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# 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

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ABSTRACT: DNA topoisomerase II is believed to be the enzyme that produces the protein-associated DNA strand breaks observed in mammalian cell nuclei treated with various intercalating agents. Two intercalators—4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, amsacrine) and 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$)—differ in their effects on protein-associated double-strand breaks in isolated nuclei. *m*-AMSA stimulates their production at all concentrations, whereas 2-Me-9-OH-E$^+$ stimulates at low concentrations and inhibits at high concentrations. We have reproduced these differential effects in experiments carried out in vitro with purified L1210 DNA topoisomerase II, and we have found that concentrations of 2-Me-9-OH-E$^+$ above 5 $\mu$M prevent the trapping of DNA–topoisomerase II cleavable complexes irrespective of the presence of *m*-AMSA. It also stimulated topoisomerase II mediated DNA strand passage, again with or without inhibitory amounts of *m*-AMSA (this result suggests that extensive intercalation by 2-Me-9-OH-E$^+$ destabilized the cleavable complexes). From these data, it is concluded that intercalator-induced protein-associated DNA strand breaks observed in intact eukaryotic cells and isolated nuclei are generated by DNA topoisomerase II and that intercalators can affect mammalian DNA topoisomerase II in more than one way. They can trap cleavable complexes and inhibit DNA topoisomerase II mediated DNA relaxation (*m*-AMSA and low concentrations of 2-Me-9-OH-E$^+$) or destabilize cleavable complexes and stimulate DNA relaxation (high concentrations of 2-Me-9-OH-E$^+$).

Intercalator-induced DNA breaks in mammalian cells (Ross et al., 1979; Zwelling et al., 1981; Pommier et al., 1984a,b) and in isolated nuclei (Pommier et al., 1982, 1984c,d) have been postulated to result from altered DNA topoisomerase II action. Results obtained with purified DNA topoisomerase II are in agreement with such a hypothesis. Intercalators stimulate topoisomerase II induced DNA strand breaks and DNA–protein links within single topoisomerase II–DNA complexes (Nelson et al., 1984; Tewey et al., 1984a,b; Minford et al., 1984). These "cleavable complexes" (Liu et al., 1983) are analogous to the protein-associated DNA breaks induced by intercalators in cells, in that (1) enzyme molecules are bound to the 5′ termini of both DNA strands at the break site (Liu et al., 1983; Sander & Hsieh, 1983; Marshall et al., 1983) and (2) DNA strands are prevented from swiveling at the break site, presumably by bound DNA topoisomerase II (Liu et al., 1983; Pommier et al., 1984a,b). Further evidence that intercalator-induced protein-associated DNA breaks observed in mammalian cells correspond to DNA topoisomerase II–DNA cleavable complexes is that the intercalator-dependent DNA–protein-linking activity from mouse leukemia (L1210) nuclei has been purified and identified as DNA topoisomerase II (Minford et al., 1984).

In the preceding paper (Pommier et al., 1985), we report that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA, amsacrine) at all concentrations stimulated the formation of DNA double-strand breaks, whereas 2-methyl-9-

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hydroxyellipticinium (2-Me-9-OH-E$^+$) stimulated their formation at low concentrations and inhibited it at high concentrations in isolated mouse leukemia (L1210) cell nuclei. We postulated that, depending on concentration, intercalators could trap DNA topoisomerase II in the cleavable complex configuration or prevent such trapping.

We now report the results of similar experiments carried out in vitro with *m*-AMSA and 2-Me-9-OH-E$^+$, using DNA topoisomerase II purified from L1210 cells (Minford et al., 1984). Since the effects of both 2-Me-9-OH-E$^+$ and *m*-AMSA on the formation of cleavable complexes by DNA topoisomerase II were analogous to those seen in isolated nuclei, the protein-associated DNA breaks produced by intercalators in mammalian cells are likely to result from altered DNA topoisomerase II action.

## Materials and Methods

*Enzymes, SV40 DNA, and Antitumor Drugs.* Mouse leukemia (L1210) DNA topoisomerase II and I were purified as described previously (Minford et al., 1984) and stored frozen at −20 °C in 30% glycerol. Supercoiled SV40 DNA (>95% form I) was purchased from Bethesda Research Laboratories (Bethesda, MD). Supercoiled $^3$H-labeled SV40 DNA (>85% form I; $2.2 \times 10^4$ dpm/μg) was purified according to the extraction procedure of Hirt (1967). 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) (NSC 249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI. *m*-AMSA stock solution was 10 mM in dimethyl sulfoxide. 2-Methyl-9-hydroxyellipticinium (2-Me-9-OH-E$^+$) was a gift from Dr. J. B. Le Pecq, Institut Gustave Roussy, Villejuif, France. 2-Me-9-OH-E$^+$ stock solution was 10 mM in glass-distilled water. Both *m*-AMSA and 2-Me-9-OH-E$^+$ stock solutions were stored frozen at −20 °C.

*Reaction Conditions for DNA Topoisomerase II Mediated DNA Cleavage and DNA–Protein Binding.* Reactions were performed in mixtures containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 15 μg/mL bovine serum albumin, pH 7.4. SV40 DNA (0.4 μg in the DNA cleavage reactions) or $^3$H-labeled SV40 DNA (40 ng in the DNA–protein binding reactions) were added first, followed by the drugs (*m*-AMSA or 2-Me-9-OH-E$^+$ or both) and, immediately thereafter, by topoisomerase II (260 ng unless otherwise indicated). Reaction volumes were 40 and 100 μL, respectively, in the DNA cleavage and the DNA–protein binding reactions.

*DNA Topoisomerase II Binding to DNA. Filter Binding Assay.* To measure total DNA binding of topoisomerase II, we used $^3$H-labeled SV40 DNA in a filter binding assay derived from that of Minford et al. (1984). It utilizes the property of poly(vinyl chloride) filters to retain protein-bound DNA in the presence of sarkosyl and 2 M NaCl, without retaining free DNA (Kohn et al., 1981). After DNA topoisomerase II reactions were stopped by a 10-fold dilution in 20 mM EDTA (pH 10) at 4 °C, each reaction mixture was deposited onto a poly(vinyl chloride) filter mounted in a Swinnex holder (Millipore Corp., Bedford, MA) (Kohn et al., 1981). Two milliliters of 20 mM EDTA (pH 10) at 4 °C was then used to rinse the filter, and the solution that was collected by gravity (EDTA fraction) was assayed for $^3$H radioactivity by liquid scintillation spectrometry. Three milliliters of LS10 (0.2% sarkosyl, 40 mM $Na_2EDTA$, 2 M NaCl, pH 10) was then deposited onto the filter and allowed to drip out. The LS10 fraction was collected and assayed for $^3$H radioactivity. Filters were processed as described previously (Kohn et al., 1981). The total binding of topoisomerase II to DNA was evaluated as

$$\text{total binding} = \frac{[\text{filter}] + [\text{LS10 fraction}]}{[\text{filter}] + [\text{LS10 fraction}] + [\text{EDTA fraction}]}$$

where [filter], [LS10 fraction], and [EDTA fraction] represent the $^3$H disintegrations per minute of the filter, the LS10 fraction, and the EDTA fraction, respectively. In the absence of any topoisomerase II, the background value for total binding was approximately 20%. Cleavable complex formation was quantified by determining the covalent binding of topoisomerase II to $^3$H-labeled SV40 DNA, evaluated as

$$\text{covalent binding} = \frac{[\text{filter}]}{[\text{filter}] + [\text{LS10 fraction}] + [\text{EDTA fraction}]}$$

In the absence of topoisomerase II the background value was less than 2%.

*DNA Topoisomerase II Mediated DNA Cleavage.* DNA topoisomerase II reactions were stopped by adding sodium dodecyl sulfate (SDS) (final concentration, 0.5%) and proteinase K (final concentration, 0.5 mg/mL). Reaction mixtures were then run into 1% agarose gels at 2 V/cm overnight. The agarose electrophoresis running buffer (40 mM Tris–acetate, 10 mM $Na_2EDTA$, pH 7.6) contained 0.1% SDS, in order to dissociate intercalator molecules from DNA. In the absence of SDS, 2-Me-9-OH-E$^+$ (but not *m*-AMSA) retarded the migration of SV40 DNA at drug concentrations greater than 5 μM. Gels were stained with 2 μM ethidium bromide (final concentration) after the SDS had been removed by three or four washes with $H_2O$. After being stained for 45–60 min, the gels were destained in 1 mM $MgSO_4$ for 5–12 h and photographed.

*DNA Topoisomerase II Mediated DNA Relaxation.* Reactions were carried out in the presence of 1 mM ATP (final concentration) and stopped with 0.5% SDS and 0.5 mg/mL proteinase K (final concentration). Agarose gel electrophoresis (see above) was used to visualize DNA topoisomers.

## Results

*Effects of m-AMSA.* *m*-AMSA stimulated the formation of presumed covalent complexes between SV40 DNA and purified DNA topoisomerase II (Figure 1). Enzyme–DNA binding was assayed by the retention of radioactive SV40 DNA on protein-adsorbing filters. The binding was probably covalent because most of the filter retention of DNA resisted 2 M NaCl and 0.2% sarkosyl.

*Effects of 2-Me-9-OH-E$^+$.* Low concentrations (up to 1 μM) of 2-Me-9-OH-E$^+$ stimulated the covalent binding of DNA topoisomerase II to SV40 DNA, but high concentrations (greater than 2 μM) inhibited binding (Figure 2). At 5 μM and above, the inhibitory effect could be seen as a lower DNA filter retention than in the absence of drug. At concentrations of 20–40 μM, although there was no covalent binding, 2-Me-9-OH-E$^+$ stimulated noncovalent binding of $^3$H-labeled SV40 DNA (Figure 2). This stimulatory effect was not observed in the absence of DNA topoisomerase II or with bovine serum albumin, which suggests that high concentrations of 2-Me-9-OH-E$^+$ enhance specifically the noncovalent binding of the enzyme to DNA while inhibiting the formation of cleavable complexes.

*Inhibition of m-AMSA-Induced Cleavable Complexes by 2-Me-9-OH-E$^+$.* We then asked whether 2-Me-9-OH-E$^+$ could inhibit not only the cleavable complexes formed by DNA

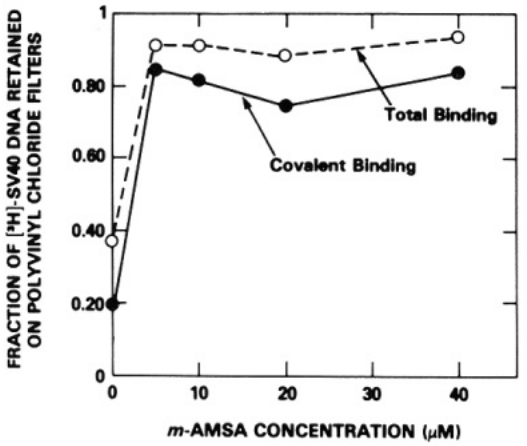


FIGURE 1: Stimulation of binding of topoisomerase II to SV40 DNA by *m*-AMSA. Reaction mixtures (100 μL) contained 260 ng of topoisomerase II, 40 ng of $^3$H-labeled SV40 DNA, and various concentrations of *m*-AMSA; incubation was for 30 min at 37 °C. Reactions were stopped by 20-fold dilution in 20 mM EDTA (pH 10) at 4 °C. Total binding (○) was assayed as the fraction of [$^3$H]DNA retained on a filter after a 2-mL wash with 20 mM EDTA (pH 10). Covalent binding (●) was assayed as the fraction retained after a 3-mL wash of the filter with 2 M NaCl, 0.2% sarkosyl, and 0.02 M EDTA, pH 10 (LS10).

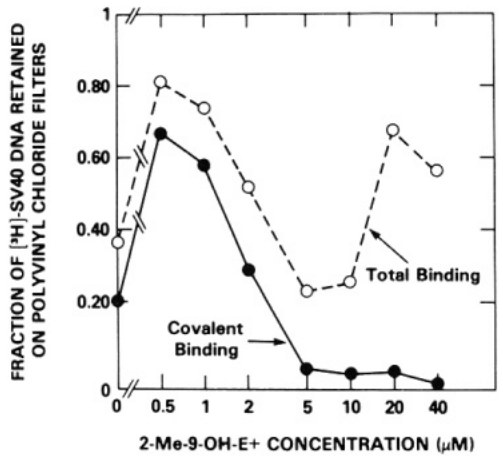


FIGURE 2: Effect of 2-Me-9-OH-E$^+$ on topoisomerase II binding to SV40 DNA. Reaction conditions as in Figure 1 except that 2-Me-9-OH-E$^+$ was used instead of *m*-AMSA. ○, total binding; ●, covalent binding.

topoisomerase II alone or DNA topoisomerase II in the presence of low concentrations of 2-Me-9-OH-E$^+$ (Tewey et al., 1984a) but also those trapped by *m*-AMSA (Pommier et al., 1985). More DNA topoisomerase II was used in the reactions than in the previous experiments, which led to the covalent retention of 75% of SV40 DNA to poly(vinyl chloride) filters in the absence of any drug (Figure 3). *m*-AMSA increased this retention to 90%. Increasing concentrations of 2-Me-9-OH-E$^+$ inhibited the cleavable complexes formed in both the absence and presence of *m*-AMSA (Figure 3) while stimulating the noncovalent binding of the enzyme to DNA. More than 5 μM 2-Me-9-OH-E$^+$ was required for inhibition. This is more than is observed in Figure 2, where 5 μM was enough to inhibit completely the cleavable complex formation.

Parallel experiments were performed in agarose gel electrophoresis (Figure 4). Topoisomerase II induced DNA double-strand breaks in the absence of any drug (lane 3). This result is consistent with those obtained in the filter binding assay (Figure 3). *m*-AMSA stimulated the formation of cleavable complexes (Liu et al., 1983), observed as DNA single- and double-strand breaks when the reaction was stopped

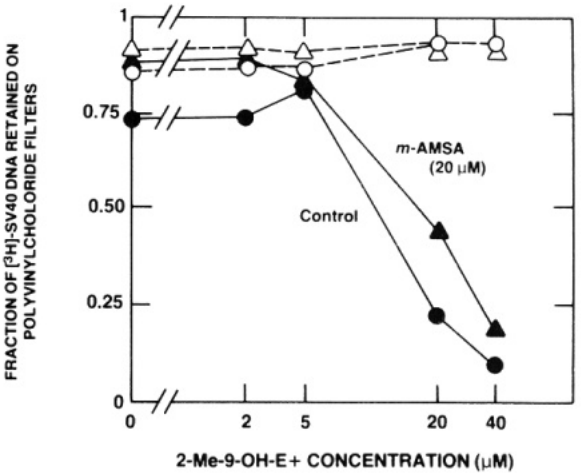


FIGURE 3: Inhibition by 2-Me-9-OH-E$^+$ of the formation of cleavable complexes in the interaction of topoisomerase II with SV40 DNA in the absence or presence of *m*-AMSA. Reaction mixtures (100 μL) contained 520 ng of topoisomerase II, 40 ng of $^3$H-labeled SV40 DNA, and various concentrations of 2-Me-9-OH-E$^+$ in the absence (●, ○) or presence of 20 μM *m*-AMSA (▲, △). Reaction conditions as in Figure 1. ○ and △, total binding; ● and ▲, covalent binding.

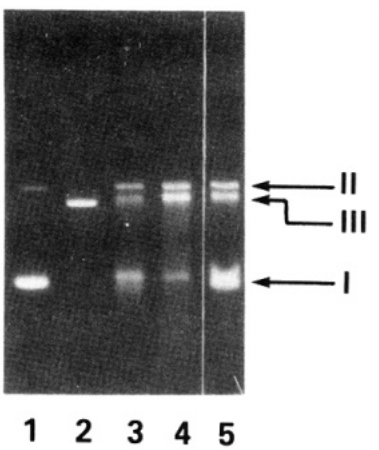


FIGURE 4: Inhibition by 2-Me-9-OH-E$^+$ of the formation of cleavable complexes in the interaction of topoisomerase II with SV40 DNA in the absence of added ATP and in the absence or presence of *m*-AMSA. Reaction mixtures (40 μL) contained 520 ng of topoisomerase II, 0.4 μg of SV40 DNA (lane 3), and 20 μM *m*-AMSA in lanes 4 and 5. Ten micromolar 2-Me-9-OH-E$^+$ was added to the reaction mixture just before *m*-AMSA in the reaction mixture of lane 5. Reactions were performed for 30 min at 37 °C and stopped by adding 0.5% SDS and 5 mg/mL proteinase K (final concentrations). Lane 1, untreated SV40 DNA; lane 2, SV40 DNA linearized with *Eco*RI. Electrophoresis was in 1% agarose gel containing 0.1% SDS.

with SDS (lane 4). Single-strand breaks generate relaxed circles ("form II"). This band was identified as nicked circles rather than relaxed closed circles by running a second-dimension electrophoresis in the presence of ethidium bromide (data not shown). A double-strand break would generate a linear full-length molecule ("form III"). In the presence of *m*-AMSA, most of the DNA complexed to DNA topoisomerase II was recovered as nicked circles or full-length linear molecules in approximately equal proportions (Figure 4, lane 4). Some of the DNA molecules probably contained two or more double-strand cuts, producing a smear running ahead of the linear bands in Figure 4. 2-Me-9-OH-E$^+$ inhibited the cleavage of supercoiled SV40 DNA produced by DNA topoisomerase II in the presence of *m*-AMSA (Figure 4, compare lanes 4 and 5). This result is in agreement with those obtained in isolated L1210 cell nuclei (Pommier et al., 1985). The inhibition of cleavable complex production by high

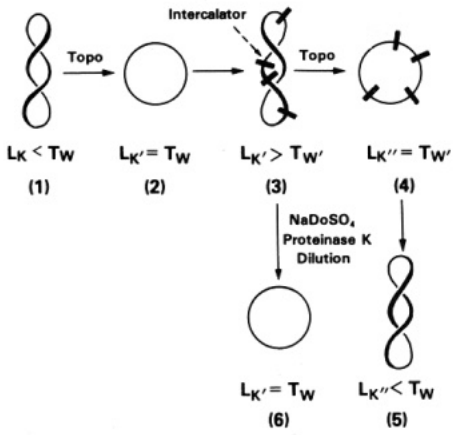


FIGURE 5: The principle of the relaxation assay of DNA by DNA topoisomerases. (1) Negatively supercoiled SV40 DNA (Lk < Tw) was reacted first with either DNA topoisomerase II or DNA topoisomerase I (topo) for 15 min. (2) Lk was thereby increased to Lk′, and the DNA relaxed (Lk′ = Tw). (3) Various concentrations of either *m*-AMSA or 2-Me-9-OH-E$^+$ were then added for the next 15 min. Intercalation reduces the twist to Tw′ and induces positive supercoiling (Lk′ > Tw′). (4) In the case of topoisomerase action, DNA supercoiling is removed by a decrease of Lk′ to Lk″ (Lk″ = Tw′). (5) Simultaneous removal of intercalators and enzyme by SDS, dilution, and proteinase K brings back Tw′ to its initial value Tw, while Lk″ remains unchanged. This results in DNA negative supercoiling (Lk″ < Tw). (6) In the case of topoisomerase inhibition, no adjustment of Lk′ occurs (inhibition of step 4), and simultaneous removal of intercalators and enzyme leads to relaxed DNA (Lk′ = Tw). Reactions were performed with 0.4 μg of SV40 DNA. ATP was added to the reaction mixtures containing DNA topoisomerase II. Lk, linking number; Tw, average DNA twist.

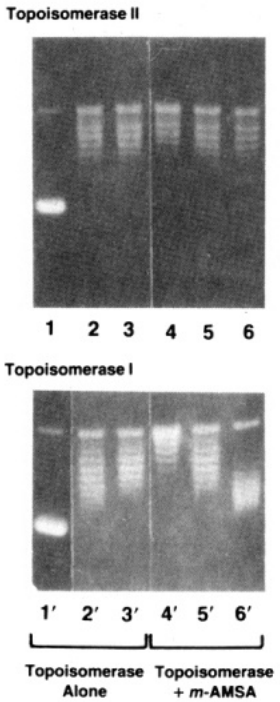


FIGURE 6: Comparative effect of *m*-AMSA on topoisomerase II and topoisomerase I induced DNA relaxation. Reaction mixtures (40 μL) contained 0.4 μg of SV40 DNA, 1 mM ATP, and either 130 ng of topoisomerase II (upper panel, lanes 2–6) or 130 ng of topoisomerase I (lower panel, lanes 2′–6′) at 37 °C. After 15 min of reaction (lanes 2 and 2′) the following concentrations of *m*-AMSA (μM) were added: lanes 3 and 3′, 0; lanes 4 and 4′, 50; lanes 5 and 5′, 80; lanes 6 and 6′, 120. Incubation was for an additional 15 min, and reactions were stopped with 0.5% SDS and 0.5 μg/mL proteinase K (final concentration). Electrophoresis was in 1% agarose gel with 0.1% SDS.

concentrations of 2-Me-9-OH-E$^+$ could result from either (1) a specific destabilization of cleavable complexes without inhibition of enzyme function or (2) an overall inhibition of enzyme binding to DNA due to a high level of intercalation and alteration of the DNA target. Our finding that high concentrations of 2-Me-9-OH-E$^+$ increased noncovalent enzyme-DNA binding while inhibiting cleavable complex detection seems to favor the former mechanism. To investigate

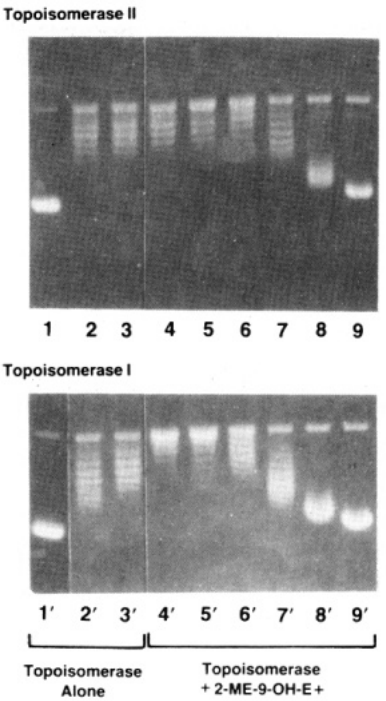


FIGURE 7: Comparative effect of 2-Me-9-OH-E$^+$ on topoisomerase II and topoisomerase I induced DNA relaxation. Reaction conditions were as in Figure 6 except that 2-Me-9-OH-E$^+$ was used instead of *m*-AMSA. Topoisomerase II, upper panel, lanes 2–9; topoisomerase I, lower panel, lanes 2′–9′. After 15 min of reaction (lanes 2 and 2′) the following concentrations of 2-Me-9-OH-E$^+$ (μM) were added: lanes 3 and 3′, 0; lanes 4 and 4′, 0.5; lanes 5 and 5′, 0.8; lanes 6 and 6′, 1.25; lanes 7 and 7′, 2; lanes 8 and 8′, 3.2; lanes 9 and 9′, 5. Incubation was for an additional 15 min, and reactions were stopped with 0.5% SDS and 0.5 μg/mL proteinase K (final concentration). Electrophoresis was in 1% agarose gel with 0.1% SDS.

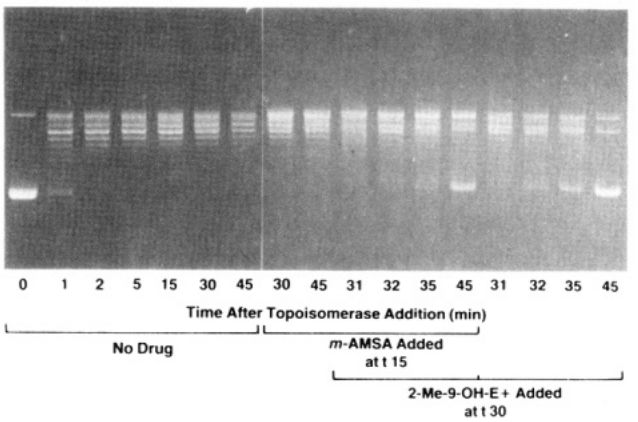


FIGURE 8: Effects of 2-Me-9-OH-E$^+$ upon topoisomerase II mediated DNA strand passage in the absence or presence of *m*-AMSA. Native SV40 DNA was first reacted with topoisomerase II (1 mM ATP, 37 °C). After 15 min, the reaction mixture was divided into two tubes containing either no drug or 20 μM *m*-AMSA (final concentration). Fifteen minutes later, each of these tubes was divided again into two tubes containing either 5 μM 2-Me-9-OH-E$^+$ (final concentration) or no 2-Me-9-OH-E$^+$. The kinetics of the effects of topoisomerase II upon SV40 in the absence or presence of intercalators was monitored by taking reaction samples to 0.5% SDS–5 mg/mL proteinase K (final concentrations). The numbers under the electrophoresis lanes are the times (in minutes) at which reaction samples were taken. Electrophoresis was in 1% agarose gel with 0.1% SDS.

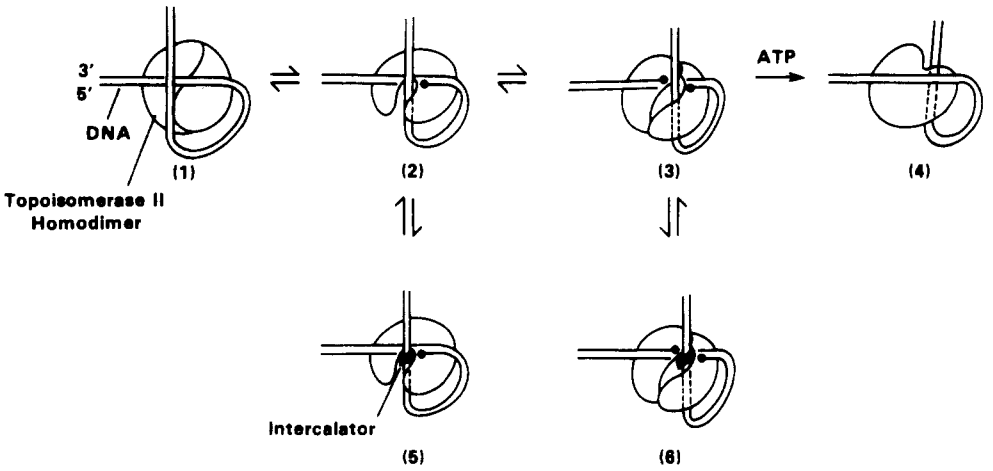


FIGURE 9: Possible interaction between DNA intercalators and DNA topoisomerase II. The normal DNA strand passing reaction of topoisomerase II is represented on the upper part of the figure. Intercalation of *m*-AMSA or 2-Me-9-OH-E$^+$ within the enzyme–DNA complex (steps 5 and 6) traps cleavable complexes with either one (step 5) or the two DNA strands of the cleavage complex interrupted (step 6). This trapping inhibits the ATP-dependent strand passage (step 4).

this possibility, we determined whether various concentrations of drug would affect the DNA strand passing activity of DNA topoisomerase II.

*Effects of m-AMSA and 2-Me-9-OH-E$^+$ upon the DNA Relaxing Activity of DNA Topoisomerase II.* Utilizing the DNA unwinding effect of intercalators, the assay was designed as described in Figure 5. As a control, L1210 DNA topoisomerase I was used. Both DNA topoisomerase I and topoisomerase II relaxed DNA supercoiling in the absence of drug. However, a few positive supercoils were present in the agarose gels (Figure 6 and 7, lanes 2, 3, 2′, 3′) because of differences in conditions between reaction mixtures and electrophoresis.

In the presence of *m*-AMSA, whose intercalation reduces DNA twist, the *net* effect of DNA topoisomerase I action should be the introduction of negative supercoils [reduction of linking number (Lk); as final effect of step 5 in Figure 5]. This is what we observed (Figure 6, lanes 4′–6′). In the case of DNA topoisomerase II, however, no reduction in Lk was observed in the presence of *m*-AMSA (Figure 6, lanes 4–6). This finding shows that *m*-AMSA inhibited the DNA strand passage activity of DNA topoisomerase II in the direction of decreasing Lk. In other experiments, we found that *m*-AMSA also inhibited topoisomerase II DNA strand passage in the increasing Lk direction (from negatively supercoiled to relaxed DNA) (Minford et al., 1984).

In the presence of 2-Me-9-OH-E$^+$, the *net* effect of DNA topoisomerase I was the introduction of negative supercoiling. 2-Me-9-OH-E$^+$ was more effective than *m*-AMSA at much lower concentrations and induced more extensive negative supercoiling (Figures 7 and 6). This is probably related to the fact that 2-Me-9-OH-E$^+$ binds to DNA more tightly than *m*-AMSA (Le Pecq et al., 1974; Baguley & Falkenhaug, 1978). In the case of topoisomerase II and high concentrations of 2-Me-9-OH-E$^+$, DNA strand passage in the decreasing Lk direction still occurs. For the same degree of intercalation (as measured by comparable changes in supercoiling; Figure 6, lane 6′, and Figure 7, lane 8′), 2-Me-9-OH-E$^+$ seemed to allow more topoisomerase II DNA strand passage than did *m*-AMSA. At low 2-Me-9-OH-E$^+$ concentrations, however, (Figure 7, lanes 4,4′, 5, and 5′), our results suggest an inhibition of DNA topoisomerase II strand passage. In other experiments, we found also that low 2-Me-9-OH-E$^+$ concentrations inhibited topoisomerase II DNA strand passage in the increasing Lk direction (data not shown). The former results lead to the following conclusions: (1) neither *m*-AMSA nor 2-Me-9-OH-E$^+$ inhibited DNA topoisomerase I; (2) *m*-AMSA inhibited (over a wide range of concentrations) the DNA strand passing activity of DNA topoisomerase II, in agreement with the results of other studies using the P4 unknotting assay (Nelson et al.,1984); (3) 2-Me-9-OH-E$^+$ inhibited topoisomerase II DNA strand passage at low concentrations, in agreement with the results of studies using the P4 unknotting assay (Tewey et al., 1984a); (4) topoisomerase II was capable of carrying out strand passage reactions in the decreasing Lk direction, and this reaction was not markedly inhibited at high concentrations of 2-Me-9-OH-E$^+$.

The next question was whether high 2-Me-9-OH-E$^+$ concentrations that prevented the trapping of cleavable complexes by *m*-AMSA would also overcome *m*-AMSA-induced inhibition of the DNA strand passage activity of DNA topoisomerase II. To answer this question, SV40 DNA was first relaxed with DNA topoisomerase II in the presence of 1 mM ATP. The kinetics of this reaction had a half-time shorter than 1 min (Figure 8). After 15 min of reaction, 20 $\mu$M *m*-AMSA was added for an additional 30 min. This concentration was chosen because it inhibited completely DNA strand passage. As expected, *m*-AMSA induced DNA breaks (increase in nicked circles and linear DNA) and did not affect the DNA topoisomer distribution (Figure 8). Addition of 5 $\mu$M 2-Me-9-OH-E$^+$ to the DNA topoisomerase II–DNA reaction mixture induced DNA supercoiling within 15 min irrespective of the presence of *m*-AMSA (Figure 8). However, the kinetics of the supercoiling reaction was slightly slower in the presence of *m*-AMSA (Figure 8). This result suggests that 5 $\mu$M 2-Me-9-OH-E$^+$ could overcome partially the inhibitory effect of *m*-AMSA on DNA topoisomerase II strand passing activity.

## Discussion

In the preceding paper (Pommier et al., 1985), we found that the production of protein-associated DNA double-strand breaks by intercalators in isolated L1210 nuclei was markedly different in the cases of *m*-AMSA and 2-Me-9-OH-E$^+$. *m*-AMSA produced DNA double-strand breaks at all concentrations whereas 2-Me-9-OH-E$^+$ produced them only at low concentrations ($\leq$10 $\mu$M) and inhibited their production at high concentrations ($\geq$20 $\mu$M). We postulated that these different effects were due to different interactions between DNA topoisomerase II and *m*-AMSA or 2-Me-9-OH-E$^+$. We

now find that the protein-associated DNA strand breaks (cleavable complexes) produced by topoisomerase II from isolated nuclei show the same drug and concentration dependence as was seen in isolated nuclei. This further supports the conclusion that the intercalator-induced protein-associated DNA breaks observed in isolated nuclei and in cells are DNA topoisomerase II cleavable complexes.

Topoisomerase II cleavable complexes can be formed reversibly by the purified enzyme in the absence of drug and added ATP (Figures 1–4; Sander & Hsieh, 1983; Liu et al., 1983). Upon ATP addition, the formation of these cleavable complexes is associated with DNA topoisomerization (Figures 6–8; Cozzarelli, 1979; Liu et al., 1980; Gellert, 1981). It is likely that DNA topoisomerase II cleavable complexes are intermediates of the DNA strand passage reaction through which the enzyme passes one intact DNA double strand through a second cleaved strand (Figure 9).

Both *m*-AMSA and 2-Me-9-OH-E$^+$ (at low concentration) increased the yield of cleavable complexes while inhibiting the DNA strand passage activity of topoisomerase II. This result is consistent with the results of Minford et al. (1984) and Nelson et al. (1984) for *m*-AMSA and of Tewey et al. (1984a) for 2-Me-9-OH-E$^+$. The trapping of protein-associated cleavable complexes by *m*-AMSA and 2-Me-9-OH-E$^+$ could correspond to the stabilization of cleavable complexes by intercalated DNA. However, intercalation by itself is not sufficient since neither the *m*-AMSA isomer, *o*-AMSA (Nelson et al., 1984), nor ethidium bromide (Tewey et al., 1984b) increases the yield of cleavable complexes. Therefore, some stereospecificity must be involved in the trapping of the protein-associated cleavage intermediates. This stereospecificity plus the fact that *m*-AMSA-induced cleavable complexes can form in linear DNA (Nelson et al., 1984) and in the presence of DNA topoisomerase I (Pommier and Kohn, unpublished results)—i.e., in the absence of long-range torsional tension (Pommier et al., 1984d)—suggests that intercalators interact closely with DNA topoisomerase II to trap cleavable complexes. One possible interaction is the intercalation of *m*-AMSA or 2-Me-9-OH-E$^+$ within the passing strand of the cleavable complex. In the case of *m*-AMSA, which traps essentially single-strand break intermediates (Zwelling et al., 1981; Minford et al., 1984), the DNA breaking–rejoining reaction of DNA topoisomerase II would be slowed down markedly, with an accumulation of both single- and double-strand break intermediates (Figure 9, steps 5 and 6). Addition of ATP to DNA topoisomerase II in the presence of *m*-AMSA did not induce DNA strand passage (Figure 6). This inhibition could result from the trapping of DNA topoisomerase II within the DNA single- and double-strand break cleavable complexes formed by *m*-AMSA (Figure 9, step 5). In the case of low 2-Me-9-OH-E$^+$ concentrations, which produce essentially DNA double-strand cleavage in mammalian cells (Zwelling et al., 1982), the trapping probably occurs at the DNA double-strand break step (Figure 9, step 6) and leads also to DNA strand passage inhibition. 2-Me-9-OH-E$^+$ has no bulky group, as does *m*-AMSA, and this may relate to the absence of trapping of single-strand break intermediates.

At high 2-Me-9-OH-E$^+$ concentration, the noncovalent binding of topoisomerase II to DNA was stimulated; hence, the inhibition of covalent complex formation may not result simply from a prevention of topoisomerase II–DNA binding. High 2-Me-9-OH-E$^+$ concentration may actually reverse the inhibition of strand passing under some circumstances. Trapping of covalent complexes by *m*-AMSA inhibited the strand passing reaction, but this inhibition was at least partially overcome when high concentrations of 2-Me-9-OH-E$^+$ were added. Low concentrations of 2-Me-9-OH-E$^+$ stimulated the production of cleavable complexes in proportion to drug concentration. This was seen in the experiments with isolated nuclei and confirmed with the purified enzyme. This result is consistent with the participation of one drug molecule per complex. The inhibition of cleavable complex formation at high 2-Me-9-OH-E$^+$ concentration, in both the isolated nuclei and purified enzyme experiments, is not consistent with a process involving only a single drug molecule. Since the inhibition only makes itself felt at high drug concentrations, this reaction probably requires the participation of two or more drug molecules. Such a process could be the result of drug-induced conformational distortion or stiffening of the DNA that must wind around the enzyme protein or form a free loop between the sites of strand cleavage and strand passage.

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# Ethidium Ion Binds More Strongly to a DNA Double Helix with a Bulged Cytosine Than to a Regular Double Helix†

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ABSTRACT: Thermodynamic parameters for ethidium intercalation were determined for the double helices formed by the oligonucleotides $dCA_6G + dCT_6G$, which form a normal helix, and $dCA_3CA_3G + dCT_6G$, which form a double helix with the middle cytosine bulged outside of the helix. Ethidium intercalation was measured by monitoring the absorbance at 260 and 283 nm as a function of temperature for a number of concentrations of ethidium. The binding to the normal helix occurs equally at all the intercalation sites, with an enthalpy of binding of −8 kcal $mol^{-1}$, an entropy of binding of −6 eu, and an equilibrium constant at 25 °C of $2.2 \times 10^4$ $M^{-1}$. The binding to the bulged double helix was considerably stronger and is consistent with a model in which the intercalation sites on either side of the bulged base bind 10 times stronger than the other sites. Thus, there are two strong binding sites on the perturbed helix with equilibrium constants for binding of $2 \times 10^5$ $M^{-1}$ at 25 °C in addition to five normal sites. Several other binding models were tested but did not fit the data satisfactorily.

Frameshift mutagenesis requires the formation of a structure in which there is an extra nucleotide on one strand of a DNA double helix. This intermediate in the mechanism of mutation is called a bulge. The probability of a frameshift mutation depends on the incorporation of an extra nucleotide into a newly synthesized strand (a plus one mutation) or on the omission of a nucleotide from the new strand (a minus one mutation). Repair of the bulged structure before replication is also important to the mutation process. The stability and conformation of the bulge affect all of these reactions. The role of intercalators in the process of frameshift mutagenesis has been hypothesized for many years (Streisinger et al., 1966). Intercalating molecules are thought to promote frameshift mutagenesis by binding preferentially to bulged structures, thereby stabilizing them and increasing the likelihood that the bulge will become incorporated into the DNA.

Ethidium ion intercalates into a complex consisting of the trinucleoside diphosphate rGpUpG and the dinucleoside phosphate rCpC, forming a mini double helix with the ethidium sandwiched between the two C-G base pairs with the bulged U outside the helix (Lee & Tinoco, 1978). It is also known that ethidium bromide binds more strongly into synthetic RNA polynucleotides that contain a small amount of A·I mismatches (Helfgott & Kallenbach, 1979). These mismatches probably form nonstandard base pairs, and although they are not bulges, the situation is relevant to the bulges. No direct measurement has been done on the magnitude or mechanism of the stabilization of a bulge by ethidium bromide. We previously studied the intercalation of ethidium bromide into the ribooligonucleotides $rCA_5G + rCU_5G$ and the corresponding deoxyribooligonucleotides $dCA_5G + dCT_5G$ (Nelson & Tinoco, 1984). Here, we discuss the extension of the work to the intercalation of ethidium ion into the deoxyribooligonucleotides $dCA_6G + dCT_6G$, which form a normal double helix, and $dCA_3CA_3G + dCT_6G$, which form a double helix with a bulged cytosine in the middle of the helix.

The structure of these normal and bulged double helices has been characterized by NMR,[1] and the thermodynamic stabilities have been studied by optical melting curves (Morden et al., 1983). It was found that the bulged cytosine is out of the helix and the two A·T base pairs on either side of the bulge stack on each other similar to the normal helix. The destabilization caused by the bulge was also determined to be due to a less favorable enthalpy of double-strand formation, with an increase in the standard free energy at 25 °C of approximately 2.9 kcal $mol^{-1}$.

## Materials and Methods

The synthesis of the oligonucleotides is discussed in Morden et al. (1983). The extinction coefficients per mole of strand at 260 nm and 25 °C were estimated from the coefficients for

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[1] Abbreviations: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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