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Inhibition of DNA Topoisomerase II Catalytic Activity by the Antiviral Agents 7-Chloro-1,3-dihydroxyacridone and 1,3,7-Trihydroxyacridone*

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ABSTRACT. Previously we reported that the antiproliferative and antiviral actions of 7-chloro-1,3 dihydroxyacridone (compound 1) and its derivatives may be mediated through the inhibition of mammalian DNA topoisomerase II. In the present work, we have extended our investigation into the mechanism of topoisomerase II inhibition by these agents. Both compound 1 and its 7-OH derivative, compound 2, inhibited topoisomerase II catalytic activity *in vitro*, yet neither agent affected the activity of topoisomerase I. DNA unwinding assays indicated that compound 1 and compound 2 bound to DNA, although no correlation was found between DNA unwinding and topoisomerase II catalytic inhibition. Neither agent enhanced topoisomerase II-mediated DNA cleavage *in vitro*; however, both compound 1 and compound 2 antagonized breaks induced by etoposide and amsacrine. Experiments indicate that interference with etoposide-stimulated breaks results from inhibition of topoisomerase II·DNA binding by compound 1. These findings suggest that compound 1 and its derivatives may represent a novel structural class of topoisomerase II catalytic inhibitors. BIOCHEM PHARMACOL **58**;4:703–708, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. DNA topoisomerase II; catalytic inhibitor; acridone; antiviral

Topoisomerase II is an essential cellular enzyme responsible for resolving topological complications introduced through cellular processes such as replication and transcription and is required for chromosome condensation and segregation [reviewed in Ref. 1]. Topoisomerase II also serves as the cellular target for several clinically used anticancer drugs and is a potentially viable target for the development of antiparasitic, antifungal, and antiviral agents [reviewed in Refs. 2–5]. Inhibitors of the enzyme generally are grouped into two classes based on their mechanism of action. One group of topoisomerase II-targeting agents referred to as poisons act by stabilizing a ternary complex between topoisomerase II and DNA. This intermediate complex, called the cleavable complex, can be trapped by the addition of a protein denaturant, resulting in double-stranded DNA breaks.

A second group of agents function by inhibiting the overall catalytic activity of topoisomerase II without stabilizing enzyme-associated cleavable complexes. Inhibition of topoisomerase II by members in this group may result from interference with DNA binding (aclarubicin) [6] or DNA cleavage (merbarone) [7], through competition with ATP binding (novobiocin) [8], or by locking the enzyme in an inactive conformation (ICRF-193) [9]. In addition to inhibiting enzyme catalytic activity, these agents act to antagonize the formation of cleavable complexes stabilized by topoisomerase II poisons.

A cell-based screening assay was developed in this laboratory in order to identify potentially novel inhibitors of topoisomerase II [10]. The synthetic compound 7-chloro-1,3-dihydroxyacridone (compound 1; structures of acridone derivatives are given in Fig. 1) was selected for further evaluation since the IC_{50} of this agent was approximately 3-fold lower against the etoposide-resistant KB/7d cell line [11] (IC_{50} 10 μM) compared with the parental KB cell line (IC_{50} 35 μM) [12]. In addition, 30 μM compound 1 inhibited *in vitro* topoisomerase II catalytic activity by 50%, corresponding well with the IC_{50} for KB cell growth inhibition. Moreover, compound 1 did not induce topoisomerase II-dependent DNA strand breaks. These results suggested that topoisomerase II may represent the intracellular target of compound 1 and also indicated that compound 1 may act on topoisomerase II in a potentially unique manner resulting in the observed inhibition of enzyme catalytic activity *in vitro*. Thus, the current studies were undertaken to identify the mechanism of topoisomerase II inhibition by compound 1. Results from this work indicate that com-

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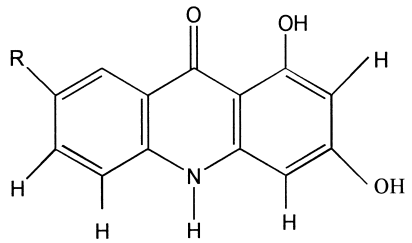

FIG. 1. Chemical structures of compound 1 and compound 2.

pound 1 functions as a catalytic inhibitor of topoisomerase II by interfering with the DNA binding step of the enzyme. Inhibition of topoisomerase II·DNA binding by the compound also resulted in the suppression of enzyme-stabilized DNA strand breaks by etoposide and amsacrine.

MATERIALS AND METHODS

Compounds 1 and 2 were synthesized as described previously [12]. Amsacrine (NSC-249992) was obtained from the National Cancer Institute. Etoposide was obtained from the Natural Products Laboratory, UNC School of Pharmacy. Human DNA topoisomerase II (p170 form) and human DNA topoisomerase I were purchased from TopoGen. Negatively supercoiled DNA was isolated from *Escherichia coli* strain HB101 transformed with plasmid pRYG or pBR322 in this laboratory. Plasmid pRYG is a pUC derivative containing a 54-bp purine/pyrimidine alternating repeat constituting a strong eukaryotic topoisomerase II binding site [13]. Both plasmids were isolated from chloramphenicol-treated cultures by the alkaline lysis method followed by banding on CsCl gradients.

DNA Topoisomerase II Catalytic Assay

Topoisomerase II catalytic activity was monitored by the relaxation of supercoiled plasmid pBR322 DNA. Assays were carried out in a final volume of 25 μL containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL of nuclease-free BSA, 0.25 μg DNA, and compounds as indicated. Reactions were initiated by the addition of 1 unit of human DNA topoisomerase II (1 unit of topoisomerase II fully relaxes 0.25 μg of supercoiled plasmid DNA in 30 min). All reactions were incubated for 30 min at 37° before termination by the addition of 5 μL of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, and 50% glycerol). Reaction mixtures were electrophoresed on 0.8% horizontal agarose gels in TBE buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0). Gels then were soaked in ethidium bromide (1 μg/mL) before illumination and photography using Polaroid Type 667 film.

DNA Unwinding Assay

The possibility that compounds 1 and 2 interact with DNA was evaluated through the use of a plasmid DNA unwinding assay [14]. In this assay, negatively supercoiled pBR322 DNA (0.25 μg/reaction) first was relaxed at 37° by incubation with 10 units of human topoisomerase I in 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.5 mM dithiothreitol, and 30 μg/mL of nuclease-free BSA for 30 min. Then drugs were added, and the reaction was continued for an additional hour. Reactions were terminated by the addition of SDS (0.5% final concentration) followed by dilution and extraction to remove drugs. Reaction mixtures were electrophoresed on 0.8% horizontal agarose gels in TBE buffer. To determine the direction of supercoiling, chloroquine (2 μg/mL) was added to the gel and running buffer. Then gels were soaked in ethidium bromide (1 μg/mL) before illumination and photography using Polaroid Type 667 film.

In Vitro *DNA Topoisomerase II Cleavage Assay*

Cleavage reactions were performed according to the procedure described by Liu *et al.* [15]. *Hin*dIII-restricted pRYG was end-labeled with [α-^{32}P]dCTP (3000 Ci/mmol; ICN Radiopharmaceuticals) using T4 polymerase and the other three unlabeled deoxyribonucleoside triphosphates. Unincorporated triphosphates were removed by Sephadex G-100 column chromatography. Topoisomerase II cleavage assays performed under coincubation conditions were carried out in a final volume of 25 μL containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL of nuclease-free BSA, 20 ng/mL of ^{32}P-labeled pRYG DNA, and compounds as indicated. Coincubation reactions were initiated by the addition of 1 unit of human DNA topoisomerase II. Cleavage assays performed under preincubation conditions were carried out by incubating enzyme and drug in the same reaction buffer at 37° for 3 min prior to the simultaneous addition of etoposide and labeled DNA. All reactions were incubated for a total of 30 min at 37° and then terminated by the addition of 1 μL of 20% SDS. Samples were treated for 2 hr at 50° with 1 μL proteinase K (25 μg/mL) before electrophoresis on 1% agarose gels in TBE buffer. Gels were dried under vacuum and autoradiographed. Levels of cleavage and cleavage interference were quantitated by scanning autoradiographs using a model GS 300 scanning densitometer (Hoefer Scientific Instruments).

Topoisomerase II·DNA Binding Assay

Binding of DNA by topoisomerase II was carried out in a final volume of 25 μL containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.5 mM dithiothreitol, 30 μg/mL of nuclease-free BSA, 20 ng/mL of ^{32}P-labeled pRYG DNA, 1 unit of human DNA topoisomerase II, and compounds as indicated. Reactions were performed under coincubation and preincubation conditions as described for the cleavage

assays. After incubating for 20 min at 37°, reactions were adjusted to 3% sucrose, 0.0025% bromophenol blue followed by electrophoresis on 0.7% agarose gels in TBE buffer at 4°. Then gels were dried under vacuum and autoradiographed. DNA binding was quantitated by densitometric scanning of autoradiographs.

RESULTS

Consistent with results previously reported by Bastow *et al.* [12], compounds 1 and 2 (Fig. 1) inhibited topoisomerase II-catalyzed relaxation of supercoiled DNA with IC_{50} values of 30 and 10 μM, respectively. In contrast, neither compound 1 nor compound 2 inhibited topoisomerase I activity at concentrations as high as 200 μM, indicating that these agents are selective for the type II enzyme (results not shown).

Many inhibitors of topoisomerase II are DNA-interactive agents. However, certain DNA binding agents can inhibit the enzyme *in vitro* as a result of their ability to intercalate or bind to the minor groove of DNA rather than through a specific interaction with the enzyme [16, 17]. Therefore, to rule out the latter possibility, DNA binding by the acridone derivatives was evaluated in a topoisomerase I-catalyzed DNA unwinding assay [14]. Using this assay, DNA unwinding by compounds 1 and 2 was evaluated along with the weakly intercalating topoisomerase II poison amsacrine for comparison. As shown in Fig. 2 (upper gel), both acridone derivatives exhibited a lower affinity for DNA than did amsacrine. Partial DNA unwinding was observed at 200 and 100 μM compound 1 with minimal unwinding detected at 50 μM. Compound 2 had a lower affinity than compound 1 for DNA, as no DNA unwinding was noted at 50 μM. Although both acridone derivatives caused partial unwinding of DNA, it is unlikely that drug·DNA binding accounts for their inhibition of topoisomerase II, since concentrations that markedly inhibited enzyme activity had little effect on DNA unwinding. In addition, compound 2, the more potent topoisomerase II inhibitor, had a lesser capacity to unwind DNA.

While the top gel in Fig. 2 demonstrates that the acridone derivatives unwind DNA, it does not indicate the direction of supercoils induced (i.e. positive or negative). Therefore, duplicate samples were electrophoresed on a second agarose gel containing 2 μg/mL of chloroquine. Chloroquine intercalates into DNA and induces supercoiling such that a negatively supercoiled plasmid first will be converted to the open circular form and then, as the concentration of chloroquine is increased, eventually will be converted to the positively supercoiled form. The result of adding chloroquine during electrophoresis is shown in the lower gel in Fig. 2. The upward shift in the topoisomers indicates that the *net* effect of the acridone derivatives (as well as amsacrine) in the DNA unwinding assay is to produce negative rather than positive DNA supercoils.

Previous studies demonstrated that compounds 1 and 2 do not stimulate topoisomerase II-mediated cleavable complex formation *in vitro*, indicating that these agents may act as catalytic inhibitors of the enzyme [12]. To better understand the mechanism of topoisomerase II inhibition by the acridone derivatives, the effects of compound 1 on etoposide-stimulated cleavable complex formation were evaluated. Similar to other antagonists of topoisomerase II, compound 1 interfered with the formation of etoposide-stimulated cleavable complexes. Upon incubating etoposide (50 μM) with increasing concentrations of compound 1, a concentration-dependent decrease in cleavable complex formation was observed (Fig. 3). Under these conditions (coincubation conditions), 200 and 100 μM compound 1 decreased etoposide-stimulated cleavage by 60%, whereas concentrations below 50 μM provided little interference with cleavable complex formation.

Interference with etoposide-stabilized cleavable complexes by topoisomerase II antagonists such as aclarubicin and merbarone is proposed to result from the formation of a drug-enzyme complex that prevents topoisomerase II from binding to or cleaving DNA [6, 7, 18]. Therefore, the above cleavage reaction procedure was altered such that topoisomerase II and compound 1 were incubated briefly prior to addition of etoposide and DNA (preincubation condi-

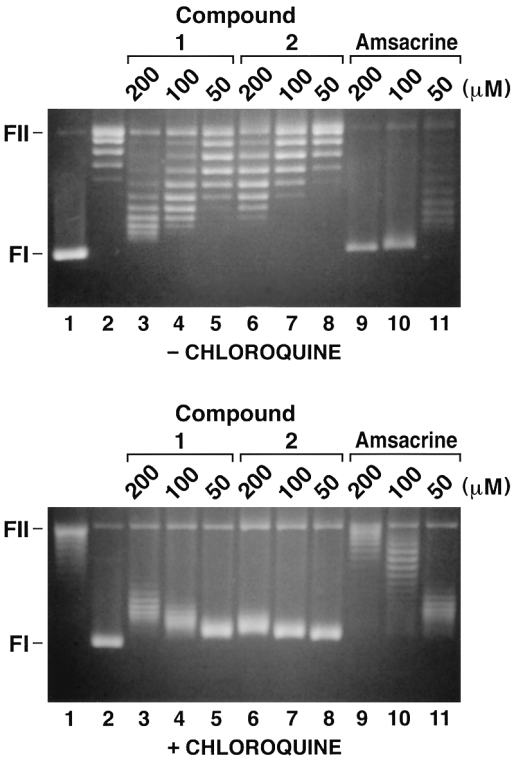

FIG. 2. DNA unwinding by compound 1, compound 2, and amsacrine. Negatively supercoiled plasmid pBR322 first was relaxed at 37° in the presence of excess (10 units/reaction) topoisomerase I. Then drugs were added, and reactions were continued for an additional 30 min. Samples were extracted to remove drugs and then were electrophoresed on a 1% agarose gel. The lower gel was run in the presence of 2 μg/mL of chloroquine. The positions of supercoiled (form I, FI) and relaxed (form II, FII) DNA are indicated.

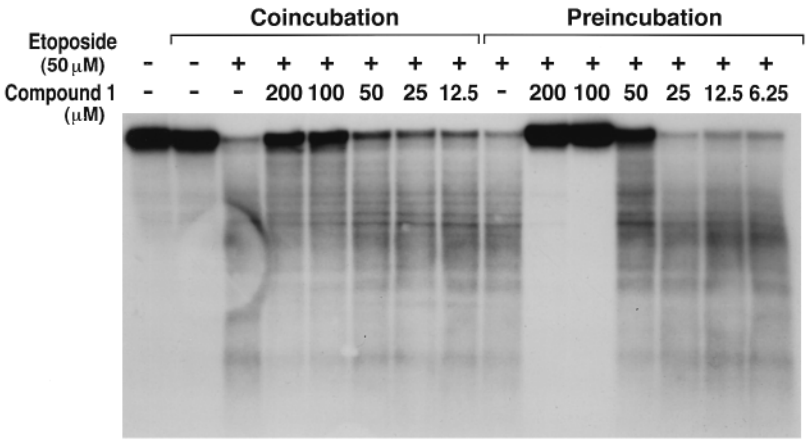

FIG. 3. Interference with etoposide-stimulated topoisomerase II-DNA complexes by compound 1 under coincubation or preincubation conditions. Under coincubation conditions, the two drugs were present simultaneously prior to adding enzyme and DNA. Under preincubation conditions, enzyme and compound 1 were incubated in reaction buffer for 3 min prior to the simultaneous addition of DNA and etoposide. Reactions were incubated for 30 min total before stopping with SDS and then were processed as described in Materials and Methods. All lanes contained topoisomerase II except for lane 1 (far left).

tions). Preincubation of topoisomerase II and compound 1 decreased the formation of etoposide-stabilized cleavable complexes to levels significantly below those observed under coincubation conditions (Fig. 3). Densitometric scanning of autoradiographs revealed that a 50% reduction in etoposide-stabilized strand breaks was achieved at approximately 60 μM compound 1. In addition, 100 μM compound 1 decreased DNA cleavage to levels below that of the enzyme and DNA alone. Interestingly, compound 1 also reduced amsacrine-stimulated DNA cleavage in a similar manner, indicating that cleavage interference is not specific to etoposide alone (results not shown).

We next altered the reaction procedure to allow the establishment of enzyme-DNA equilibrium prior to the addition of either (A) etoposide followed by compound 1, or (B) compound 1 followed by the addition of etoposide. Results under both experimental conditions are shown in Fig. 4. The addition of compound 1 to the pre-equilibrated mixture containing topoisomerase II and DNA followed by etoposide resulted in a level of cleavage interference slightly below that observed under preincubation conditions (condition B, Fig. 4). However, by pre-equilibrating enzyme and DNA and then adding etoposide prior to compound 1, no interference with cleavable complex formation was observed even at 200 μM compound 1 (condition A, Fig. 4). These results indicate that compound 1 interferes with topoisomerase II prior to or during cleavage. When the topoisomerase II-DNA equilibrium is shifted in favor of cleavage (as is the case when etoposide is added before compound 1), little or no cleavage interference by

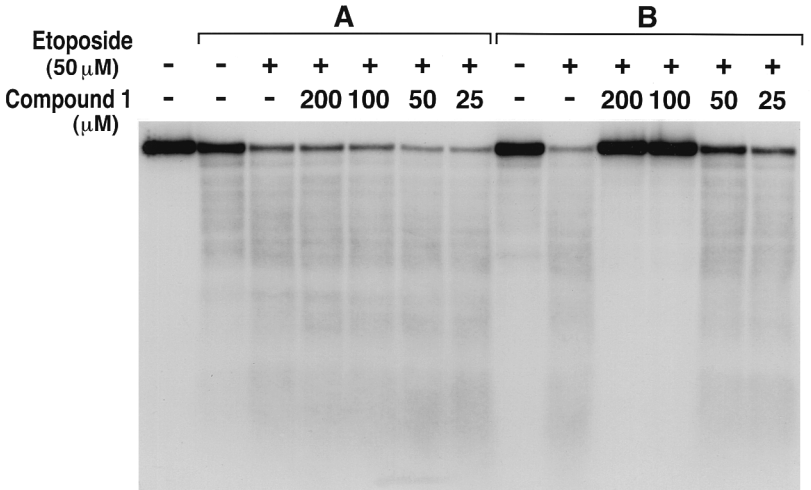

FIG. 4. Effect of drug order of addition on etoposide-stimulated topoisomerase II·DNA complex formation. In condition A, enzyme, DNA and etoposide were incubated for 5 min prior to adding compound 1. In condition B, enzyme, DNA and compound 1 were incubated for 5 min prior to adding etoposide. All reactions were incubated for 30 min total before stopping with SDS and then were processed as described in Materials and Methods. All lanes contained topoisomerase II except for lane 1 (far left).

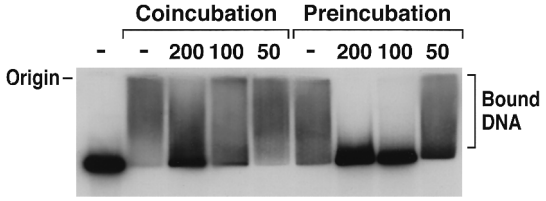

FIG. 5. Effect of compound 1 on topoisomerase II·DNA binding. For coincubation reactions, enzyme, DNA, and compound 1 were incubated at 37° for 20 min prior to electrophoresis on a 1% agarose gel. For preincubation reactions, compound 1 and enzyme were incubated for 3 min before the addition of DNA and then continued for an additional 17 min. The concentrations of compound 1 (200, 100, and 50) are given in micromolar. Topoisomerase II-bound DNA remained at the origin or exhibited reduced electrophoretic mobility. All lanes contained topoisomerase II except for lane 1 (far left).

compound 1 is observed. In contrast, when the equilibration of topoisomerase II and DNA is performed in the absence of etoposide, a low level of covalent DNA-enzyme binding occurs. Consequently, compound 1 effectively interferes with cleavable complex formation when added prior to etoposide.

Results from the cleavage interference experiments indicate that compound 1 may interfere with the ability of topoisomerase II to bind to DNA, the initial step of the topoisomerase II catalytic cycle. Therefore, the effect of compound 1 on topoisomerase II·DNA binding was assessed by a gel electrophoretic mobility shift assay. Results from this assay (Fig. 5) revealed a pattern of DNA·topoisomerase II binding inhibition by compound 1, which closely paralleled its ability to interfere with etoposide-stimulated cleavable complex formation. Coincubation of compound 1 with topoisomerase II and DNA led to a concentration-dependent, yet incomplete decrease in enzyme-DNA interaction, whereas preincubation of compound 1 and topoisomerase II prior to the addition of DNA led to the complete loss of DNA binding at both 200 and 100 μM compound 1 (Fig. 5).

DISCUSSION

Several topoisomerase II-targeting agents interact with DNA, although DNA binding is not required for inhibition of catalytic activity (e.g. merbarone, ICRF-193) or for cleavable complex formation (e.g. etoposide). The acridone derivatives were evaluated for their ability to bind DNA to determine if the effects of these agents occurred solely due to DNA binding rather than as a result of an effect on the enzyme or the enzyme·DNA complex. Although compounds 1 and 2 do interact weakly with DNA to induce negative supercoiling, several lines of evidence indicate that the interaction of the acridone derivatives with DNA does not account for their topoisomerase II inhibitory actions. First of all are the contrasting effects of these agents on topoisomerase I and topoisomerase II catalytic activity. While both acridone derivatives inhibited topoisomerase II catalytic activity at low micromolar concentrations (< 30 μM), neither agent inhibited topoisomerase I catalytic activity even at concentrations as high as 200 μM. Second, although compounds 1 and 2 interact with DNA, no correlation was found between their ability to bind DNA and their ability to inhibit topoisomerase II catalytic activity. For example, compound 2 inhibited topoisomerase II catalytic activity at a 2-fold lower concentration than compound 1, yet compound 1 exhibited a relatively higher affinity for DNA than compound 2. Finally, compound 1 more effectively antagonized etoposide-stimulated cleavable complex formation and inhibited enzyme·DNA binding as well as topoisomerase II catalytic activity (results not shown) when preincubated with topoisomerase II rather than when coincubated with enzyme and DNA. These observations indicate that the acridone derivatives primarily interact with the enzyme rather than with DNA. Consistent with this fact, inhibition of topoisomerase II catalytic activity by compounds 1 and 2 could be reversed by increasing the concentration of enzyme in the reaction mixture (results not shown).

The steps in the reaction mechanism of topoisomerase II can be isolated *in vitro* and therefore allow for evaluation at different stages of the catalytic cycle of the enzyme. This method of evaluation has provided some insight into the mechanism of enzyme inhibition by compound 1. The fact that compound 1 did not stabilize topoisomerase II-mediated strand breaks suggested that it may interact with the enzyme in a unique manner, resulting in inhibition of catalytic activity. Gel electrophoretic mobility shift assays demonstrated that compound 1 inhibits topoisomerase II·DNA binding. By interfering with this reaction step, compound 1 prevents the stimulation of enzyme-mediated cleavable complex formation in the presence of the topoisomerase II poisons etoposide and amsacrine.

Numerous structurally disparate compounds have been identified as topoisomerase II inhibitors. Interestingly, although several such agents inhibit enzyme function through distinct mechanisms, they apparently do so by interacting at similar or overlapping domains on the enzyme [7, 19, 20]. For example merbarone, which impedes the cleavage reaction of topoisomerase II, purportedly shares a common enzyme binding site with cleavage enhancing poisons such as etoposide and amsacrine. Similar to merbarone, compound 1 reduced etoposide-stimulated cleavage most effectively when incubated with the enzyme·DNA complex prior to the addition of etoposide and was least effective when added to the enzyme II·DNA complex after etoposide. Although it cannot be ruled out that compound 1 and etoposide may bind to a partially overlapping site on the enzyme, or within the cleavable complex, the strong correlation between cleavage interference and topoisomerase II·DNA binding inhibition under both preincubation and coincubation conditions indicates that compound 1 inhibits topoisomerase II activity by interfering with the initial non-covalent interaction between the enzyme and its DNA substrate.

Although merbarone and compound 1 apparently affect different steps in the topoisomerase II catalytic cycle (i.e. cleavage and DNA binding, respectively), results presented here indicate that both agents function as catalytic inhibitors of topoisomerase II. Merbarone is currently under investigation in phase II clinical trials, indicating that catalytic inhibitors of topoisomerase II hold promise as potentially useful therapeutic agents.

Compounds 1 and 2, as well as other acridone congeners, are unique among known topoisomerase II inhibitors in that they are selective inhibitors of herpes simplex virus replication in cell culture systems [12, *]. The question of whether these agents inhibit intracellular topoisomerase II activity is of interest for the rational development of acridone derivatives as antiviral or antitumor agents.

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