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EFFECT OF MINOR GROOVE BINDING DRUGS ON MAMMALIAN TOPOISOMERASE I ACTIVITY

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Abstract—Three minor groove binding drugs, distamycin A, bisbenzimide (Hoechst 33258) and 4′,6-diamidino-2-phenylindole (DAPI), were examined for their abilities to modulate the activity of topoisomerase I purified from L1210 cells. At 0.5 and 1.0 μM, distamycin stimulated topoisomerase I relaxation of supercoiled DNA by 38 and 13%, respectively, while increasing the drug concentration above 2.0 μM resulted in inhibition. Inhibition was reversible. Complete relaxation could be achieved even in the presence of inhibitory concentrations of distamycin if the incubation time with topoisomerase I was increased from 7.5 to 120 min. The velocity of topoisomerase I mediated relaxation was reduced by 2 μM distamycin at DNA levels ranging from 350 to 2000 ng/reaction. Hoechst 33258 and DAPI inhibited topoisomerase I relaxation in a concentration-dependent manner. Hoechst 33258 and distamycin were equivalent in their abilities to inhibit topoisomerase I, whereas DAPI had a lesser effect (e.g. relaxation was reduced by 50% with 2.7 μM distamycin and 2.8 μM Hoechst 33258 compared to 5 μM DAPI). This study suggests that ligand binding in the minor groove can be a factor in the regulation of topoisomerase I activity.

Topoisomerase I is a DNA directed enzyme which alters helical structure via the rapid induction and rejoining of single-strand breaks. This activity regulates the degree of DNA supercoiling by changing the linking number in steps of one [for review, see Refs 1 and 2]. Such changes in topology are essential during RNA [2] and possibly DNA [3] synthesis in order to facilitate movement along the DNA strand of the respective RNA and DNA polymerases [4, 5]. The topoisomerase I reaction requires the formation of at least two enzyme–DNA intermediates: a non-cleavable and a cleavable complex [6]. Inhibition of this reaction is manifested by decreased relaxation and appearance of single-strand breaks.

Despite extensive studies on the role of topoisomerase I in metabolism, studies on modulation of enzyme activity are limited. Such modulation may occur by several different mechanisms. These include direct effects on the enzyme itself, alterations at the level of the enzyme–DNA complex, or specific interactions with the DNA substrate. Direct inhibition of enzyme has been reported for ATP and other nucleoside triphosphate analogues [7, 8]. Heparin has also been shown to bind topoisomerase I, effecting inhibition of both relaxation and cleavage activities [9]. Inhibition at the level of the enzyme–DNA complex is induced by the plant alkaloid camptothecin and its analogues [6]. This agent does not bind either the enzyme or DNA alone. Rather, it traps the enzyme covalently on the DNA, resulting in formation of DNA–protein crosslinks and single-strand breaks. Inhibition of relaxation of supercoiled DNA by topoisomerase I is also observed in the presence of camptothecin. To date, this interaction at the level of the topoisomerase I "cleavable complex" is unique to camptothecin.

Reports of agents which inhibit topoisomerase I by interaction with DNA include the intercalators 9-aminoacridine and ethidium bromide [10]. These drugs inhibit topoisomerase I induced relaxation but have no effect on strand cleavage. However, intercalators induce major changes in helical structure such as base unstacking and DNA unwinding. Thus, identification of specific sites on DNA responsible for topoisomerase I effects is hampered.

While intercalators cause major structural distortions, ligands which interact with DNA by groove binding effect only minor changes. Steric, electrostatic and hydrophobic factors modulate the binding of these agents to the outside of the helix [11]. Interestingly, most small molecule ligands associate not with the major but with the minor groove, possibly because the "fit" of such molecules is a good match for the width of the minor groove [11]. The related antibiotics, netropsin and distamycin, are A-T specific minor groove binders which have been examined for their effects on superhelicity. Earlier, netropsin was found to increase the linking number of negatively supercoiled DNA treated with topoisomerase I; distamycin showed no such effects [12]. However, both drugs have been shown to alter DNA-directed enzyme reactions, such as DNAse I cleavage [13–15] and RNA polymerase transcription [16, 17]. Although DNAse I cleavage was inhibited at the site of drug binding, it was enhanced at regions flanking these sites. Similarly, drug effects on early transcription by RNA polymerase were distinguishable from effects on elongation, suggesting alterations at specific sites on the template. These observations are more readily explained by drug interaction with DNA than with the enzyme.

Thus, minor groove binding agents cause little change in DNA structure other than insertion of a small molecule into the minor groove while effecting

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significant alterations in activities of DNA directed enzymes. It was therefore of interest to determine whether topoisomerase I would be similarly affected. This study examined the effect of distamycin A on topoisomerase I mediated relaxation of supercoiled DNA. Two other DNA binding dyes, 4',6-diamidino-2-phenylindole (DAPI) and bisbenzimide (Hoechst 33258), were also tested to determine whether effects observed with distamycin were common to other minor groove binders. Above 2 μM, all three agents inhibited activity in a dose-dependent manner, suggesting their utility as modulators of topoisomerase I activity.

MATERIALS AND METHODS

DNA. Purified closed circular pLTL-1 plasmid DNA was initially obtained from Dr K. Yamamoto (University of California, San Francisco). It was derived by insertion of the HSV-tK gene flanked by MMTV-LTR sequences into pBR322 [18]. The plasmid was isolated in large amounts by transformation of *Escherichia coli* strain MM294 according to published procedures [19] by Dr R. Beckmann of this laboratory.

Topoisomerase I. Topoisomerase I was purified by hydroxylapatite chromatography of L1210 nuclear extracts according to published procedures [20]. Briefly, 3×10^9 L1210 cells were washed with cold phosphate-buffered saline, then resuspended in 40 ml of Buffer A (2 mM $MgCl_2$, 5 mM potassium phosphate, pH 7.0, 0.1 mM EDTA*, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF) and adjusted to 0.35% Triton X-100. After 15 min, the suspension was homogenized and nuclei were pelleted by centrifugation at 500 *g* for 7 min. The pellet was resuspended in 10 ml of Buffer A, adjusted to 4 mM EDTA and 0.375 M NaCl, and gently agitated for 15 min at 4° whereupon polyethylene glycol was added to a final concentration of 9%. After an additional 40 min at 4°, the sample was centrifuged at 20,000 *g* for 30 min. The supernatant fraction was applied to a 10-ml hydroxylapatite column, washed with 100 ml of 0.2 M potassium phosphate, pH 7.0, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF and eluted with a 100-ml gradient of 0.2–0.8 M potassium phosphate, pH 7.0, in 10% glycerol, 1 mM dithiothreitol and 1 mM PMSF. The fractions containing topoisomerase I which eluted at 0.5–0.6 M potassium phosphate readily relaxed supercoiled pLTL-1 DNA in the absence of ATP (see conditions for enzyme reaction below) but were unable to decatenate kinetoplast DNA [21]. One unit was sufficient to effect complete relaxation of 0.5 μg of supercoiled pLTL-1 DNA in 30 min at 37°.

Topoisomerase I relaxation reaction and electrophoresis. Unless otherwise noted, topoisomerase I reactions (25 μl) contained 0.35 μg pLTL-1 in assay buffer (50 mM Tris–HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml bovine serum albumin) and drug as indicated. After addition of 3 units of topoisomerase I, samples were incubated for 7.5 min at 37°. Reactions were adjusted to 0.5% SDS and digested with 50 μg/ml proteinase K at 37° for 30 min. Samples were electrophoresed on horizontal 1% agarose gels for 18 hr at 30 V in 40 mM Tris–acetate, pH 8.3, 1 mM EDTA buffer. Gels were stained with 0.5 μg/ml ethidium bromide and photographed with a Polaroid CU-5 Land Camera. Topoisomerase I activity was determined by measuring the amount of relaxed Form I produced. This value was obtained by scanning the DNA bands on the polaroid negative using an Helena Quik Scan densitometer.

Chemicals. Distamycin A and DAPI were purchased from Sigma Chemical Co. (St Louis, MO). Hoechst 33258 was obtained from Aldrich Chemical Co. (Milwaukee, WI). These drugs were dissolved in water at 1 mM concentrations and stored at −20°. Dithiothreitol, proteinase K, bovine serum albumin, PMSF, Triton X-100, polyethylene glycol and agarose were also obtained from Sigma. Hydroxylapatite was from Bio-Rad (Richmond, CA). All other chemicals used were reagent grade.

RESULTS

Netropsin was shown by others to effect significant changes in the linking number of DNA while the structurally similar minor groove binder distamycin did not [12]. That distamycin was itself unable to alter superhelical structure prompted its usage for initial studies of minor groove binder effects on topoisomerase I. Figure 1a is a photo of a representative gel showing the effects of distamycin A on the relaxation of supercoiled pLTL-1 DNA by topoisomerase I purified from L1210 cells. In this system, DNA was either fully relaxed (Form I') or fully supercoiled (Form I) with no intermediate forms observed. That relaxed Form I and not nicked closed circular Form II DNA was produced under these conditions was confirmed by electrophoresis of these samples in the presence of 0.5 μg/ml ethidium bromide. All ethidium-treated samples migrated with supercoiled Form I pLTL-1 as predicted for topologically relaxed DNA (data not shown). When topoisomerase I mediated relaxation was measured, no inhibition was observed below 2 μM distamycin. However, a progressive decrease in relaxed Form I occurred between 2 and 10 μM distamycin (lane 2 vs lanes 3–8). Distamycin alone was unable to alter DNA electrophoretic mobility (lane 1 vs lanes 9–12). Figure 1b shows a graphic representation of distamycin effects on topoisomerase I activity. At low distamycin concentrations (0.5 and 1 μM), some enhancement of topoisomerase I catalysis (38 and 13% respectively) was observed. However, relaxation was reduced by 50% with 2.7 μM and completely inhibited with 10 μM distamycin.

Whether complete relaxation of distamycin-treated DNA could be achieved after prolonged incubation with topoisomerase I was next examined. Figure 2 shows the percent Form I relaxed in the presence or absence of 2 μM distamycin at incubation times between 0.5 and 120 min. With 3 units of topoisomerase I and 0.35 μg pLTL-1 DNA, the reaction was linear for at least 10 min at 37°. Over this time period,

* Abbreviations: EDTA, ethylenediaminetetraacetic acid, disodium salt; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; and SDS, sodium dodecyl sulfate.

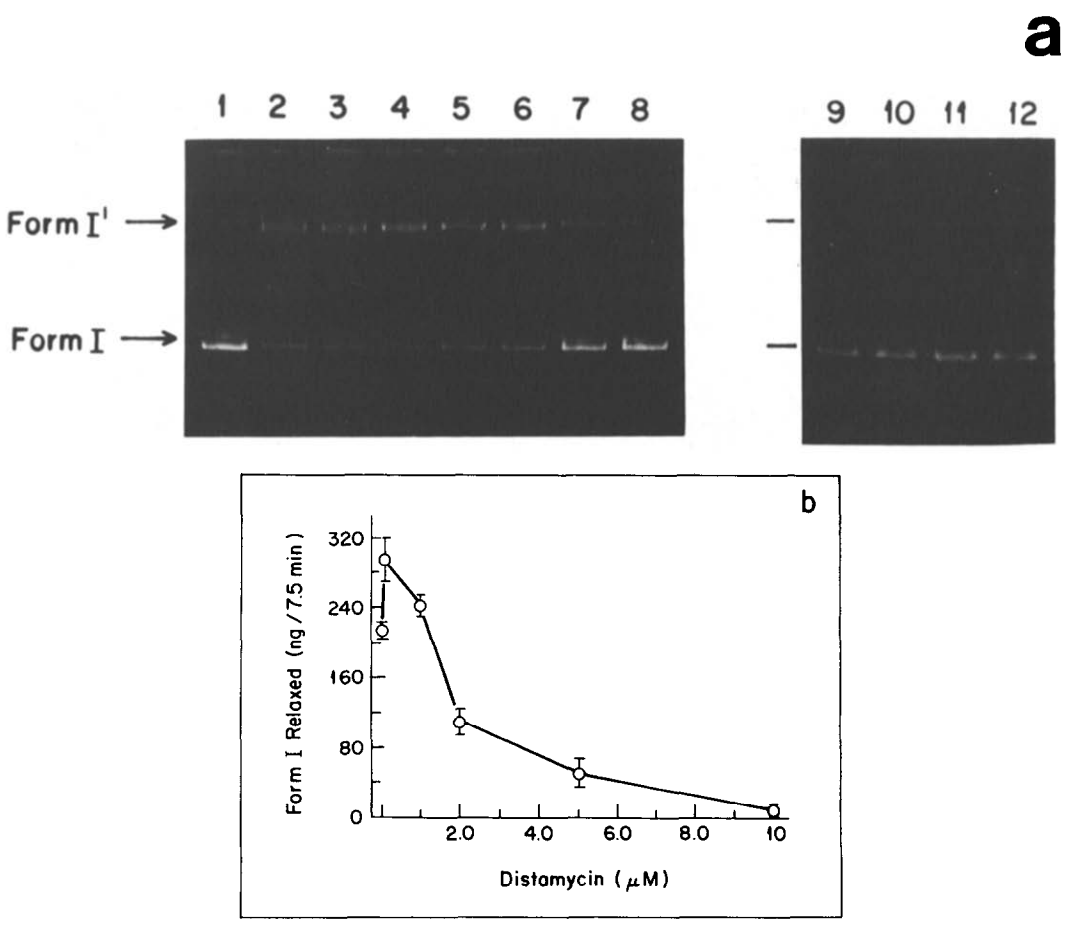

Fig. 1. Effect of distamycin on relaxation of supercoiled pLTL-1 DNA by topoisomerase I. Enzyme reactions, gel electrophoresis and analysis were performed as described in Materials and Methods. (a) Electrophoretic pattern. Lanes: (1) no distamycin, no topoisomerase I; (2) topoisomerase I alone; (3–8) 0.1, 0.5, 1.0, 2.0, 5.0 and 10 μM distamycin, respectively, and topoisomerase I; (9–12) 0.5, 1, 2 and 10 μM distamycin, no topoisomerase I. (b) Topoisomerase I relaxed Form I DNA produced in the presence of increasing distamycin concentrations. Each point in this and subsequent graphs represents six to eight samples from at least three separate experiments.

the percentage Form I relaxed was 1.5- to 3-fold higher in the absence than in the presence of distamycin. The relaxation rate of both distamycin-treated and untreated samples decreased between 15 and 60 min of incubation. Supercoiled Form I was exhausted by 60 min in the absence of drug, whereas 25% still remained in the distamycin-treated samples. Relaxation continued with reduced velocity in these samples yielding 93% conversion of Form I to the relaxed state by 2 hr. Thus, despite a decrease in the rate of relaxation, distamycin-treated samples were capable of attaining nearly complete relaxation by 2 hr.

The effect of increasing concentrations of DNA on the amount of Form I relaxed by topoisomerase I in the presence and absence of 2 μM distamycin is shown in Fig. 3. In the presence of distamycin, velocity was reduced compared to control over a substrate pLTL-1 DNA range of 350 to 1400 ng supercoiled Form I. Samples containing 2.0 μg DNA were also analyzed, and relaxation of distamycin-treated samples was similarly reduced (data not shown). If depletion of substrate alone was responsible for enzyme inhibition, increasing the substrate would be expected to reverse the distamycin effect. Such a reversal was not observed over the range of DNA concentrations tested. However, increasing the DNA above 2000 ng/25-μl reaction resulted in decreased velocity in both the presence and absence of distamycin, limiting further analysis.

Two other minor groove binders tested showed topoisomerase I inhibitory effects similar to distamycin. Figure 4 is a photo of a representative gel comparing the relaxation of DNA by topoisomerase I alone (lane 2) and topoisomerase I plus distamycin (lanes 3–5), Hoechst 33258 (lanes 6–8) or DAPI (lanes 9–11). As with distamycin, no effect on migration of supercoiled Form I pLTL-1 was observed in the presence of DAPI or Hoechst 33258 alone (data not shown). Above 2 μM, progressive inhibition of relaxation with increasing concentrations of all three drugs was readily apparent. When a series of experiments comparing these

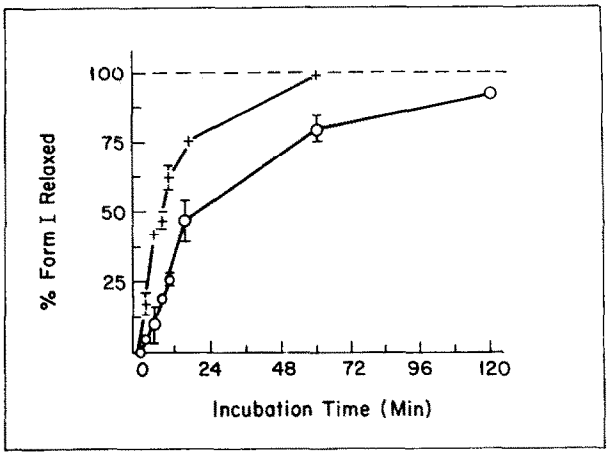

Fig. 2. Time course of distamycin inhibition of topoisomerase I mediated relaxation of DNA. Enzyme assays were performed and analyzed as described in Materials and Methods except that incubation times ranged between 0.5 and 120 min. Key: topoisomerase I alone (+—+); and topoisomerase I plus 2 μM distamycin (O—O).

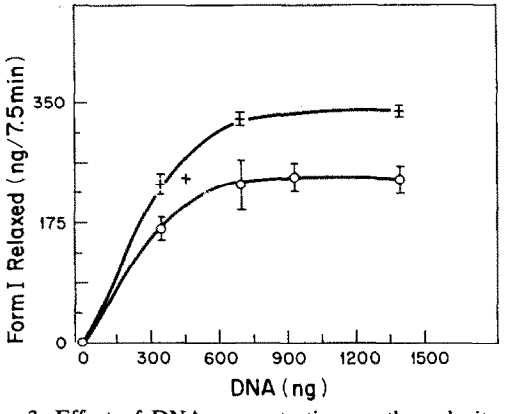

Fig. 3. Effect of DNA concentration on the velocity of topoisomerase I mediated relaxation of DNA. Assays were performed and analyzed as described in Materials and Methods except that DNA was varied from 350 to 1400 ng. The data were corrected for the amount of form II (nicked circular DNA) present in the control which migrated in the same position as relaxed Form I'. Substrate DNA values shown reflect the actual amount of supercoiled Form I present in the reaction with topoisomerase I. Key: topoisomerase I alone (+—+); and topoisomerase I plus 2 μM distamycin (O—O).

Table 1. Comparative effects of minor groove binders on topoisomerase I mediated relaxation of supercoiled DNA*

% Maximum relaxation	Drug (μM) Distamycin	Hoechst 33258	DAPI
100	0	0	0
70	2	2	2
50	2.7	2.8	5
25	3.8	4.2	7.3

* Topoisomerase I reactions were performed as described in Materials and Methods, with concentrations of distamycin, DAPI or Hoechst 33258 ranging from 0.5 to 25 μM. Maximum relaxation was that obtained by topoisomerase I alone in the absence of drug and equaled 60% conversion of Form I to Form I'.

ligands was analyzed, the data presented in Table 1 were obtained. Distamycin and Hoechst 33258 were equivalent in their abilities to reduce relaxation. Relaxation was reduced to 50% of control levels by 2.7 μM distamycin and 2.8 μM Hoechst 33258, versus 5 μM DAPI. Similarly, reduction to 25% of control required 3.8 μM distamycin and 4.2 μM Hoechst 33258 compared to 7.3 μM DAPI. Thus, all three minor groove binders tested altered topoisomerase I activity, albeit with somewhat differing potencies.

DISCUSSION

This report describes the effect on topoisomerase I activity of a class of drugs which bind specifically to the minor groove of DNA. Inhibition of topoisomerase I was observed with three of these drugs: distamycin A, DAPI and Hoechst 33258. Similar effects on this enzyme have been reported elsewhere for the intercalators, 9-aminoacridine and ethidium bromide. Pommier *et al.* [10] have shown that these intercalators prevent topoisomerase I from entering its "processive catalytic cycle" resulting in topoisomers that are either fully relaxed or fully supercoiled. Even in the presence of intercalators, complete relaxation by topoisomerase I could be attained upon long-term incubation. The current report showed that minor groove binders also inhibited topoisomerase I in an all or nothing manner (no intermediate topoisomers were observed) and that inhibition by distamycin A was reversible upon long-term incubation.

The topoisomerase I inhibition reported for intercalators correlated directly with drug-induced DNA unwinding. By contrast, minor groove binders do not intercalate and cannot unwind DNA. Thus, though

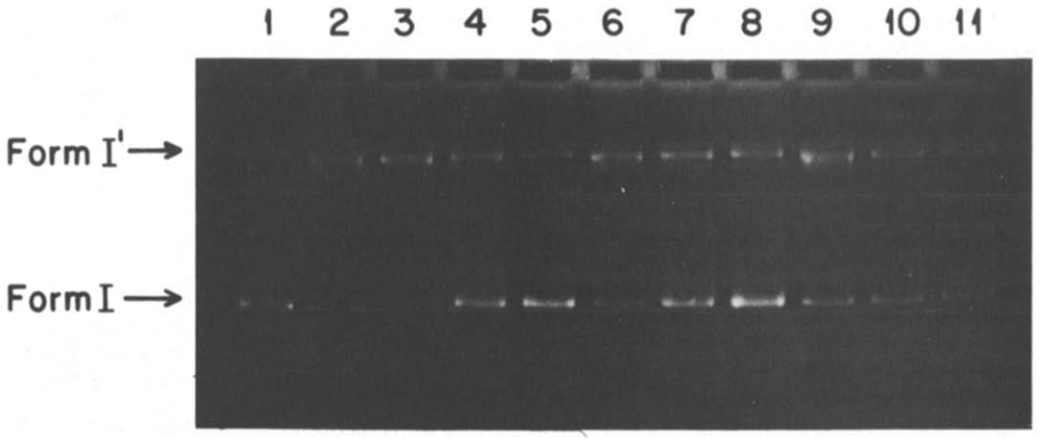

Fig. 4. Electrophoretic analysis of minor groove ligand effects on relaxation of pLTL-1 DNA by topoisomerase I. Assays were performed and analyzed as described in Materials and Methods, with various concentrations of single drugs. Lanes: (1) no drug, no topoisomerase I; (2) topoisomerase I alone; (3–5) 2.0, 5.0 and 10 μM distamycin, respectively, and topoisomerase I; (6–8) 2.0, 5.0 and 10 μM Hoechst 33258, respectively, and topoisomerase I; (9–11) 2.0, 5.0 and 10 μM DAPI, respectively, and topoisomerase I.

effects on topoisomerase I activity are similar, alternate changes in DNA structure must be proposed for the minor groove drug-binding effect observed.

Parameters associated with drug binding to the minor groove include binding to A-T base pair rich regions, the ability to destabilize the B-form of DNA, extrusion of water molecules, and induced widening of the minor groove, as well as spatial and electrostatic interactions [11, 22]. Any of these may be responsible for alterations in topoisomerase I catalysis. While the three drugs tested all bind to the minor groove, some differences have been noted. Although, in general, runs of AT base pairs are preferred, tolerance for alternate base sequences vary. For example, DAPI binds TpA better than Hoechst 33258, whereas Hoechst and distamycin tolerate GC base pairs more readily than DAPI [23]. The number of base pairs occupied also varies with the ligand: DAPI binding to 3 or 4 versus 5 for distamycin and Hoechst 33258 [15, 23]. On the basis of size, DAPI should have the least effect on the helix, while changes in width of the minor groove have been reported for both Hoechst and netropsin, an antibiotic similar to distamycin [23–25]. Our results showing that DAPI was less effective in inhibiting topoisomerase I than either distamycin or Hoechst 33258 may be due to the size of the drug molecule and/or differences in base sequence requirements noted above.

Besides inhibition, some enhancement of topoisomerase I activity was noted at low distamycin A concentrations. Whether stimulation or inhibition was observed could depend on the degree to which saturation of drug-binding sites has occurred. Low drug levels might interact with only a portion of the available nucleotides effecting a "localized distortion" and "opening up" of the helix to an enzyme such as topoisomerase I and enhancement of its activity. At higher drug levels, these changes could be sufficient to decrease topoisomerase I association with the substrate resulting in decreased catalysis.

Such results are reminiscent of previous reports detailing minor groove binder effects on other DNA-directed enzymes, among them RNA polymerase [16, 17], DNAse I [13, 15, 23, 26] and S_1 nuclease [27]. Bruzik *et al.* [16] found that formation of the *E. coli* RNA polymerase–DNA open complex is stimulated at a low distamycin concentration (0.08 μM), while increasing distamycin above 0.2 μM results in inhibition of transcription initiation. They attributed these effects to high and low affinity distamycin binding sites on the DNA. Straney and Crothers [17] also observed inhibition of this enzyme with 0.7 μM distamycin and concluded that association of RNA polymerase with DNA is altered by the drug. Some effect of minor groove binders on DNAse I activity would be expected since this enzyme itself probably cleaves from the direction of the minor groove [11]. Indeed, inhibition of DNAase I at the drug-binding site accompanied by enhanced cleavage at neighboring regions has been reported for distamycin [13], netropsin [15, 26], DAPI and Hoechst 33258 [23]. This increased cleavage was attributed to drug-induced alteration in the nucleotides under attack. Enhancement of cleavage by the single-strand specific S_1 nuclease has also been reported with netropsin [27].

In an attempt to extrapolate the enzyme data to conditions which more approximate the *in vivo* situation, preliminary studies on distamycin effects in nuclei were performed [28]. In this system, DNA from L1210 nuclei treated with the topoisomerase I specific inhibitor camptothecin exhibits DNA–protein crosslinks and single-strand breaks. When distamycin alone was reacted with nuclei under conditions identical to those which permit camptothecin-induced DNA lesions, no crosslinking or strand breakage was observed. Distamycin was, however, a potent inhibitor of camptothecin-associated DNA damage. Thus, at least in nuclei, the mode of action of distamycin is distinct from that of camptothecin which binds to and inhibits topoisomerase I at the

level of the topoisomerase I–DNA complex. Distamycin-induced alterations of nuclear DNA could account for the decrease in camptothecin-associated cleavable complex observed.

Effects of minor groove binders on L1210 topoisomerase II have also been noted in this laboratory (J. Woynarowski *et al.*, manuscript to be submitted). At low levels (1–2 μM), distamycin markedly stimulated topoisomerase II activity, whereas the effect of DAPI or Hoechst 33258 was negligible. Above 5 μM, a progressive inhibition of topoisomerase II with increasing concentrations of all three minor groove binders was observed, with DAPI having less of an effect than either Hoechst 33258 or distamycin A. Despite basic differences in their mechanism of action, both topoisomerases share a common substrate, supercoiled DNA, and alterations in this substrate induced by ligand binding is a likely explanation for such similar effects on both enzymes.

In conclusion, minor groove binding drugs affect topoisomerase I activity. Recently, a strong preference by topoisomerase I for cleavage at an A-T base pair located within an AT-rich hexadecamer has been reported [29]. Distamycin also binds preferentially to AT sequences [24] and may induce changes in topoisomerase I association with its preferred recognition sequence. Whether the inhibition of topoisomerase I observed in the current report is due to drug binding to specific sequences remains to be determined. Future studies will attempt to further characterize the effect of minor groove binders on topoisomerase I activity both *in vitro* and in subcellular (nuclei) and cellular assay systems.

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