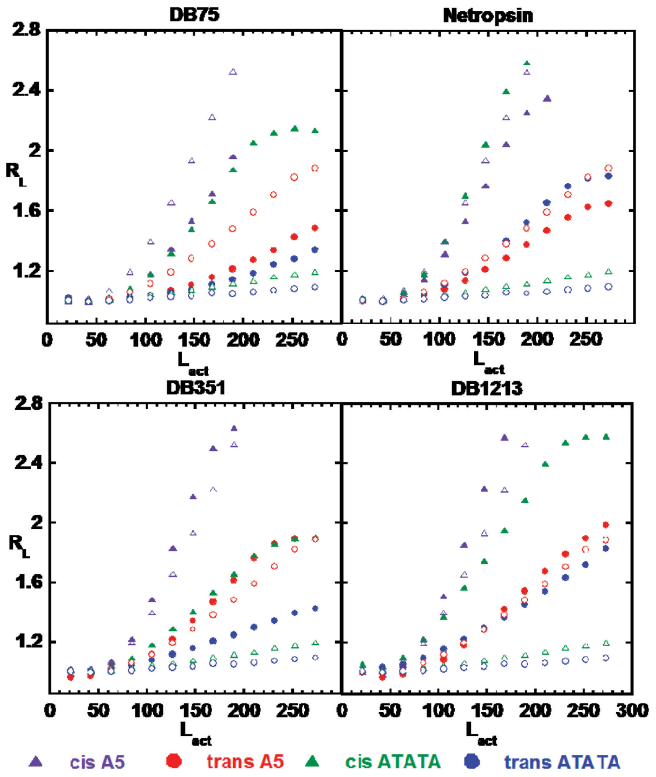

Figure 3. Panels for relative length (R_L) plotted as a function of actual length (L_{act}) for the four ligated *cis* and *trans* duplexes in the absence of compound (open shape) and the presence of four different compounds (solid shape). The compound described in each panel is indicated at the top of the panel.

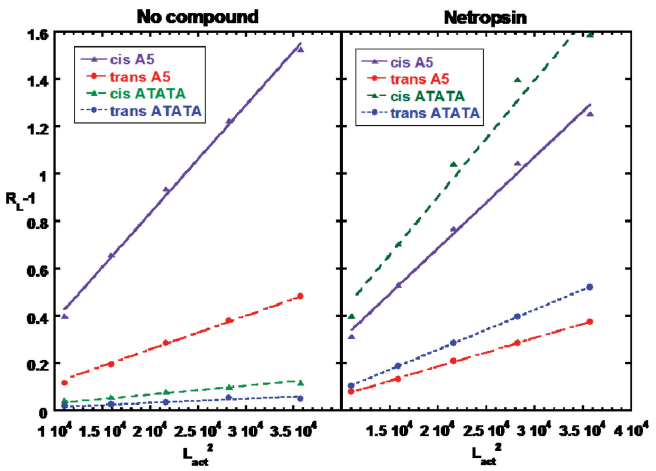

Figure 4. Example plots of actual length squared (L_{act}^2) versus relative length minus one ($R_L - 1$) are shown in the absence of compound (left) and in the presence of netropsin (right) for *cis* A_5 (purple triangles), *trans* A_5 (red circles), *cis* ATATA (green triangles) and *trans* ATATA (blue circles).

then the values obtained for C_r for each duplex can be converted to estimates of curvature angle (8) (Table 1).

DB75. A gel experiment with DB75 and each of the duplexes is shown in Figure 2. Plots of R_L versus L_{act} for ladders both with and without compound make it possible to compare mobility changes in the ligation ladders due to the presence of compound (Figure 3). DB75 induces changes in *cis* A5 and *cis* ATATA that are opposite. The compound induces changes in *trans* A5 and *trans* ATATA that are similar to their *cis* counterparts but smaller in magnitude. The reduction in mobility for *cis* ATATA is quite large and the smaller increase in mobility of *cis* A5 is just enough that these two DNAs migrate almost identically with one another in the presence of DB75. In terms of degrees, this is a reduction in curvature of 4° for the *cis* A5 tract and a surprising increase from 5° to 13° for the *cis* ATATA tract (Table 1).

DB351 and DB1213. PAGE results (Supplementary Figure 1A and B) and plots of R_L versus L_{act} (Figure 3) were obtained for the thiophene (DB351) and selenophene (DB1213) analogs of DB75. Both compounds reduce the mobility of the *cis* ATATA ladder. Unlike DB75, however, which slightly straightens *cis* A5, DB1231 and DB351 both reduce the mobility of this sequence and thus slightly increase curvature. As noted above, when DB75 complexes with the two *cis* sequences their mobilities become almost identical, even though the free DNAs are quite different. Though the compounds are structurally very similar to DB75, DB351 and DB1213 do not cause these two sequences to migrate similarly. DB351 decreases the mobility of *cis* ATATA the least, DB75 is intermediate and DB1213 induces the largest change, which corresponds to an increase in bend angle from 5° in the absence of ligand to 12°, 13° and 16°, respectively, in the presence of ligand (Table 1).

Netropsin. Because netropsin and related polyamide analogs have been used in a large number of studies of minor groove interactions, this compound was tested for comparison to the diamidine derivatives. Netropsin also induces significant effects on the mobilities of the DNAs. These effects are similar to those for DB75 and are sequence dependent, as can be seen in plots of R_L versus L_{act} (Figure 4). Netropsin increases the mobility of *cis* A5, straightening the duplex to 16°. Of the compounds investigated here, it causes the largest decrease in the mobility of *cis* ATATA, bending the duplex to an impressive angle of ~19° (Table 1). Netropsin is able to make this intrinsically unbent DNA migrate as if it were as bent as an A5 tract.

Determining the direction of bending with mixed sequence DNA

The A5 tract bends DNA in the direction of the minor groove (27). We have ligated a mixed sequence duplex (Supplementary Figure 2), which has one A5 and one ATATA, to determine the direction that compounds bend ATATA. Results for the mixed sequence DNA without compound, based on the reference A5 tract, show that ATATA also bends DNA in the direction of the minor groove. The C_r values for A5 and ATATA are 1.0 and 0.29, respectively (Table 1). If bending is in the same direction (additive), the predicted C_r value for mixed DNA without compound is ~0.64 [(1.0 + 0.29)/2]. Alternatively, if the direction of bend for these two

Table 1. Relative curvature values (C_r) and calculated bend angle (Θ) for free DNA and complexes

	cis A5		*cis* ATATA		*trans* A5		*trans* ATATA		*cis* A5-ATATA*	
	C_r[a]	Θ°	C_r[a]	Θ°	C_r[a]	Θ°	C_r[a]	Θ°	C_r[a]	C_{pred}
No compound	1.00	18	0.29	5	0.56	10	0.20	4	0.64	0.64
DB75	0.77	14	0.74	13	0.36	6	0.30	5	0.77	0.76
DB351	1.08	19	0.66	12	0.62	11	0.41	7	0.83	0.87
DB1213	1.13	20	0.88	16	0.58	10	0.55	10	0.97	1.00
Netropsin	0.91	16	1.04	19	0.48	9	0.57	10	0.87	0.98

[a]Error in C_r is less than ± 0.03.
*Mixed sequence; C_{pred} is predicted C_r for ATATA if bending in the same direction as A5; see text for details.

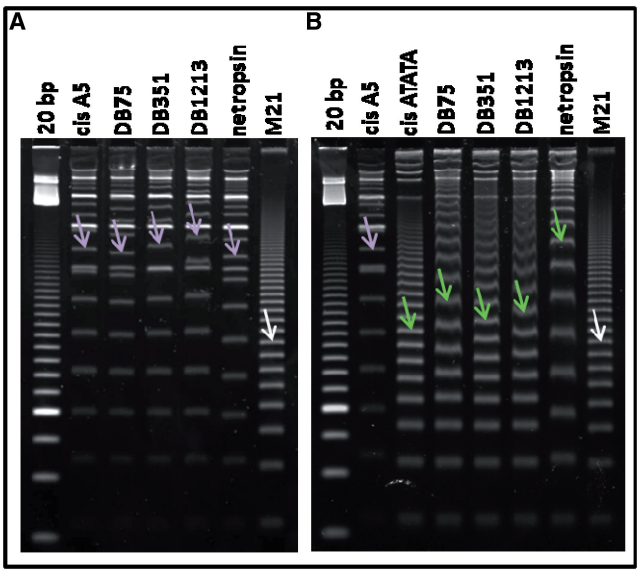

Figure 5. PAGE of *cis* A5 (**A**) and *cis* ATATA (**B**) ligation ladders incubated with the indicated compound at a ratio of 2:1 (compound to binding site) prior to electrophoresis. Compounds are not in the gel in this experiment. Lanes containing *cis* A5 and *cis* ATATA are provided as references and were not incubated with compound. Arrows indicate 105-bp bands. DNA sequences are shown in Figure 2.

sequences is in opposite directions the expected C_r would be ~0.36 [(1.0 − 0.29)/2]. In a gel with the mixed sequence the observed C_r is 0.64 (Supplementary Figure 2), indicating that A5 and ATATA are bent in the same direction.

Since we have determined the bend angle of both sequences when bound by compound, the mixed A5–ATATA sequence can be used to determine if these sequences are both bent in the same direction when complexed. DB75 bends both *cis* A5 and *cis* ATATA by approximately the same amount (Figure 2 and Table 1). If both sequences are bent in the same direction, then the observed C_r for the mixed sequence will be approximately equal to C_r for the A5 or ATATA only sequences in the presence of compound. If, however, ATATA bends in a direction opposite that of A5, the bends will tend to cancel one another resulting in an overall curvature similar to the *trans* sequence. The C_r for the mixed sequence in the presence of DB75 is 0.77, which is close to values for *cis* A5 and *cis* ATATA with DB75 (Table 1, Supplementary Figure 2B), as predicted if the direction of bend is the same for both AT-tracts. Similar experiments were also conducted for DB351, DB1213 and netropsin (Table 1). The direction of bend induced by these compounds was also found to be toward the minor groove.

Ligation ladders as a qualitative topological screening tool

The effects of DB75, DB351, DB1213 and netropsin on *cis* A5 and *cis* ATATA in the presence and absence of compound were compared in gels cast with no ligand in them. The question to be answered in these experiments is whether effects of compounds on DNAs containing specific binding sites can be determined in an accurate but qualitative manner. A number of bands exhibited compound induced mobility shifts, allowing a comparison of compound induced changes in both A-tract sequences on a single gel (Figure 5).

Beginning at 126 bp, an increase in mobility due to the presence of DB75 can be seen when compared to the *cis* A5 ladder without compound (Figure 5A). As in the quantitative experiments, DB75 had an effect on mobility opposite that of DB351 and DB1213; DB351 and DB1213 decreased the mobility of the ligation ladders containing the A5-binding site, with DB1213 inducing a larger shift than DB351. Netropsin produced an increase in the mobility of the *cis* A5 duplex greater than DB75 and at lower molecular weight. Interestingly, the only bands that did not shift were the circular ligation products.

There are obvious visual differences in gels containing the bound *cis* A5 sequence and those containing the bound *cis* ATATA sequence (Figure 5B). The *cis* ATATA–complex bands are somewhat distorted, which probably results from partial dissociation of compounds from this sequence and the large difference in mobility of the free and bound *cis* ATATA sequence. This band distortion phenomenon only appeared in lanes where compound was present, but was almost non-existent in the lane containing netropsin, the compound with the highest binding constant. The band distortion is not due to a localized increase in gel temperature, with subsequent DNA melting, since temperature was held constant using a circulation temperature control system. Were melting occurring, the *cis* ATATA DNA alone would be expected to have a lower melting temperature than the

DNA–compound complexes, yet the DNA alone does not show band distortion. Mobility shifts were seen at lower molecular weight multimers of *cis* ATATA than in the experiment with *cis* A5 duplexes. As was the case with *cis* A5, there was no observable change in the mobility of circular bands due to the presence of compound.

Even when there was no ligand in the gel, sequence and compound dependent shifts similar to those seen in the qualitative experiments were apparent. Netropsin induced mobility changes in *cis* ATATA that made this intrinsically unbent DNA migrate at almost the same rate as the intrinsically bent *cis* A5 sequence. The direction and order of greatest change in mobility also remained consistent between these two types of experiments; netropsin changed *cis* ATATA topology the most, followed by DB75, DB1213 and DB351.

The qualitative results are in agreement with the experiments using gels cast with ligand in them and clearly show that the screening method is useful for comparing the topological effects of a variety of compounds on specific sequences in a single gel.

DISCUSSION

The antiparasitic diamidines evaluated here target AT rich DNA sequences in kinetoplast minicircle DNA of trypanosomes and leishmania, and cause degradation of the kinetoplast structure with cell death (1–3). Since DNA topology is clearly important to kDNA function and kinetoplast structure, a hypothesis for the biological action of these compounds is that they induce topological changes in the kDNA upon complex formation which could lead to DNA degradation. For these reasons, we have initiated an evaluation of compound effects on ligated AT DNA sequences, which are good general models for strongly bent and relatively straight AT base pair rich sites in kDNA. The topological analysis with repeated identical binding sites, as reported here, simplifies the quantitative evaluation of effects at a specific sequence while improving comparative analysis at different binding sites.

Surprisingly, in spite of considerable interest in bending in DNA complexes, there has been no systematic analysis of minor groove compound structural effects on DNA topology. Cons and Fox (23) found that distamycin caused a slight increase in mobility of a restriction fragment that contained a bent kDNA fragment with primarily A-tracts, but a slight decrease for non-A-tract DNA. Very small effects on DNA topology were also observed with the intercalators ethidium and actinomycin. Le Pecq and coworkers evaluated the interaction of a number of intercalators and minor groove binders with bent and unbent DNA fragments by using polyacrylamide gels, without compound in them, and by AFM (21,28). Their results with distamycin are in qualitative agreement with those of Cons and Fox (23). Hansma and Bruice (28) also used AFM to evaluate the effects of distamycin on a bent kDNA fragment and random sequence DNA and their results are in general agreement with those described above. In the work mentioned above, changes induced in DNA by distamycin were evaluated using a kDNA fragment from plasmid pPK201/CAT. This curved, ~200 bp, *Crithidia fasciculata* kDNA fragment contains mostly A-tracts. The relatively small changes in mobility observed by Cons and Fox are in agreement with our results which found only small changes in A5. Zewail-Foote and Hurley found that anticancer minor groove alkylators caused topological changes in DNA that appear to be critical to their anticancer activity (22). The wedge-shaped ecteinascidin anticancer compound, for example, alkylates a specific sequence in the minor groove and bends the helix toward the major groove. The distorted complex is proposed to trap an intermediate during the attempted repair of the DNA adduct (29).

As a first step to obtain information on possible topological effects in antiparasitic action of heterocyclic cationic minor groove binders, we have used a closely related group of diamidines with DNAs containing a phased A-tract and an alternating AT sequence and whose binding affinities for these sequences are known. To our knowledge, this is the first quantitative report of curvature determination of an ATATA DNA sequence and, as might be expected, the DNA is significantly less curved than the A5 tract. The general A-tract sequence (Figure 2) has been used in a number of topological studies (8–10,12,30,31) and our results for the A5 *cis* and *trans* sequences are in good agreement with published work on identical sequences (9). If the value of 18° is used for the curvature angle of the A5 tract (8,32), then the relative curvature angle for ATATA in an identical sequence context is only 5° and in the same direction as the A5 bend. These values then serve as the reference for comparison of compound binding effects on the topology of the two DNA sequences. The *trans* sequences of A5 and ATATA generally change in the same direction as the *cis* sequences but by a smaller amount. For that reason, compound effects on the *trans* sequences will not be discussed in detail.

Addition of DB75 and netropsin to the *cis* A5 ladder clearly demonstrates that both cause an increase in mobility. There are two alternative explanations for this mobility change; the compounds may be straightening the bent A-tracts, or they may be slightly changing the helical repeat, dephasing the A-tracts. Since the *trans* A5 sequence is already significantly dephased, an increase in the mobility of the A5 ladder is probably due to a decrease in the curvature of the A5 A-tracts. Additional experiments with 20-, 21- and 22-bp duplexes would be required in order to definitively differentiate between dephasing and straightening to account for the mobility increase (8).

Based on 18° for unbound A5 tracts, the DB75-bound site would have a reduced curvature of 14° due to either straightening or dephasing of the A-tracts. The well-studied polyamide, netropsin, also increases the mobility of the A5 sequence, but the change is small with a relative curvature of 16°. As described above, straightening of bent DNAs from kinetoplast DNA has also been reported for the polyamide, distamycin, and it might seem from these results that minor groove binders generally remove curvature from bent DNAs. Despite their structural similarity to DB75, however, the thiophene and seleneophane diamidines have the opposite effect on A5, increasing the

bending angle to 19° and 20°, respectively. These two compounds cause a decrease in mobility that can only be explained by an increase in curvature at the A-tract, since a change in helical phasing in a *cis* sequence can only increase mobility. The larger Se/S may be causing an additional distortion or opening of the groove which could account for the larger bending by DB351 and DB1213. All of these angle changes at a single A-tract, however, are relatively small and the largest change, for DB75, is only 20% of the unbound value.

Possibly because of the relatively small changes on adding minor groove agents to A-tract sequences, it is generally believed that minor groove binding does not have a significant effect on DNA structure. Addition of the diamidines to *cis* ATATA, however, illustrates that small structural changes are not a general rule. DB75, for example, causes a large effect on topology of ATATA that is opposite to the effect on A5. The 5° curvature angle for the unbound DNA is increased to 13° when DB75 is bound and the bend is into the minor groove as it is with the A5 complex. The magnitude of the bend angle for the ATATA complex is close to the value for A5 with bound DB75 and these two topologically quite different DNAs in the free state have almost identical mobilities in the presence of DB75 (Figure 2). Netropsin also causes large bending of ATATA with a change to a relative angle of 19°, slightly greater than value of unbound A5, apparently more bent than the classical A-tract. ATATA with DB1213 has a 16° relative curvature that is intermediate between DB75 and netropsin while DB351 has the smallest relative curvature angle, 12° in the ATATA complex. Even this lowest relative curvature value, however, is 225% of the value for the unbound DNA. Thus, all compounds studied to date decrease the mobility of ATATA, consistent with a general induced bending on complex formation and the induced effects are considerably larger than those observed with A5.

It is worth considering what structural changes on binding could explain the different compound induced effects at these closely related AT DNA sequences. A number of studies have indicated that A-tract sequences are narrow and highly hydrated (33–35). Compounds can bind to such sites with displacement of bound water and ions but with little required conformational change as they fit into the groove on complex formation. Such small induced conformational changes would cause slight increases or decreases in DNA mobility that would depend on small variations in local interactions due to different compound–DNA contacts and interacting groups. The minor groove in alternating AT sequences is wider and less strongly hydrated (36). In order to interact with such a site, a minor groove binder would have to decrease the groove width to form a minor groove cleft of appropriate width for strong van der Waals contacts with the bound compound. For example, the crystal structure of netropsin bound to an ATAT containing sequence showed a narrowed minor groove (37). Since the compounds in Figure 1 are similar linked-conjugated systems, they would all require similar topological changes in the ATA TA sequence for strong binding. The PAGE results with A TATA show that all of the compounds reported here induce bending at that site, which is consistent with minor groove narrowing upon complex formation.

Although quantitative analysis of curvature requires that small molecules be included in the gel matrix and lower buffer chamber, published results suggest that qualitative analysis, for example, for screening of compounds for different effects could be accomplished simply by adding compound to a DNA ladder followed by PAGE. The quantitative results reported here give us a basis to test the screening method for correct reporting of topological changes on minor groove compound binding. As can be seen in Figure 5, accurate topological changes are reported for all compounds in the screening gel with both A5 and ATATA ladders. DB75, for example, causes an increase in mobility of A5 while DB1213 causes a decrease relative to unbound DNA in the same experiment. Both compounds, on the other hand, cause a mobility decrease in the ATATA ladder, in agreement with the quantitative results. DB75 has the lowest association binding constant of the compounds under investigation here, about $2 \times 10^7\,M^{-1}$ under the PAGE conditions and it clearly reports correct values in the screening assay. This suggests that at least down to a K of approximately $1 \times 10^7\,M^{-1}$ the screening assay should provide an accurate qualitative analysis of compound induced topological changes. We are currently using this assay with other DNAs and compounds.

The initial question of this paper, can AT specific minor groove agents cause significant changes in DNA topology, is now answered in the affirmative. The primary source of the compound induced bending, the ATATA DNA sequence, is somewhat unexpected given the extensive literature on A-tract bending, particularly in the kinetoplast minicircles of *Trypanosoma brucei* mitochondria. Initial analysis of trypanosome sequences indicates, however, that the ~1000 bp minicircles have over 20 ATAT sequences, 8–10 of which are ATATA sequences (see for example EMBL accession nos M15321–15324). Given that there are thousands of minicircles in the kinetoplast, a fairly small error rate in replication could cause synergistic destruction of the kinetoplast as a whole with breakdown of cell replication. The minicircles must be opened for replication and both the original and daughter molecules must be resealed as replication proceeds. Our gel results show that the mobilities of circular DNAs are relatively unaffected by compound binding. This suggests that they could be opened with little effect by the compounds and replicated. The topological effects would appear in the open circles, however, and could create, at least a slight, inhibition of resealing of the circles. With thousands of kDNAs a small error rate could quickly lead to a biological catastrophe at the kinetoplast and cell death. Such effects would be unique to the highly interlocked kinetoplast minicircles and would not be seen in nuclear DNA. This difference in effects in host and parasite DNAs could explain the low toxicity of the minor groove binding compounds. This is simply a model that explains all of the currently available results but it clearly requires additional testing and refinement.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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