Merbarone Inhibits the Catalytic Activity of Human Topoisomerase IIα by Blocking DNA Cleavage*

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Merbarone is a catalytic inhibitor of topoisomerase II that is in clinical trials as an anticancer agent. Despite the potential therapeutic value of this drug, the mechanism by which it blocks topoisomerase II activity has not been delineated. Therefore, to determine the mechanistic basis for the inhibitory action of merbarone, the effects of this drug on individual steps of the catalytic cycle of human topoisomerase IIα were assessed. Concentrations of merbarone that inhibited catalytic activity >80% had no effect on either enzyme-DNA binding or ATP hydrolysis. In contrast, the drug was a potent inhibitor of enzyme-mediated DNA scission (in the absence or presence of ATP), and the inhibitory profiles of merbarone for DNA cleavage and relaxation were similar. These data indicate that merbarone acts primarily by blocking topoisomerase II-mediated DNA cleavage. Merbarone inhibited DNA scission in a global (rather than site-specific) fashion but did not appear to intercalate into DNA or bind in the minor groove. Since the drug competed with etoposide (a cleavage-enhancing agent that binds directly to topoisomerase II), it is proposed that merbarone exerts its inhibitory effects through interactions with the enzyme and that the drug shares an interaction domain on topoisomerase II with cleavage-enhancing agents.

Topoisomerase II is the target for some of the most active anticancer drugs used in the treatment of human malignancies (1–6). Among the topoisomerase II-targeted agents currently in clinical use are etoposide, teniposide, doxorubicin, mitoxantrone, and amzasacrine. These drugs kill cells in an unusual fashion. Rather than inhibiting the overall catalytic activity of the type II enzyme, they act by increasing levels of topoisomerase II-mediated DNA cleavage, thus converting this essential enzyme into a potent cellular toxin (1, 3, 5, 7–10). Hence, to distinguish their unique mechanism of action, they are referred to as topoisomerase II “poisons” (11).

A second class of drugs that affect the activity of topoisomerase II also appears to have clinical potential (2, 5, 7). In contrast to poisons, these agents act by inhibiting the catalytic activity of the enzyme and display no ability to stimulate DNA cleavage. Originally, topoisomerase II “catalytic inhibitors” were defined by antibacterial compounds such as novobiocin and coumarmycin (12). These coumarin-based drugs block the DNA strand passage activity of the prokaryotic type II enzyme, DNA gyrase, by interfering with the ability of the enzyme to bind its ATP cofactor (13–17).

Recently, catalytic inhibitors that display high activity against eukaryotic type II topoisomerases have been described. These are typified by drugs such as merbarone (18), ICRF-193 (19), aclarubicin (20), fostriecin (21), staurosporine (22), and mitomodine (23), which reflect a variety of inhibitory mechanisms. Aclarubicin, for example, blocks binding of the enzyme to its DNA substrate, the initial step of the topoisomerase II catalytic cycle (24) (see Fig. 12). In contrast, ICRF-193 blocks the final step of the catalytic cycle, ATP hydrolysis (25). This action traps topoisomerase II on the DNA in its closed clamp form and prevents both enzyme release and regeneration.

One of the catalytic inhibitors of topoisomerase II that has generated the most interest is merbarone (18, 26–28) (see Fig. 1). This thiobarbituric acid derivative was originally shown to inhibit the type II enzyme by Drake et al. (18) and has been the subject of phase II clinical cancer trials (29–32). In addition to its inhibitory effects, merbarone has been shown in vitro and in cultured cells to attenuate the DNA cleavage-enhancing properties of topoisomerase II poisons such as teniposide and amzasacrine (18, 33). Although the cytotoxic actions of merbarone correlate with its ability to block topoisomerase II function (18, 33, 62), the mechanism by which this drug inhibits the enzyme has never been demonstrated. Therefore, to determine the basis of merbarone inhibition, the effects of the drug on individual steps of the catalytic cycle of human topoisomerase IIα were characterized. Results indicate that merbarone is a specific inhibitor of topoisomerase II-mediated DNA scission.

**EXPERIMENTAL PROCEDURES**

Human topoisomerase IIα was expressed in Saccharomyces cerevisiae (34) and purified by the protocol of Kingma et al. (35). Yeast topoisomerase IIα was isolated from S. cerevisiae by the procedure of Elsea et al. (36) as modified by Burden et al. (37). Drosophila melanogaster topoisomerase II was purified from embryonic Kc cells as described by Shelton et al. (38). Calf thymus topoisomerase I was purchased from Life Technologies, Inc. Negatively supercoiled pBR322 DNA was prepared as described (39). Hepes was obtained from Boehringer Mannheim; proteinase K and SDS were from Merck; bacteriophage T4 polynucleotide kinase, Klenow DNA polymerase, and restriction endonucleases were from New England Biolabs, Escherichia coli uracil DNA glycosylase, [γ-32P]ATP (~6000 Ci/mmole), and [γ-32P]ATP (~3000 or ~6000 Ci/mmole) were from Amersham Pharmacia Biotech; etoposide and ellipticine (stored at 4 °C as 20 mM stock solutions in 100% Me2SO) as well as ethidium bromide were from Sigma; and fluorescein phosphoramidite was from Glen Research. Merbarone was the generous gift of Dr. Randall K. Johnson (SmithKline Beecham) and was stored at 4 °C as a 20 mM stock solution in 100% Me2SO. All other chemicals were analytical reagent grade.

**DNA Relaxation**—DNA relaxation assays were based on the procedure of Osheroff et al. (40). Reactions contained 3 nM human topoisomerase IIα, 5 nM negatively supercoiled pBR322 DNA, and 1 mM ATP in a total of 20 μl of reaction buffer (50 mM Tris-HCl (pH 7.9), 135 mM
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KCl, 10 mM MgCl₂, 0.5 mM NaEDTA, and 2.5% glycerol. One micromolar of merbarone (or Me₂SO for control reactions) was included, so that the final [Me₂SO] was 5% (v/v). Reactions were started by the addition of topoisomerase II, incubated 10 min at 37 °C, and stopped by the addition of 3 μl of 0.77% SDS, 77 mM NaEDTA (pH 8.0). Alternatively, assays contained deoxyribonuclease I (1.6 mg/ml), and reactions were incubated 20 min at 37 °C. Cleavage products were removed at intervals up to 16 min and spotted on polyethyleneimine-impregnated thin layer cellulose chromatography plates (J. T. Baker Inc.). Plates were developed by chromatography in freshly made 400 mM NH₄HCO₃ and analyzed by autoradiography. Radioactive areas were quantitated by phosphorimaging.

DNA Cleavage—DNA cleavage reactions were carried out as described previously (42), either in the absence or presence of a nucleoside triphosphate. Assays contained 300 nM human topoisomerase IIa, negatively supercoiled pBR322 DNA (5 nM in reactions that lacked nucleoside triphosphate or contained 1 mM ATP), and 0–200 μM merbarone (5% final [Me₂SO]) in a total of 20 μl of reaction buffer. Reactions were started by the addition of topoisomerase II. Following a 6-min incubation at 37 °C, cleavage intermediates were trapped, resolved by electrophoresis, and quantitated as described above for relaxation assays.

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Merbarone DNA Binding—The effect of merbarone on topoisomerase II DNA binding was characterized by two independent techniques. In the first, an electrophoretic mobility shift assay was employed (41). Reactions were carried out in 20 μl of reaction buffer containing 10 nM negatively supercoiled pBR322 DNA and 0–300 nM human topoisomerase IIa in the presence or absence of 100 μM merbarone (5% final [Me₂SO]). Reactions were incubated at 37 °C for 6 min, loaded directly onto a 1% agarose gel, and subjected to electrophoresis in 1× TAE buffer (40 mM Tris acetate [pH 8.3], 2 mM EDTA) containing 0.5 μg/ml ethidium bromide. DNA bands were visualized and photographed as described above for relaxation assays.

Alternatively, the effect of merbarone on topoisomerase II DNA binding was monitored by fluorescence anisotropy. Assays were performed at 25 °C on an ISS FCS2 spectrofluorometer. A 40-mer double-stranded oligonucleotide (same as utilized for AP site cleavage described below, except that the uracil was replaced by guanine) with fluorescein incorporated at the 5′ terminus of the top strand was synthesized on an Applied Biosystems DNA synthesizer. A uracil (shown below in bold) was incorporated into the bottom strand. The sequences of the top and bottom strands, respectively, were 5′-TGAGATCTACAACTATGGCTGTCACTCATCCTGCGACCCGT-3′ and 5′-ACCGTGGAGGAGTCAACGTGAGTCGAGTTAGATTTCGTT-3′.

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Topoisomerase II-mediated DNA cleavage was monitored by fluorescence anisotropy in the presence of fluoresceinated DNA substrate. Reaction products were resolved by electrophoresis, and quantitated by scanning photographic negatives with an E-C apparatus model EC910 scanning densitometer in conjunction with a Molecular Dynamics PhosphorImager system.

Cleavage of a DNA Substrate Containing an APurinic Site—A double-stranded 40-mer DNA oligonucleotide (residues 87–126 of plasmid pBR322 (43)) that contained a position-specific apurinic site (44) was used in the context of a preexisting topoisomerase II cleavage site was employed (45, 46). To this end, complementary 40-base oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. A uracil (shown below in bold) was incorporated into the bottom strand. The sequences of the top and bottom strands, respectively, were 5′-TGAGATCTACAACTATGGCTGTCACTCATCCTGCGACCCGT-3′ and 5′-ACCGTGGAGGAGTCAACGTGAGTCGAGTTAGATTTCGTT-3′. Sites of topoisomerase II-mediated DNA cleavage are denoted by the addition of an asterisk. Sites of uracil-DNA glycosylase (44) are indicated with a bar. Imaging and data analysis were performed using a Molecular Dynamics PhosphorImager system.

ATP Hydrolysis—ATPase assays were performed as described by Osheroff et al. (40). ATP hydrolysis reactions contained 50 nM human topoisomerase IIa, 100 nM apurinic site-containing 40-mer DNA, and 0–400 μM merbarone (5% final [Me₂SO]) in a total of 20 μl of 10 mM Hepes-HCl (pH 7.9), 135 mM KCl, 5 mM MgCl₂, 0.1 mM NaEDTA, and 2.5% glycerol. Reactions were started by the addition of topoisomerase II, incubated 10 min at 37 °C, and stopped by the addition of 2 μl of 4% SDS and 2 μl of 250 mM NaEDTA. Proteinase K was added (2 μl of 1.6 mg/ml), and reactions were incubated 20 min at 37 °C. Cleavage products were ethanol-precipitated twice and resuspended in 5 μl of 1.5% agarose gel containing 2 μg/ml ethidium bromide, 0.05% bromphenol blue, 0.05% xylene cyanole FF. Products were subjected to electrophoresis in a denaturing 7% urea, 14% polyacrylamide sequencing gel in TBE buffer. The gel was fixed in 10% methanol, 10% acetic acid for 30 s and dried. DNA cleavage products were quantitated by analysis on a PhosphorImager.

The abbreviations used are: APP(NH)P, adenyl-5′-yl imidophosphate; bp, base pair.
buffer. The oligonucleotide employed for this study was the same as utilized for AP site cleavage (see above), except that the uracil was replaced by guanine.

**RESULTS**

**Inhibition of Topoisomerase II Catalytic Activity by Merbarone**—Merbarone (Fig. 1) is a catalytic inhibitor of topoisomerase II (18, 27, 28) that displays curative activity against some murine cancer models (26, 50) and is currently in human clinical trials (29–32). The effects of this drug on the activity of human topoisomerase IIα (which represents the major topoisomerase II isoform present in rapidly proliferating human cells) as well as the type II enzymes from Drosophila and yeast are shown in Fig. 2. Merbarone was considerably more effective against the mammalian type II enzyme than it was against topoisomerase II from lower eukaryotes. While the drug inhibited relaxation of negatively supercoiled pBR322 plasmid DNA by human topoisomerase IIα with an IC50 of ~40 μM, little inhibition was observed with either the Drosophila or yeast enzyme up to 100 μM merbarone. The IC50 values for these latter type II enzymes were ~350 and ~700 μM merbarone, respectively (not shown).

Although the inhibition of topoisomerase II by merbarone is believed to contribute to its efficacy against cancer cells, the mechanism by which this drug blocks the function of the type II enzyme is not known. Therefore, to determine the basis for merbarone action, the ability of this drug to inhibit individual steps of the catalytic cycle (41, 51) (see Fig. 12) of human topoisomerase IIα was assessed.

**Merbarone Does Not Impair Topoisomerase II-DNA Binding**—It has been suggested that merbarone inhibits the catalytic activity of topoisomerase II by blocking its ability to bind to its DNA substrate (33). To test this hypothesis, two independent approaches were utilized to characterize the effects of the drug on enzyme-DNA binding. One hundred micromolar merbarone, a concentration that inhibited catalytic activity ~80%, was employed for both.

In the first approach, interactions between human topoisomerase IIα and negatively supercoiled pBR322 DNA were monitored by an electrophoretic mobility shift assay. As determined by the upshift of bound DNA to the gel origin, merbarone did not impair topoisomerase II-DNA interactions (Fig. 3). If anything, levels of DNA binding appeared to be slightly higher in the presence of the drug.

In the second approach, interactions between human topoisomerase IIα and a double-stranded 40-mer oligonucleotide that contained a single cleavage site were monitored by fluorescence anisotropy. As seen in Fig. 4, merbarone had no effect on enzyme-oligonucleotide binding. The apparent KD values calculated in the absence or presence of the drug were 43 ± 9 or 45 ± 10 nM topoisomerase IIα, respectively.

**Merbarone Blocks Topoisomerase II-mediated DNA Cleavage**—Since merbarone does not interfere with enzyme-DNA binding, its effects on other steps of the topoisomerase II catalytic cycle were assessed in order to delineate the mechanism by which it exerts its inhibition.

The step that immediately follows DNA binding is pre-strand passage DNA cleavage (i.e. scission monitored in the absence of a nucleoside triphosphate (41, 51)). As shown in Fig. 5 (bottom panel), merbarone strongly inhibited this reaction step (IC50 ~50 μM). A similar inhibition was observed for post-strand passage DNA cleavage (i.e. scission monitored in the presence of the nonhydrolyzable ATP analog, APP(NH)P (41, 51)) (not shown). Finally, the effects of merbarone on DNA scission were determined in the presence of ATP, conditions that support the overall catalytic activity of the enzyme. The IC50 of the drug in ATP-containing reactions was identical to that determined in the absence of a nucleoside triphosphate (Fig. 5, top and bottom panels).

The concentration of merbarone required to inhibit topoisomerase II-mediated DNA cleavage by 50% was similar to the IC50 value (~40 μM) for its inhibition of enzyme-catalyzed DNA relaxation. The concentration of merbarone required to inhibit topoisomerase II-mediated DNA cleavage by 50% was similar to the IC50 value (~40 μM) for its inhibition of enzyme-catalyzed DNA relaxation.
relaxation. This finding strongly suggests that the primary mechanism by which merbarone inhibits the overall catalytic activity of human topoisomerase IIa is by blocking DNA cleavage.

Merbarone Does Not Inhibit Topoisomerase II-catalyzed ATP Hydrolysis—Coumarin-based topoisomerase II inhibitors as well as a number of DNA cleavage-enhancing topoisomerase II poisons impair interactions between the enzyme and ATP (40, 52–55). However, the fact that the IC₅₀ for merbarone inhibition of DNA cleavage is unaffected by ATP implies that this drug does not interfere with ATP utilization. To address this issue directly, the effects of merbarone on the ATPase activity of human topoisomerase IIa were determined (Fig. 6). Even at a drug concentration of 200 μM, no inhibition of ATP hydrolysis was observed. This result is similar to that reported recently by Hammonds and Maxwell (55) and supports the conclusion that merbarone is specific for topoisomerase II-mediated DNA scission.

Global Inhibition of Topoisomerase II-mediated Cleavage by Merbarone—Topoisomerase II poisons act in a site-specific fashion and dramatically alter the spectrum of DNA sites cleaved by the enzyme (2, 4, 56, 57). However, it is not known whether catalytic inhibitors of the enzyme act in a DNA site-specific manner. To this end, the effects of merbarone on site-specific DNA scission mediated by human topoisomerase IIa were characterized (Fig. 7).

A uniquely end-labeled 3609-bp fragment of pBR322 DNA was employed for this experiment. Under the conditions utilized, 10 sites of topoisomerase II-mediated scission were observed. Relative levels of DNA cleavage were normalized to site 8 at 100 μM merbarone (relative cleavage = 1). The standard deviations of two independent experiments are represented by error bars.

Interactions between Merbarone and DNA—Since chemical interactions between merbarone and DNA might be expected to compromise the ability of the drug to inhibit DNA cleavage, the effects of merbarone on DNA binding were determined (Fig. 4). Merbarone has no effect on topoisomerase II-DNA binding in a fluorescence anisotropy assay. Samples contained 5 nM fluorescein end-labeled double-stranded 40-mer DNA oligonucleotide and either 0 μM (●) or 100 (○) μM merbarone. The fluorophore was excited at 430 nm, and emission was monitored through a 530-nm cut-off filter. Anisotropies were determined over a concentration range of 0–50 nM human topoisomerase IIa (Topo II).

Merbarone does not affect topoisomerase II-mediated ATP hydrolysis. ATPase reactions contained 50 nM human topoisomerase IIa, 40 nM pBR322 plasmid DNA, and 1 mM [γ-³²P]ATP. A time course of ATP hydrolysis was performed at 0 μM (●) or 200 μM (○) merbarone. Three independent assays were carried out for each drug concentration. Error bars depict standard deviations.

Merbarone inhibits topoisomerase II-mediated DNA cleavage in a global manner. DNA cleavage reactions contained an end-labeled 3609-bp fragment derived from pBR322 (6 nM), 300 nM human topoisomerase IIa, 1 mM ATP, and either 0 μM (●) or 100 μM (☺) merbarone. Ten topoisomerase II-mediated DNA cleavage sites were observed. Relative levels of DNA cleavage were normalized to site 8 at 100 μM merbarone (relative cleavage = 1). The standard deviations of two independent experiments are represented by error bars.

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Fig. 4. Merbarone has no effect on topoisomerase II-DNA binding in a fluorescence anisotropy assay. Samples contained 5 nM fluorescein end-labeled double-stranded 40-mer DNA oligonucleotide and either 0 μM (●) or 100 (○) μM merbarone. The fluorophore was excited at 430 nm, and emission was monitored through a 530-nm cut-off filter. Anisotropies were determined over a concentration range of 0–50 nM human topoisomerase IIa (Topo II).

Fig. 5. Topoisomerase II-mediated DNA cleavage is blocked by merbarone. Cleavage reactions contained 300 nM human topoisomerase IIa (Topo II), pBR322 plasmid DNA (5 nM for –ATP cleavage, 10 nM for +ATP cleavage), and 0–200 μM merbarone. The gel shown in the top panel depicts the effects of merbarone on enzyme-mediated DNA cleavage in the presence of 1 mM ATP. Double-stranded DNA cleavage converts negatively supercoiled plasmid (form I, FII) to linear molecules (form III, FIII). The position of nicked circular DNA (form II, FII) is shown for reference. The bottom panel quantitates the effects of merbarone on DNA cleavage as described above in the absence (●) or presence (○) of ATP. Standard deviations of 2 to 3 independent assays under each condition are shown as error bars.

Fig. 6. Merbarone does not affect topoisomerase II-mediated ATP hydrolysis. ATPase reactions contained 50 nM human topoisomerase IIa, 40 nM pBR322 plasmid DNA, and 1 mM [γ-³²P]ATP. A time course of ATP hydrolysis was performed at 0 μM (●) or 200 μM (○) merbarone. Three independent assays were carried out for each drug concentration. Error bars depict standard deviations.

Fig. 7. Merbarone inhibits topoisomerase II-mediated DNA cleavage in a global manner. DNA cleavage reactions contained an end-labeled 3609-bp fragment derived from pBR322 (6 nM), 300 nM human topoisomerase IIa, 1 mM ATP, and either 0 μM (●) or 100 μM (☺) merbarone. Ten topoisomerase II-mediated DNA cleavage sites were observed. Relative levels of DNA cleavage were normalized to site 8 at 100 μM merbarone (relative cleavage = 1). The standard deviations of two independent experiments are represented by error bars.
Compounds that alter the gross structure of DNA either by intercalation or by minor groove binding can have dramatic effects on the activity of type II topoisomerases (1, 2, 58–61). It is possible that merbarone induces its global inhibition of enzyme-mediated DNA cleavage by one of these two mechanisms. Therefore, two approaches were utilized to determine whether this was the case.

First, the ability of merbarone to intercalate into DNA was determined by a topoisomerase I-catalyzed unwinding assay (Fig. 8). In the presence of a strongly intercalative drug such as ellipticine, 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. As seen in Fig. 8, 100 μM merbarone also had no effect on the topological state of the plasmid. To ensure that this latter result reflected a lack of DNA intercalation rather than an inhibition of topoisomerase I by the drug, negatively supercoiled DNA was utilized as the initial substrate for assays (not shown). Once again, relaxed plasmid resulted after treatment with topoisomerase I in the presence of 100 μM merbarone. These findings strongly suggest that merbarone is not intercalative in nature.

Second, the ability of merbarone to displace ethidium bromide from DNA was determined by a fluorescence emission assay. The DNA-bound form of ethidium has a significantly stronger fluorescence emission than does free ethidium; thus, displacement of ethidium from DNA can be monitored by a decrease in fluorescence signal (48, 49). Moreover, since ethidium bromide intercalates into DNA through interactions in the minor groove, this assay is capable of detecting drugs that either intercalate or bind in the minor groove of DNA.

As seen in Fig. 9, 100 μM merbarone was incapable of displacing 1 μM ethidium bromide. Less than 6% displacement was observed at 200 μM merbarone (not shown). In contrast, the intercalative drug amsacrine readily dislodged the bound fluorophore (IC50 ~ 50 μM). Taken together, these results indicate that if merbarone does in fact bind to DNA, it neither intercalates nor interacts with the minor groove.

**Figure 8**

**Merbarone does not intercalate into DNA.** The ability of merbarone to intercalate into DNA was investigated using a topoisomerase I-catalyzed DNA unwinding assay. Relaxed pBR322 DNA (generated by treatment of negatively supercoiled pBR322 DNA (form I, FII) with topoisomerase I) was incubated with 100 μM merbarone, etoposide, or ellipticine, or with Me2SO as a control (Topo I control), in the presence of topoisomerase I. A negatively supercoiled plasmid control (DNA Control) is shown. The position of nicked circular DNA molecules (form II, FII) is indicated.

**Figure 9**

**Merbarone does not displace ethidium from the minor groove of DNA.** The ability of merbarone to interact with the minor groove of DNA was determined by a fluorescence-based ethidium displacement assay. Samples contained 1 μM ethidium bromide and 5 nt double-stranded 40-mer DNA oligonucleotide. Increasing concentrations of merbarone (●) or amsacrine (○) were added, and ethidium fluorescence at 605 nm (λmax) was monitored (510 nm excitation wavelength).

**Figure 10**

**Etoposide-enhanced topoisomerase II-mediated DNA cleavage is attenuated by merbarone.** DNA cleavage reactions contained 75 nM human topoisomerase IIa, 10 nM pBR322 plasmid, and 1 mM ATP. Each reaction included etoposide (20 μM) and merbarone (100–400 μM). The order of drug addition was varied such that the enzyme/DNA complex was incubated with Etoposide First, Merbarone First, or the two drugs simultaneously (Etoposide/Merbarone). Cleavage levels are reported relative to cleavage (set at 100%) in control reactions which contained etoposide in the absence of merbarone. Error bars represent standard deviations of 2 to 6 data sets.

Consistent with previous reports, merbarone diminished the stimulation of topoisomerase II-mediated DNA cleavage by etoposide (Fig. 10). Furthermore, merbarone curtailed the cleavage-enhancing effects of an apurinic site located within a topoisomerase II recognition sequence (Fig. 11). Thus, merbarone is capable of attenuating cleavage enhancement induced both by anticancer drugs and by lesions that are an intrinsic part of the genetic material.

Although the above results are compatible with the fact that merbarone blocks the DNA cleavage step of the topoisomerase II catalytic cycle, they shed little light on potential relationships between this catalytic inhibitor and topoisomerase II poisons. However, by comparing Fig. 5 with Figs. 10 and 11, it is clear that higher concentrations of merbarone were required to diminish cleavage in the presence of etoposide or a DNA...
lesion. This finding at least suggests that beyond its inhibition of DNA scission, merbarone may compete with DNA cleavage-enhancing drugs for a binding site within the topoisomerase II-DNA complex.

Therefore, to dissect further the mechanism by which merbarone attenuates the actions of etoposide, an order of addition experiment was performed (Fig. 10). The diminution of cleavage was greatest when the enzyme-DNA complex was incubated with merbarone prior to the addition of etoposide and was largely overcome when the poison was added first. An intermediate degree of cleavage attenuation was observed when etoposide and merbarone were added simultaneously. These data suggest that the actions of the poison and the catalytic inhibitor on human topoisomerase IIα are mutually exclusive and imply that they may act within an overlapping interaction domain on the enzyme.

**DISCUSSION**

Merbarone is a catalytic inhibitor of human topoisomerase IIα that exhibits clinical potential as an anticancer agent (18, 26–32, 50). This drug disrupts chromosome separation during mitosis (33, 62–64) in a manner that is reminiscent of temperature-sensitive mutations in topoisomerase II (65–67) or treatment of cells with ICRF-193 (68, 69), a specific catalytic inhibitor of the enzyme (70, 71). Furthermore, merbarone blocks the stimulation of DNA cleavage by topoisomerase II poisons in cultured cells (33). Taken together, these findings provide strong (albeit circumstantial) evidence that topoisomerase II is an important in vivo target for merbarone.

Despite the potential therapeutic value of merbarone, the mechanism by which it inhibits the catalytic activity of topoisomerase II has never been delineated. Based on the fact that merbarone blocks the actions of topoisomerase II poisons, it has been suggested that this compound acts by obstructing enzyme-DNA binding (33). On the contrary, results of the present study indicate that this is not the case. Rather, it appears that merbarone inhibits topoisomerase II specifically by blocking enzyme-mediated DNA cleavage.

This conclusion was derived by dissecting the actions of merbarone on the individual steps of the topoisomerase II catalytic cycle. Concentrations of merbarone that inhibited catalytic activity ≥80% had no effect on either enzyme-DNA bind-

![FIG. 11. Merbarone decreases topoisomerase II-mediated cleavage at a DNA site that contains an apurinic lesion. A double-stranded 40-mer DNA oligonucleotide that contained an apurinic site located within a topoisomerase II cleavage site was utilized as the cleavage substrate. Reactions contained 100 nM oligonucleotide, 150 nM human topoisomerase IIα, and 0–400 μM merbarone. The Control reaction (assigned a relative cleavage value = 1) contained no merbarone and employed an oligonucleotide lacking an apurinic site. Standard deviations of 8 independent data sets are presented as error bars.](image)

![FIG. 12. Effects of inhibitors on the catalytic cycle of topoisomerase II. The topoisomerase II homodimer (shown in green) is modeled after the crystal structure reported by Berger et al. (72). The catalytic cycle of the enzyme has been described previously as a series of six individual reaction steps (1, 51). Step 1, topoisomerase II binds its DNA substrate (41, 73, 74). Step 2, a transient enzyme-linked double-stranded break is formed in the “cleavage” helix (shown in yellow) (75, 76). Step 3, ATP binding induces a conformational change in the enzyme (77) that converts topoisomerase II into a “protein clamp” on the DNA (41, 78). Concomitant with this structural reorientation, the “passage” helix (shown in purple) is translocated through the break in the cleavage helix (40). Step 4, the enzyme religates the break in the cleavage helix (41). Step 5, upon ATP hydrolysis, the protein clamp opens (41, 78), allowingStep 6, release of the DNA and the initiation of a new round of catalysis (41). Drugs that inhibit topoisomerase II catalytic function act at different steps of the catalytic cycle. Aclarubicin is an anthracycline that blocks enzyme-DNA binding, the initial step of the cycle (24). Staurosporine, a tyrosine kinase inhibitor that also acts on topoisomerase II, inhibits DNA cleavage and ATP interactions (22). Novobiocin and coumermycin, coumarin-based drugs that are primarily active against prokaryotic type II topoisomerases (15), block binding of ATP to the enzyme (40, 52). ICRF-193 is a bis(2,6-dioxopiperazine) derivative that blocks ATP hydrolysis, an action that traps the enzyme on DNA in its closed clamp form (25). Finally, as described in the present study, merbarone specifically blocks topoisomerase II-mediated cleavage of its DNA substrate.](image)
ing or ATP hydrolysis. In contrast, the drug was a potent inhibitor of DNA scission mediated by human topoisomerase IIα (in the absence or presence of ATP), and the IC_{50} values of merbarone for DNA cleavage and relaxation were similar.

It was not possible to assess the effects of merbarone on the DNA strand passage step of the catalytic cycle due to drug inhibition of DNA cleavage (the step that immediately precedes strand passage (see Fig. 12)). If merbarone is affecting the strand passage step, it is not doing so by interfering with topoisomerase II-ATP interactions, the primary mechanism that is employed by several other inhibitory drugs (15, 40, 52–55).

A number of chemical compounds that inhibit the catalytic activity of type II topoisomerases do so by distorting the gross structure of DNA. This is most often seen with drugs that intercalate or bind in the minor groove of the genetic material (58–61). As assessed by two independent DNA binding assays, merbarone does not appear to act in this manner. Instead, it seems likely that this drug exerts its effects on topoisomerase II catalytic activity by a specific interaction with either the enzyme or the enzyme-DNA complex. This suggestion is supported by two lines of evidence. First, merbarone competes for action with etoposide, a DNA cleavage-enhancing drug that enters the topoisomerase II-DNA complex primarily through its interactions with the enzyme (37). Second, merbarone displays a dramatic species dependence, inhibiting human topoisomerase IIα with a potency that is vastly greater than that for either the yeast or Drosophila type II enzymes.

A number of drugs that inhibit the catalytic activity of topoisomerase II have been identified. Although detailed studies have not been carried out for all of these agents, the available data indicate that catalytic inhibitors act by a variety of mechanisms (depicted in Fig. 12). Depending upon which step(s) of the topoisomerase II catalytic cycle is targeted by a specific agent, the cellular effects of that drug could be significantly different. For example, a compound such as aclacinomycin, which disrupts topoisomerase II-DNA binding (24), would undermine both the catalytic and structural roles of the enzyme. In contrast, drugs such as merbarone, which act by blocking DNA cleavage, or camptothecins, which act by blocking enzyme-ATP interactions (40, 52), would only impact the catalytic functions of topoisomerase II. Since IC_{50} values act by blocking ATP hydrolysis, it not only inhibits catalytic activity but also traps the enzyme on the DNA in its “closed clamp” form (25). This in turn might generate a physical barrier on the chromosome that impedes the actions of other DNA enzymes. Finally, in addition to their differential cellular effects, drugs that act at or before the DNA cleavage step are likely to attenuate the actions of topoisomerase II poisons. Clearly, the “mechanistic fingerprint” of a catalytic inhibitor could seriously impact its therapeutic potential.

Several compounds that inhibit the activity of topoisomerase II are under consideration as anticancer agents. Although these drugs are categorized under the general “umbrella” of catalytic inhibitors, they exert their effects by a variety of mechanisms. On the basis of the present study, merbarone acts by blocking the DNA cleavage reaction of topoisomerase II. This is the first drug found to specifically inhibit this reaction step and defines a new mechanistic class of catalytic inhibitors.

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