Structural Insight into a Quinolone-Topoisomerase II-DNA Complex

FURTHER EVIDENCE FOR A 2:2 QUINOBENZOXAZINE-Mg$^{2+}$ SELF-ASSEMBLY MODEL FORMED IN THE PRESENCE OF TOPOISOMERASE II*

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Quinobenzoxazine A-62176, developed from the antibacterial fluoroquinolones, is active in vitro and in vivo against murine and human tumors. It has been previously claimed that A-62176 is a catalytic inhibitor of mammalian topoisomerase II that does not stabilize the cleaved complex. However, at low drug concentrations and pH 6–7, we have found that A-62176 can enhance the formation of the cleaved complex at certain sites. Using a photocleavage assay, mismatched sequences, and competition experiments between psorospermin and A-62176, we pinpointed the drug binding site on the DNA base pairs between positions +1 and +2 relative to the cleaved phosphodiester bonds. A 2:2 quinobenzoxazine-Mg$^{2+}$ self-assembly model was previously proposed, in which one drug molecule intercalates into the DNA helix and the second drug molecule is externally bound, held to the first molecule and DNA by two Mg$^{2+}$ bridges. The results of competition experiments between psorospermin and A-62176, as well as between psorospermin and A-62176 and norfloxacin, are consistent with this model and provide the first evidence that this 2:2 quinobenzoxazine-Mg$^{2+}$ complex is assembled in the presence of topoisomerase II. These results also have parallel implications for the mode of binding of the quinolone antibiotics to the bacterial gyrase-DNA complex.

The type II topoisomerases (topo II)$^1$ are enzymes that regulate the topological state of DNA (1, 2). These enzymes function by making transient double-stranded DNA breaks (or “gates”) and allowing another DNA helix to pass through these breaks. At the DNA cleavage step, topo II is covalently linked to the 5′-phosphoryl end of the broken DNA by DNA-enzyme transesterification, then following strand passage, the DNA gate is resealed by topo II. Eukaryotic topo II has been identified as the molecular target of a number of potent anticancer drugs, such as the anthracyclines, acridines, and epipodophyllotoxins. Many of these topo II inhibitors, collectively known as topo II poisons, interfere with the breakage-rejoining reactions of topo II by trapping the covalent reaction intermediate, which is called the cleavable complex or, more accurately, the cleaved complex (3–8). Other topo II inhibitors, known as topo II suppressors, inhibit the catalytic activity of topo II without trapping the cleaved complex (8).

The quinobenzoxazine compounds (typified by 1-(3-aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4H-quinol[2,3,4-i,j][1,4]benzoxazine-5-carboxylic acid (A-62176); Fig. 1) were initially developed from antibacterial fluoroquinolones (typified by norfloxacin, Fig. 1) by scientists at Abbott Laboratories (9–11). These compounds are active against a number of human and murine cancer cell lines including the multidrug resistant P388/ADR line in vitro and several murine and human tumors in vivo (9). An initial study has revealed that the quinobenzoxazines are potent inhibitors of mammalian topo II, both in vitro and in vivo (12). According to this study, the quinobenzoxazines appear to belong to the class of topo II suppressors that interfere with the catalytic activity of the topo II at a step prior to the formation of the cleaved complex.

The quinobenzoxazines, which are structurally related to fluoroquinolones, possess a planar tetracyclic ring in place of the fused bicyclic ring of fluoroquinolones (Fig. 1). The extended flat aromatic ring structure of quinobenzoxazines enables them to form a stable intercalation complex with the DNA helix (12), while the more limited aromaticity of the fluoroquinolone norfloxacin prevents the formation of a stable intercalation complex with duplex DNA (13, 14). Preliminary data from Abbott Laboratories showed that DNA binding of quinobenzoxazine A-62176, like norfloxacin, is Mg$^{2+}$-dependent (15). Further biophysical and electrophoretic studies on the ternary complex between the quinobenzoxazine-Mg$^{2+}$ complex and DNA have provided evidence for a 2:2 quinobenzoxazine-Mg$^{2+}$ self-assembly complex, in which one quinobenzoxazine molecule is intercalated into the DNA helix and the second drug molecule is externally bound, held together by two Mg$^{2+}$ bridges (17). In this proposed ternary complex, the externally bound quinobenzoxazine molecule can be replaced by norfloxa- cin to form mixed-structure dimers on DNA, an observation that is supported by the additive effects observed on the DNA binding of quinobenzoxazines when norfloxacin is added. This 2:2 quinobenzoxazine-Mg$^{2+}$ self-assembly complex has elements of both the Shen model (13, 14), which involves a self-assembly complex, and the Palumbo model (15, 16), which proposed a phosphate-Mg$^{2+}$-drug complex.

To further characterize the molecular basis of the anticancer activity exhibited by the quinobenzoxazines, the interaction of quinobenzoxazine A-62176 with the topo II-DNA complex has been investigated. In contrast to the previous findings that A-62176 catalytically inhibits topo II without stabilizing the cleaved complex (12), we have determined that A-62176 possesses a dual mechanism at pH 6–7. At one topo II cleavage site, A-62176 enhances the cleaved complex formation at low drug concentrations but inhibits it at high drug concentrations, and at another topo II cleavage site, A-62176 inhibits the cleaved complex formation at both low and high drug concent-

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‡ The abbreviations used are: topo II, topoisomerase II; AppNHp, β,γ-imidoadenosine 5’-triphosphate; A-62176, 1-(3-aminopyrrolidin-1-yl)-2-fluoro-4-oxo-4H-quinol[2,3,4-i,j][1,4]benzoxazine-5-carboxylic acid.
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Photocleavage Reactions—The 5'-32P-labeled DNA was incubated with Drosophila topo II in 20 μl of a reaction buffer (10 mM imidazole-HCl (pH 6.0), 10 mM MgCl₂, 50 mM KCl, and 1 mM App(NH)p) in the presence of 0.5 μM A-62176. The samples were loaded onto a 24-well Titertek microtiter plate (ICN) on top of a Pyrex glass shield and irradiated for 1 h with an 85-watt xenon lamp placed under the Pyrex glass. (Pyrex glass is used to filter out the UV light under 300 nm, thereby eliminating DNA damage caused directly by UV irradiation.) During the irradiation, the Titertek plate was turned three times to eliminate the light heterogeneity. Reactions were terminated by adding 10 μg of calf thymus DNA, followed by heating at 95 °C for 15 min in the presence of 20 mM Tris-HCl (pH 6.0), 10 mM MgCl₂, 50 mM KCl, and 1 mM App(NH)p containing various amounts of either A-62176, norfloxacin, or both. The incubation reactions proceeded at 30 °C for 10 min, followed by addition of psorospermin (10 μM final concentration). The reactions were 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 induces strand breakage at the drug modification sites (20). The samples were extracted with phenol/chloroform followed by ethanol precipitation.

Competition Experiments—The 5'-32P-labeled DNA was incubated with Drosophila topo II in a buffer (10 mM imidazole-HCl (pH 6.0), 10 mM MgCl₂, 50 mM KCl, and 1 mM App(NH)p) containing various amounts of either A-62176, norfloxacin, or both. The incubation reactions proceeded at 30 °C for 10 min, followed by addition of psorospermin (10 μM final concentration). The reactions were 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 induces strand breakage at the drug modification sites (20). The samples were extracted with phenol/chloroform followed by ethanol precipitation.

Protocol Electrophoresis and Quantification—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 using a PhosphorImager and ImageQuant 4.1 software from Molecular Dynamics.

RESULTS

The Effect of A-62176 on the DNA Cleavage Produced by Topo II at Sites A and B of the 80-mer DNA

Experiments were designed to determine the effect of A-62176 on the cleavage of DNA produced by topo II. The 80-mer shown in Fig. 2 was originally used in a DNase I footprinting study, in which the enlarged region was protected by Drosophila topo II, and only one topo II cleavage site (site A) was detected (21). However, in the present study, it was discovered that this fragment contains two adjacent topo II cleavage sites (sites A and B in Fig. 3, lane 1), although the intensity of the cleavage is much less at site B than at site A. Under similar conditions to those used in the DNase I study (in the presence of App(NH)p, a nonhydrolyzable ATP, and at pH 6), the intensity of the topo II-mediated cleavage at site A decreased as the concentrations of A-62176 were increased. Although the intensity of the cleavage at site B was initially enhanced, it also eventually decreased at high drug concentrations (lanes 2–7 in Fig. 3A). This reflects the typical bell-shaped curve for intercalative topo II poisons (8), as shown in Fig. 3B. In a previous study, it was proposed that the quinobenzoxazines are catalytic inhibitors of mammalian topo II and do not trap the cleaved complex (12). In accordance with the results of this study, we also found that at pH 7.5 and above, the intensity of topo II-mediated cleavage at both sites A and B decreased with increasing amounts of A-62176 (data not shown). However, at a lower pH (pH 6–7) and using App(NH)p, A-62176 can enhance Drosophila topo II-mediated DNA cleavage at site B at low drug concentrations (Fig. 3 at pH 6, and data at pH 7 not shown), which is typical of intercalative topo II poisons.

In order to determine whether the use of App(NH)p is critical for A-62176 to behave like a topo II poison, ATP was used instead of App(NH)p in a repetition of the topo II cleavage assay described above, and the results are shown in Fig. 4. From a comparison of Figs. 3 and 4, it can be seen that A-62176 behaves the same with ATP as it does with App(NH)p, although the topo II-mediated cleavage level at site B reaches the maximum at about 0.2 μM of A-62176 with ATP instead of the 1 μM found with App(NH)p. These results demonstrate that, at low drug concentrations and pH levels between 6 and 7,
A-62176 enhances DNA cleavage by topo II at site B and reduces cleavage at site A, regardless of whether ATP or App(NH)p is used.

Evidence That A-62176 Intercalates at the $+1$ and $+2$ Sites

The Effect of Mismatched Base Pairs on Topo II Cleavage at Sites A and B—Recently, Bigioni and co-workers (22) observed that mismatches introduced at the $+4$, $+3$, $+2$, or $+1$ positions reduced or abolished the topo II-mediated cleavage, whereas at $+1$ and $+2$ positions, the topo II-mediated cleavage increased (see Fig. 2 legend for number definition). Since A-62176 can stimulate the topo II-mediated DNA cleavage at site B, it seemed possible that A-62176 might have a similar effect at site B as seen with the mismatched sequences. To test this hypothesis, four sets of oligonucleotide sequences were synthesized to contain mismatched base pairs at the $+1$, $+2$, $+3$, or $+4$ positions of site B (Fig. 5A). The topo II cleavage assays were performed on all four mismatched sequences (Fig. 5B). In comparison to the wild type sequence (lane W), mismatches at either the $+1$ (lane M1) or $+2$ (lane M2) positions enhance the topo II-mediated DNA cleavage at site B and abolish (in the case of M1) or reduce (in the case of M2) the topo II cleavage at site A. On the other hand, mismatches at $+3$ (lane M3) and $+4$ (lane M4) positions have little effect on topo II-mediated DNA cleavage at site B. These results demonstrate that mismatched base pairs at either $+1$ or $+2$ positions at site B can mimic the A-62176 effect on topo II-induced cleavage at site A and B, suggesting that A-62176 either introduces or stabilizes a DNA distortion that is similar to a mismatch.

In the Presence of Topo II, A-62176 Produces Enhanced DNA Photocleavage at Site B—A photocleavage assay was used to determine the binding sites of A-62176 in the topo II-DNA complex. A-62176 has been shown to photocleave DNA around the drug intercalating site (2). Upon UV irradiation, DNA is cleaved through a free radical mechanism, leading to several DNA breakage products. The major products detectable in the gel electrophoresis after heating with piperidine are 3'-phosphate termini products, which co-migrate with the Maxam-Gilbert sequencing products in the polyacrylamide gel electrophoresis (23).

The DNA photocleavage ability of A-62176 was used to locate its binding site in the topo II-DNA complex, as shown in Fig. 6A. In the absence of topo II, there is a significant amount of
DNA photocleavage by A-62176 (lanes 5–7 in Fig. 6A). In the presence of topo II, the overall amount of photocleavage of DNA by A-62176 decreased (lanes 8–10) as the amount of topo II was increased, possibly due to the increased amount of glycerol (50%) present in the protein storage buffer, since glycerol is known to quench the free radical reaction. However, in comparison with the control (lanes 5–7), the photocleavage of the guanine at the +2 position (arrows in Fig. 6A) was enhanced (lanes 8–10). In comparison with other photocleavage sites (i.e., bands II and III), the enhanced site (band I) was clearly amplified 1.5-fold (or 50% enhancement) in lanes 8–10 while the other bands were slightly diminished (Fig. 6D). These enhanced bands could not have come from the products generated by topo II-induced DNA cleavage, since those products associated with site B (lane 4) travel a half base farther than the enhanced photocleavage products in the gel.\(^3\) In fact, with an enlarged scan of this region (lane 10 in Fig. 6C), the residue of this topo II cleavage band can be detected as a "shoulder" of the guanine peak that has the enhanced photocleavage.

\(^3\) Photoreaction with DNA alone shows no DNA cleavage under our conditions (lane 2 in Fig. 6A). In the presence of topo II, the enhanced bands detected in the gel could not have come from the products generated by topo II-induced DNA cleavage for two reasons. First, the reaction in lane 3, which is similar to that in lane 12 but without A-62176, showed a reversal of most topo II cleavage products upon heat-piperidine treatment. Second, with adenine at its 3'-end, the topo II cleavage product at site B should have traveled one base farther than the enhanced photocleavage product with cytosine at its 3'-end in the polyacylamide gel electrophoresis (Fig. 2). However, the DNA cleavage product produced by topo II has a 3'-hydroxyl terminus, which migrates one-half base less than the 3'-phosphate product at the same position in the gel. Therefore, the topo II cleavage product of site B on the top strand travels a half base farther than the photoproduct with enhanced cleavage in the gel.
FIG. 6. Photocleavage of DNA by A-62176. A, the photocleavage reaction of A-62176 was done as described under “Experimental Procedures” with (lanes 3 and 4 and 8–10) and without Drosophila topo II (lanes 1 and 2, 5–7, and 11 and 12), except in lane 4, where topo II cleavage reaction was used. After the topo II-DNA incubation, the reaction in lane 4 was subjected to SDS-proteinase K digestion instead of piperidine-heat treatment, as described in the topo II cleavage reaction (“Experimental Procedures”). Lane 1 contains DNA without any treatment, and lane 2 contains DNA with UV irradiation and heat-piperidine treatment. Lanes 3 and 4 contain 120 units of topo II but no A-62176. Lanes 5–7 contain 1.5, 3, and 6 µl of topo II dilution buffer (10 mM sodium phosphate, pH 7.1, 50 mM NaCl, 0.2 mM dithiothreitol, 0.1 mM EDTA, 0.5 mg/ml bovine serum albumin, 10% glycerol), respectively. These three lanes were used to detect whether the glycerol (10%) and dithiothreitol (0.2 mM) in the dilution buffer will inhibit the photocleavage of DNA by A-62176, and it turns out there is little difference between these three lanes. Lanes 8–10 contain 30, 60, and 120 units of Drosophila topo II, respectively. Lanes AG and TC contain the Maxam-Gilbert sequencing reactions. B and C, PhosphorImager scans of lanes 4, 5, and 8–14 of A. Since the total amount of cleavage was reduced in lanes 8–10, the scales of these three lanes were enlarged to match lane 5 (control). D, bands I, II, and III, indicated by the arrows in B, were quantitated using a PhosphorImager and analyzed using ImageQuant software (Molecular Dynamics). The intensity (arbitrary unit) of each band was determined from the volume of each band normalized by the total cleavage products in each lane.
photocleavage. This result demonstrates an increased A-62176 binding to the guanine around the +2 position of cleavage site B in the presence of topo II, indicating that topo II induces a DNA conformational change that creates a high affinity site for binding of A-62176.

A-62176 Produces Enhanced Photocleavage at Site B in Mismatched Sequences M1 and M2—To shed further light on the nature of the structural distortion of DNA induced by topo II, mismatched sequences M1 and M2 were used in the photocleavage reaction. These two sequences, which have a mismatched base pair at either the +1 or +2 position at site B, have been shown to mimic the A-62176 effect on the topo II-induced cleavage at sites A and B (see above). In the absence of topo II, A-62176 produced enhanced photocleavage at the guanine at the +2 position of site B for both M1 and M2 oligos (lanes 11 and 12 in Fig. 6, A, B, and D), which corresponds to the same guanine that showed an enhanced photocleavage by A-62176 in the presence of topo II. These results demonstrate that positioning a mismatched base pair at either the +1 or +2 position at site B mimics the structural change in DNA induced by topo II, suggesting that a similar structural perturbation, possibly a base pair opening, is introduced at the +1 and +2 positions by topo II when it binds to DNA.

Competition between Psorospermin and A-62176 for Binding at Site B—Psorospermin, a potent DNA alkylating antitumor agent, has been shown to intercalate into the DNA helix and alkylate N-7 of guanine at the 3′ side of the intercalation site (20). In the presence of topo II, the reactivity of psorospermin is enhanced more than 25-fold with the guanine at the +4′ position at site B (24). A DNA strand breakage assay was used to locate the position of alkylation of DNA by the epoxide of psorospermin upon intercalation to the base pairs between the +1 and +2 positions at site B within the topo II-DNA complex (24). Psorospermin has subsequently been used to probe the binding sites for m-AMSA (24). In the present study, this same assay was performed to determine the binding site of A-62176 on DNA in the presence of topo II.

The A-62176 photocleavage studies have demonstrated that topo II produces a structural distortion at the +1 and +2 positions at site B that creates a binding pocket for A-62176. As an independent method for determining the specific location of A-62176, a competition study between psorospermin and A-62176 was carried out (Fig. 7). As demonstrated previously, psorospermin displayed relatively weak DNA reactivity at a 10 μM concentration in the absence of topo II (Fig. 7A, lane 5). However, in the presence of topo II, the psorospermin alkylation of the guanine at the +4′ position of site B (band 2) was greatly enhanced (lane 6), while the reactivity of psorospermin...
with the guanines at other positions (such as band 1 and band 3) remained unchanged (24). As the concentrations of A-62176 were increased (lanes 7–11), the amount of psorospermin alkylation at site B decreased (band 2), as shown by the reduction of the strand breakage product, while the reactivity of psorospermin with the guanines at other position (bands 1 and 3) showed little if any change (Fig. 7B). Since A-62176 is a DNA-interactive intercalator, this competition effect may be due to its nonspecific inhibition of topo II binding to DNA at site B. However, in this experiment, nonhydrolyzable ATP was used.
Under these conditions, a concentration of A-62176 (up to 10 μM) can enhance the topo II-mediated cleavage (Fig. 3). Therefore, these results indicate that A-62176 can specifically compete with psorospermin for intercalation between positions +1 and +2 in the presence of topo II. For comparison, norfloxacin was used instead of A-62176 in a parallel experiment. In contrast to the intercalating A-62176, the nonintercalating norfloxacin showed no competition with psorospermin (lanes 12–16). In fact, quantitative data showed that norfloxacin produced a small but significant enhancement of the psorospermin alkylation at site B (Fig. 7B). Overall, these results strongly support the idea that A-62176 specifically interacts with DNA at the site where psorospermin binds in the presence of topo II (i.e., between positions +1 and +2).

**The Cooperative Binding of A-62176 and Norfloxacin in the Presence of Topo II as Evidence for the Assembly of a 2:2 Drug-Mg²⁺ Complex on the Topo II-DNA Complex**

In a previous study, it was proposed that a 2:2 drug-Mg²⁺ complex forms a "heterodimer complex" with respect to DNA, in which one A-62176 molecule is intercalated into DNA and a second A-62176 molecule is externally bound, held to the first molecule by two Mg²⁺ bridges (17). In this complex, on the basis of DNase I and viscometry studies, externally bound A-62176 molecules can be replaced by norfloxacin, demonstrating that norfloxacin and A-62176 bind to DNA in a cooperative manner. In the present study, competition experiments between psorospermin and A-62176, in combination with norfloxacin, were used to determine if the heterodimer complex is also formed in the presence of topo II. The psorospermin alkylation of guanine at the +4' position of site B in lane 5 (Fig. 8A) is greatly enhanced in the presence of topo II (compared with lane 4). As the concentrations of A-62176 were increased (lanes 6–11), the amount of alkylation by psorospermin at the same positions decreased. The effects of 20 or 100 μM of norfloxacin in combination with A-62176 on psorospermin alkylation are shown in lanes 12–18 and 19–25, respectively. At face value, it appears that norfloxacin has no effect on psorospermin alkylation in combination with A-62176 (Fig. 8B, graph II). However, when the enhancement effect of norfloxacin alone on psorospermin alkylation (lanes 12 and 19 in Fig. 8A; Fig. 8B, graph I) is taken into account, it is evident that norfloxacin does show an additive effect with A-62176. If the enhancement effect by norfloxacin alone on psorospermin alkylation is subtracted from the combination effect, as shown in Fig. 8B (graph III), A-62176, in combination with 20 μM norfloxacin, shows an additive effect on the inhibition of psorospermin alkylation. This additive effect is even more dramatic when 100 μM of norfloxacin is used. Hence, A-62176 and norfloxacin bind to the topo II-DNA complex in a cooperative manner, which is consistent with the 2:2 drug-Mg²⁺ model originally proposed for the binary complex between the quinobenzoxazines and DNA (17).

**DISCUSSION**

The results presented in this paper demonstrate that A-62176 possess a dual mechanism at pH 6–7; at one topo II cleavage site (site B), A-62176 is an intercalative topo II poison, while at another topo II cleavage site (site A), A-62176 is a catalytic inhibitor of topo II. Using a combination of photocleavage assay, mismatched sequences, and competition experiments between psorospermin and A-62176, we have shown that topo II creates a high affinity intercalation site for A-62176 between the base pairs at positions +1 and +2 at site B. Our data are in accord with our previously proposed 2:2 quinobenzoxazine-Mg²⁺ heterodimer complex model (17).

A-62176 Has Two Mechanisms of Action: Topo II Poison at Site B and Catalytic Inhibitor at Site A—Permana and co-workers have demonstrated that A-62176 is a strong catalytic inhibitor of mammalian topo II that does not increase topo II-mediated DNA strand breakage, suggesting that A-62176 should inhibit topo II reactions at a step prior to the formation of the cleaved complex (12). However, our results show that at pH 6–7, A-62176 can be both a topo II poison and a catalytic inhibitor. At one topo II cleavage site, A-62176 enhances the cleaved complex formation at low drug concentrations but inhibits it at high drug concentrations. At another topo II cleavage site, A-62176 inhibits the cleaved complex formation at both low and high drug concentrations. Our experimental conditions differed from Permana’s study in two respects. First, in their paper, plasmid DNA was used to study overall drug effects on the DNA cleavage by topo II, whereas in our study, oligonucleotides were used in order to determine drug effects on the individual site. Second, our experiments were performed at pH 6–7, whereas in Permana’s study, pH 7.5 was used. Hence, our results may not be contradictory to Permana’s study, which concluded that A-62176 has an overall catalytic inhibitory effect on topo II (12).

When Binding to DNA at Site B, Topo II Induces a Conformational Change in DNA That Creates a High Affinity Inter- nalization Site for A-62176 between Base Pairs +1 and +2—Previous studies have suggested that topoisomerases can create high affinity DNA intercalation sites for drugs by producing structural changes in DNA. One example of this phenomenon is T4 topo II, which creates preferential binding sites for m-AMSA at the –1 and +4’ positions in the immediate vicinity of the topo II cleavage gate (25). Likewise, psorospermin shows an unusually high reactivity at the +4’ position within the gate site in the presence of Drosophila topo II (Fig. 9C) (24). In the case described here, both the photocleavage and topo II cleavage results suggest that an enhanced binding of A-62176 occurs in the presence of Drosophila topo II. In addition, these results indicate that a DNA base pair distortion, which resembles the mismatches, may occur when topo II binds to site B, and this distortion might be recognized and stabilized by A-62176.

Competition experiments (Fig. 7) provide further evidence that A-62176 intercalates between the base pairs at the +1 and +2 positions in the presence of topo II. Our results have demonstrated that the intercalating A-62176 can compete with psorospermin for the DNA intercalation pocket induced by topo II, while the nonintercalating analogue norfloxacin is unable to do so. Data from the competition experiments are consistent with our previous studies, which suggested that intercalating and nonintercalating topo II poisons bind to different sites in the topo II-DNA complex (24). Since psorospermin intercalates into the base pairs between the +1 and +2 positions of site B (Fig. 9C), in order to compete with psorospermin alkylation, A-62176 can intercalate between the same base pairs as psorospermin (i.e., the position between +1 and +2) or, in accordance with the nearest-neighbor exclusion principle for DNA intercalators (26, 27), between the adjacent base pairs (i.e., the positions between –1 and +1 or +2 and +3). Our topo II cleavage and photocleavage studies indicate that the structural changes induced by topo II at positions +1 and +2 create a preferential binding site for A-62176. Therefore, it is likely that the intercalations of both A-62176 and psorospermin into the same base pairs between +1 and +2 may account for the competition between these two drugs (Fig. 9). On the contrary, norfloxacin cannot compete with psorospermin, suggesting that the planar tetracyclic ring, which is the intercalative moiety of A-62176, is critical for the competition.

A-62176 Is Likely to Interact with the Topo II-DNA Complex
at a Step prior to the Strand Cleavage—It has been proposed that the stimulation effects of topo II poisons on topo II-mediated cleavage might be related to the cleavage-rejoining reaction of the enzyme (22, 28). A-62176, a strong DNA intercalator, appears to have the same effect, blocking the religation process of the topo II. However, subsequent studies found that base pairing within the overhang is not required for the religation process of topo II (29). In addition, a number of eukaryotic quinolones, such as CP-115,953, have been shown to enhance the topo II-mediated cleavage by increasing the forward cleavage reaction (30). Norfloxacin can stimulate the DNA binding of mutant topo IV that lacks the strand cleavage activity (31). Similarly, psorospermin alkylation is enhanced at the topo II cleavage gate without Mg2+, which is required for enzyme-mediated DNA cleavage (18). These results suggest that the DNA conformational change induced by topo II occurs preceding the strand cleavage event. Therefore, it is possible that A-62176 binds to the topo II-DNA complex at a step prior to strand cleavage and accelerates the strand cleavage by stabilizing the structural distortion so that the activation energy required for strand cleavage is reduced. A similar mechanism has been suggested for the mechanism of quinolone action (31).

The Experimental Findings Are Consistent with a 2:2 Quinobenzoxazine-Mg2+ Heterodimer Complex, Which Can Assemble in the Presence of Topo II—The results of previous studies have led to a proposal of a 2:2 quinobenzoxazine-Mg2+ heterodimer complex model on duplex DNA, in which one quinobenzoxazine molecule serves as an intercalator and the other quinobenzoxazine molecule binds externally, held to the first drug molecule by two Mg2+ ions (Fig. 9A) (17). However, this model was proposed based on the experimental evidence on the drug-DNA binary complex in the absence of topo II. Our results (Fig. 8) show that A-62176 and norfloxacin have cooperative effects in the competition with psorospermin in the presence of topo II, indicating that this heterodimer complex may also assemble in the topo II-DNA complex (Fig. 9, A and B). In preliminary studies, in vitro experiments using norfloxacin with a quinobenzoxazine, we have also observed a dose-dependent enhancement of the cytotoxicity of the quinobenzoxazine compound by norfloxacin.4

Implications for Drug Design—The 2:2 quinobenzoxazine-Mg2+ and 1:1:2 quinobenzoxazine-norfloxacin-Mg2+ heterodimer models have important implications for future drug design, not only for eukaryotic topo II but also for gyrase, since norfloxacin is known to be a gyrase inhibitor. The proposed drug-Mg2+ heterodimers have two moieties; one of them interacts exclusively with DNA through intercalation and the other binds externally to DNA (Fig. 9, A and B). The results presented in this paper suggest that topo II induces transient structural distortion, possibly unwinding, that is captured or stabilized by the binding of A-62176 at the topo II cleavage gate. Therefore, the binding of the 2:2 A-62176-Mg2+ heterodimer to the topo II-DNA complex includes not only the intercalation of one A-62176 molecule to the partially unwound DNA base pairs at the gate, but also the interaction of the externally bound A-62176 molecule to topo II. This model provides substantial information for drug design.

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