0026-895X/89/020177-06$02.00/0

MOLECULAR PHARMACOLOGY, 35:177–182

Modulation of Topoisomerase II Catalytic Activity by DNA Minor Groove Binding Agents Distamycin, Hoechst 33258, and 4′,6-Diamidine-2-phenylindole

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Received September 12, 1988; Accepted November 10, 1988

SUMMARY

The effects of distamycin, Hoechst 33258, and 4′,6-diamidine-2-phenylindole (DAPI) on the catalytic activity of topoisomerase II from L1210 cells were determined. These compounds were used as model agents capable of AT-specific binding in the minor groove of DNA while producing no profound long-range alterations to the DNA structure. Two types of reactions catalyzed by topoisomerase II were examined, relaxation of supercoiled DNA and decatenation of highly catenated DNA. Distamycin at low concentrations (0.2–2 μM) substantially stimulated relaxation of supercoiled pBR322 DNA. Higher drug levels (25–50 μM) resulted in a potent inhibition of relaxation. At the stimulatory concentrations of distamycin, only completely relaxed reaction products were observed, as in the absence of the drug. The onset of inhibition (caused by 5–10 μM distamycin) was accompanied by the appearance of partially relaxed intermediates. Similar inhibition of relaxation was observed for Hoechst 33258 and DAPI but, unlike distamycin, these agents produced only marginal stimulation of relaxation when added in low noninhibitory concentrations. Another reaction of topoisomerase II, decatenation of catenated kinetoplast DNA, was also inhibited by distamycin, Hoechst 33258, and DAPI at concentrations similar to those inhibiting the relaxation reaction. This study demonstrates that agents binding to the minor groove of DNA represent a new class of drugs interfering with topoisomerase II and provides possibilities for modulation of this important enzyme.

DNA topoisomerase II (EC 5.99.1.3) is an enzyme responsible for DNA topological conversions, which occur by passage of a duplex DNA through a transient double strand break (for review see Refs. 1 and 2). Recently, mammalian topoisomerase II has attracted considerable attention as a new target for antitumor agents. A number of drugs were shown to interfere with this enzyme by stabilization of a reaction intermediate, the cleavable complex (1, 2). At this stage of reaction, the enzyme remains covalently bound to the 5′ ends of the DNA in the induced break (1, 2). Stabilization of the cleavable complex by drugs such as *m*-AMSA or VM-26 is accompanied by inhibition of the catalytic activity of topoisomerase II (3, 4).

Although drug-induced stabilization of the cleavable complex has been extensively studied, very little is known about alternative mechanisms of interference with the catalytic activity of topoisomerase II. To date, only certain intercalating drugs, such as *o*-AMSA or ethidium bromide, were shown to inhibit topoisomerase II-induced strand passing without stabilizing the cleavable complex (3–5). Furthermore, even for these intercalating agents, the basis for the interference with the enzyme is not precisely known. Although intercalators induce long-range changes (unwinding) in DNA structure, their effects on the catalytic activity of topoisomerase II are not correlated with their unwinding potentials (4).

In contrast to the intercalators, there exists a class of compounds that bind to the minor groove of the DNA and associate tightly with the DNA surface while producing little or no distortion of the helix apart from the binding site (6–8). Moreover, minor groove binders can affect the actions of other DNA-processing enzymes. For example, an antibiotic, distamycin, was reported to stimulate (9) and inhibit (10) transcription, presumably by altering the interaction of RNA polymerase with the DNA. A related drug, netropsin, enhanced DNA digestion by nuclease S1 (11). At the same time, distamycin was found to be unable to stabilize the cleavable complex of topoisomerase II in nuclei (12).

In this study, we characterized the effects of the minor groove binders distamycin, Hoechst 33258, and DAPI (Fig. 1) on two

This study was supported by American Cancer Society Grant CH-293 and National Cancer Institute Grant CA-24538.
Preliminary data were presented at the Second Conference on DNA Topoisomerases in Cancer Chemotherapy, October 17–19, 1988, New York, NY.

ABBREVIATIONS: AMSA, 4′-(9-acridinylamino)-methanesulfon-anisidide; DAPI, 4′,6-diamidine-2-phenylindole; DTT, dithiothreitol; PMSF, phenyl methyl sulfonyl fluoride; form I, supercoiled circular DNA; form I_R, relaxed covalently closed circular DNA; form II, relaxed nicked circular DNA; kDNA, kinetoplast DNA; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

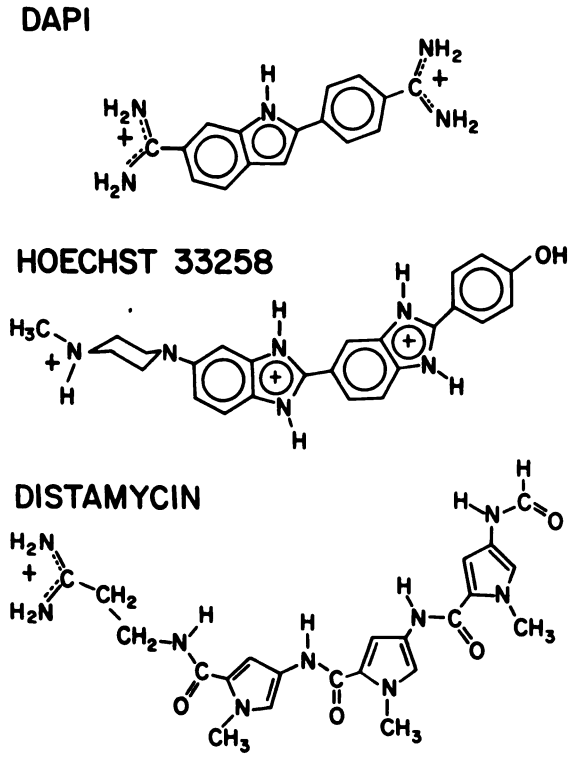

Fig. 1. The structures of the studied minor groove binders.

types of reactions catalyzed by topoisomerase II isolated from L1210 cells. All three agents were found to strongly inhibit both relaxation of supercoiled DNA and decatenation of highly catenated kDNA. In addition, distamycin at low concentrations (0.2–2 μM) appeared to stimulate the relaxation activity of topoisomerase II. Thus, DNA minor groove-binding agents can be regarded as a new class of drugs interfering with topoisomerase II.

Materials and Methods

Drugs and biochemicals. Distamycin and DAPI were obtained from Sigma Chemical Co. (St. Louis, MO). Hoechst 33258 [2(4-hydroxyphenyl)-5[5-(4-methylpiperazine-1-yl)-benzimidazol-2-yl]benzimidazole] was from Aldrich Chemical Co. (Milwaukee, WI). Stock solutions of the groove binders were made in water and stored at −20°. DTT, PMSF, polyethylene glycol 8000, bovine serum albumin, and Triton X-100 were from Sigma. Proteinase K was obtained from Boehringer-Mannheim (Indianapolis, IN), agarose from FMC Corporation (Homer City, PA), and hydroxylapatite (Biogel HT) from Bio-Rad (Richmond, CA).

Enzyme isolation. Partially purified topoisomerase II was isolated from L1210 cells according to the slightly modified procedure of Miller *et al.* (13). Briefly, exponentially growing L1210 cells (3×10^9) were washed with cold phosphate-buffered saline, with 1 mM PMSF, and resuspended in nuclei isolation buffer (2 mM $MgCl_2$, 5 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) supplemented with 0.35% (v/v) Triton X-100. After 15 min on ice, the suspension was homogenized in a Dounce homogenizer (three strokes) and the nuclei were collected by centrifugation (1200 rpm, 10 min) and washed once with nuclei isolation buffer. The final pellet was resuspended in nuclei isolation buffer, which was supplemented with 4 mM EDTA and 0.375 M NaCl. Nuclei were gently agitated for 15 min at 0°, followed by the addition of polyethylene glycol (9%, v/v, final concentration) and additional incubation for 40 min at 0°. The samples were centrifuged at 20,000 × *g* for 30 min and the supernatant (nuclear extract) was fractionated on a hydroxylapatite column. The column (1.5 × 8 cm) was washed with 100 ml of 0.2 M potassium phosphate, pH 7.0, in 10% (v/v) glycerol, 1 mM DTT, and 1 mM PMSF. The wash removed >90% of protein present in the nuclear extract, as judged by absorbance at 280 nm. Next, the column was eluted with a linear gradient (100 ml) of 0.2–0.8 M potassium phosphate, pH 7.0, with 10% glycerol, 1 mM DTT, and 1 mM PMSF. Topoisomerase II activity in the column fractions was monitored by the decatenation assay (see below). Topoisomerase I activity in these fractions was determined by the relaxation assay, as described below for topoisomerase II but carried out in the absence of ATP. Topoisomerase II eluted at approximately 0.4 M phosphate. The enzyme used in this study was "functionally pure," in that it contained neither topoisomerase I activity (as indicated by its inability to relax pBR322 DNA in the absence of ATP) nor endonucleolytic activity (inasmuch as there was no production of nicked or linear DNA in the relaxation assay). The enzyme was stored at −80° in 50%, v/v, glycerol with 0.005% Triton X-100. The specific activity of the enzyme preparation was $>2 \times 10^4$ units/mg, one unit being defined as the quantity of the enzyme that fully relaxed 0.15 μg of pBR322 DNA in 15 min at 37°. This specific activity corresponded to at least 2 orders of magnitude purification, as compared with the initial nuclear extract.

Relaxation of pBR322 DNA. The assay, based on published procedures (13, 14), utilized pBR322 DNA (Boehringer-Mannheim), which normally contained approximately 80% of supercoiled form I and 20% of relaxed nicked form II. Unless otherwise indicated, the reactions (25 μl final volume) contained 0.15 μg of pBR322 DNA in 50 mM Tris·HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and 30 μg/ml bovine serum albumin. After addition of drugs and topoisomerase II (normally 0.5 units), the samples were incubated at 37° for 10 min. The reactions were terminated with 0.5% sodium dodecyl sulfate and 50 μg/ml proteinase K, followed by addition of EDTA (10 mM final concentration) and incubation for 30 min at 37°. The samples were electrophoresed on 1% agarose gel in Tris-acetate buffer, for 18 hr at 30 V, as described previously (15). Gels were stained with ethidium bromide (0.5 μg/ml) and photographed on a UV transilluminator. The negatives were scanned and the amount of DNA in individual bands was determined with a Bio-Rad gel scanner.

Decatenation of kDNA. Highly catenated kDNA from *Crithidia fasciculata* was isolated as described elsewhere (16). The decatenation assay was based on the procedure described by others (17). The reaction mixtures were similar to the relaxation assay, except that 0.3 μg of kDNA and 1.5 units of enzyme were used. The reactions were terminated and analyzed by agarose gel electrophoresis as described for the relaxation assay. The reaction was evaluated based on generation of decatenated minicircles. Because highly catenated material does not enter the gel, it cannot be quantitated.

Results and Discussion

This study determined whether the catalytic activity of topoisomerase II could be altered by model drugs known to bind tightly and specifically in the minor groove of DNA. The selected compounds included distamycin, Hoechst 33258, and DAPI (Fig. 1). Each of these agents is known to interact with A or T sequences (18, 19) in the minor groove while producing little or no long-range distortion of the double helix (3, 20–22). The size of drug binding sites on the DNA is proportional to the length of drug molecules. The distamycin molecule occupies five base pairs whereas Hoechst 33258 and DAPI occupy four and three base pairs, respectively (20–22).

Initially, we characterized the effects of distamycin on the relaxation of pBR322 DNA by topoisomerase II. Fig. 2A shows that in the absence of drug (*lanes 2* and *3*) the enzyme can convert supercoiled DNA (form I) into relaxed circular DNA (form I_R), which comigrates with the nicked DNA (form II) present in the DNA preparation. Analysis of the samples under

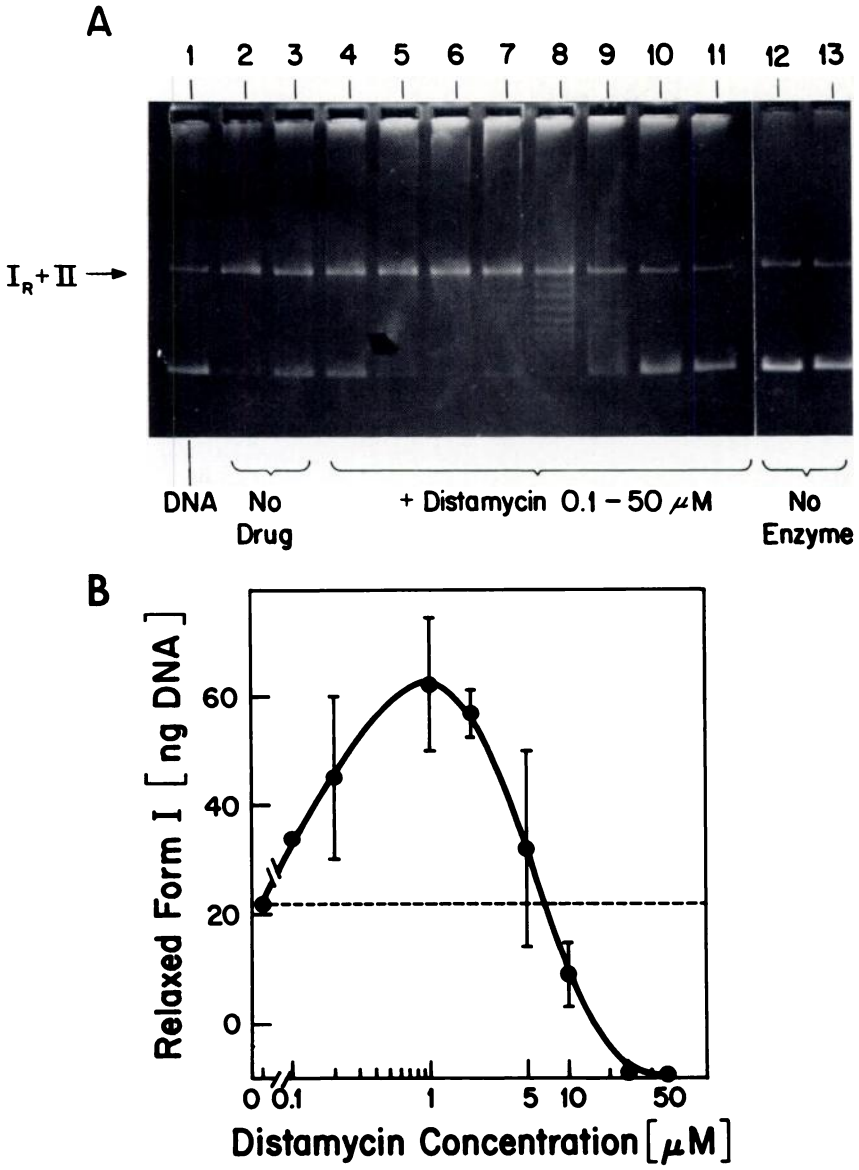

Fig. 2. The effects of distamycin on relaxation of supercoiled pBR322 DNA by topoisomerase II. A, Agarose gel electrophoresis. *Lane 1*, untreated pBR322 DNA; *lanes 2* and *3*, DNA incubated with 0.75 and 0.5 units of topoisomerase II, respectively, for 10 min at 37°; *lanes 4–11*, as *lane 3*, except that distamycin was added at 0.1, 0.2, 1, 2, 5, 10, 25, and 50 μM, respectively; *lanes 12* and *13*, DNA incubated with 2 or 25 μM distamycin, respectively, in the absence of enzyme. B, Quantitation based on formation of form I_R as determined by scanning agarose gels. The results represent mean values (± standard error) from three to six independent experiments.

conditions separating form I_R from form II (on a gel containing 30 μg/ml chloroquine) confirmed that the amount of form II DNA remained unchanged upon the action of the enzyme, alone or in the presence of distamycin (data not shown). Furthermore, there was no induction of linear DNA (form III) and no changes were seen in the absence of ATP (data not shown). Thus, the observed changes in DNA distribution reflect only relaxation catalyzed by topoisomerase II. The reaction was linear for at least 30 min with DNA concentration ranging from 3 to 24 μg/ml.

In the absence of topoisomerase II, distamycin had no effect on the electrophoretic migration of pBR322 DNA (Fig. 2A, *lanes 12* and *13*). However, addition of the drug with the enzyme resulted in profound alteration of the relaxation reaction. Low concentrations of distamycin (0.2–2 μM) brought about a substantial stimulation of the production of form I_R with a concurrent decrease in form I (Fig. 2A, *lanes 3–5*). In contrast, high levels of distamycin (25 and 50 μM) resulted in complete inhibition of topoisomerase II-induced topological conversions of pBR322 DNA (Fig. 2A, *lanes 10* and *11*). Quantitation of these effects (expressed as generation of form I_R) over a range of distamycin concentration (Fig. 2B) clearly indicates that distamycin is capable of both stimulating and inhibiting relaxation activity of topoisomerase II. Maximal stimulation (at 1–2 μM distamycin) corresponded to approximately 60% of the initial amount of form I being completely relaxed, compared with 25% in the absence of drug. At higher drug levels, the amount of relaxed form I decreased to 8% with 10 μM and to background levels with 25 μM distamycin.

In addition to altering the amount of relaxed DNA produced, distamycin apparently affected the character of the reaction. In the absence of the drug as well as with the stimulatory concentrations of distamycin, virtually no intermediate bands (partially relaxed form I) were seen (Fig. 2A, *lanes 3–7*). This indicates that relaxation proceeded in a processive manner, i.e., a supercoiled DNA was relaxed completely by an enzyme molecule before this enzyme relocated onto another DNA molecule (14). In contrast, further increases in drug concentration (corresponding to the onset of the inhibitory effect) produced distinct intermediate bands indicative of a distributive enzyme

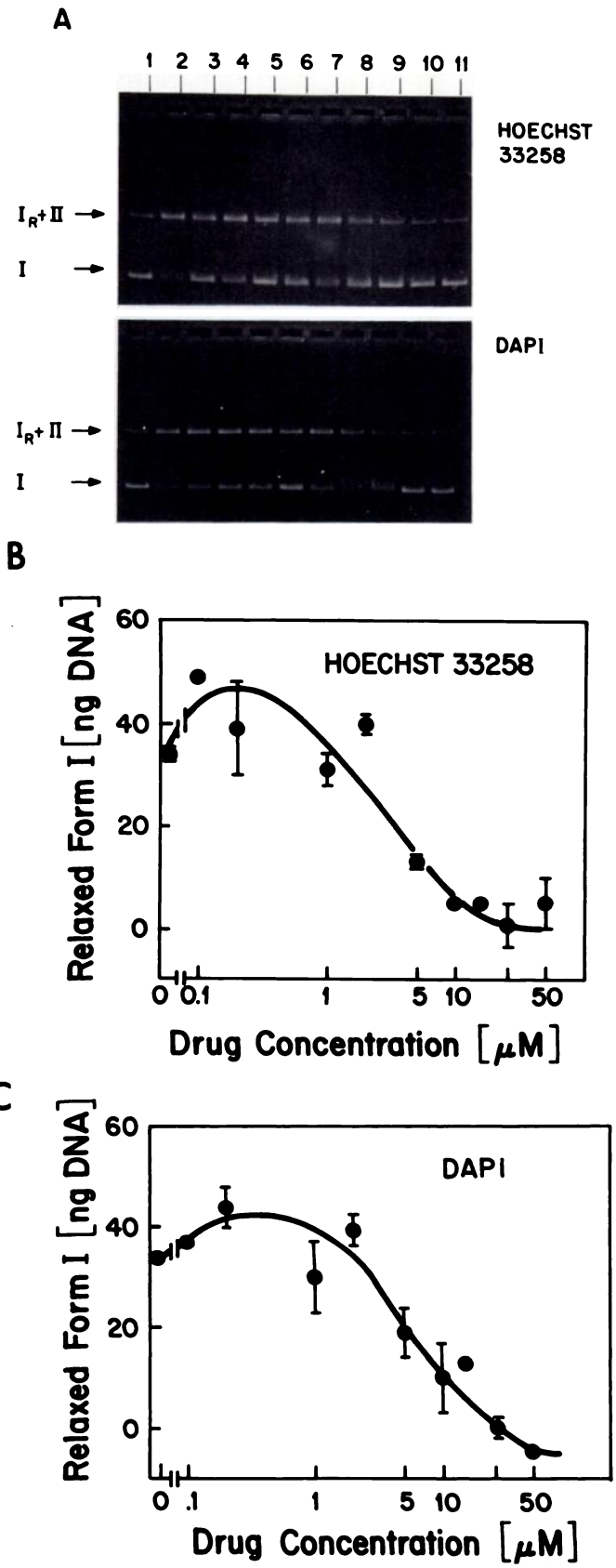

Fig. 3. The effects of Hoechst 33252 and DAPI on relaxation of supercoiled pBR322 DNA by topoisomerase II. A, Agarose gel electrophoresis. *Lane 1*, untreated pBR322 DNA; *lanes 2* and *3*, DNA incubated with 0.75 and 0.5 units of topoisomerase II, respectively, for 10 min at 37°; *lanes 4–11*, as *lane 3*, except that Hoechst 33258 or DAPI was added at 0.1, 0.2, 1, 2, 5, 10, 25, and 50 μM, respectively. B and C, quantitation based on formation of form I_R as determined by scanning agarose gels. The results represent mean values (± standard error) from two independent experiments except for data for drugs at 0.1 and 50 μM, which are from a single determination.

reaction (14), in which the enzyme initiates but does not complete the relaxation on several target DNA molecules. For example, at 5 or 10 μM distamycin, 66% and 70%, respectively, of the initial amount of form I was converted to intermediates.

The influence of various reaction conditions, including the order of addition, on distamycin action was also examined. In a typical experiment the drug was allowed to bind to the DNA before enzyme addition. However, addition of the enzyme before distamycin failed to overcome the inhibition (data not shown). Other reaction conditions examined, specifically DNA concentration and reaction time, affected the balance between stimulation and inhibition by distamycin. Stimulation was seen only at low DNA concentrations (3–6 μg of DNA/ml). For example, 2 μM distamycin, which produced 185% stimulation of relaxation at 6 μg of DNA/ml, yielded 30% and 39% inhibition at 12 and 24 μg of DNA/ml, respectively. Longer reaction times (20–30 min) led to a diminished inhibitory ability of distamycin (at 10 or 25 μM). This suggests that distamycin slows but does not completely block the reaction of topoisomerase II. The stimulation of relaxation by the drug (at 2 μM) was less affected by the extended (up to 30 min) incubation.

Hoechst 33258 and DAPI were used to determine whether the effects of distamycin on topoisomerase II represent a common feature among DNA minor groove-binding agents. Both compounds appeared to affect relaxation of pBR322 DNA by the enzyme (Fig. 3). Whereas Hoechst 33258 and DAPI produced only minor, if any, stimulation of relaxation when present at low concentrations, higher drug levels (5–50 μM) caused a dose-dependent inhibition of enzyme activity (Fig. 3, B and C). Moreover, the relaxation reaction in the presence of low concentrations of DAPI (2–5 μM) resulted in generation of partially relaxed intermediates (Fig. 3A). At 5 μM DAPI, 33% of form I was converted to intermediates, compared with only 19% being fully relaxed. As with distamycin (Fig. 1), these data indicate the transition from processive to distributive enzyme action during the onset of inhibition. For Hoechst 33258, the transition from noninhibitory to inhibitory conditions occurred without obvious generation of reaction intermediates.

Another type of reaction catalyzed by topoisomerase II is decatenation of highly catenated DNA, which ultimately yields single minicircles (17). The effects of minor groove binders on the decatenation activity of topoisomerase II were assayed using highly catenated DNA from kinetoplasts of *C. fasciculata* (16). In this assay, released minicircles are separated by electrophoresis from catenated DNA, which does not enter the gel (Fig. 4). Distamycin effects on decatenation were similar to those on the relaxation reaction. Although the reaction conditions (DNA concentration and enzyme activity) were selected primarily for the detection of inhibition, low concentration of the drug slightly stimulated the release of uncatenated circles (Fig. 4, *lane 4*). Increased drug concentrations (5–10 μM) brought about inhibition of the decatenation. At 25 μM distamycin, the decatenation reaction was blocked completely (Fig. 4, *lanes 7* and *8*). Hoechst 33258 and DAPI were also inhibitory in the decatenation assay, exhibiting potency similar to distamycin (data not shown). As in the relaxation assay, distamycin in-

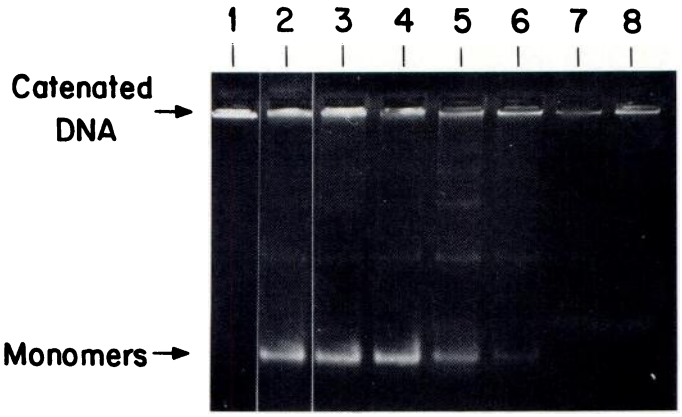

Fig. 4. The effects of distamycin on decatenation of kDNA by topoisomerase II, determined by agarose gel electrophoresis. *Lane 1*, untreated kDNA; *lane 2*, DNA incubated with 1.5 units of topoisomerase II for 15 min at 37°. *Lanes 3–8*, as *lane 2*, except that distamycin was added at 1, 2, 5, 10, 25, and 50 μM, respectively.

TABLE 1

Effects of minor groove binders on catalytic activity of topoisomerase II

	Drug Concentration Resulting in		
	Inhibition of relaxation[a]	Stimulation of relaxation[b]	Inhibition of decatenation[a]
		μM	
Distamycin	12	1–2 (+++)	5–10
Hoechst 33258	10	0.1–2 (+/–)	5–10
DAPI	25	0.2–2 (+/–)	10–25

[a] Almost complete inhibition (>90%).
[b] Maximal effect. The relative extent of stimulation is shown in parenthesis; (+/–) corresponds to less than 25% stimulation.

duced a change from processive reaction, when only fully decatenated monomeric minicircles were formed, to distributive reaction, indicated by a series of partially decatenated oligomers (Fig. 4, *lanes 5* and *6*). A similar transition was observed with DAPI and Hoechst 33258 (data not shown).

The effects of distamycin, Hoechst 33258, and DAPI on the decatenation reaction with the partially purified enzyme were qualitatively and quantitatively similar to those obtained with the crude nuclear extract as a source of topoisomerase II (data not shown). Thus, it seems unlikely that the observed effects on catalytic activity of topoisomerase II were due to an indirect effect on another protein that might interact with topoisomerase II.

The summarized results (Table 1) indicate that all the studied groove binders, distamycin, Hoechst 33258, and DAPI, are inhibitors of topoisomerase II catalytic activity in two types of reactions. No substantial differences in the ability of these agents to inhibit the relaxation reaction were observed, although DAPI was consistently somewhat less active than distamycin and Hoechst 33258. Likewise, all three drugs showed a similar potency to inhibit the decatenation reaction. Inhibition of decatenation was caused by approximately the same drug levels as inhibition of relaxation (Table 1).

One interesting difference among the studied agents is a profound stimulatory potency of distamycin in the relaxation reaction, as compared with weak, if any, enhancement by Hoechst 33258 and DAPI (Table 1). Because stimulation by distamycin was dependent on the DNA concentration, it is likely that stronger stimulation by both Hoechst 33258 and DAPI would occur with different concentrations of DNA or enzyme. Alternatively, an ability to stimulate the relaxation may be unique to distamycin, possibly due to the size of the drug binding site [five base pairs compared with four and three base pairs for Hoechst 33258 and DAPI, respectively (20–22)].

The observed effects of minor groove binders on topoisomerase II are consistent with other observations for these agents. First, parallel investigation in this laboratory demonstrated that distamycin, as well as Hoechst 33258 and DAPI, inhibited catalytic activity of another topological enzyme, topoisomerase I (23). Second, a recent independent study (24) showed that distamycin is able to stimulate and inhibit topoisomerase II-mediated cleavage of linearized pBR322 induced by an epipodophyllotoxin derivative, VM-26. In addition, the dual effect of distamycin on topoisomerase II resembles the effects of the drug on transcriptional proteins; although several studies reported inhibition of transcription by distamycin (Refs. 7 and 10 and references therein), stimulation of transcription initiation was also observed (9).

The exact mechanism by which distamycin, Hoechst 33258, and DAPI stimulate or inhibit topoisomerase II remains to be elucidated. It is possible that both stimulation and inhibition originate from specific localized drug interactions with the DNA, as proposed for the interference of minor groove binders with other DNA processive enzymes (7, 9, 11, 18). Local alterations in DNA structure, such as bending of the DNA induced by distamycin (20), might generate sites of high affinity for the enzyme, which could lead to stimulation at a lower concentration of binding agent. At higher levels of drug bound to DNA, many sites on DNA would become inaccessible to the enzyme, resulting in the inhibition of the enzymatic reaction. The observed stimulatory and inhibitory effects of distamycin are consistent with such interpretation. Moreover, transition from a processive to a distributive reaction implies that, in the presence of moderately inhibitory concentrations of distamycin, the enzyme remains functional and can bind to the DNA and initiate its relaxation. The relaxation, however, cannot be completed.

One might expect that drugs binding more tightly to the DNA minor groove would be more potent as inhibitors of topoisomerase. An antitumor drug, CC-1065, binds covalently in the minor groove, in addition to noncovalent interaction (25). We found that this agent inhibits topoisomerase II at concentrations approximately 1 order of magnitude lower than the noncovalently binding drugs examined in this investigation.[1]

This study indicated that the minor groove binders distamycin, Hoechst 33258, and DAPI may be regarded as a new class of agents interfering with topoisomerase II. These findings warrant further investigation. Details of the molecular nature of the stimulatory and inhibitory effects, particularly how minor groove binders alter the interaction between topoisomerase II and DNA, remain to be elucidated. On the other hand, the ability of groove binders to alter the catalytic activity of topoisomerase II provides new opportunities for modulation of this enzyme. In a follow-up study, we explored the latter possibility and demonstrated that the minor groove binders can modulate topoisomerase II-mediated DNA lesions induced in nuclei by the clinical antitumor drugs VM-26 and *m*-AMSA (12).

[1] J. M. Woynarowski and T. A. Beerman, unpublished observation.

Acknowledgments

The authors would like to thank Dr. Bonnie Glisson for a culture of *C. fasciculata*, Dr. Eros Ferrazzi for isolation of kDNA, and Dr. John McGuire for helpful advice. Skillful technical assistance of Loretta S. Gawron is also acknowledged.

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