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Topoisomerase II-mediated site-directed alkylation of DNA by psorospermin and its use in mapping other topoisomerase II poison binding sites

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ABSTRACT **Psorospermin is a plant natural product that shows significant *in vivo* activity against P388 mouse leukemia. The molecular basis for this selectivity is unknown, although psorospermin has been demonstrated to intercalate into DNA and alkylate N7 of guanine. Significantly, the alkylation reactivity of psorospermin at specific sites on DNA increased 25-fold in the presence of topoisomerase II. In addition, psorospermin trapped the topoisomerase II-cleaved complex formation at the same site. These results imply that the efficacy of psorospermin is related to its interaction with the topoisomerase II–DNA complex. Because thermal treatment of (N7 guanine)–DNA adducts leads to DNA strand breakage, we were able to determine the site of alkylation of psorospermin within the topoisomerase II gate site and infer that intercalation takes place at the gate site between base pairs at the +1 and +2 positions. These results provide not only additional mechanistic information on the mode of action of the anticancer agent psorospermin but also structural insights into the design of an additional class of topoisomerase II poisons. Because the alkylation site for psorospermin in the presence of topoisomerase II can be assigned unambiguously and the intercalation site inferred, this drug is a useful probe for other topoisomerase poisons where the sites for interaction are less well defined.**

Psorospermin, a natural product isolated from roots and stem bark of the African plant *Psorospermum febrifugum*, is mechanistically related to the pluramycin family of antitumor antibiotics (Fig. 1) and has been shown to exhibit significant activity *in vitro* against various tumor cell lines and *in vivo* against P388 mouse leukemia (1, 2). Several studies have revealed that the pluramycins intercalate into the DNA helix and covalently modify guanine at the N7 position in the major groove through an epoxide-mediated electrophilic addition (3–7). Using high-field NMR and gel electrophoresis experiments, Hansen *et al.* (8) were able to show that these two compounds react with DNA in a similar fashion. However, despite the similarity of their mechanism of covalent modification of DNA, distinct differences exist between psorospermin and the pluramycin class of compounds in terms of sequence selectivity, relative alkylation reactivity, and orientation of the chromophore at the intercalation site (8).

Protein–DNA complexes are the molecular targets of a number of antitumor agents (9). It has been demonstrated that pluramycin reactivity is enhanced at a specific site downstream of the TATA box, immobilizing the TATA box-binding protein on DNA (10). Studies using an alkylating analogue of camptothecin have suggested that the camptothecins inhibit topoisomerase I by binding at the enzyme–DNA interface (11). A radioisotope-labeled quinolone was found to form a complex with DNA and gyrase together, but not with either component alone (12). In a study carried out by Permana *et al.* (13), simian virus 40 DNA isolated from psorospermin-treated cells was cross-linked to a variety of proteins, including DNA topoisomerases I and II. Although the molecular mechanisms responsible for the biological activities of psorospermin are still unknown, this result suggests that the antitumor activity of psorospermin may be related to its interaction with the DNA–topoisomerase II complex.

DNA topoisomerase II is a nuclear enzyme required for the regulation of the topological status of DNA in eukaryotic cells (14). This enzyme functions by passing a double-stranded DNA helix through a transient double-stranded break site (or "gate") generated by the enzyme and then resealing the gate. During the DNA cleavage step, topoisomerase II is covalently linked to the 5′ phosphoryl end of the broken DNA. Eukaryotic topoisomerase II has been identified as the target of a number of potent anticancer drugs, such as the anthracyclines, acridines, and epipodophyllotoxins (15–18). Most of these topoisomerase II inhibitors, known as poisons, interfere with the breakage–rejoining reaction of the enzyme by trapping the covalent reaction intermediate, which is called the cleavable complex (15) or, more accurately, the cleaved complex. These cleaved complexes block other cellular enzyme functions and eventually lead to cell death.

In this study, it is shown that in the presence of topoisomerase II, psorospermin has an unusually high reactivity with DNA at the gate site, whereas the reactivity of pluramycin decreases at the same position. In addition, the topoisomerase II-mediated site-directed psorospermin alkylation traps the topoisomerase II-cleaved complex at the same site. This finding has important implications for the mode of action of psorospermin. Finally, this selective alkylation of DNA by psorospermin at the topoisomerase II gate site proves to be a useful tool for mapping the binding sites of other topoisomerase II poisons on DNA.

MATERIALS AND METHODS

Materials, Enzymes, and Drugs. Etoposide and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) were purchased from TopoGene (Columbus, OH), and psorospermin was a generous gift from John M. Cassady (Ohio State University). Electrophoretic reagents (acrylamide, *N, N′*-methylenebisacrylamide, and ammonium persulfate) were from J. T. Baker, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was from Fisher. T4 polynucleotide kinase, *Drosophila* topoisomerase II, and [γ-^{32}P]ATP were purchased from Amersham.

Preparation and End-Labeling of Oligonucleotides. The 80-base oligonucleotides were synthesized on an Expedite 8900 nucleic acid synthesis system (PerSeptive Biosystems, Framingham, MA) using the phosphoramidite method. The oligonucleotides were eluted out of the column by using aqueous ammonia

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Abbreviation: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

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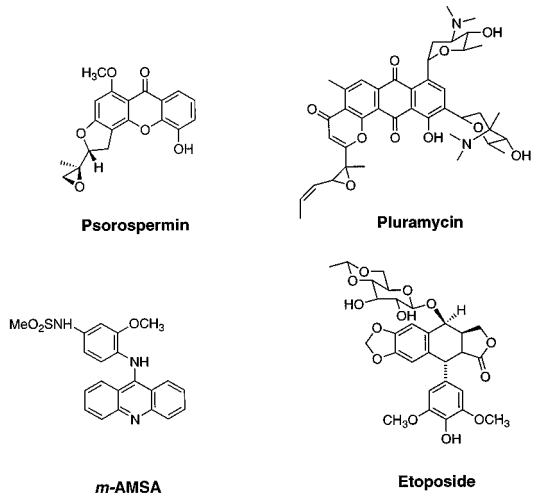

FIG. 1. Structures of pluramycin and the topoisomerase II inhibitors used in this study.

and deprotected by heating at 75°C for 1 hr, followed by 12% denaturing polyacrylamide gel purification. The 5′ end-labeled single-stranded oligonucleotides were obtained by incubating the oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP at 37°C for 1 hr. The labeled strands were then annealed with the complementary strands and purified on an 8% native polyacrylamide gel.

Strand-Breakage Assay. The 5′ ^{32}P-labeled DNA was incubated with *Drosophila* topoisomerase II in 20 μl of reaction buffer A (10 mM imidazole·HCl, pH 6.0/10 mM $MgCl_2$/50 mM KCl/1 mM ATP) at 30°C for 10 min before 10 μM (final concentration) psorospermin or 0.5 μM pluramycin was added to the mixture. The reaction was continued for an additional 5 min and then terminated by adding 5 μg of calf thymus DNA followed by heating at 95°C for 15 min. In the presence of piperidine, this procedure results in strand breakage at the drug modification site (8). The samples were subjected to phenol/chloroform extraction and ethanol precipitation.

Topoisomerase II Cleavage Reactions. The 5′ ^{32}P-labeled DNA was incubated with *Drosophila* topoisomerase II (10–20 units) in 20 μl of reaction buffer A at 30°C for 10 min in the presence of various amount of psorospermin, *m*-AMSA, and etoposide. Reactions were terminated by adding SDS to 1% of the final concentration, and topoisomerase II was removed by proteinase K digestion (100 μg/ml) at 42°C for 1 hr followed by phenol/chloroform extraction and ethanol precipitation.

Gel Electrophoresis and Quantification. All of the samples were loaded onto a 12% denaturing sequencing gel. The dried gels were exposed on both x-ray film and phosphor screen. Imaging and quantification were performed by a PhosphorImager and IMAGEQUANT 4.1 software from Molecular Dynamics.

RESULTS

Topoisomerase II Directs the Site-Directed Alkylation of DNA by Psorospermin but Not by Pluramycin. Experiments were designed to test the reactivity of psorospermin with the topoisomerase II–DNA complex. The DNA substrate shown in Fig. 2*D* originally was used in a DNase I footprinting study in which the enlarged region in Fig. 2*D* was protected by *Drosophila* topoisomerase II (19). In this earlier study, only one topoisomerase II cleavage site (site A) was detected in this sequence; however, in the present study, it was discovered that this DNase I-protected region contains two adjacent topoisomerase II cleavage sites (sites A and B in Fig. 3), although the intensity of the cleavage is much lower at site B (lane 1 in Fig. 3). The 5′ end-labeled DNA was incubated with various amounts of *Drosophila* topoisomerase II to form the topoisomerase II–DNA complex. Psorospermin then was added to the reaction mixture followed by piperidine-heat treatment to generate the strand-breakage products resulting from depurination of the N7-alkylating guanine (8). For comparison, pluramycin was used instead of psorospermin in a parallel experiment. In the absence of topoisomerase II, psorospermin (lane 8 in Fig. 2*A* and arrows, which indicate the alkylated guanines) displays a significant difference in reactivity with DNA in comparison to pluramycin (lane 1); i.e., the amount of DNA strand-cleavage products using 0.5 μM pluramycin (lane 1) is much greater than that produced by 10 μM psorospermin (lane 8). This result agrees with our previous study, in which psorospermin was shown to be between 1/10 and 1/100 as reactive as the most reactive pluramycins (8). However, in the presence of increasing amounts of topoisomerase II, only the alkylation by psorospermin at the topoisomerase II cleavage site B was enhanced (lanes 9–12 vs. lanes 2–5). To precisely define the location of the base showing the enhanced alkylation at the topoisomerase II cleavage site, negative and positive numbers (. . . −2, −1, +1, +2, +3, +4, +5, +6. . .) were used to identify the nucleotides at the 3′ and 5′ termini of the topoisomerase II-cleaved phosphodiester bonds for the top strand and, correspondingly, (. . . +6′, +5′, +4′, +3′, +2′, +1′, −1′, −2′. . .) for the bottom strand (Fig. 2*D*). According to this nomenclature, the base showing enhanced alkylation (Fig. 2*A*, ∗) is a guanine located at the +4′ position of cleavage site B (Fig. 2*D*). Quantitative analysis using a PhosphorImager shows a 25-fold enhancement of psorospermin reactivity at this site (Fig. 2*C*, ●). A similar site of increased psorospermin reactivity was found at the guanine on the top strand at the +4 position of site A (Fig. 2*D*; Fig. 2*B*, ∗), although the magnitude of the increased reactivity was only 3-fold. The corresponding guanine on the top strand at site B shows only slightly increased reactivity (Fig. 2*B*, #). Psorospermin alkylation reactivity at other positions is either unchanged or shows slightly diminished reactivity in the presence of topoisomerase II (Fig. 2*D*). However, the guanines located 3′ to the guanine with enhanced alkylation on the bottom strand are underestimated because of the strand breakage site nearer to the 5′ label. Despite the reduction of psorospermin alkylation reactivity at some positions, the total DNA alkylation of both the top and bottom strands was significantly increased (Fig. 2*C*, ▲). In contrast to psorospermin, pluramycin alkylation of the guanine at the +4′ position of cleavage site B is significantly inhibited as the amount of topoisomerase II increases (Fig. 2*A*, lanes 1–5). In addition, several guanines close to the topoisomerase II cleavage sites are protected from pluramycin alkylation, as shown in Fig. 2*D*.

The enhanced cleavage bands detected in the gel (Fig. 2*A*) could not have come from the products generated by the topoisomerase II-induced DNA cleavage. The topoisomerase II cleavage sites on the bottom strand are either four bases 5′ (site A) or three bases 3′ (site B) to the guanine that shows the enhanced alkylation. Using SDS and proteinase K, which trap the enzyme-induced cleavage (20–22), instead of the piperidine-heat treatment, we found that psorospermin did not induce any new topoisomerase II cleavage sites (Fig. 3). Moreover, addition of excess salt to the topoisomerase II cleavage reactions reversed most topoisomerase II cleavage products (data not shown) but did not affect the alkylation sites. This result indicates that these topoisomerase II-mediated DNA cleavage products stimulated by psorospermin are reversible. The absence of these products in the gel after piperidine-heat treatment suggests that they were reversed by heat treatment, which is consistent with results from a previous study that showed that elevated temperatures can reverse the cleaved complex (23).

Psorospermin Enhances the DNA Cleavage Produced by Topoisomerase II at Site B, but Reduces It at Site A. Because topoisomerase II greatly enhances the psorospermin alkylation of the guanine at the +4′ position of site B, it was important to determine the effect of psorospermin on the topoisomerase II-mediated DNA cleavage. In the absence of psorospermin, the intensity of the topoisomerase II-mediated DNA cleavage is

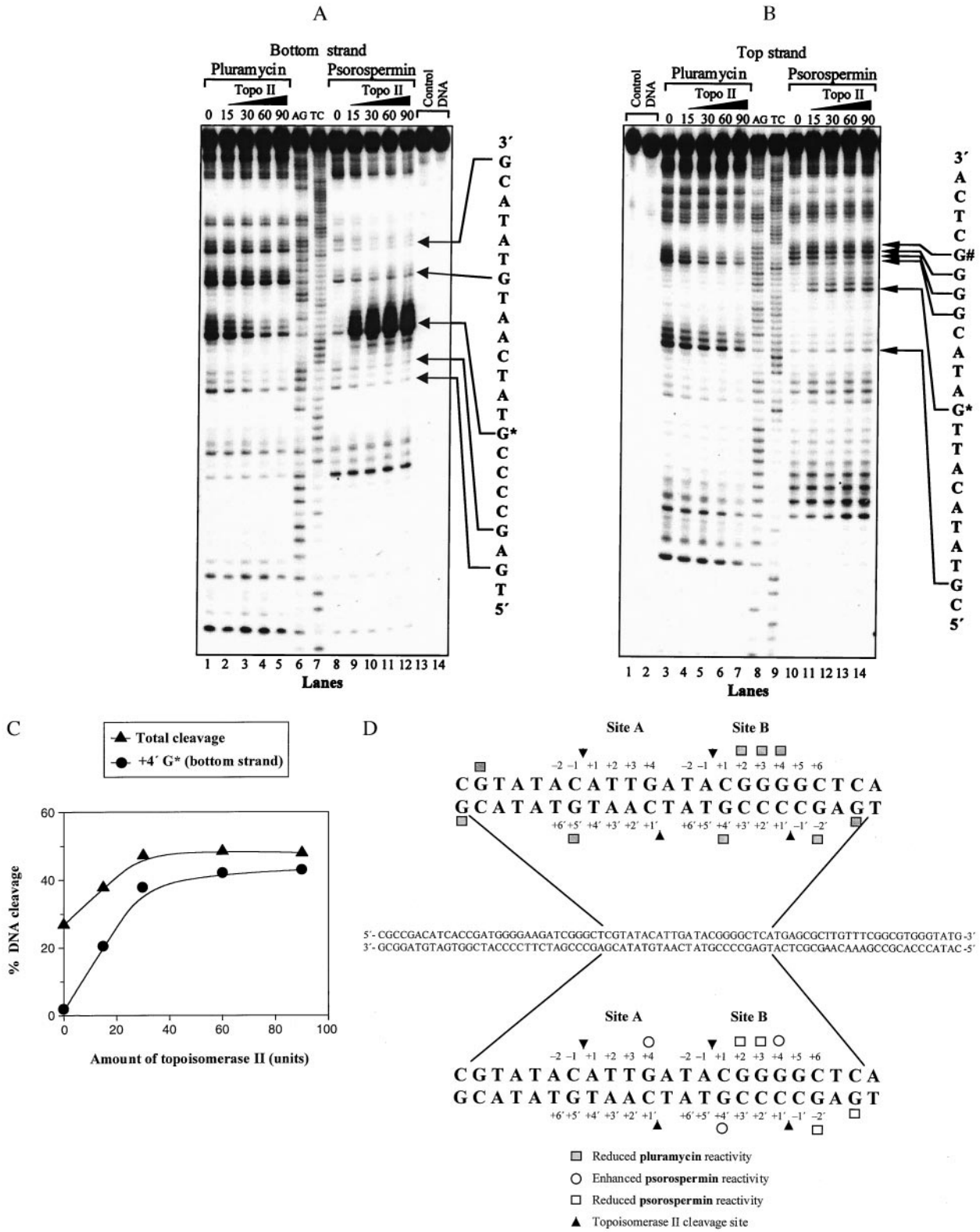

FIG. 2. Effect of topoisomerase II on the extent of alkylation of DNA by psorospermin and pluramycin. (*A* and *B*) Autoradiograms of 12% denaturing polyacrylamide gels showing the DNA strand-breakage patterns of the bottom (*A*) and top (*B*) strands produced by psorospermin and pluramycin. Lanes 13 and 14 in *A* and lanes 1 and 2 in *B* are control DNA with and without heat treatment, respectively. Lanes 6 and 7 in *A* and lanes 8 and 9 in *B* contain the Maxam–Gilbert sequencing reactions of AG and TC. Lanes 1–5 in *A* and lanes 3–7 in *B* contain 0.5 μM pluramycin, respectively. Lanes 8–12 in *A* and lanes 10–14 in *B* contain 10 μM of psorospermin, respectively. Lanes 1 and 8 in *A* and lanes 3 and 10 in *B* are in the absence of protein. Lanes 2 and 9, 3 and 10, 4 and 11, and 5 and 12 in *A* and lanes 4 and 11, 5 and 12, 6 and 13, and 7 and 14 in *B* contain 15, 30, 60, and 90 units of *Drosophila* topoisomerase II, respectively. (*C*) Quantification was performed by using IMAGEQUANT 4.1 software from Molecular Dynamics. Percentage of total DNA cleavage produced by psorospermin was determined from the volume of the cleavage bands (sum of the top and bottom strands for the "total cleavage") divided by the total volume in each lane (sum of the top and bottom strands for the "total cleavage"). (*D*) The 80-bp oligonucleotide used in this study. This sequence originally was used in the DNase I footprinting studies of *Drosophila* topoisomerase II carried out by Lee *et al.* (19).

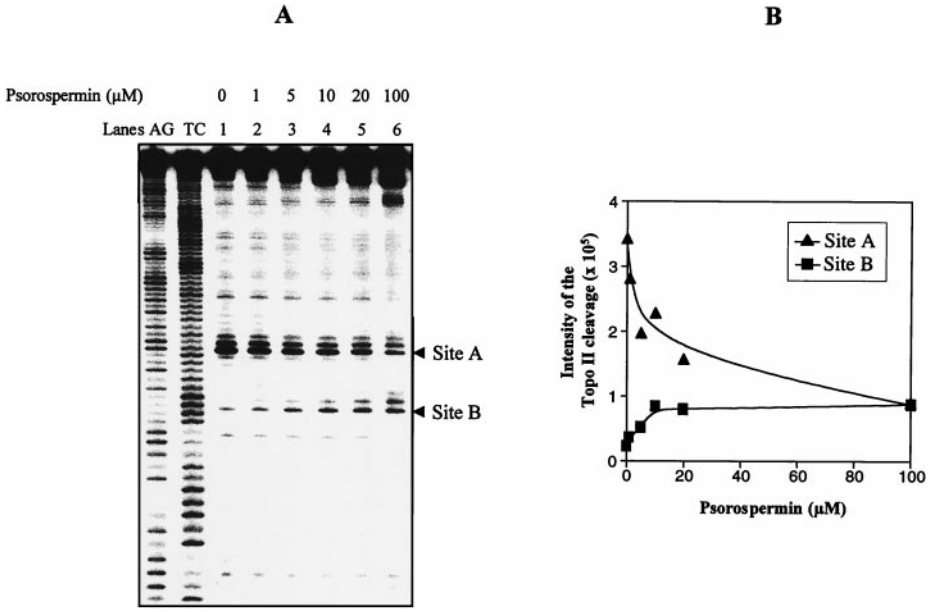

FIG. 3. Effect of psorospermin on DNA cleavage by topoisomerase II. (*A*) Autoradiogram of a 12% denaturing polyacrylamide gel showing the topoisomerase II-mediated cleavage pattern of the bottom strand (Fig. 2*D*) in the presence of psorospermin. Lanes AG and TC contain the Maxam–Gilbert sequencing reactions. Lane 1 contains no drug. Lanes 2–6 contain 1, 5, 10, 20, and 100 μM psorospermin, respectively. (*B*) Topoisomerase II cleavage products indicated by the arrows in *A* were quantitated using a PhosphorImager and analyzed by using IMAGEQUANT 4.1 software (Molecular Dynamics). The intensity of the topoisomerase II-mediated cleavage was determined from the volume of the bands (indicated by arrows in *A*) normalized by the total radioactivity in each lane.

much lower at site B than site A (Fig. 3*A*, lane 1). As the concentration of psorospermin was increased, the topoisomerase II-mediated DNA cleavage at site A was decreased, whereas the cleavage at site B was enhanced (Fig. 3*A*, lanes 2–6). The psorospermin-induced DNA cleavage by topoisomerase II reaches a maximum of 3-fold at a 10 μM drug concentration (Fig. 3*B*). This result suggests that psorospermin alkylation at site B traps the topoisomerase II–DNA complex at this site. On the other hand, the cleaved-complex formation at site A was reduced in the presence of psorospermin, despite the 3-fold enhancement of psorospermin alkylation at site A. Sites A and B are 3 base pairs away from each other, and *Drosophila* topoisomerase II binds a region of approximately 23 bp, based on the results of a DNase I footprinting experiment (19). Therefore, it is likely that sites A and B are competing with each other for topoisomerase II binding, and the 25-fold enhancement of the psorospermin alkylation at site B dominates this competition.

In the Presence of Topoisomerase II, *m*-AMSA, but Not Etoposide, Competes with Psorospermin Alkylation of DNA. Because psorospermin shows enhanced reactivity for certain guanines at the topoisomerase II cleavage sites, and the covalently modified guanine can be precisely located by a heat strand-breakage assay (7), this drug is potentially an excellent tool for mapping the binding sites of other topoisomerase II inhibitors on DNA. Two classes of topoisomerase II inhibitors were chosen: *m*-AMSA, an intercalative agent, and etoposide, a nonintercalative agent (15, 16). Both drugs are clinically used cancer chemotherapeutic agents. The effects of *m*-AMSA and etoposide on topoisomerase II-mediated DNA cleavage are shown in Fig. 4. As the concentration of *m*-AMSA was increased, the DNA cleavage produced by topoisomerase II at site B was enhanced, whereas the cleavage at site A was diminished (Fig. 4*A*, lanes 2–6) compared with that in the absence of drug (Fig. 4*A*, lane 1). In the case of etoposide, the topoisomerase II-mediated DNA cleavage at both sites A and B were enhanced in the presence of the drug (Fig. 4*B*, lanes 8–12). This result demonstrates that both *m*-AMSA and etoposide are topoisomerase II poisons at site B.

The results of individual competition experiments between psorospermin and *m*-AMSA and etoposide in the presence of

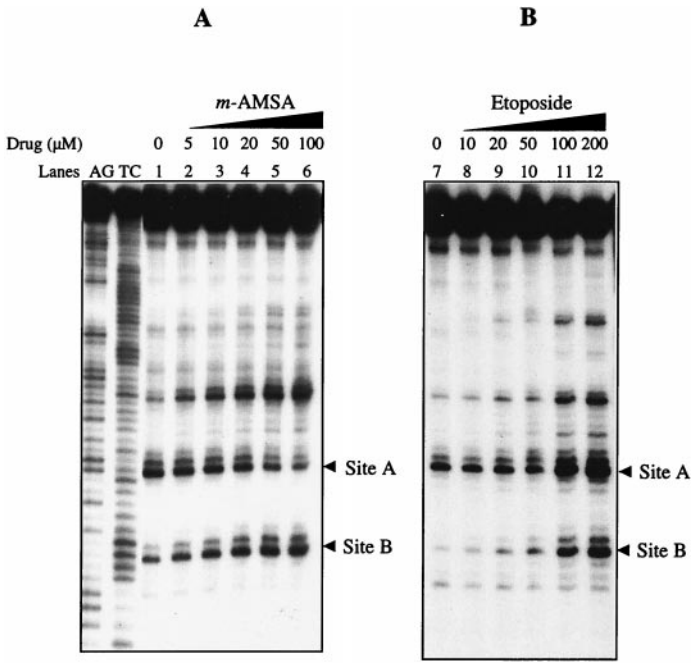

FIG. 4. Effect of *m*-AMSA and etoposide on DNA cleavage by topoisomerase II. (*A*) Lanes AG and TC contain the Maxam–Gilbert sequencing reactions. Lane 1 contains no drug. Lanes 2–6 contain 5, 10, 20, 50, and 100 μM *m*-AMSA, respectively. Because *m*-AMSA was dissolved into dimethyl sulfoxide, which is made up of 10% of the reaction buffer, control reaction without drug (lane 1) contains 10% of dimethyl sulfoxide as well, which slightly enhances the DNA cleavage produced by topoisomerase II (compare with lane 7 in *B*). (*B*) Lane 7 contains no drug. Lanes 8–12 contain 10, 20, 50, 100, and 200 μM etoposide, respectively.

topoisomerase II are shown in Fig. 5*A*. In these experiments, the topoisomerase II–DNA complex was formed in the presence of topoisomerase II poisons before psorospermin was added to the reaction mixture. As the concentration of *m*-AMSA was increased (Fig. 5*A*, lanes 7–11), the amount of guanine alkylation by psorospermin decreased, as shown by the reduction of the strand breakage product (Fig. 5 *A* and *B*). In contrast, when the nonintercalating etoposide was used (Fig. 5*A* lanes 12–16) there was little change in the amount of alkylation by psorospermin. These results suggest that in the presence of topoisomerase II, whereas *m*-AMSA intercalates into DNA on the same or adjacent base pair as psorospermin, etoposide binds to a site distinct to psorospermin.

DISCUSSION

The results reported here demonstrate that psorospermin alkylation of DNA is significantly enhanced in the presence of topoisomerase II. In a previous study, Snapka *et al.* (13) showed that simian virus 40 DNA from psorospermin-treated cells has a very high density of abasic sites. These abasic sites are proposed to arise from psorospermin modification of guanine at the N7 position followed by the loss of alkylated bases (8). Simian virus 40 DNA was found to form cross-links with a number of proteins, including topoisomerase I and II, in the presence of psorospermin (13). Because amino groups of proteins can react with abasic sites to form Schiff bases, resulting in covalent protein–DNA cross-links, it was suggested that the topoisomerase–DNA cross-links formed in the presence of psorospermin were nonspecific. However, the results reported here suggest that these protein–DNA cross-links derive from the specific interaction between psorospermin and the topoisomerase II–DNA complex. Psorospermin reactivity with DNA is dramatically increased at certain topoisomerase II cleavage sites (i.e., site B in Fig. 2*D*). In addition, psorospermin enhances DNA cleavage by topoisomerase II at the same site, indicating that psorospermin is a topoisomerase II poison that traps the cleaved complex, resulting in protein–DNA cross-links. Because abasic sites can stimulate DNA cleavage by topoisomerase II when the lesions are located within the 4-base cleavage overhang (24–26), psorospermin–DNA adducts and the abasic sites resulting from depurination both could contribute to the enhanced topoisomerase II cleavage. However, depurination was not observed under the experimental conditions described here (unpublished results). Therefore, psorospermin–DNA adduct formation most likely is the cause of the topoisomerase II poisoning, although we cannot exclude the possibility that depurination can occur *in vivo*, resulting in protein–DNA cross-links. These results also suggest that these protein–DNA cross-links may be the major contributors to the antitumor activity of this compound. Topoisomerase II binding to DNA at the gate site produces high-affinity binding sites that presumably lead to enhanced psorospermin alkylation, which then traps the topoisomerase II cleaved complex and produces higher levels of protein–DNA cross-links. Because rapidly proliferating or neoplastic cells usually contain elevated levels of topoisomerase II (27–29), psorospermin may be more cytotoxic to these cells than to normal cells.

Because pluramycin is mechanistically related to psorospermin and is 10–100 times more reactive to DNA than psorospermin (8), it was surprising that pluramycin alkylation of DNA was inhibited in the presence of topoisomerase II. The most likely explanation is that the geometry of the DNA intercalation binding pocket induced by topoisomerase II binding at the gate site is favorable for psorospermin alkylation but unfavorable for pluramycin, possibly because of the bulky carbohydrate substituents of pluramycin and/or the geometry of intercalation. In accord with these proposals, high-field NMR studies have revealed that pluramycin intercalates into DNA perpendicular to the adjacent base pairs, which places the sugar substituents into the minor groove of DNA. In contrast, psorospermin, which lacks the minor-groove sugar substituents, positions the intercalating chromophore in an orientation parallel to the base pairs to maximize the stacking interactions (8). These different modes of psorospermin and pluramycin precovalent interaction with DNA presumably dictate the different alkylating reactivities in the topoisomerase II cleavage sites and explain the differential effects of the two drugs in the presence of topoisomerase II.

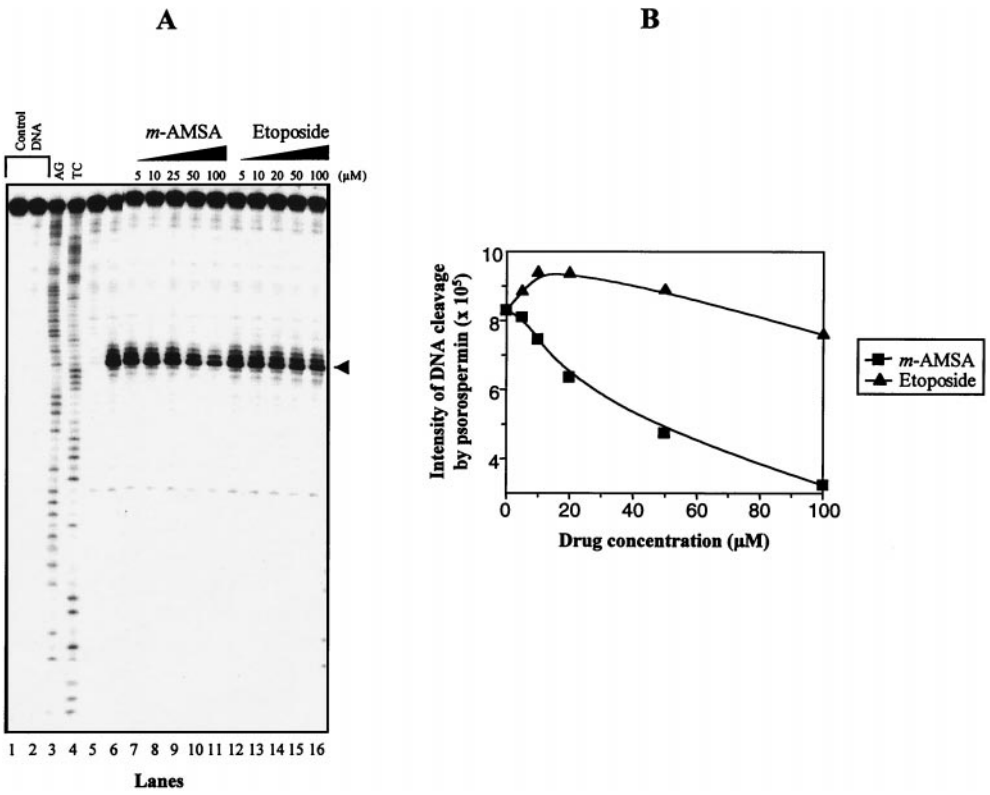

FIG. 5. Results of competition experiments between psorospermin and the topoisomerase II inhibitors *m*-AMSA and etoposide. (*A*) The *Drosophila* topoisomerase II–DNA complex was formed as described in *Materials and Methods* in the presence of various amounts of *m*-AMSA and etoposide. Psorospermin (10 μM) then was added to the reaction mixture, and the reaction continued for another 5 min followed by heat strand breakage (8). Lanes 1 and 2 are control DNA without and with heat treatment, respectively. Lanes 3 and 4 contain the Maxam–Gilbert sequencing reactions for AG and TC. Lane 5 contains DNA and psorospermin only. Lane 6 contains DNA, psorospermin, and topoisomerase II without inhibitor. Lanes 7–11 and 12–16 contain 5, 10, 25, 50, and 100 μM *m*-AMSA and etoposide, respectively. (*B*) Strand breakage products indicated by the arrowhead in *A* were quantitated using a PhosphorImager and analyzed by using IMAGEQUANT 4.1 software (Molecular Dynamics). The intensity of the DNA cleavage produced by psorospermin was determined from the volume of the bands (indicated by the arrowhead in *A*) normalized by the total radioactivity in each lane.

A number of previous studies have suggested that high-affinity DNA binding sites for drugs are created by topoisomerase II, which induces DNA conformational change on binding to DNA. For example, gyrase (bacterial topoisomerase II) and bacterial topoisomerase IV produce DNA distortions (possibly unwinding) that introduce the high-affinity binding sites for the quinolones (12, 30–33). Likewise, T4 topoisomerase II creates preferential binding sites for *m*-AMSA at the two sites in the immediate vicinity of the gate (34). Similarly, psorospermin appears to take advantage of the DNA distortion produced by binding of topoisomerase II. The intercalation and enhanced covalent alkylation site for psorospermin in the presence of topoisomerase II are within the gate site, with alkylation occurring at the +4′ position. Finally, psorospermin is a topoisomerase II poison that traps the enzyme–DNA complex, suggesting that psorospermin also may interact with topoisomerase II or, alternatively, distort the DNA on covalent reaction to slow down the religation step (35).

The competition experiments (Fig. 5) demonstrate that whereas the DNA intercalating topoisomerase II poison *m*-AMSA can compete with psorospermin alkylation at site B, the nonintercalating etoposide is unable to do so. Because *m*-AMSA is a DNA intercalator, this competition effect may be caused by either its intercalation into DNA on the same or adjacent base pair as psorospermin or nonspecific inhibition of topoisomerase II binding to DNA at site B. However, *m*-AMSA can enhance the topoisomerase II-mediated DNA cleavage at site B up to at least a 100 μM concentration (Fig. 4*A*), eliminating the possibility that *m*-AMSA prevents the nonspecific binding of topoisomerase II to DNA at site B. Hence, the competition between *m*-AMSA and psorospermin indicates that both of them bind to the DNA intercalation pocket induced by topoisomerase II.

Both the high-field NMR and gel electrophoresis studies show that psorospermin intercalates between the DNA base pairs and alkylates the guanine to the 3′ side of the intercalation site (8). On the basis of these results, the tricyclic xanthone chromophore of psorospermin is located within the topoisomerase II–DNA complex between the +1 and +2 positions of the gate. Psorospermin is unique among the topoisomerase II poisons, because most of them interact with the −1 and +1 positions or the +2 and +3 positions (16). To compete with psorospermin alkylation, *m*-AMSA either could intercalate between the same base pairs as psorospermin (the position between +1 and +2) or, in accord with the nearest-neighbor exclusion principle for DNA intercalators (36, 37), between the adjacent base pairs (i.e., the positions between −1 and +1 or +2 and +3). Several mutational analysis and sequence-selectivity studies of *m*-AMSA (38–40) have shown that *m*-AMSA has base preferences at the −1 and +1 positions, suggesting that it interacts primarily with these two base pairs. Recently, a photoactive analogue of *m*-AMSA was shown to alkylate the bases at both the −1 and +4′ positions, direct evidence for *m*-AMSA positioning between the −1 and +1 base pairs (34). Therefore, to account for the competition between the covalently anchored psorospermin and the intercalating topoisomerase II poison *m*-AMSA, we propose that the nearest-neighbor exclusion effect, in which *m*-AMSA intercalates between the −1 and +1 positions, excludes psorospermin intercalation between the neighboring base pairs (i.e., +1 to +2 positions). Conversely, etoposide, which does not intercalate between the base pairs, may trap the cleaved complex by other mechanisms. Moreover, a recent study has suggested that etoposide induces the topoisomerase II–DNA cleaved complex through direct enzyme–drug (rather than DNA–drug) interaction (41).

In summary, in the presence of topoisomerase II, the anticancer agent psorospermin shows an unusually high reactivity with DNA within the gate site, whereas the structurally related pluramycins show diminished alkylation at the same position. Furthermore, psorospermin would have enhanced cytotoxicity to cell lines that have elevated levels of topoisomerase II. Psorospermin is unique among topoisomerase II poisons in two respects: it intercalates between the base pairs at the +1 to +2 position of the gate site, and it forms a covalent adduct. The use of psorospermin as a structural probe to map the drug binding sites within the topoisomerase II–DNA complex has demonstrated that the intercalating topoisomerase II poison *m*-AMSA interacts with the base pairs surrounding the topoisomerase II-cleaved phosphodiester bonds, whereas the nonintercalating agent etoposide binds to a different site. These results provide important insights for the design of a new generation of DNA-intercalative topoisomerase II poisons.

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