Azatoxin Is a Mechanistic Hybrid of the Topoisomerase II-Targeted Anticancer Drugs Etoposide and Ellipticine†

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ABSTRACT: One approach to broadening the diversity of topoisomerase II-targeted anticancer agents is to generate novel compounds by combining structural elements of drugs known to stimulate enzyme-mediated DNA cleavage. The first agent to emerge from such a rational drug design is azatoxin, a hybrid drug that fuses chemical structures from etoposide and ellipticine. Since these drugs differ significantly in their structural and mechanistic attributes, azatoxin may preferentially retain the functional properties of one of these two drugs, behave as a hybrid molecule, or act as a novel pharmacophore. Therefore, the properties of azatoxin were characterized to determine relationships between its mechanism of action and those of its parent compounds. Azatoxin, like etoposide, binds to DNA in a nonintercalative fashion. However, similar to ellipticine, the drug has no effect on enzyme-mediated DNA religation and apparently stimulates scission primarily by enhancing cleavage complex formation. Depending on the species of topoisomerase II examined, the cleavage potency of azatoxin resembles that of either of its chemical parents. Furthermore, out of 43 DNA cleavage sites analyzed, ∼90% of those induced by azatoxin are shared with either etoposide, ellipticine, or both drugs. Finally, competition studies indicate that azatoxin interacts with topoisomerase II in the enzyme domain utilized by etoposide and ellipticine. Taken together, these results strongly suggest that azatoxin is a mechanistic hybrid of its parent compounds and shares functional properties with both drugs.

Topoisomerase II is one of the most widely utilized and most successful targets for the chemotherapeutic treatment of human malignancies (*1*−*7*). Anticancer drugs directed toward this enzyme act in an unusual fashion. Rather than blocking the essential cellular functions of topoisomerase II, these drugs increase levels of covalent enzyme-cleaved DNA complexes that are requisite intermediates in the catalytic DNA strand passage cycle of the enzyme (*1*−*10*). As a result, topoisomerase II-targeted drugs "poison" the enzyme and convert it to a cellular toxin that creates double-stranded breaks in the genome of treated cells (*1*−*7, 11*−*14*).

Despite the host of structurally diverse anticancer agents that act on topoisomerase II, many of the currently available drugs are limited by toxic side effects, pharmacokinetics, or acquired resistance (*1, 5, 14*−*19*). Consequently, there is a need for the development of novel chemotherapeutic agents directed toward this enzyme. One approach to designing new topoisomerase II-targeted compounds takes advantage of previously recognized drug classes and combines structural units from existing agents in an effort to enhance productive interactions within the enzyme·drug·DNA ternary complex (*20*). This type of rational drug design led to the development of azatoxin (Figure 1), a drug that displays potent activity against topoisomerase II and mammalian cells and currently is in preclinical development (*20*−*24*).

Azatoxin is a structural hybrid of the topoisomerase II-targeted drugs etoposide and ellipticine (*20, 21, 25*) (Figure 1). This compound contains portions of the epipodophyllotoxin ring system of etoposide (which is believed to interact with both the minor groove of DNA and the enzyme) (*26*) fused to the planar indole ring of ellipticine (which intercalates in DNA) (*27*−*29*). In addition to their structural differences, the two parent drugs display different DNA binding characteristics (etoposide is nonintercalative and ellipticine is intercalative) (*26*−*29*) and utilize different mechanisms to increase double-stranded DNA cleavage by topoisomerase II. While etoposide enhances enzyme-mediated DNA scission primarily by inhibiting religation of the cleaved substrate, ellipticine increases scission primarily by stimulating the formation of enzyme−DNA cleavage complexes (*30*−*32*).

The mechanistic basis for azatoxin action has not been established. Given the structural and mechanistic differences between etoposide and ellipticine, azatoxin may preferentially retain the functional properties of one of these two drugs, behave as a hybrid molecule, or act as a novel pharmacophore. Therefore, in order to establish relationships between the actions of azatoxin and its parent compounds, the effects of this drug on the DNA cleavage and religation reactions of topoisomerase II were compared to those of etoposide and ellipticine. Results indicate that azatoxin is a mechanistic hybrid of its parent compounds and displays functional attributes of both drugs.

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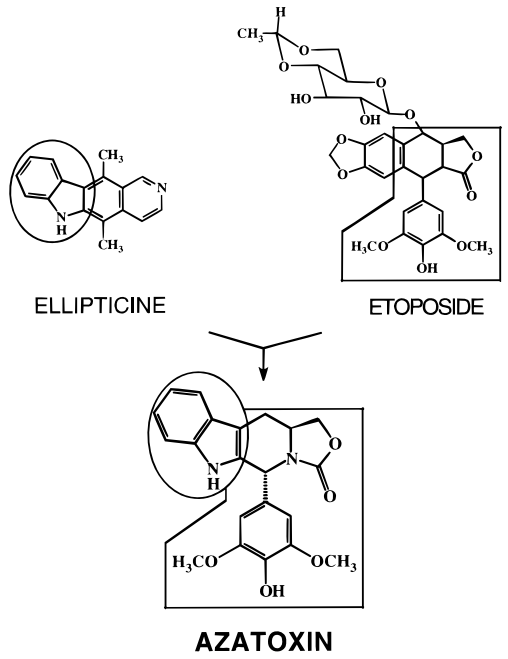

FIGURE 1: Scheme depicting the design of azatoxin. The indole ring of ellipticine (circled) was fused to a portion of the epipodophyllotoxin ring system of etoposide (boxed). The lactone in the epipodophyllotoxin structure was changed to a urethane to prevent potential epimerization and bioinactivation as observed for etoposide.

EXPERIMENTAL PROCEDURES

Calf thymus topoisomerase I was purchased from GIBCO BRL. Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (*33*) and purified by the protocol of Kingma et al. (*34*). *Drosophila melanogaster* topoisomerase II was purified from embryonic Kc cells as described by Shelton et al. (*35*). Wild type and mutant (H1012Y) yeast topoisomerases II were isolated from *S. cerevisiae* by the procedure of Elsea et al. (*36*) as modified by Burden et al. (*37*). Negatively supercoiled pBR322 DNA was prepared as described (*38*). Azatoxin was synthesized by the method of Leteurtre et al. (*39*) and stored at room temperature as a 20 mM stock in DMSO. Etoposide and ellipticine were purchased from Sigma and stored as 20 mM stocks in DMSO at room temperature and 4 °C, respectively. Ciprofloxacin (obtained from Bayer) was stored as a 40 mM stock in 0.1 N NaOH at room temperature and diluted to 8 mM with 10 mM Tris-HCl (pH 7.9) immediately prior to use. Tris was obtained from Sigma; proteinase K and SDS were from Merck; [γ-^{32}P]ATP (~6000 Ci/mmol) was from Amersham; and restriction endonucleases, calf intestinal alkaline phosphatase, and T4 polynucleotide kinase were from New England Biolabs.

DNA Intercalation. A topoisomerase I unwinding assay was used to monitor the intercalation of drugs into plasmid DNA (*40*). Reactions contained 5 nM relaxed or supercoiled pBR322 plasmid DNA and 10 units of topoisomerase I, and assays were carried out in the presence or absence of 100 μM drug in 40 μL of 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 30 μg/mL BSA. Following a 15 min incubation at 37 °C, reaction mixtures were treated with 3 μL of 250 mM EDTA and extracted with phenol−chloroform. Samples were treated with 2 μL of 2.5% SDS and subjected to electrophoresis in 1% agarose gel in 40 mM Tris−acetate buffer (pH 8.3), 2 mM EDTA. DNA bands were stained with 1 μg/mL ethidium bromide, visualized by UV light, and photographed through Kodak 23A and 12 filters with Polaroid type 665 positive/negative film.

Topoisomerase II-Mediated DNA Cleavage. The effects of drugs on the DNA cleavage reaction of topoisomerase II were examined over a drug concentration range of 0−150 μM. Cleavage assays containing *Drosophila* topoisomerase II were performed as described by Osheroff and Zechiedrich (*41*). Briefly, 5 nM negatively supercoiled pBR322 DNA was incubated with 100 nM topoisomerase II in 20 μL of assay buffer [10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 2.5% glycerol] at 30 °C for 6 min. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (15 mM). Following enzyme digestion by proteinase K, DNA products were analyzed by agarose gel electrophoresis as described above. DNA bands were quantitated by scanning negatives with an E-C Apparatus Model EC910 scanning densitometer using Hoefer GS-370 Data System software. Under the conditions employed, the intensity of bands in the negative was proportional to the amount of DNA present. Double-stranded cleavage of the plasmid substrate was monitored by conversion of negatively supercoiled molecules to linear products.

DNA cleavage mediated by human topoisomerase IIα was performed at 37 °C for 10 min in 20 μL of 50 mM Tris-HCl (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5 mM EDTA, and 2.5% glycerol. DNA cleavage mediated by wild-type or H1012Y mutant yeast topoisomerase II was performed at 28 °C for 6 min in 20 μL of 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, and 2.5% glycerol, and reactions were stopped by the addition of SDS to a final concentration of 0.5%. In all other respects, assays containing human or yeast type II topoisomerases were identical to those described above for the *Drosophila* enzyme.

The effects of the quinolone ciprofloxacin on the ability of azatoxin to enhance DNA cleavage mediated by *Drosophila* topoisomerase II were determined as described by Elsea et al. (*42*). In all cases, ciprofloxacin (0−800 μM) and azatoxin (50 or 100 μM) were present in reaction mixtures prior to the addition of topoisomerase II.

Topoisomerase II-Mediated DNA Religation. DNA religation mediated by *Drosophila* topoisomerase II was monitored by the procedure of Robinson and Osheroff (*31*). Cleavage/religation equilibria were established as described in the preceding section. Religation was initiated by shifting the temperature from 30 °C to 55 °C and stopped at the indicated times by the addition of SDS. The apparent first-order rate of religation was determined by quantifying the loss of the linear DNA cleavage product.

Mapping of Drug-Induced DNA Cleavage Sites. A uniquely end-labeled 564 base pair DNA substrate was prepared from pBR322 (residues 375−939) as described previously (*37*). Briefly, pBR322 plasmid DNA (40 μg) was digested with restriction endonuclease *Eag*I and dephosphorylated with calf intestinal alkaline phosphatase. The DNA was phosphorylated with T4 polynucleotide kinase and 10 μM [γ-^{32}P]ATP and treated with restriction endonuclease *Bam*HI, and the digest was resolved by electrophoresis on a 5% nondenaturing polyacrylamide gel. The 564-mer was detected by UV shadowing, excised, eluted overnight at room

temperature with 2.5 M ammonium acetate, precipitated with ethanol, and resuspended in water.

Sites of drug-induced DNA cleavage generated by *Drosophila* topoisomerase II were determined by the procedure of Knab et al. (*43*) as modified by Burden et al. (*37*). Cleavage reactions contained 1.4 nM (25 ng) labeled DNA, 3.5 nM (60 ng) topoisomerase II, and 20 μM ellipticine, 100 μM azatoxin, 100 μM etoposide, or 1% DMSO (drug diluent) in a total of 50 μL of assay buffer. (The lower concentration of ellipticine reflects the level of drug that produced maximal DNA cleavage enhancement with *Drosophila* topoisomerase II.) After incubating at 30 °C for 10 min, cleavage complexes were trapped by the addition of SDS (1% final concentration), and topoisomerase II was digested for 30 min at 45 °C with proteinase K in the presence of EDTA (15 mM final concentration). DNA cleavage products were precipitated twice with ethanol, dried, and resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF. Samples were subjected to electrophoresis in 8% denaturing polyacrylamide sequencing gels (*43*), fixed in 10% methanol/10% acetic acid for 10 min, and dried. Reaction products were analyzed using a Molecular Dynamics PhosphorImager. DNA sequence ladders of the cleavage substrate were generated by the dideoxynucleotide method (*44*).

RESULTS

Azatoxin is a novel topoisomerase II-targeted drug that was generated by combining structural moieties from etoposide and ellipticine (*20*,*39*). Despite the fact that both of these latter compounds kill cells by increasing levels of topoisomerase II-mediated DNA scission (*45*−*47*), they display differences in their mode of DNA binding, species specificity, mechanism of cleavage enhancement, and DNA sequence selectivity (*1*, *4*, *26*−*31*, *47*). Therefore, a series of experiments was carried out to determine whether azatoxin preferentially retains the functional properties of one of its parent compounds, acts as a mechanistic hybrid of the two, or represents a novel pharmacophore.

Azatoxin·DNA Binding Mode. The parent compounds of azatoxin interact with DNA by different binding modes; while etoposide is nonintercalative in nature, ellipticine binds to DNA in an intercalative fashion (*26*−*29*). Although a previous study reported that azatoxin was nonintercalative with respect to DNA (*39*), the highest drug concentration used (1 nM) was several orders of magnitude below the range in which azatoxin displays activity against topoisomerase II. Since ellipticine also fails to show DNA unwinding at this low concentration (data not shown), the binding mode of azatoxin was reexamined at 100 μM, a concentration that induces high levels of topoisomerase II-mediated DNA cleavage (see Figure 3).

A topoisomerase I-catalyzed DNA unwinding assay was utilized to address this issue (Figure 2). In the presence of an intercalative drug such as ellipticine (lane 5), a net negative supercoiling of relaxed DNA substrate was induced following treatment with the type I enzyme. Conversely, no unwinding was observed in the presence of the nonintercalative drug etoposide (lane 3). As seen in lane 4, azatoxin had no effect on the topological state of the plasmid. Since the drugs did not block the DNA relaxation activity of topoisomerase I under the conditions employed (data not shown), these results confirm that azatoxin is nonintercalative in nature, even at concentrations that induce significant levels of DNA scission.

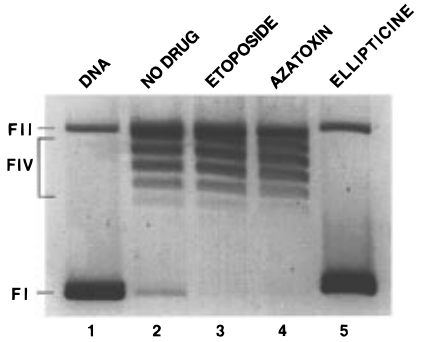

FIGURE 2: Azatoxin is nonintercalative with respect to DNA. A topoisomerase I unwinding assay was utilized, and products were resolved by electrophoresis on an agarose gel. Lane 1, supercoiled pBR322 standard; lane 2, relaxed DNA standard in the absence of drug; lanes 3−5, relaxed DNA incubated with topoisomerase I in the presence of 100 μM etoposide, azatoxin, or ellipticine, respectively. The positions of negatively supercoiled DNA (form I; FI), nicked circular plasmid molecules (form II; FII), and relaxed DNA (form IV; FIV) are indicated. Azatoxin had no effect on the catalytic activity of topoisomerase I under the experimental conditions utilized.

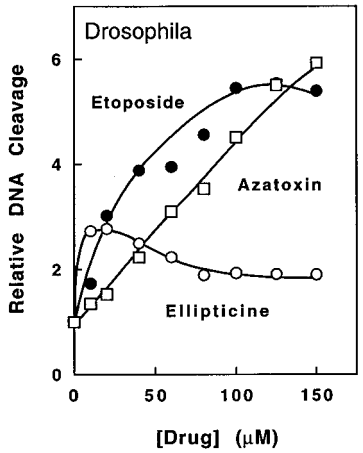

FIGURE 3: Effects of azatoxin on DNA cleavage mediated by *Drosophila* topoisomerase II. Results for 0−150 μM ellipticine (○), etoposide (●), and azatoxin (□) are shown. Data for each drug represent an average of three independent assays. Levels of DNA cleavage observed in the absence of drug were set to 1.0.

DNA Cleavage Enhancement. Over a broad range of topoisomerase II species, etoposide stimulates enzyme-mediated DNA cleavage to a greater extent than does ellipticine. As seen in Figures 3−5, cleavage enhancement with 100 μM etoposide was severalfold higher than observed with ellipticine in assays that utilized *Drosophila* or yeast topoisomerase II or the α isoform of the human enzyme.

To compare the activity of azatoxin with that of its parent compounds, the ability of the drug to stimulate DNA cleavage mediated by these enzymes was characterized. The DNA cleavage efficacy of azatoxin closely resembled that of etoposide in assays that utilized *Drosophila* topoisomerase II (Figure 3) or human topoisomerase IIα (Figure 4). However, in marked contrast, the cleavage profile of azatoxin with the wild-type yeast enzyme was similar to that of ellipticine (Figure 5, left panel).

To further assess the properties of azatoxin, its ability to stimulate DNA scission mediated by top2H1012Y, a mutant

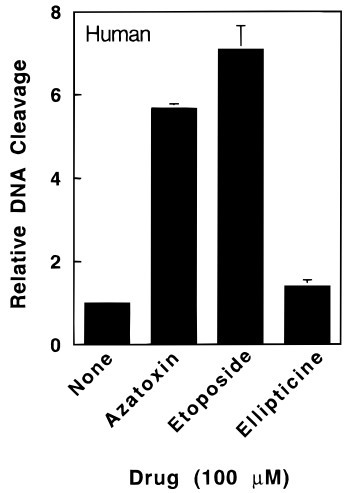

FIGURE 4: Effects of azatoxin on DNA cleavage mediated by human topoisomerase IIα. Results for 100 μM azatoxin, etoposide, and ellipticine represent the average of three independent assays for each drug. Standard deviations are represented by error bars.

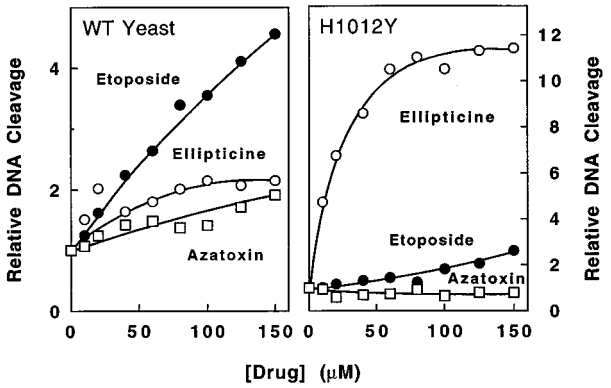

FIGURE 5: Effects of azatoxin on DNA cleavage mediated by wild-type (left panel) and H1012Y mutant (right panel) yeast topoisomerase II. Results for ellipticine (○), etoposide (●), and azatoxin (□) are shown over a range of 0−150 μM in assays with each enzyme. The plots represent data for each drug from three independent experiments. Levels of DNA cleavage observed in the absence of drug were set to 1.0.

yeast type II topoisomerase selected for altered drug sensitivity (*36*), was examined. The H1012Y mutation distinguishes between drug classes, and results in an enzyme that is highly resistant to etoposide, but is severalfold hypersensitive to ellipticine (*36*) (Figure 5, right panel). As seen in Figure 5, top2H1012Y was completely resistant to azatoxin. Thus, the ability of azatoxin to enhance DNA cleavage mediated by the mutant enzyme resembles that of etoposide, despite the fact that the actions of this drug against wild-type yeast topoisomerase II are similar to ellipticine. Taken together, these results indicate that azatoxin exhibits cleavage enhancement properties that are characteristic of both of its chemical parents.

DNA Religation. Topoisomerase II poisons increase levels of DNA cleavage by two different mechanisms. Anticancer agents such as etoposide act primarily by inhibiting the ability of the enzyme to religate cleaved DNA, and drugs such as ellipticine act primarily by enhancing the formation of topoisomerase II−DNA cleavage complexes (*1, 4, 30, 31, 47*). As illustrated in Figure 6, these two mechanisms can be readily distinguished by determining the effects of drugs on the rate of enzyme-mediated DNA religation. While etoposide decreases the apparent first-order rate of religation

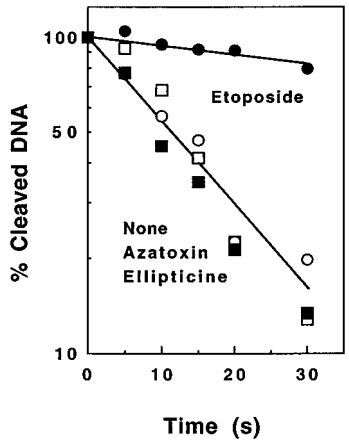

FIGURE 6: Effects of azatoxin on DNA religation mediated by *Drosophila* topoisomerase II. Results were obtained over a 0−30 s time course following initiation of religation by shifting reaction mixtures from 30 to 55 °C in the presence of drug concentrations that yielded maximal DNA cleavage enhancement. Data represent at least two independent assays containing no drug (■), or 20 μM ellipticine (○), 100 μM etoposide (●), or 125 μM azatoxin (□). The percent linear DNA was arbitrarily set at 100% at time zero.

mediated by *Drosophila* topoisomerase II by an order of magnitude, ellipticine shows no ability to inhibit the reaction.

The effects of azatoxin on the rate of topoisomerase II-mediated DNA religation also are shown in Figure 6. Under conditions that generated levels of scission identical to etoposide, the drug had no discernible effect on the ability of the enzyme to religate cleaved DNA. Therefore, like ellipticine, azatoxin appears to stimulate DNA scission primarily by enhancing the formation of cleavage complexes.

DNA Cleavage Specificity. Etoposide and ellipticine induce topoisomerase II-mediated DNA scission with different nucleic acid sequence specificities. While etoposide preferentially stimulates cleavage at sites that contain a cytosine immediately upstream from the scissile bond (*i.e.*, at the −1 position), ellipticine prefers sequences with a thymine in the corresponding position (*5, 48, 49*). A previous study noted similarities between the DNA cleavage pattern induced by azatoxin and epipodophyllotoxin derivatives (*39*); however, the sequence specificity of the drug was never compared directly to both etoposide and ellipticine.

To further elucidate the cleavage characteristics of azatoxin, 43 sites induced by this drug were analyzed (Figure 7). Azatoxin shared ∼90% of its cleavage sites with ellipticine (10/43 sites), etoposide (18/43), or both drugs (11/43). Only 4 sites induced by azatoxin were unique to this agent (sites 28, 34, 38, and 43), and poor cleavage enhancement was observed at 3 of these sequences. Thus, it appears that azatoxin shares specificity patterns with its chemical parents.

Azatoxin Interaction Domain on Topoisomerase II. Several DNA cleavage-enhancing anticancer drugs share a common site of action on topoisomerase II (*42, 50, 51*). As determined by a series of drug competition studies, the functional interaction domains for quinolones, amsacrine, genistein, and ellipticine all appear to overlap that of etoposide (*42, 50, 51*).

The fact that the H1012Y mutation alters the sensitivity of topoisomerase II to azatoxin suggests that this drug shares the interaction domain on the enzyme utilized by etoposide,

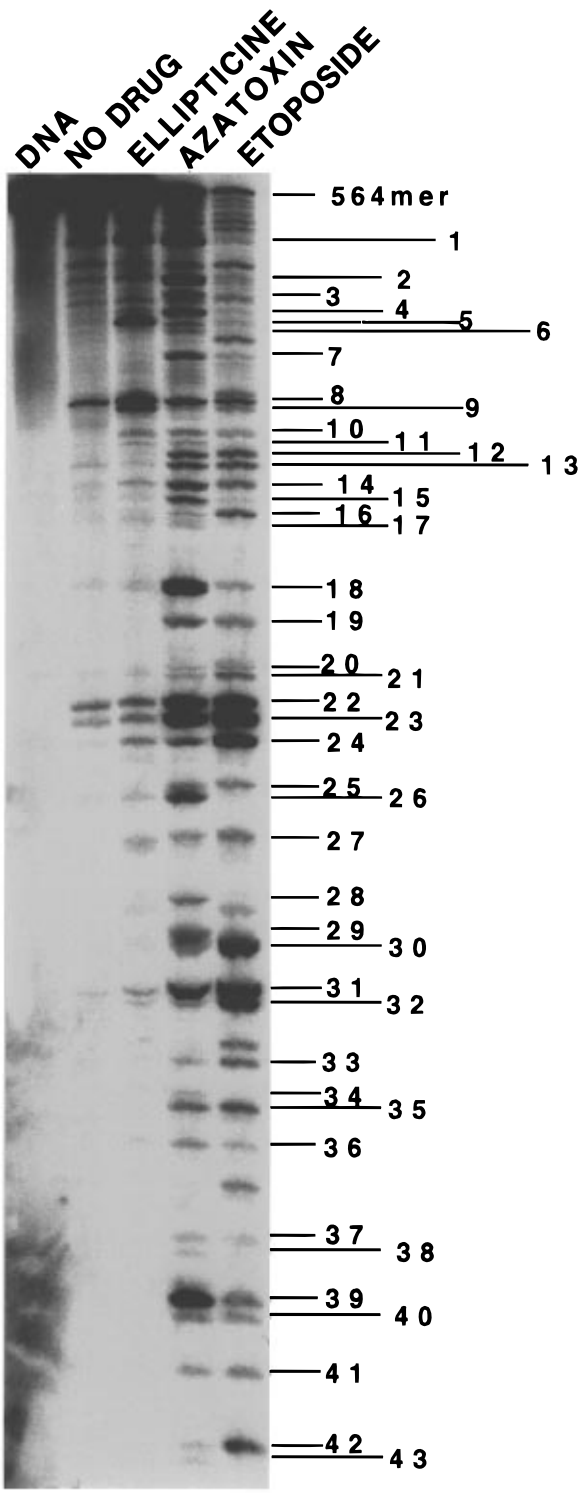

FIGURE 7: Cleavage site map of azatoxin-induced DNA cleavage mediated by *Drosophila* topoisomerase II. Bands resulting from 43 sites of azatoxin-induced DNA cleavage were resolved on a denaturing polyacrylamide gel and visualized by PhosphorImager analysis. Lanes from left to right correspond to a DNA standard (DNA), DNA cleavage mediated by the enzyme in the absence of drug (NO DRUG), or in the presence of 20 μM ellipticine, 100 μM azatoxin, or 100 μM etoposide, respectively.

ellipticine, and other DNA cleavage-enhancing agents. To determine whether this is the case, an assay based on competition with the antibacterial quinolone, ciprofloxacin, was employed (*42*). Although ciprofloxacin is a poor enhancer of DNA cleavage mediated by eukaryotic type II topoisomerases, it is a competitive inhibitor of etoposide and other topoisomerase II-targeted anticancer agents (*42*).

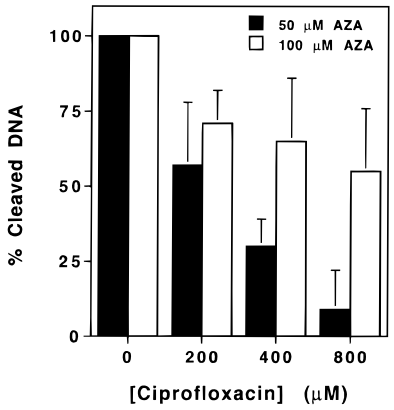

FIGURE 8: Inhibition of azatoxin-induced topoisomerase II-mediated DNA cleavage by ciprofloxacin. Closed and open bars represent the averages of 3−5 independent experiments carried out in the presence of 50 or 100 μM azatoxin, respectively. The percent of DNA cleavage mediated by *Drosophila* topoisomerase II in assays that contained azatoxin but lacked ciprofloxacin was set at 100%. Standard deviations are indicated by error bars.

As seen in Figure 8, ciprofloxacin attenuated azatoxin-induced DNA scission. Approximately 300 μM quinolone inhibited the reaction by 50% in the presence of 50 μM azatoxin. This IC_{50} value is comparable to that determined for competition with 50 μM etoposide (*42*). When the azatoxin concentration was increased to 100 μM, the IC_{50} for ciprofloxacin rose to ~800 μM, providing further evidence that these two drugs compete for the same site of action on topoisomerase II. These data indicate that azatoxin, which shares mechanistic properties with both etoposide and ellipticine, also shares a common interaction domain on the type II enzyme.

DISCUSSION

Although drugs targeted to topoisomerase II represent some of the most effective chemotherapeutic agents used for the treatment of human malignancies (*1*−*7*), the persistent threat from cancer necessitates the ongoing development of new drug classes. One approach to broadening the diversity of topoisomerase II-targeted anticancer drugs is to generate novel compounds by combining structural features of drugs known to stimulate enzyme-mediated DNA cleavage (*20*, *39*). The first agent to emerge from such a rational drug design is azatoxin, a hybrid drug that fuses chemical structures from etoposide and ellipticine (*20*, *39*).

Etoposide and ellipticine differ significantly in their functional attributes (*1*, *4*). Therefore, the properties of azatoxin were characterized to determine relationships between its mechanism of action and those of its parent molecules. Azatoxin retains the nonintercalative DNA binding mode of etoposide, but similar to ellipticine, stimulates DNA scission primarily by enhancing cleavage complex formation. Furthermore, depending on the species of topoisomerase II examined, the cleavage potency of azatoxin resembles that of either compound. Finally, the vast majority of DNA cleavage sites induced by azatoxin are shared with either etoposide, ellipticine, or both drugs, and azatoxin appears to interact with topoisomerase II in the common site of action utilized by etoposide and ellipticine. Taken together, these results strongly suggest that azatoxin is a mechanistic hybrid of its parent compounds and shares functional properties with both.

Considering that the major portion of azatoxin is comprised of structural elements from etoposide (Figure 1), it is surprising that this compound has no effect on topoisomerase II-mediated DNA religation. Thus far, the only three cleavage-enhancing drugs that have been demonstrated to inhibit religation are etoposide, teniposide, and amsacrine (*1*, *4*, *30*, *31*, *52*, *53*) (D. A. Burden, and N. Osheroff, unpublished result). The one feature common to these three compounds is the presence of a ring system in the "variable substituent domain" (*i.e.*, the ring position of the sugar moiety in etoposide, Figure 1) (*20*). Since azatoxin and the other drugs (examined to date) that lack cyclic components in this domain appear to stimulate topoisomerase II-mediated DNA scission primarily by enhancing cleavage complex formation, it is possible that structural elements within the variable substituent domain are responsible for the inhibition of DNA religation.

Along with their structural diversity, topoisomerase II-targeted anticancer agents display considerable mechanistic individuality (*1*, *4*, *51*). At the present time, it is not obvious how the structure of these drugs determines the DNA binding mode, cleavage specificity, or the mechanism of DNA cleavage enhancement. Furthermore, it is less clear how the above properties ultimately impact the clinical efficacy of these agents. By utilizing a combinatorial approach and merging structural and functional properties of different drug classes, it may be possible to develop novel topoisomerase II-targeted drugs with improved therapeutic properties. Results of the present study indicate that the chemical marriage of etoposide and ellipticine has yielded an offspring with a unique "mechanistic signature." Although the clinical fate of azatoxin has yet to be determined, the initial success of this chemical recombination opens the door for similar experiments in rational drug design.

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