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Mechanism of antitumor drug action: Poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide

(protein–DNA complex)

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ABSTRACT **The intercalative acridine derivative 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), but not its isomer *o*-AMSA, is a potent antitumor drug that in mammalian cells stimulates the formation of DNA strand breaks that are characterized by tightly bound proteins. Using purified mammalian DNA topoisomerases, we have analyzed the effects of these antitumor drugs on topoisomerase–DNA interactions. The antitumor drug *m*-AMSA dramatically stimulates the formation of a topoisomerase II–DNA complex that is detected on protein-denaturant treatment: both single- and double-stranded DNA breaks are produced and a topoisomerase II subunit is linked covalently to each 5′ end of the broken DNA strands. The noncytotoxic isomer, *o*-AMSA, which does not induce significant amounts of DNA breaks in cultured cells, exhibits a correspondingly smaller effect in stimulating formation of the complex *in vitro*. The agreement between *in vitro* and *in vivo* studies suggests that mammalian DNA topoisomerase II may be the primary target of *m*-AMSA and that the drug-induced complex formation between topoisomerase II and DNA may be the cause of cytotoxicity and other effects such as DNA sequence rearrangements and sister-chromatid exchange.**

A large number of antitumor drugs are known to interact with DNA *in vitro* (for review, see ref. 1). Despite their success in treating certain tumors, the cellular mechanisms of the actions of these drugs are still unknown (1). Among these antitumor drugs, many of them interact with DNA by an intercalative mode (1). A cellular phenomenon has been noted that seems to be unique to these intercalative antitumor drugs. After treatment of mammalian cells with these antitumor drugs [e.g., daunomycin, adriamycin, ellipticine, actinomycin D, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)], DNA breakage can be demonstrated by several techniques (2–7). Both single- and double-strand breaks have been observed and their ratio varies depending on the particular drug used. Usually, DNA cleavage is quickly reversed on removal of the drug. The cleavage products have protein tightly (perhaps covalently) associated with the broken DNA ends (2–7). The synthetic antitumor drug *m*-AMSA, being a weak DNA intercalator (1), is particularly interesting because similar DNA strand breaks can be shown to occur in both cultured mammalian cells and isolated nuclei (6, 8–12). Furthermore, it has been shown that the 5′ ends of the cleavage product formed by *m*-AMSA treatment are blocked by proteins (13). The suggestion that a DNA topoisomerase may be involved has been made (3, 5, 6, 13).

Recently, it was shown that a tight complex is formed between purified eukaryotic DNA topoisomerase II and DNA (14, 15). Mammalian DNA topoisomerase II and DNA can form a tight complex that, on protein-denaturant treatment, produces protein-linked DNA breaks (15). This complex (henceforth referred to as the "cleavable complex") is operationally defined by the detection method used. One of its unusual features is its reversibility with respect to low-temperature and high-salt treatment (15). The DNA product after low-temperature or high-salt treatment is identical to the original DNA with respect to its circularity and linking number (15). It is likely that the putatively broken DNA (staggered by four bases with each 5′ protruding end covalently linked to the enzyme monomer) (14, 15) is fully protected by the bound enzyme so that relative rotation of the two broken ends is inhibited. Many properties of this complex suggested to us that mammalian DNA topoisomerase II may be involved in the action of these intercalative antitumor drugs. In this communication, we report our initial studies indicating that mammalian DNA topoisomerase II may be the primary target for at least one intercalative antitumor drug, *m*-AMSA.

MATERIALS AND METHODS

Materials. Homogeneous preparations of DNA topoisomerase I and II from both calf thymus and HeLa cells were used (unpublished results). *Drosophila* DNA topoisomerase II and *Escherichia coli* gyrase were gifts from T. S. Hsieh and Martin Gellert, respectively. *m*-AMSA (NSC 249992) and its isomer *o*-AMSA (NSC 156306) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Both drugs were dissolved in dimethyl sulfoxide at 2 mg/ml and used fresh.

Topoisomerase Assays and DNA-Strand Cleavage Conditions. Type II DNA topoisomerase activity was assayed using the P4 unknotting assay described previously (16). The reaction mixture for the P4 unknotting assay was 50 mM Tris·HCl, pH 7.5/100 mM KCl/10 mM $MgCl_2$/1 mM ATP/0.5 mM EDTA/0.5 mM dithiothreitol containing bovine serum albumin at 30 μg/ml, DNA at 20 μg/ml, and various amounts of a type II DNA topoisomerase. DNA-strand cleavage reactions were carried out in the same reaction mixture. Special care was made to maintain the reaction mixture at 37°C during the addition of a protein denaturing solution (usually 1% $NaDodSO_4$) to stop the cleavage reaction, because temperature has a large effect on the extent of cleavage complex formation (15).

3′-End Labeling of *Hin*dIII-Cut pBR322 DNA. 3′-End labeling was done as described (15). Briefly, pBR322 DNA was cut with *Hin*dIII restriction endonuclease and then labeled with [α-^{32}P]dATP by using the large fragment of *E. coli* DNA polymerase I and unlabeled dCTP/dGTP/dTTP at 15°C for 1 hr. Unincorporated triphosphates were removed

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Abbreviations: *m*-AMSA and *o*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and -*o*-anisidide, respectively.

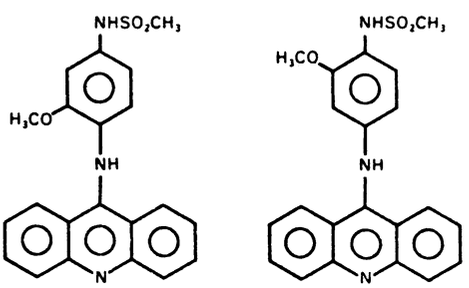
by two cycles of ethanol precipitation in the presence of 2 M ammonium acetate (17).

Quantitative Precipitation of the Covalent Enzyme-DNA Complex. The precipitation method was essentially that described previously (15). Briefly, reaction mixtures (50 μl each) containing 50 ng of calf thymus DNA topoisomerase II, 25 ng of 3′-end- or 5′-end-labeled pBR322 DNA, and various concentrations of drugs were incubated at 37°C for 30 min. The reactions were then terminated by the addition of 100 μl of a prewarmed (37°C) solution of 0.2 M NaOH/2% $NaDodSO_4$/5 mM EDTA containing salmon sperm DNA (0.5 mg/ml; Sigma), and this mixture was incubated at 37°C for 10 min. Then, the covalent enzyme-DNA complexes (DNA was single-stranded because of alkali denaturation) were precipitated by the addition of 50 μl of 0.25 M KCl/0.4 M HCl/0.4 M Tris, pH 7.9. Precipitation was allowed to occur at 0°C for 10 min. The precipitate was collected by centrifugation in an Eppendorf centrifuge for 15 min at 4°C and washed once by heating to 65°C for 10 min in 200 μl of 10 mM Tris·HCl, pH 7.9/100 mM KCl/1 mM EDTA containing carrier DNA at 100 μg/ml and bovine serum albumin at 50 μg/ml. After cooling on ice and recentrifugation, the pellet was suspended in 200 μl of H_2O, dissolved by heating to 65°C, and transferred to a vial containing 4 ml of scintillation fluid for determination of radioactivity.

Gel Electrophoresis. To demonstrate the presence of the covalent enzyme-DNA complex in the native gel, 0.1% $NaDodSO_4$ was included in the Tris/borate/EDTA (TBE) electrophoresis buffer (17). Alkaline agarose gel electrophoresis was carried out as described (17).

RESULTS

The Antitumor Drug *m*-AMSA Stimulates Formation of the Cleavable Complex. A number of antitumor drugs were tested, using purified mammalian DNA topoisomerase II, for their ability to stimulate DNA cleavage *in vitro*. *m*-AMSA was one of the more potent drugs that stimulated formation of the cleavable complex. Its isomer *o*-AMSA (Fig. 1), which is dramatically less potent in both cytotoxic effect on tumor cells and induction of protein-linked DNA breaks *in vivo* (6, 18), was also much less potent in stimulating the cleavable complex formation *in vitro*.



m-AMSA *o*-AMSA

A typical assay for screening the antitumor drugs *in vitro* is shown in Fig. 1. Supercoiled plasmid DNA pBR322 (lane A) was treated with calf thymus DNA topoisomerase II (lane B) (HeLa DNA topoisomerase II gave similar results) in the presence of various amounts of *m*-AMSA or *o*-AMSA. As the concentration of *m*-AMSA is increased (from 0.5 μg/ml in lane C to 128 μg/ml in lane G), the closed circular form is progressively converted to the open circular and linear forms. On the other hand, treatment with *o*-AMSA in the same concentration range had very little effect (lanes H–L). Several pieces of evidence indicate that the observed DNA cleavage is due to the cleavable complex formation between calf thymus DNA topoisomerase II and DNA rather than to nucleases or chemical degradation (19). The cleavage occurred within seconds after the mixing of topoisomerase II, DNA, and *m*-AMSA. Prolonged incubation did not significantly increase DNA cleavage. Similar to the cleavable complex described previously (15), high-salt treatment of the reaction mixture after the first incubation aborted the cleavage (data not shown). Furthermore, topoisomerase II is found covalently linked to the 5′ end of all the DNA cleavage products (see below). DNA cleavage by mammalian DNA topoisomerase I and *E. coli* DNA gyrase is refractory to *m*-AMSA treatment *in vitro*. *Drosophila* DNA topoisomerase II is only very weakly sensitive to *m*-AMSA (data not shown).

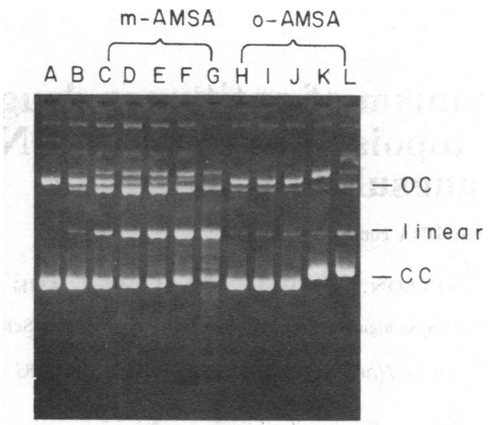


FIG. 1. Gel electrophoresis assay of stimulation of formation of the cleavable complex between mammalian DNA topoisomerase II and DNA. Reaction mixtures (20 μl each) were as described in *Materials and Methods*. Calf thymus DNA topoisomerase II (10 μg/ml) and pBR322 DNA (20 μg/ml) were present in samples B–L. After 10 min of incubation at 37°C, reactions were terminated by addition of 4 μl of 5% $NaDodSO_4$ proteinase K at 2 mg/ml. Proteinase K digestion was done at 37°C for 30 min. Reaction mixtures were then analyzed by 1.2% agarose gel electrophoresis. Concentrations of *m*-AMSA were 0.5, 2.0, 8.0, 32, and 128 μg/ml for samples C–G, respectively. Samples H–L were the same as samples C–G except that *o*-AMSA was used instead. Sample A is the pBR322 control. Sample B has topoisomerase II but no drug. CC, closed circular DNA; OC, open circular DNA.

Detection of the Cleavable Complex by a Quantitative Precipitation Method. To quantitate the cleavage reaction, a procedure that selectively precipitates covalent protein-DNA complexes has been developed (ref. 15; see *Materials and Methods*). Using end-labeled DNA, we denatured the DNA product after the reaction to monitor polarity-specific protein-DNA linkage. To determine whether topoisomerase II is linked to the 5′ end of the cleavage product, we used DNA substrates labeled at either the 3′ or 5′ ends. Radioactive label was recovered in the precipitated protein-DNA complex only when 3′-end-labeled DNA substrate was used, suggesting that topoisomerase II is covalently linked to the 5′ end of the broken DNA (data not shown). The results of the precipitation assay when 3′-end-labeled DNA was used as the substrate are shown in Fig. 2. Under the reaction conditions used, *m*-AMSA stimulated formation of the covalent enzyme-DNA complex more than 25-fold at the highest concentration (62.5 μg/ml) tested (the actual stimulation is much greater than 25-fold because of the nonlinearity of the curve). *o*-AMSA was no more than 5% as effective as *m*-AMSA in stimulating complex formation.

Mapping of *m*-AMSA-Stimulated Cleavage Sites. Although the precipitation method is quantitative, it measures only the overall stimulation. To study the site specificity, we analyzed the cleavage products on gels. The cleavage sites on pBR322 were mapped by agarose gel electrophoresis (Fig. 3). Several interesting features were noted: (*i*) Most if not all

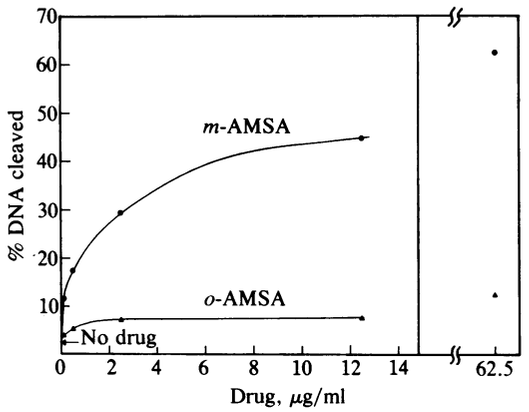


FIG. 2. Quantitative precipitation assay for formation of the cleavable complex in the presence of *m*-AMSA and *o*-AMSA.

of the cleavage sites induced by *m*-AMSA treatment (lane C) were present in the untreated sample (lane B). The four sites most extensively induced by *m*-AMSA treatment mapped around nucleotides 3380, 2790, 1630, and 560 on the pBR322 map. (*ii*) Different sites on pBR322 were stimulated to different extents by *m*-AMSA. For example, the four most-induced sites on pBR322 DNA were stimulated at least 100-fold by *m*-AMSA at 2 μg/ml whereas some other sites were only very weakly stimulated (the intensities of the bands were quantitated by densitometric scanning). To show that

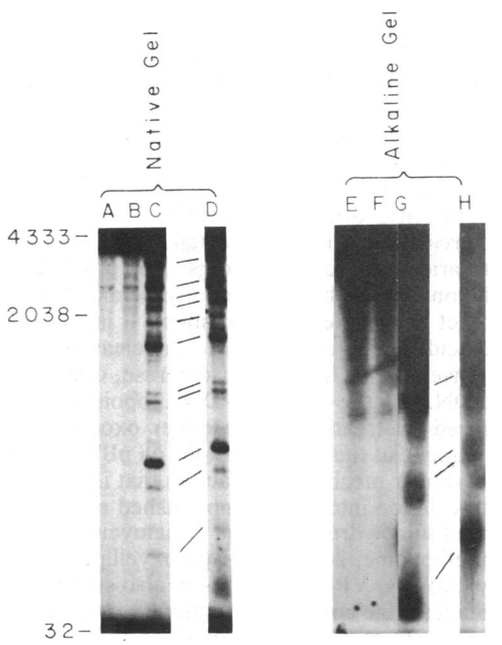


FIG. 3. Mapping of the *m*-AMSA-stimulated topoisomerase II cleavage site on pBR322 DNA. 3'-End-labeled pBR322 DNA was cut with *Eco*RI to generate one large fragment (4333 base pairs) and one small fragment (32 base pairs) both of which are uniquely labeled at one end only. Because of its small size, the 32 base pair fragment was not separated from the large fragment. Reaction mixtures contained labeled DNA at 10 μg/ml, calf thymus DNA topoisomerase II at 12 μg/ml, and *m*-AMSA at 2 μg/ml. Samples were analyzed on a 1.2% native agarose gel (containing 0.1% $NaDodSO_4$) and on a 1.2% alkaline agarose gel. Lanes A and E, no topoisomerase, no drug; B and F, with topoisomerase but no drug; C and G, with topoisomerase and *m*-AMSA at 2 μg/ml; D and H, as for lanes C and G except that proteinase K treatment was omitted. The mobility shift due to the presence of the covalently bound protein is indicated by a solid line.

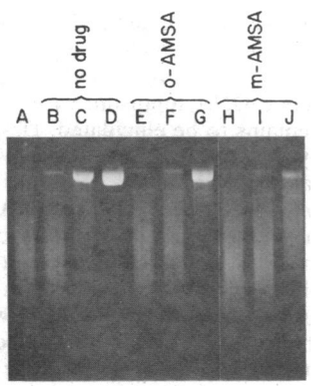


FIG. 4. Inhibition of the strand-passing activity of calf thymus DNA topoisomerase II by *m*-AMSA and *o*-AMSA. The strand-passing activity of calf thymus DNA topoisomerase II was monitored by the P4 unknotting assay. Three topoisomerase II dilutions were used for each drug (all at 20 μg/ml). Lanes B, E, and H, 12 ng/ml; lanes C, F, and I, 60 ng/ml; lanes D, G, and J, 300 ng/ml. Lane A was a DNA control (no enzyme).

all the cleavage products were associated with tightly bound topoisomerase, we compared the gel mobilities of the bands with and without protease treatment (15). When the cleavage products with (lane C) and without (lane D) proteinase K treatment were compared in a detergent-containing agarose gel, the retardation of the migration rates of the protein-DNA complexes was apparent, consistent with the idea that topoisomerase II is covalently linked to each cleavage product.

Topoisomerase Is Linked to the 5′ Termini of Cleaved DNA. To confirm that the *m*-AMSA-induced cleavable complex is structurally the same as the cleavable complex identified previously in the absence of the drug (15), we analyzed the cleavage products with and without protease treatment on an alkaline agarose gel (Fig. 3). Since the radioactive label is at the 3′ ends of the DNA, the mobility shift observed for the cleavage products with (lane G) and without (lane H) protease treatment is consistent with the presence of a covalently linked protein at the 5′ terminus of each broken DNA single strand. The covalent linkage between mammalian DNA topoisomerase II and DNA was also determined to be phosphotyrosine by ^{32}P transfer to protein and direct determination of the labeled amino acid by using high-voltage paper electrophoresis and thin layer chromatography (unpublished results).

Inhibition of the Strand-Passing Activity of Mammalian DNA Topoisomerase II by *m*-AMSA. To test whether *m*-AMSA can inhibit the catalytic activity of mammalian DNA topoisomerase II, the unknotting activity of topoisomerase II was assayed in the presence of *o*-AMSA (Fig. 4, lanes E–G) and *m*-AMSA (lanes H–J). Both drugs inhibited the strand-passing activity of DNA topoisomerase II with *m*-AMSA being reproducibly more effective than *o*-AMSA in inhibiting the topoisomerase activity at 20 μg/ml. Unwinding measurements using topoisomerase I and relaxed pBR322 DNA indicated that about one *m*-AMSA molecule was intercalated for every 60 base pairs of DNA under our assay conditions (data not shown). It is thus not clear whether the inhibitory effect of these antitumor drugs reflects the altered cleavable complex or drug intercalation into DNA.

DISCUSSION

Type II DNA topoisomerases are characterized by their ability to catalyze topological crossing of two DNA segments (20–25). Studies in prokaryotic cells have shown that DNA topoisomerase II (DNA gyrase) is involved in DNA replica-

tion, gene expression, and recombination. Its catalytic function is to generate DNA superhelical tension, which has been found both *in vivo* and *in vitro* (reviewed in refs. 21 and 22). Whether eukaryotic DNA topoisomerase II is a true DNA gyrase *in vivo* remains to be elucidated. Despite this uncertainty, it is likely that eukaryotic DNA topoisomerase II has similar biological functions.

The effect of *m*-AMSA on eukaryotic DNA topoisomerase II is reminiscent of the effect of oxolinic acid (or nalidixic acid) on bacterial DNA topoisomerase II (21, 22). In the presence of oxolinic acid, DNA gyrase forms a complex with DNA, which can be activated by treatment with $NaDodSO_4$ to produce double-strand breaks in DNA. Detailed analyses of the broken complex have revealed that the break is a four-base stagger and that a *gyrA* subunit is covalently linked to each 5′ protruding end of the break (21, 22). The formation of this complex *in vivo* in the presence of oxolinic acid presumably poisons DNA gyrase on the DNA template and thus inhibits cell growth (21, 22, 26). The stimulation of mammalian DNA topoisomerase II to form a similar complex by *m*-AMSA treatment and the cytotoxic effect of *m*-AMSA probably represent a eukaryotic analog of this bactericidal effect of oxolinic acid.

Based on current knowledge about the topoisomerase-DNA complex, a simple model for the mammalian DNA topoisomerase II-DNA interaction is presented in Fig. 5. In this model, only the initial steps of the strand-passing mechanism are considered. The ATPase-mediated strand-crossing event that presumably follows these initial steps is not illustrated for reasons of simplicity. Based on the present methods of detection, we assume that at least two forms of topoisomerase-DNA complexes exist that are at equilibrium. The cleavable complex (Fig. 5*C*) refers to the complex described above that can be detected by treatment with protein denaturants such as $NaDodSO_4$ or alkali. Mammalian DNA topoisomerase II is most likely a homodimer (M_r, 120,000–170,000) (14, 27). On protein-denaturant treatment, the cleavable complex falls apart in such a manner that one topoisomerase subunit is covalently linked through a tyrosine residue to each 5′ phosphoryl end of the double-strand break (unpublished results). The double-strand break is staggered by four bases with protruding 5′ ends (15). The "noncleavable complex" (Fig. 5*B*) refers to the complex in which the interaction between mammalian DNA topoisomerase II and DNA is noncovalent. Treatment of the noncleavable complex with protein denaturants leads to dissociation of the topoisomerase from DNA. Normally, the equilibrium favors formation of the noncleavable complex and only a very small fraction of the topoisomerase can be trapped as the cleavage product by protein-denaturant treatment. The antitumor drug *m*-AMSA may alter this equilibrium by acting as an allosteric effector or by forming a topoisomerase-AMSA-DNA ternary complex. In either case, the equilibrium is shifted toward the cleavable complex in the presence of *m*-AMSA. This model can explain the reversibility of the drug action and the effects of temperature and salt on the cleavable complex (7, 9–12, 15).

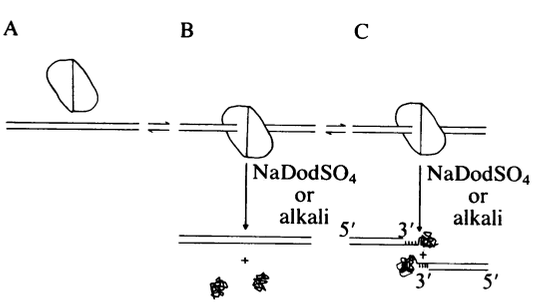


FIG. 5. Model for the initial steps in the topoisomerase II-catalyzed DNA strand-passing reaction. (*A*) Unbound topoisomerase. (*B*) Noncleavable complex. (*C*) Cleavable complex.

It is attractive to hypothesize that, similar to oxolinic acid (26), *m*-AMSA may alter the state of the cleavable complex such that it may have a higher probability of falling apart *in vivo* either spontaneously or because some other interaction such as a fast moving replication fork, helicases in motion, or other nuclear proteins. This type of double-strand break, in which one native protein subunit is tightly associated with each end, may abort DNA replication and thus cause cytotoxicity or DNA rearrangement and sister-chromatid exchange (28). It is conceivable that the alteration of the cleavable complex by *m*-AMSA may also result in inhibition of the ATP-dependent strand-passing function of topoisomerase II. Indeed, *m*-AMSA is reproducibly a stronger inhibitor of the strand-passing activity of mammalian DNA topoisomerase II than *o*-AMSA (Fig. 4). However, since both drugs are DNA intercalators, we do not know whether this inhibition is a direct result of the altered cleavable complex or drug intercalation into DNA. It is interesting that the ATP-dependent strand-passing activity of type II DNA topoisomerases are sensitive to DNA intercalators (unpublished results). The cytokinetic effect of *m*-AMSA is a block in the G_2 phase of the cell cycle (29). This block may be related to drug inhibition of the strand-passing activity of DNA topoisomerase II. However, we think that the activity inhibition is not related to the cell killing action of the antitumor drug.

How does *m*-AMSA alter the cleavable complex formed between DNA topoisomerase II and DNA? Clearly, the alteration is not due to the torsional stress introduced because both supertwisted and relaxed DNA produce the same number of breaks after *m*-AMSA treatment (unpublished results). Intercalation alone cannot explain the specificity of *m*-AMSA in stimulating the cleavable complex formation, because both *m*-AMSA and *o*-AMSA weakly intercalate into DNA under our assay conditions and the difference in the extent of intercalation is within a factor of 2 (unpublished results). Specific drug-enzyme interaction, in addition to DNA intercalation, must be essential for the antitumor functions of various intercalative drugs. It is possible that drug intercalation may inhibit DNA topoisomerase II independent of its effect on the cleavable complex. It is interesting that oxolinic acid, which can stimulate the cleavage reaction of DNA gyrase and T4 DNA topoisomerase, does not intercalate into DNA. In the case of T4 DNA topoisomerase, cleavage induced by treatment with either oxolinic acid or *m*-AMSA mapped at the same positions on pBR322 DNA, suggesting a similar mechanism of action that is independent of drug-intercalation into DNA (unpublished results).

Other antitumor drugs such as adriamycin, 5-iminodaunorubicin, ellipticine, 2-methyl-9-hydroxyellipticine, and epipodophyllotoxins, VP-16 and VM-26, also stimulate the formation of cleavable complex formation *in vitro* (unpublished results). Consistent with our proposition that stimulation of the cleavable complex formation is responsible for cytotoxicity, 2-methyl-9-hydroxyellipticine, which is more cytotoxic than ellipticine, is also much more efficient in stimulating the cleavable complex formation *in vitro* (unpublished results). Stimulation of the cleavable complex formation thus seems to be a general phenomenon for a variety of antitumor drugs. Topoisomerase cleavage sites produced by antitumor drugs from different chemical classes (e.g., anthracyclines, ellipticines, and acridines), however, are quite different. Mammalian DNA topoisomerase II may thus be a multidrug target that responds differently to antitumor drugs from different chemical classes. To test whether mammalian DNA topoisomerase II is the primary cytotoxic target of these antitumor drugs, additional genetic and biochemical studies are neces-

sary. The present correlation studies suggest a possible screening procedure for new antitumor drugs using the *in vitro* topoisomerase cleavage reaction.

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