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Effects of Morpholinyl Doxorubicins, Doxorubicin, and Actinomycin D on Mammalian DNA Topoisomerases I and II

KARSTEN WASSERMANN, JUDITH MARKOVITS, CHRISTINE JAXEL, GIOVANNI CAPRANICO, KURT W. KOHN, and YVES POMMIER

Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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SUMMARY

The effect of cyanomorpholinyldoxorubicin, morpholinyldoxorubicin, doxorubicin, and Actinomycin D were studied on purified mouse leukemia (L1210) DNA topoisomerases I and II. DNA unwinding and cross-linking were also studied. It was found that 1) morpholinyldoxorubicin, cyanomorpholinyldoxorubicin, and Actinomycin D (but not doxorubicin) stimulated DNA topoisomerase I-induced cleavage at specific DNA sites; 2) only doxorubicin and Actinomycin D stimulated DNA cleavage by DNA topoisomerase II; 3) at higher drug concentrations, DNA intercalators suppressed enzyme-mediated DNA cleavage induced by DNA topoisomerase I, as well as topoisomerase II; 4) only cyanomorpholinyldoxorubicin produced DNA-DNA cross-links; no DNA unwinding could be observed; and 5) DNA intercalation (unwinding) potency of morpholinyldoxorubicin was about 2-fold less than that of doxorubicin. The data indicate that some DNA intercalators are not only inhibitors of DNA topoisomerase II but act also on DNA topoisomerase I. The stabilization of cleavage intermediates by intercalators may have a common mechanism for DNA topoisomerase I and DNA topoisomerase II.

A number of anticancer drugs are believed to exert their cytotoxic activity by binding to DNA and by inhibiting DNA topoisomerases (1). DNA topoisomerases are nuclear enzymes that interconvert topological isomers of DNA by breaking and resealing phosphodiester bonds (for review see Refs. 2 and 3). Type I topoisomerase changes the DNA linking number in steps of one by transient cuts of a single DNA strand and type II topoisomerase in steps of two by transient cuts of both strands of duplex DNA (2, 3). In higher eukaryotes, DNA topoisomerase I is enriched in the nucleolus (4) and its activity seems tightly associated with RNA polymerase I (5). DNA topoisomerase I is also enriched in transcribed genes (6, 7) and has been shown to relax DNA supercoiling and torsional tension in transcribing DNA domains (8). Therefore, there is good evidence that DNA topoisomerase I is involved in gene transcription.

The interaction of the anthracycline antibiotic DOX with DNA has been extensively studied. DOX binds to DNA by intercalation (9) and its antitumor activity has been associated with the production of protein-concealed DNA strand breaks (10) as a result of DNA topoisomerase II poisoning (11). *N*-Alkylated derivatives of DOX have been synthesized in an attempt to avoid cardiac toxicity and to improve the therapeutic activity of the drug. Two analogues in this series of derivatives, MRA-CN and MRA (Fig. 1), have been found to be more cytotoxic than DOX (12–14). These two derivatives have been reported to preferentially inhibit ribosomal gene transcription (15). This inhibition appears to result from drug interaction with the nucleolar ribosomal DNA template and not from an interaction with the RNA polymerase I itself. This was also observed after exposure to the intercalating antitumor drug ActD (Fig. 1), but not after exposure to DOX (15). Taken together with the observations concerning topoisomerase I, these recent data on the morpholinyl anthracyclines suggest a relationship between the drug effects on ribosomal gene transcription and topoisomerase I.

In the present study, we have examined the DNA effects of MRA-CN, MRA, DOX, and ActD in the presence and absence of purified mouse leukemia L1210 DNA topoisomerases I and II. We report that 1) MRA, DOX, and ActD bind to DNA by intercalation, 2) MRA-CN cross-links DNA, 3) MRA, MRA-CN, and ActD, but not DOX, stimulate topoisomerase I-mediated DNA cleavage at specific DNA sites, 4) DOX and ActD, but neither MRA-CN nor MRA, stimulate DNA cleavage by DNA topoisomerase II, and 5) high drug concentrations tend to suppress DNA cleavage, probably as a result of sufficient

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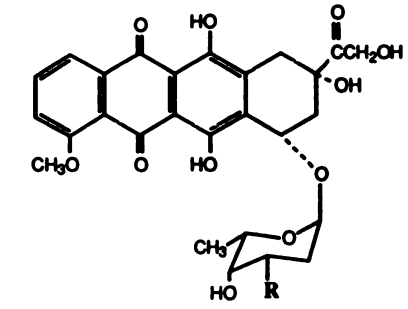
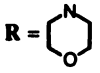
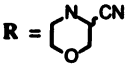
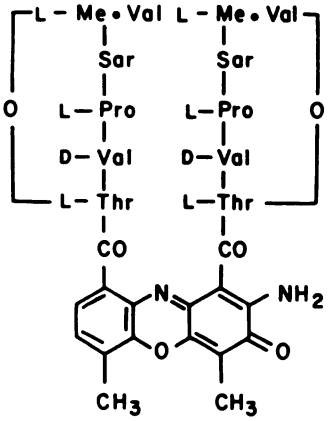
ABBREVIATIONS: DOX, doxorubicin; MRA-CN, 3′-deamino-3′-(3-cyano-4-morpholinyl)-doxorubicin; MRA, 3′-deamino-3′-(4-morpholinyl)-doxorubicin; ActD, actinomycin D; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.



R = NH_2 Doxorubicin (DOX)

R =  Morpholino doxorubicin (MRA)

R =  Cyanomorpholino doxorubicin (MRA-CN)



Actinomycin D ACT-D

Fig. 1. Structures of the morpholinyl anthracyclines. DOX, and ActD used in the present study.

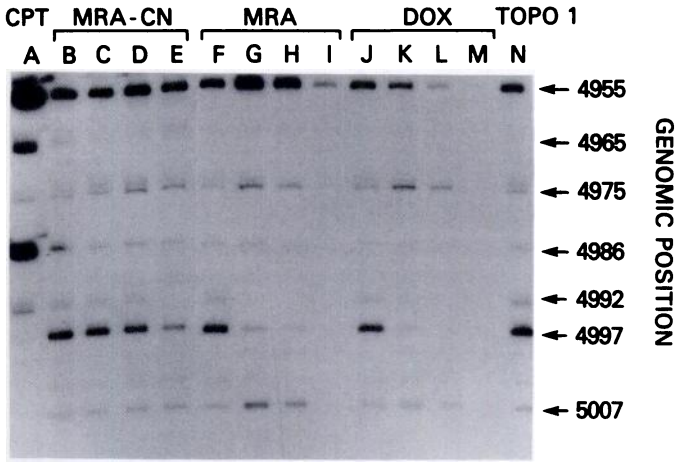


Fig. 2. DNA sequence of topoisomerase I-mediated cleavage of SV40 DNA induced by camptothecin, MRA-CN, MRA, and DOX. Reactions were performed with the 4912–5049 uniquely ^{32}P-3′-end labeled *Fok*I fragment (137 base pairs). DNA fragments were reacted with topoisomerase I in the absence or in the presence of various drugs, as described in Materials and Methods. The DNA topoisomerase I-mediated cleavage sites are marked at the *right*. Topoisomerase I reactions in the presence of drug are shown as follows: *lane A*, 10 μM camptothecin; *lanes B–E*, 0.1, 1, 3, and 10 μM MRA-CN; *lanes F–I*, 0.1, 1, 3, and 10 μM MRA; *lanes J–M*, 0.1, 1, 3, and 10 μM DOX; and *lane N*, topoisomerase I alone.

DNA intercalation. The data suggest that the stabilization of cleavage intermediates by intercalators may have a common mechanism for mammalian DNA topoisomerases I and II.

Materials and Methods

DNA, enzymes, and drugs. SV40 DNA (>90% form I), *Ban*I and *Hpa*II restriction endonucleases, and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). *Fok*I restriction endonuclease and Klenow polymerase were purchased from Biolabs and Pharmacia, respectively. Polyacrylamide and [α-^{32}P]dGTP were purchased from Bio-Rad, Inc. (Richmond, CA) and New England Research Products (Boston, MA), respectively. Autoradiography was performed with XAR-5 film (Eastman Kodak Company, Rochester, NY).

Type I and type II DNA topoisomerases were purified from mouse leukemia (L1210) cells, as described previously (16, 17).

MRA-CN, MRA, DOX, and ActD were obtained through the Developmental Therapeutics Program, Division of Cancer Treatment, Na-

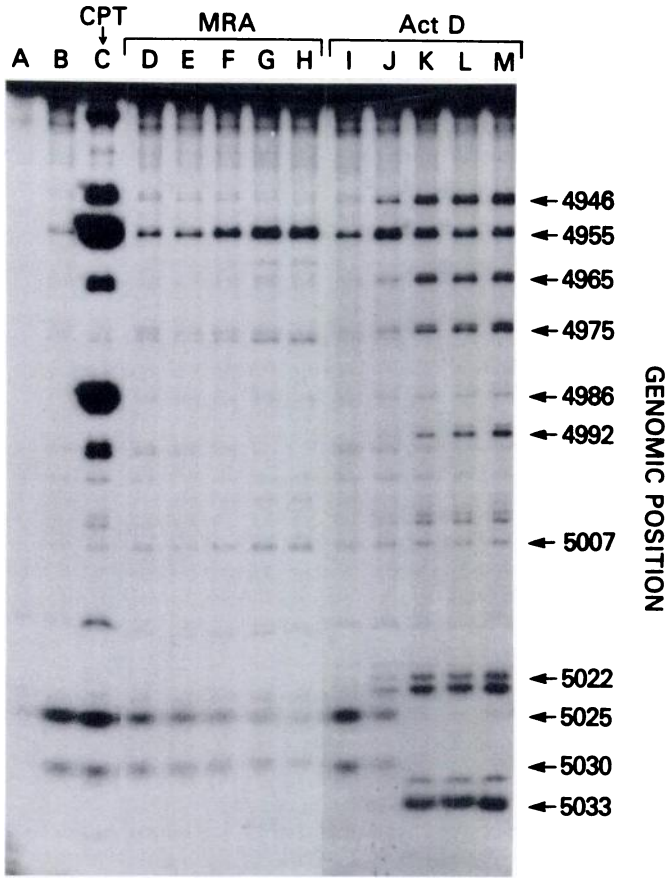


Fig. 3. Effect of camptothecin, MRA, and ActD on sequence-specific cleavages of topoisomerase I. Topoisomerase I cleavage reactions were carried out as described in Materials and Methods, in the presence or absence of various drugs. Reactions were performed with the 4912–5049 uniquely ^{32}P-3′-end labeled *Fok*I fragment (137 base pairs). The genomic positions of cleavage sites are marked at the *right*. *Lane A*, the intact fragment. Topoisomerase I reactions are shown as follows: *lane B*, topoisomerase I alone; in the presence of drug, *lane C*, 10 μM camptothecin; *lanes D–H*, 0.1, 0.2, 0.5, 1, and 2 μM MRA; and *lanes I–M*, 1, 2, 5, 10, and 20 μM ActD.

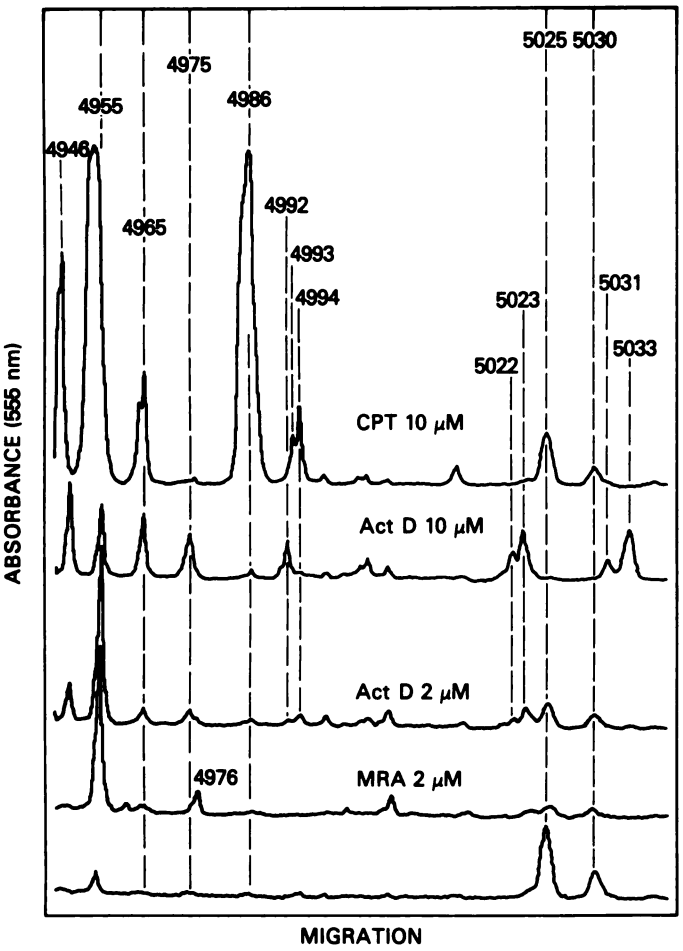


Fig. 4. Genomic localization of topoisomerase I-mediated cleavage of SV40 DNA produced in the absence and presence of camptothecin (*CPT*), ActD, and MRA. *Lanes B*, *C*, *H*, *J*, and *L* of the autoradiogram shown in Fig. 3 were scanned with a Beckman DU-8B densitometer interfaced with a computer for data collection and analysis.

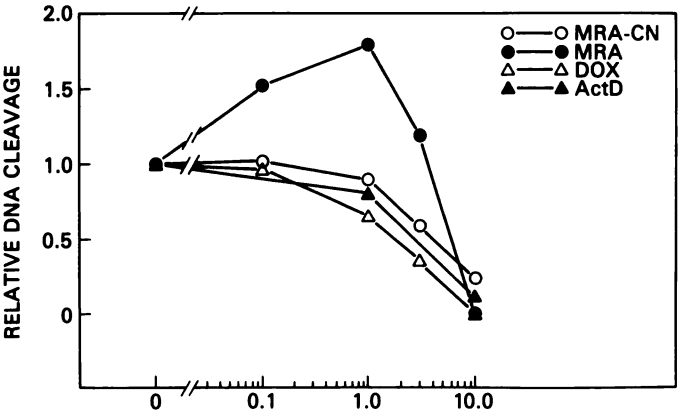


Fig. 5. Effects of MRA-CN, MRA, DOX, and ActD on DNA cleavage induced by camptothecin. Reactions were performed with ^{32}P-3′-end labeled *Ban*I-*Hpa*II fragment of SV40 DNA and the samples were electrophoresed on a 1% agarose gel, as described in Materials and Methods. DNA cleavage at the major camptothecin cleavage site of SV40 DNA (site 4955) was quantified by autoradiography scanning, using a Beckman DU-8B densitometer.

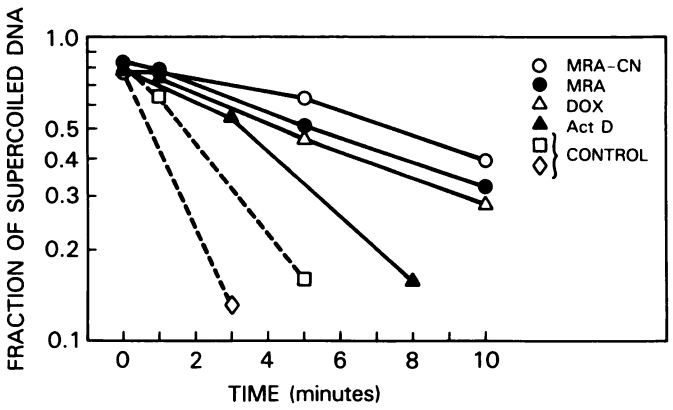


Fig. 6. Inhibition of DNA topoisomerase I catalytic activity by MRA-CN, MRA, DOX, and ActD. SV40 DNA was reacted with limited amounts of topoisomerase I in the absence (□, control for MRA-CN, MRA, and DOX; ◇, control for ActD) or presence of the drugs. Drug concentrations were 5 μM MRA-CN, 1 μM MRA; 1 μM DOX, and 2 μM ActD. Photographic negatives of agarose gels were scanned with a Beckman DU-8B densitometer and the fraction of native supercoiled SV40 DNA was calculated.

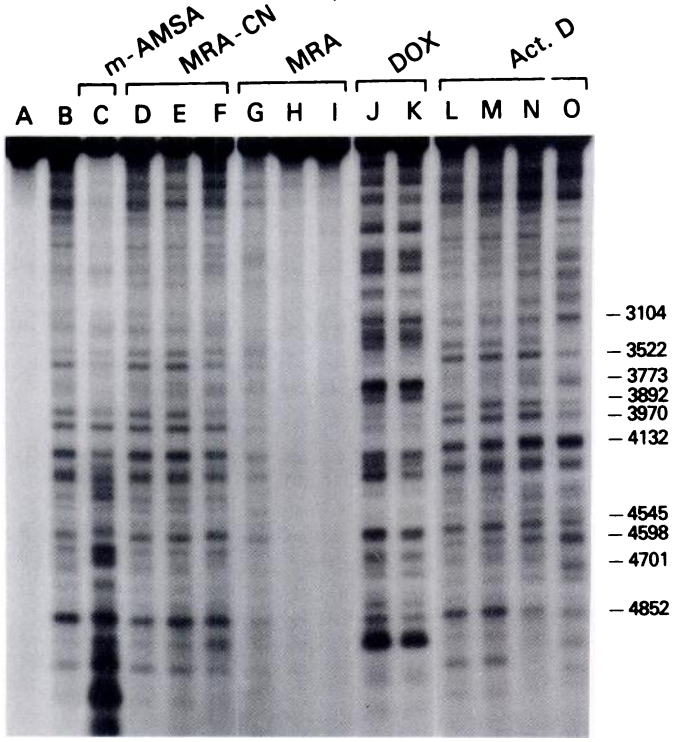


Fig. 7. Effects of MRA-CN, MRA, DOX, and ActD on topoisomerase II-mediated cleavage of SV40 DNA. The long *Ban*I-*Hpa*II SV40 DNA fragment (5191 base pairs) that had been ^{32}P-end labeled at the 3′-terminus of the *Ban*I cutting site (*lane A*, control) was reacted with topoisomerase II either alone (*lane B*) or in the presence of various drugs, as described in Materials and Methods. The DNA topoisomerase II-mediated cleavage sites are marked at the *right*. Topoisomerase II reactions in the presence of drug are shown as follows: *lane C*, 10 μM *m*-AMSA; *lanes D–F*, 0.2, 0.5, and 1 μM MRA-CN; *lanes G–I*, 0.2, 0.5, and 1 μM MRA; *lanes J* and *K*, 0.5 and 1 μM DOX; *lanes L–O*, 0,5, 1, 2, and 5 μM ActD.

tional Cancer Institute. Drugs were kept frozen at −20° as stock solutions in 95% ethanol.

Labeling procedures of SV40 DNA. ^{32}P-end labeled SV40 DNA was prepared as follows: first, the DNA was linearized with *Ban*I restriction endonuclease at position 295 of the genome and its termini were labeled with [α-^{32}P]dGTP and Klenow polymerase and then ^{32}P-end labeled DNA was cut with *Hpa*II restriction endonuclease at position 347. Such a procedure generates two ^{32}P-3′-end labeled frag-

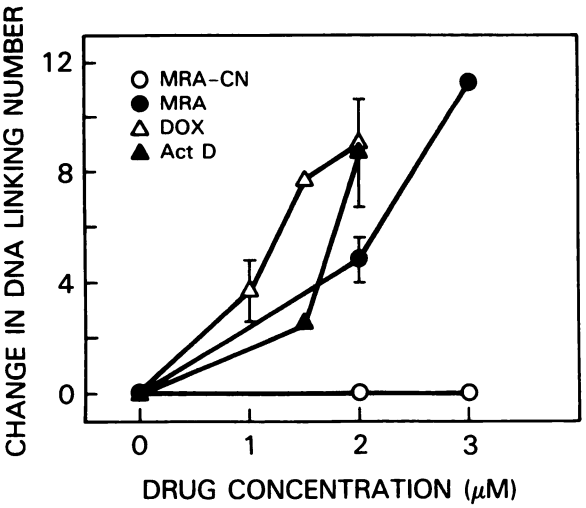


Fig. 8. DNA unwinding produced by MRA-CN, MRA, DOX, and ActD. Topoisomerase I-relaxed SV40 DNA was reacted with drugs in the presence of excess enzyme. Reactions were stopped by adding sodium dodecyl sulfate and proteinase K and were run in 1% agarose TAE buffer gels. Drug-induced DNA unwinding was determined by computing changes in DNA linking number produced by topoisomerase I (see Materials and Methods).

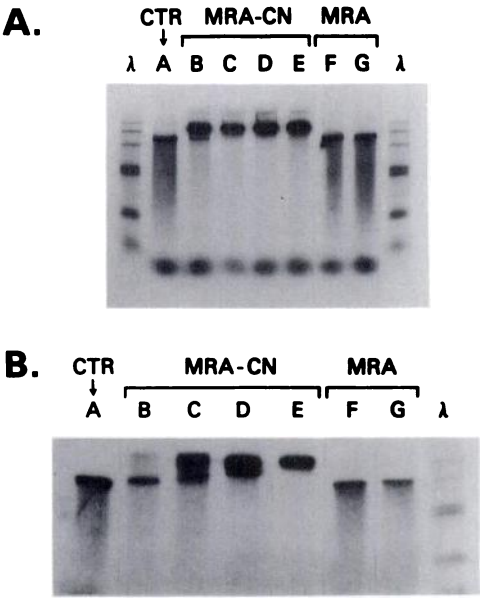


Fig. 9. DNA interstrand cross-link formation by MRA-CN. ^{32}P-5′-end labeled *Hpa*II SV40 DNA (A) or ^{32}P-5′-end labeled *Hind*III pBR322 DNA fragments (B) were incubated with MRA-CN or MRA at 37° and analyzed by agarose gel electrophoresis under denaturing conditions, as described in Materials and Methods. A, An example of dose-dependent DNA interstrand cross-link formation. *Lane A*, control; *lanes B–D*, 1, 2.5, and 5 μM MRA-CN: *lane E*, as *lane D*, without sodium dodecyl sulfate; *lanes F* and *G*, 5 and 10 μM MRA, λ, *Hind*III marker. B, The rate of DNA cross-link formation. *Lane A*, control; *lanes B–D*, reactions with 5 μM MRA-CN for 5, 10, and 30 min; *lane E*, as *lane D*, with sodium dodecyl sulfate; *lanes F* and *G*, reactions for 30 min with 0.5 μM and 5 μM MRA, respectively; λ, *Hind*III marker.

ments, one of 5191 base pairs and the other of 52 base pairs. Therefore, any DNA fragment longer than 53 base pairs could be localized unequivocally in the SV40 genome, because only one strand remains ^{32}P-labeled (18).

In order to prepare the smaller DNA fragment (137 base pairs) containing the major topoisomerase I cleavage site (18), SV40 DNA was cut with *Fok*I restriction endonuclease and 3′-end labeled with [α-^{32}P]dGTP, and the fragment between positions 4912 and 5049 of the genome was isolated by electroelution after electrophoresis on either a 6% or a 8% polyacrylamide gel. This fragment will be referred as *Fok*I DNA. Because only one of the DNA termini (nucleotide 5049) of the 4912–5049 fragment was labeled, the 4912-5049 fragment could be used directly for DNA sequencing.

Topoisomerase cleavage reactions. Reactions were performed in 30 μl of reaction buffer (0.01 M Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, 15 μg/ml bovine serum albumin), with approximately 5 ng of ^{32}P-3′-end labeled *Ban*I-*Hpa*II fragment or *Fok*I fragment and the indicated amounts of L1210 DNA topoisomerase I. DNA topoisomerase II reactions were performed in 30 μl of reaction buffer supplemented with 1 mM ATP, and ^{32}P-3′-end labeled *Ban*I-*Hpa*II fragments were reacted with excess topoisomerase II. The DNA fragments were reacted with the indicated drug concentrations at 37° for 5 min in reaction buffer in order to obtain binding equilibrium before addition of DNA topoisomerase I or II and further incubation at 37° for 30 min. Reactions were stopped by addition of sodium dodecyl sulfate and proteinase K (Merck, Darmstadt, FRG) (1% and 0.5 mg/ml final concentrations, respectively) and incubation for an additional 30 min at 37°. Samples for agarose gel electrophoresis were run into 1% gels in 1× TBE buffer (2.8 V/cm for 15 hr) after an appropriate volume of 10× loading buffer (0.3% bromophenol blue, 16% Ficoll, 0.01 M Na_2HPO_4) was added to each sample. *Fok*I DNA fragments were purified by phenol-chloroform extractions, ethanol precipitated, resuspended in Maxam Gilbert loading buffer (80% formamide, 0.1 M NaOH, 1 mM Na_2EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue), counted, and heated at 80–90° for 1–2 min before loading of equal counts onto a 6% wedge-shaped polyacrylamide sequencing gel, as described previously (18). At the end of electrophoresis, gels were transferred to 3MM paper sheets, dried, and autoradiographed with Kodak XAR-5 film. Autoradiographs were scanned with a Beckman DU-8B densitometer connected to a computer in order to store and analyze the data and to determine the DNA cleavage patterns and the genomic localization of topoisomerase-mediated DNA breaks (19).

DNA relaxation by topoisomerase I. Topoisomerase reactions were performed with 0.4 μg of native SV40 DNA in 30 μl of reaction buffer and stopped as described above. Samples were then subjected to agarose gel electrophoresis in 1% gels made in Tris-acetate-EDTA buffer (0.04 M Tris·acetate, pH 7.6, 0.01 M Na_2EDTA) containing 0.1% sodium dodecyl sulfate in order to remove drug-bound DNA that would otherwise retard DNA migration (20). Gels were run at 1 V/cm overnight, washed extensively in H_2O in order to remove sodium dodecyl sulfate, and then stained with 1 μM ethidium bromide for 45 min. After an additional 45-min destaining in 1 mM Mg_2SO_4, the DNA was visualized under UV light and photographed within the linear range of Polaroid type 55 film. Each lane of the film negative was scanned with a Beckman DU-8B densitometer interfaced with a computer in order to subtract baselines, plot the data, calculate area under selected regions of curves, and determine automatically the center of the topoisomer distribution of each densitometer tracing (21). This determination was performed according to the algorithm of Kolb and Buc (22). Briefly, it was assumed that each topoisomer distribution followed a Gaussian distribution (23, 24). For a given topoisomer distribution, the most intense band (A) was selected; its number of superhelical turns ['change in linking number' (Fig. 8)] (N_A) was calculated by the band-counting method, and its intensity (I_A) was determined as its maximum height. The same was done for the bands immediately below (B), and above (C). The superhelical density of B and C are ($N_A + 1$) and ($N_A - 1$), respectively. The superhelical density corresponding to the center of the Gaussian distribution of topoisomers was then:

$$i + N_A$$

where i was calculated as follows:

$$i = (\log I_B - \log I_C)/[2(2\log I_A - \log I_B - \log I_C)]$$

DNA interstrand cross-linking. DNA interstrand cross-link re-

TABLE 1

Topoisomerase I cleavage sites induced by Act D in the 4912–5049 *FokI* fragment of SV40 DNA.

```
                                  4946        4955        4965        4975
                                   |           |           |           |
GTTGCATCCCAGAAGCCTCCAAAGT CAGGTTGAT GAGCATATTT TACTCCATCT TCCATTTTCT

   4992                5007                5022 5023       5032 5033
    |                   |                   | |             | |
TGTACAG AGTATTCATTTTCTT CATTTTTTCTTCATC T   CCTCCTTTA T CAGGATGAAACTCCTTGCAT
```

TABLE 2

Effects of the morpholinyl anthracyclines MRA-CN and MRA, DOX, and Act D on mammalian topoisomerase I and II; relationship to DNA intercalation and DNA interstrand cross-linking

	DNA cleavage		DNA intercalation	DNA interstrand cross-linking
	Topoisomerase I	Topoisomerase II		
MRA-CN	(+)	–	–	+
MRA	+	–	+	–
DOX	–	++	++	–
Act D	+	+	++	–

actions were performed with ^{32}P-5′-end labeled *Hpa*II-*KpN*I fragments of SV40 DNA and *Hind*III-*Eco*RI fragments of pBR322 DNA in 30 μl of reaction buffer at 37° and were stopped by addition of 3 μl of alkaline loading buffer (0.5 M NaOH, 10 mM Na_2EDTA, 25% Ficoll, 0.25% bromocresol green). Samples were then subjected to alkaline agarose gel electrophoresis in 1% gels made in 1 mM Na_2EDTA, 30 mM NaOH solution. Gels were run at 1 V/cm overnight. At the end of electrophoresis, gels were transferred to 3MM paper sheets, dried, and autoradiographed with Kodak XAR-5 film.

Results

DNA cleavage induced by DNA topoisomerase I in the presence of MRA-CN, MRA, DOX and ActD; comparison with camptothecin. The *Fok*I DNA fragment selected to study the effects of the morpholinyl derivatives (MRA-CN and MRA), DOX, and ActD was chosen because it contains the major DNA topoisomerase I cleavage site induced by camptothecin in the whole SV40 genome (18).

The effects of the three anthracyclines upon topoisomerase I-mediated DNA cleavage was first studied over a wide concentration range (Fig. 2). In the absence of drug, DNA topoisomerase I produced two major cleavage sites at positions 4955 and 4997 (Fig. 2, *lane N*). Camptothecin selectively enhanced DNA cleavage at positions 4955, 4965, and 4986, whereas it suppressed cleavage at position 4997 (Fig. 2, *lane A*). This result is in agreement with previous studies (18). None of the anthracyclines induced new topoisomerase I cleavage sites but rather changed their global distribution in a dose-dependent fashion. MRA stimulated cleavage at positions 4955, 4975, and 5007 (Fig. 2, *lanes G* and *H*). Only the first of these three sites was commonly enhanced by camptothecin and MRA, whereas the last two were enhanced only by MRA. The optimum MRA concentration for cleavage was about 1–2 μM (Fig. 2, *lanes G* and *H*). Under these conditions, cleavage at position 4997 was consistently suppressed. Global suppression of topoisomerase I-mediated DNA cleavage was produced by higher MRA concentrations (Fig. 2, *lane I*). MRA-CN and DOX differed from MRA in that 1) topoisomerase I-mediated DNA cleavage was only weakly increased and 2) suppression of cleavage required lower concentrations of DOX than MRA and was only modest with MRA-CN (Fig. 2). These results indicate that MRA induced topoisomerase I-mediated DNA cleavage and suggest that, as in the case of DNA topoisomerase II (19, 20, 25), strong DNA intercalators suppress topoisomerase I-mediated DNA cleavage.

Induction of topoisomerase I-mediated DNA cleavage by MRA was analyzed further by using lower drug concentrations, and less enzyme in order to reduce background DNA cleavage, and running longer gels in order to extend the DNA sequencing range (Figs. 3 and 4). The effects of ActD were also studied. As observed in Fig. 2, MRA stimulated topoisomerase I cleavage at sites 4955, 4975, and 5007 (Fig. 3, *lanes D–H*; Fig. 4). ActD also induced DNA cleavage in the presence of DNA topoisomerase I (Fig. 3, *lanes I–M*; Fig. 4). However, MRA and ActD differed in that 1) their patterns of DNA cleavage were different and also differed from that of camptothecin (Figs. 3 and 4) and 2) no suppression of DNA cleavage was observed at high ActD concentrations (Fig. 3, *lanes L* and *M*).

Effects of MRA-CN, MRA, DOX, and ActD on DNA cleavage induced by camptothecin. The effects of the anthracycline derivatives and ActD upon camptothecin-induced DNA cleavage were studied by incubation of 3′-end labeled SV40 DNA with drugs for 5 min and then addition of camptothecin and DNA topoisomerase I to the reaction mixtures. DNA cleavage at the major camptothecin cleavage site of SV40 DNA (site 4955) was quantified by autoradiography scanning. All four drugs inhibited the production of cleavage by camptothecin at concentrations above 1–3 μM (Fig. 5). In addition, DNA cleavage was overall increased at low MRA concentrations. These concentrations were of the same order of magnitude as those required to produce maximal stimulation of topoisomerase I-mediated breaks in the absence of camptothecin (see Figs. 2 and 3).

Inhibition of DNA topoisomerase I catalytic activity by MRA-CN, MRA, DOX, and ActD. DNA topoisomerase I catalytic activity was assayed by monitoring the relaxation kinetics of native supercoiled SV40 DNA in the presence of limited amounts of enzyme (21, 26) (Fig. 6). In the absence of drug (control), DNA relaxation was first order, with a half-time of approximately 1.5 min (Fig. 6). At the early time points, the DNA was either fully relaxed or supercoiled, confirming the processive nature of the reaction (21). In the presence of MRA and ActD, enzyme catalytic activity remained first order but was partially inhibited (half-time of relaxation for MRA and ActD, 6.3 and 3 min, respectively). Inhibition of topoisomerase I catalytic activity was also produced by DOX and MRA-CN (half-lifes, 6.3 and 10 min, respectively), although neither drug induced marked DNA cleavage (Fig. 2). This observation is in agreement with results obtained with acridine derivatives

(21) and suggests that a high level of drug binding to DNA is responsible for inhibition of topoisomerase I catalytic activity.

DNA cleavage induced by DNA topoisomerase II in the presence of MRA-CN, MRA, and ActD. DOX and ActD have been shown previously to poison mammalian DNA topoisomerase II by trapping the cleavage intermediates of enzyme reactions (11). We found similar results (Fig. 7, *lanes J–O*). However, the induction of DNA cleavage by ActD was rather modest when compared with the effects of *m*-AMSA (Fig. 7, *lane C*) or DOX (Fig. 7, *lanes J* and *K*). At 1 μM ActD (Fig. 7, *lane M*), cleavage was stimulated at sites 4132, 4545, and 4852, whereas at higher drug concentrations cleavage was globally redistributed as a result of suppression of preexisting cleavage sites and selective induction of others. Five micromolar ActD suppressed cleavage at sites 3522, 3892, 3970, 4545, and 4852 and enhanced cleavage at sites 4132 and 4598. It is interesting to note that site 4132 is located in the nuclear matrix attachment region of SV40 DNA (27). Neither MRA-CN nor MRA (Fig. 6, *lanes D–I*) stimulated topoisomerase II-mediated DNA cleavage; both compounds were studied in a concentration range of 0.05–2 μM (not shown). Rather, MRA suppressed topoisomerase II-mediated DNA cleavage globally, even at concentrations as low as 0.2 μM (Fig. 7, *lane G*). In the presence of MRA-CN, only a weak suppression of DNA cleavage could be observed at 1 μM (Fig. 7, *lane F*). Suppression of topoisomerase II-mediated DNA cleavage has been found with most DNA intercalators at sufficient drug concentrations and has been attributed to high drug intercalation (19, 20, 25).

Measurement of drug intercalation into DNA. Drug intercalation was measured by using a DNA topoisomerase I unwinding assay (21) (Fig. 8). These experiments were done in the presence of excess DNA topoisomerase I, at which no detectable inhibition of the catalytic activity would influence the relaxation assay. MRA, DOX, and ActD produced drug concentration-dependent DNA topoisomer shifts indicative of changes in DNA linking number as a result of topoisomerase I action on drug-unwound DNA. MRA-CN, however, produced no detectable DNA unwinding in this assay but rather retarded the migration of relaxed DNA at high concentrations (5 μM) (not shown). Furthermore, MRA-CN did not produce DNA nicking even at 100 μM drug concentration, as evaluated from sequencing gel experiments (data not shown).

Using the topoisomerase I unwinding assay, we determined experimentally the topoisomerase I-mediated DNA linking number change (see Materials and Methods). For MRA and DOX, change of DNA linking number was approximately proportional to the drug concentration, and the DNA unwinding potency of DOX was approximately 2-fold that of its analog MRA. Although ActD also exhibited a clear unwinding of supercoiled SV40 DNA, a nonlinear relationship was consistently observed, indicative of a possible high affinity cooperative binding mode (28, 29). Thus, ActD only showed minor unwinding activity at lower concentrations, whereas it strongly unwound DNA at higher concentrations.

DNA interstrand cross-link formation by MRA-CN. Alkaline elution studies of MRA-CN-treated cells have suggested that the drug could form DNA-DNA cross-links (14). This possibility was tested directly (Fig. 9). 5′-End labeled DNA fragments were incubated with MRA-CN or MRA and analyzed by agarose gel electrophoresis under denaturing conditions. Fig. 9A shows the example of dose-dependent DNA interstrand cross-link formation by MRA-CN but not by MRA. The observed alteration in DNA migration probably measured DNA-DNA interstrand cross-link formation rather than reversible drug effects on DNA structure, because similar retardation of DNA fragments in the gel electrophoresis were observed when the samples were incubated with sodium dodecyl sulfate before electrophoresis (compare Fig. 9, *lanes D* and *E*) and because similar results have been reported independently (30). The formation of DNA interstrand cross-links was rapid and maximal within 30 min of drug incubation (Fig. 9B).

Discussion

The present study is the first demonstration that the DNA intercalator MRA inhibits mammalian DNA topoisomerase I by inducing enzyme-mediated DNA strand breaks. Thus, it is possible that the DNA single-strand breaks induced by MRA in cells (14, 31) could be due to topoisomerase I inhibition. Hence, the mechanism(s) of the antitumor activity of MRA is probably different from that of its parent compound, DOX, which acts selectively on DNA topoisomerase II (11, 32, 33). MRA appears as the second DNA intercalator after ActD (34), for which induction of topoisomerase I-mediated DNA breaks can be demonstrated. However, unlike ActD (Fig. 7 and Ref. 11), MRA did not induce topoisomerase II-mediated DNA breaks.

The inhibitory effect of MRA and ActD on topoisomerase I may be important for the preferential inhibition of ribosomal gene transcription reported for these agents (15). The following arguments support this possibility: 1) topoisomerase I is enriched in nucleoli (4), 2) topoisomerase I cleavage sites map to linker regions of ribosomal RNA genes (7) and to the DNase I hypersensitive sites flanking these genes (35), 3) topoisomerase I and RNA polymerase I have been found to be tightly complexed both *in vitro* and *in vivo* (5), 4) the topoisomerase I poison camptothecin induces a rapid cessation of the synthesis of the 45 S rRNA precursor (36), and 5) DOX, which does not induce topoisomerase I-mediated DNA breaks, has no selective inhibitory effect on rRNA transcription. Thus, our present findings suggest a possible relationship between the potent inhibitory effect of MRA and ActD on rRNA transcription and topoisomerase I poisoning by these drugs.

ActD has been shown to exhibit a binding preference for the 3′ side of guanine residues and, more selectively, to the 5′-GC dinucleotide (28). Nevertheless, ActD enhanced topoisomerase I-mediated DNA breaks at some distance from GC sequences (Fig. 3; Table 1). It is possible that binding of ActD to GC sequences could result in propagated DNA structural alterations (37), which would stabilize topoisomerase I-DNA complexes nearby. A comparable effect on the modulation of topoisomerase II cleavage sites by distamycin has recently been reported and interpreted as resulting from a propagated DNA distortion (38). The enhancement of nearby topoisomerase I-mediated DNA cleavage when ActD is present may also be explained by a nonclassical binding of ActD (29) to sequences other than GC. Using the topoisomerase I unwinding assay, ActD exhibited a nonlinear pattern of DNA unwinding (Fig. 8), suggesting cooperative binding. This result is in agreement with several studies demonstrating positive cooperativity for the binding of ActD to DNA (28, 29), without the requirement of GC sequences (29).

DNA intercalation is clearly not sufficient for poisoning of

topoisomerase I and II complexes. Although both MRA and DOX appeared to unwind DNA similarly, only MRA, and not DOX, stimulated topoisomerase I-mediated cleavage. Whether the presence of a bulky morpholinyl substituent on the daunosamine sugar of DOX could interfere with topoisomerase I remains to be determined. The analysis is further complicated by the finding that substitution with an α-cyano group on the morpholinyl moiety in MRA-CN resulted in a decreased ability of this derivative to intercalate into DNA and to poison topoisomerase I. In fact, the DNA interactions of MRA-CN appear very different from those of MRA and DOX (Table 2). The presence of a cyano substituent on the morpholinyl moiety of MRA induces the formation of DNA-DNA cross-links in purified DNA (Fig. 9 and Ref. 30). Nevertheless, a consistent finding for all intercalators studied so far is the suppressed topoisomerase I-mediated DNA cleavage at sufficiently high drug concentrations. This was seen for DOX, MRA, and ActD in the present study and had been reported previously for acridine derivatives (21). Probably, at high drug concentrations, DNA intercalation suppresses topoisomerase I-mediated DNA cleavage by modifying the DNA substrate. Clearly, it appears that DNA-intercalating agents can inhibit topoisomerase I activity by two different mechanisms, 1) poisoning the enzyme by trapping it on the DNA as a cleavable complex and 2) inhibiting the enzyme from ever binding to DNA when drug concentrations are high enough. These effects would be consistent with those of DNA intercalators upon topoisomerase II.

In summary, our studies show that some antitumor intercalators are not only inhibitors of DNA topoisomerase II but act also on topoisomerase I. This may suggest that stabilization of cleavage intermediates by some intercalators may have a common mechanism for DNA topoisomerase I and DNA topoisomerase II, which may strongly depend on their structure and interaction specificity with the DNA.

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References

1. Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **58**:351–375 (1989).
2. Wang, J. C. Recent studies of DNA topoisomerases. *Biochim. Bipohys. Acta.* **909**:1–9, (1987).
3. Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **54**:665–697 (1985).
4. Muller, M. T., W. P. Pfund, V. B. Mehta, and D. K. Trask. Eukaryotic type I topoisomerase is enriched in the nucleolus and catalytically active on ribosomal DNA. *EMBO J.* **4**:1237–1243 (1985).
5. Rose, K. M., J. Szopa, F. S. Han, Y. C. Cheng, A. Richter, and U. Scheer. Association of DNA topoisomerase I and RNA polymerase I: a possible role for topoisomerase I in ribosomal gene transcription. *Chromosoma (Berl.)* **96**:411–416 (1988).
6. Fleischmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, J. C. Wang, and S. C. Elgin. *Drosophila* DNA topoisomerase I is associated with transcriptionally active regions of the genome. *Proc. Natl. Acad. Sci. USA* **81**:6958–6962 (1984).
7. Culotta, V., and B. Sollner-Webb. Sites of topoisomerase I action on *X. laevis* ribosomal chromatin: transcriptionally active rDNA has an approximately 200 bp repeating structure. *Cell* **52**:585–597 (1988).
8. Tsao, Y. P., H. Y. Wu, and L. F. Liu. Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. *Cell* **56**:111–118 (1989).
9. Quigley, G. J., A. H. Wang, G. Ughetto, G. Van der Marel, J. H. Van Boom, and A. Rich. Molecular structure of an anticancer drug-DNA complex: daunomycin plus d (CpGpTpApCpG). *Proc. Natl. Acad. Sci USA* **77**:7204–7208 (1980).
10. Ross, W. E., D. L. Glaubiger, and K. W. Kohn. Protein-associated DNA breaks in cells treated with Adriamycin or ellipticine. *Biochim. Biophys. Acta* **519**:23–30 (1978).
11. Tewey, K. M., T. C. Rose, L. Yang, B. D. Halligan, and L. F. Liu. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science (Wash. D. C.)* **226**:466–468 (1984).
12. Sikic, B. I., M. N. Ehsan, W. J. Harker, N. F. Friend, B. W. Brown, R. A. Newman, M. P. Hacker, and E. M. Acton. Dissociation of antitumor potency from anthracycline cardiotoxicity in a doxorubicin analog. *Science (Wash. D. C.)* **228**:1544–1546 (1985).
13. Acton, E. M., G. L. Tong, C. W. Mosher, and R. L. Wolgemuth. Intensely potent morpholinyl anthracyclines. *J. Med. Chem.* **27**:639–645 (1984).
14. Wassermann, K., L. A. Zwelling, T. D. Mullins, L. E. Silberman, B. S. Andersson, M. Bakic, E. M. Acton, and R. A. Newman. Effects of 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin and doxorubicin on the survival, DNA integrity, and nucleolar morphology of human leukemia cells *in vitro*. *Cancer. Res.* **46**:4041–4046 (1986).
15. Wassermann, K., R. A. Newman, F. M. Davis, T. D. Mullins, and K. M. Rose. Selective inhibition of human ribosomal gene transcription by the morpholinyl anthracyclines cyanomorpholinyl- and morpholinyldoxorubicin. *Cancer. Res.* **48**:4101–4106 (1988).
16. Minford, J., Y. Pommier, J. Filipski, K. W. Kohn, D. Kerrigan, M. Mattern, S. Michaels, R. Schwartz, and L. A. Zwelling. Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**:9–16 (1986).
17. Pommier, Y., D. Kerrigan, R. E. Schwartz, J. A. Swack, and A. McCurdy. Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer. Res.* **46**:3075–3081 (1986).
18. Jaxel, C., K. W. Kohn, and Y. Pommier. Topoisomerase I interaction with SV40 DNA in the presence and absence of camptothecin. *Nucleic Acids Res.* **16**:11157–11170 (1988).
19. Pommier, Y., J. Covey, D. Kerrigan, W. Mattes, J. Markovits, and K. W. Kohn. Role of DNA intercalation in the inhibition of purified mouse leukemia (L1210) DNA topoisomerase II by 9-aminoacridines. *Biochem. Pharmacol.* **36**:3477–3486 (1987).
20. Pommier, Y., J. K. Minford, R. E. Schwartz, L. A. Zwelling, and K. W. Kohn. Effects of the DNA intercalators 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and 2-methyl-9-hydroxyellipticinium on topoisomerase II mediated DNA strand cleavage and strand passage. *Biochemistry* **24**:6410–6416 (1985).
21. Pommier, Y., J. M. Covey, D. Kerrigan, J. Markovits, and R. Pham. DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators. *Nucleic Acids Res.* **15**:6713–6731 (1987).
22. Kolb, A., and H. Buc. Is DNA unwound by the cyclic AMP receptor protein? *Nucleic Acids Res.* **10**:473–485 (1982).
23. Depew, R. E., and J. C. Wang. Conformational fluctuations of DNA helix. *Proc. Natl. Acad. Sci. USA* **72**:4275–4279 (1975).
24. Richardson, S. M. H., T. C. Boles, and N. R. Cozzarelli. The helical repeat of underwound DNA in solution. *Nucleic Acids Res.* **16**:6607–6616 (1988).
25. Tewey, K. M., G. L. Chen, E. M. Nelson, and L. F. Liu. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J. Biol. Chem.* **259**:9182–9187 (1984).
26. Jaxel, C., K. W. Kohn, M. C. Wani, M. E. Wall, and Y. Pommier. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer. Res.* **49**:1465–1469 (1989).
27. Pommier, Y., P. N. Cockerill, K. W. Kohn, and W. Garrard. Identification within the simian virus 40 genome of a chromosomal loop attachment site that contains topoisomerase II cleavage sites. *J. Virol.* **64**:419–423 (1990).
28. White, R. J., and D. R. Phillips. Transcriptional analysis of multisites drug-DNA dissociation kinetics: delayed termination of transcription by actinomycin D. *Biochemistry* **27**:9122–9132 (1988).
29. Snyder, J. G., N. G. Hartman, B. Langlois d'Estantoit, O. Kennard, D. P. Remeta, and K. J. Breslauer. Binding of actinomycin D to DNA: evidence for a nonclassical high affinity binding mode that does not require GpC sites. *Proc. Natl. Acad. Sci. USA* **86**:3968–3972 (1989).
30. Jesson, M. I., J. B. Johnston, E. Robotham, and A. Begleiter. Characterization of the DNA-DNA cross-linking activity of 3′-(cyano-4-morpholinyl)-3′-deaminoadriamycin. *Cancer. Res.* **49**:7031–7036 (1989).
31. Jesson, M. I., J. B. Johnson, C. D. Anhalt, and A. Begleiter. Effects of 3′-(3-cyano-4-morpholinyl)-3′-deaminoadriamycin and structural analogues on DNA in HT-29 human colon carcinoma cells. *Cancer. Res.* **47**:5935–5938 (1987).
32. Capranico, G., F. Zunino, K. W. Kohn, and Y. Pommier. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA affinity and cytotoxicity. *Biochemistry* **29**:562–569 (1990).
33. Lau, D. H., A. D. Lewis, and B. I. Sikic. Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. *J. Natl. Cancer Inst.* **81**:1034–1038 (1989).
34. Trask, D. K., and M. T. Muller. Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc. Natl. Acad. Sci. USA* **85**:1417–1421 (1988).
35. Bonven, B. J., E. Gocke, and O. Westergaard. A high affinity topoisomerase I binding sequence is clustered at DNAse I hypersensitive sites in *Tetrahymena* R-chromatin. *Cell* **41**:541–551 (1985).
36. Zhang, H., J. C. Wang, and L. F. Liu. Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes. *Proc. Natl. Acad. Sci. USA* **85**:1060–1064 (1988).
37. Huang, Y.-Q., R. P. Rehfuss, S. R. LaPlante, E. Boudreau, P. N. Borer, and M. J. Lane. Actinomycin D-induced DNase I cleavage enhancement caused

by sequence-specific propagation of an altered DNA structure. *Nucleic Acids Res.* **16**:11125–11139 (1988).
38. Fesen, M., and Y. Pommier. Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamycin in simian virus 40 DNA. *J. Biol. Chem.* **19**:11354–11359 (1989).

Send reprint requests to: Yves Pommier, Laboratory of Molecular Pharmacology, Building 37, Room 5C27, National Cancer Institute, NIH, Bethesda, MD 20892.