Induction of Topoisomerase II-Mediated DNA Cleavage by a Protoberberine Alkaloid, Berberrubine†

Sun Ahe Kim,‡ Young Kwon,‡ Jung Han Kim,§ Mark T. Muller,|| and In Kwon Chung*,‡

Departments of Biology and Biotechnology, Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea, and Department of Molecular Genetics, Ohio State University, Columbus, Ohio 43210

Received May 12, 1998; Revised Manuscript Received August 12, 1998

ABSTRACT: Topoisomerase II is the cytotoxic target for a number of clinically relevant antitumor drugs. Berberrubine, a protoberberine alkaloid which exhibits antitumor activity in animal models, has been identified as a specific poison of topoisomerase II in vitro. Topoisomerase II-mediated DNA cleavage assays showed that berberrubine poisons the enzyme by stabilizing topoisomerase II-DNA cleavable complexes. Subsequent proteinase K treatments revealed that berberrubine-induced DNA cleavage was generated solely by topoisomerase II. Topoisomerase II-mediated DNA religation with elevated temperature revealed a substantial reduction in DNA cleavage induced by berberrubine, to the extent comparable to that of other prototypical topoisomerase II poison, etoposide, suggesting that DNA cleavage involves stabilization of the reversible enzyme−DNA cleavable complex. However, the step at which berberrubine induces cleavable complex may differ from that of etoposide as revealed by the difference in the formation of the intermediate product, nicked DNA. This suggests that berberrubine's primary mode of linear formation may involve trapping nicked molecules, formed at transition from linear to covalently closed circular DNA. Unwinding of the duplex DNA by berberrubine is consistent with an intercalative binding mode for this compound. In addition to the ability to induce the cleavable complex mediated with topoisomerase II, berberrubine at high concentrations was shown to specifically inhibit topoisomerase II catalytic activity. Berberrubine, however, did not inhibit topoisomerase I at concentrations up to 240 μM. Cleavage sites induced by topoisomerase II in the presence of berberrubine and etoposide were mapped in DNA. Berberrubine induces DNA cleavage in a site-specific and concentration-dependent manner. Comparison of the cleavage pattern of berberrubine with that of etoposide revealed that they share many common sites of cleavage. Taken together, these results indicate that berberrubine represents a new class of antitumor agent which exhibits the topoisomerase II poison activity as well as catalytic inhibition activity and may have a potential clinical value in cancer treatment.

DNA topoisomerases are nuclear enzymes that are able to break and reseal the sugar−phosphate backbone bonds of DNA and adjust the topological states of the DNA helix (*1*−*4*). Eukaryotic DNA topoisomerase II catalyzes the ATP-dependent relaxation of negative and positive supercoils, knotting, unknotting, catenation, and decatenation of DNA by passing the double-stranded DNA helix through a transient double-stranded break and then resealing the strand break. The mechanism of topoisomerase II activity involves DNA cleavage, strand passage, and religation, succeeded by enzyme turnover with the aid of ATP (*5, 6*). During this cycle, the enzyme covalently binds to DNA forming an intermediate called topoisomerase II−DNA covalent cleavable complex (*1*−*4*).

Eukaryotic DNA topoisomerase II is the cellular target for a variety of active agents currently used for the treatment of human cancers (*1, 7*−*9*). By stabilizing the covalent enzyme-associated DNA complexes formed between topoisomerase II and DNA, these drugs shift the DNA cleavage/religation equilibrium of the enzyme reaction toward cleavage. These drugs are able to convert a biological intermediate in topoisomerase II activity into a lethal one, ultimately leading to cell death, and thus act as cellular poisons (*1, 7*−*10*). Since the cellular level of topoisomerase II in proliferating cells is higher than that of quiescent cells, these deleterious aspects of enzyme confer the selective sensitivity of proliferative tumor cells to the cytotoxic effects of these drugs (*1, 7, 9*). Antitumor drugs, despite their apparent structural diversity, show common properties in stabilization of the cleavable complexes (*1, 8*). The families of topoisomerase II poisons include both DNA intercalative agents, for example, adriamycin, *m*-AMSA,[1] ellipticine, and mitoxantrone, and nonintercalative agents, for example, etoposide and teniposide. Unlike the topoisomerase II poisons, some

† Supported by a grant from the KOSEF 95K2-0401-00-01-5 through the Bioproducts Research Center at Yonsei University, Korea.

* To whom correspondence should be addressed at the Department of Biology, College of Science, 134 Shinchon-dong, Yonsei University, Seoul 120-749, Korea. Tel: 822-361-2660. Fax: 822-312-5657. E-mail: topoviro@bubble.yonsei.ac.kr.

‡ Department of Biology, Yonsei University.

§ Department of Biotechnology, Yonsei University.

|| Ohio State University.

[1] Abbreviations: *m*-AMSA, 4′-(9-acridinyl-amino)methanesulfon-*m*-anisidide (amsacrine); k-DNA, kinetoplast DNA.

10.1021/bi9810961 CCC: $15.00
Published on Web 10/29/1998

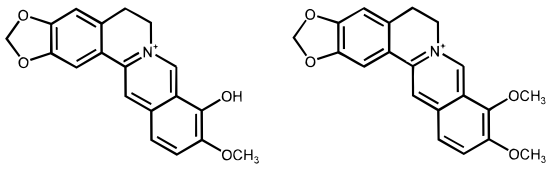

FIGURE 1: Chemical structures of protoberberine alkaloids, berberrubine, and berberine.

antitumor drugs have been reported to inhibit topoisomerase II activity without significantly stabilizing cleavable complexes. These drugs, including ICRF-193, merbarone, and quinobenoxazines, inhibit DNA topoisomerase II activity at a step prior to the formation of the cleavable complex and thus act as antagonists of DNA topoisomerase II poisons (*11*−*15*). Such precleavage catalytic inhibition may also play a role in the cytotoxicity and anticancer activity of DNA topoisomerase II poisons (*15*). Agents identified as poisons and/or catalytic inhibitors have proven to be useful in understanding the mechanisms of the topoisomerase II-catalyzed reactions in addition to their clinical use in cancer chemotherapy.

Although many topoisomerase II-targeted agents have been identified for treatment of certain types of human cancer, complications such as resistance in solid tumors and subsequent genetic changes severely limit the efficacy of DNA topoisomerase II poisons (*16*). For these reasons, the development of new drugs that supplement such problems is needed to improve clinical cancer chemotherapy. In tumor cells selected for resistance to topoisomerase II-targeted drugs, the most common mechanism of drug resistance involves (1) enhanced drug efflux associated with the expression of either P-glycoprotein (*17*) or the multiresistance protein MRP (*18, 19*) and (2) reduced formation of cleavable complexes due to the expression of decreased amounts or activities of topoisomerase II (*20*−*24*). Although the relationship between topoisomerase II level and drug sensitivity in tumor cells expressing altered topoisomerase II has been described, many questions concerning the role of topoisomerase II in the development of multidrug resistance still remain.

Berberrubine (see Figure 1 for the structure) is an isoquinoline alkaloid isolated from *Berberis vulgaris L.* and is readily derived from berberine (*25*). Previous studies showed that berberrubine and its derivatives exhibit antitumor activity in mouse models, and a hydroxyl group at the 9-position of berberrubine is essential for the manifestation of antitumor activity (*26, 27*). Although these studies have clarified the relationship between chemical structure and antitumor activity of berberrubine and its derivatives, the mechanism of action of berberrubine remained unclear. In the screening of the plant alkaloids for their ability to induce topoisomerase II-mediated DNA cleavage in vitro, we found that berberrubine shows a potent activity as DNA topoisomerase II poison; however, despite much resemblance in chemical structure (Figure 1), other protoberberine alkaloids such as berberine and palmatine did not act on topoisomerase II, although some are reported to target topoisomerase I (*28*). This observation is consistent with the previous report that some of the protoberberine alkaloids isolated from *Coptis* rhizomes induce DNA topoisomerase-mediated DNA cleavage (*29*). In this report, we have investigated possible mechanisms by which berberrubine stabilizes topoisomerase II-mediated cleavable complex in vitro as well as inhibits catalytic activity of topoisomerase II. The results imply the potency of berberrubine as a new class of antitumor agent.

EXPERIMENTAL PROCEDURES

Materials. Purified human topoisomerase I, topoisomerase II, and kinetoplast DNA (k-DNA) were obtained from TopoGEN, Inc. (Columbus, Ohio). The purity of topoisomerase II was verified by polyacrylamide gel electrophoresis; a single intense band of 170 kDa was identified, and lower molecular weight bands were undetectable (data not shown). Berberine was purchased from Sigma, and other topoisomerase II inhibitors, etoposide, *m*-AMSA, and adriamycin, were from TopoGEN. The berberrubine was derived from berberine as described (*25*) and kept as a 10 or 40 mM stock solution in DMSO. Supercoiled plasmid DNA was purified using standard methods. Restriction enzymes and other DNA-modifying enzymes were purchased from Promega. Radioactive nucleotides were from Amersham International.

Topoisomerase II Cleavage Reactions. The cleavage reactions contained 0.3 μg of pBS (Stratagene) and cleavage buffer (30 mM Tris-HCl, pH 7.6, 60 mM KCl, 8 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 3 mM ATP, 30 μg/mL bovine serum albumin) in a total volume of 20 μL. Topoisomerase II inhibitors were added as specified in each experiment, and the reactions were initiated by adding 8 units of purified human topoisomerase II. After incubation for 30 min at 37 °C, the cleavage complexes were trapped by addition of 2 μL of 10% SDS followed by topoisomerase digestion with proteinase K for 30 min at 45 °C. The reaction products were purified with phenol/chloroform extraction and electrophoresed on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide for 2 h at 0.25 V/cm. The amount of DNA products was quantified by densitometric analysis using Eagle Eye II (Stratagene). For mapping cleavage sites, topoisomerase II cleavage reactions were performed on a whole plasmid DNA, linearized with *Hin*dIII, and run into a 1% agarose gel containing 0.5 μg/mL ethidium bromide. The DNA was transferred onto nylon membrane, hybridized with ^{32}P-labeled *Hin*dIII−*Pvu*II fragment, and autoradiographed (*30*). For sequencing of topoisomerase II cleavage sites, cleavage reactions were carried out with uniquely end-labeled DNA fragments which were prepared with T4 polynucleotide kinase and [γ-^{32}P]-ATP as described elsewhere (*31*). Cleavage sites were analyzed on 8% denaturing polyacrylamide gels, along with chemical sequencing markers to locate the sites.

Topoisomerase II-Mediated DNA Religation. Religation reactions contained 0.3 μg of pBS DNA, 8 units of topoisomerase II, and 100 μM either berberrubine or etoposide in a total of 20 μL of cleavage buffer. The reactions were incubated for 30 min at 37 °C, and the topoisomerase II-mediated religation was induced by shifting the temperature from 37 to 65 °C. Religation reactions were stopped at various times by addition of SDS to 1%. Following topoisomerase II digestion with proteinase K for 30 min at 45 °C, the products were phenol/chloroform extracted and electrophoresed on a 1.2% agarose gel containing 0.5 μg/

mL ethidium bromide. The amount of DNA products was quantified by densitometric analysis.

DNA Unwinding Analysis. DNA intercalative property of berberrubine was investigated by incubating a mixture of supercoiled and relaxed pBS DNA as substrates and various amounts of drugs in 20 μL of relaxation buffer (30 mM Tris-HCl, pH 7.6, 60 mM KCl, 8 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 30 μg/mL bovine serum albumin). The reactions were initiated by the addition of an excess amount of eukaryotic topoisomerase I (8 units/reaction). After incubation for 30 min at 30 °C, the DNA samples were trapped by the addition of 2 μL of 10% SDS followed by phenol/chloroform extraction twice and electrophoresed on a 1.2% agarose gel in the presence of 0.94 μg/mL chloroquine. DNA unwinding was indicated by a shift of the topoisomers.

Kinetoplast DNA Decatenation Assays. The k-DNA decatenation reactions were performed in a total volume of 20 μL of 50 mM Tris-HCl, pH 7.6, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg/mL bovine serum albumin containing 0.13 μg of k-DNA, 1 unit of purified human topoisomerase II, and specified amounts of the test compounds. After incubation for 30 min at 37 °C, the reactions were stopped by the addition of 5 μL of 5% sarcosyl, 0.025% bromophenol blue, and 25% glycerol, and the products were analyzed on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. One unit of topoisomerase II activity is the amount of enzyme required to convert 250 ng of k-DNA substrate into the decatenated product during 15 min of incubation at 37 °C in a 20 μL reaction.

RESULTS

Formation of Topoisomerase II-Mediated DNA Cleavage by Berberrubine. To examine the effects of berberrubine and berberine on the activity of human topoisomerase II, we carried out the topoisomerase II cleavage reactions with these compounds. The results indicate that berberrubine is able to induce the formation of double-stranded breaks (linear DNA) in DNA at 100 μM concentration as did etoposide (Figure 2, compare lane 3 with 9). Etoposide also enhanced the ability of enzyme to mediate the formation of nicked circular DNA (compare lane 9 with 10). In contrast, berberrubine stimulated the formation of only linear DNA but not nicked circular DNA (compare lane 3 with 4). Despite the structural similarity between berberine and berberrubine, DNA cleavage activity of berberine was much weaker than that of berberrubine (lane 6). In the absence of topoisomerase II, berberrubine and berberine did not induce detectable changes in the DNA substrate (lanes 5 and 8). Since berberrubine is a novel topoisomerase II-targeted compound, it is important to ensure that the DNA cleavage induced by berberrubine was solely mediated by topoisomerase II. The formation of covalent cleavable complexes between topoisomerase II and DNA is one of the crucial steps in enzyme activity (*1*−*4*). Such covalently bound complexes do not comigrate with free DNA in the agarose gel if proteinase K treatment is omitted. In the absence of proteinase K treatment, the cleavable complexes failed to appear at the linear DNA position (lanes 4 and 10).

We examined the effect of drug concentration on the formation of double-stranded breaks in DNA. As shown in

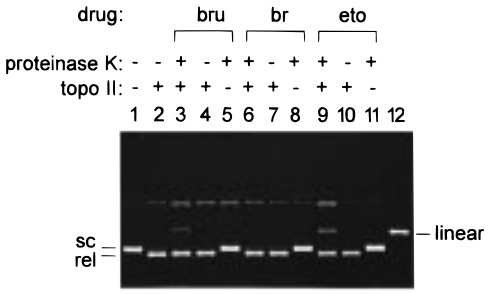

FIGURE 2: DNA cleavage stimulated by berberrubine is mediated by topoisomerase II. DNA cleavage assays were carried out with 100 μM berberrubine (lanes 3−5), berberine (lanes 6−8), or etoposide (lanes 9−11) as described in the Experimental Procedures. The reaction products were resolved on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide: lane 1, supercoiled input DNA (sc); lane 2, relaxed DNA (rel); lane 12, linear DNA: lanes 3, 6, and 9, DNA cleavage products were digested with proteinase K; lanes 4, 7, and 10, DNA cleavage products were not digested with proteinase K; lanes 5, 8, and 11, the cleavage reactions were carried out in the absence of topoisomerase II. The bands above the linear position indicate open circular DNA: bru, berberrubine; br, berberine; eto, etoposide.

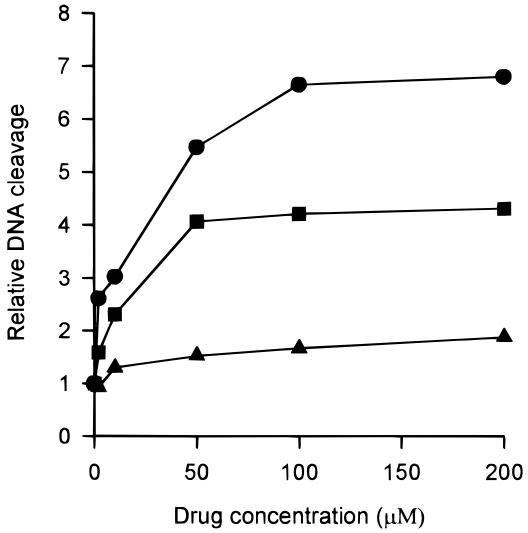

FIGURE 3: Effects of drug concentration on the formation of topoisomerase II-mediated DNA cleavage. DNA cleavage assays were carried out in the presence of increasing concentrations of drugs. The reaction products were analyzed on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide. The formation of linear DNA was expressed as a function of the concentration of berberrubine (squares), berberine (triangles), or etoposide (circles) on the basis of densitometric analysis. The relative level of DNA cleavage was arbitrarily set to 1 in the absence of drug. Data represent the average of three independent experiments.

Figure 3, DNA cleavage was measured by the increase in linear DNA in drug-containing reactions. Both berberrubine and etoposide showed a dose-dependent increase in topoisomerase II-mediated DNA double-stranded breaks. Levels of DNA breakage plateaued at drug concentrations above 100 μM. As measured from the linear range (between 10 and 50 μM) of the drug concentration curve, berberrubine was about 70%−80% as potent as etoposide in the formation of topoisomerase II-mediated DNA cleavage. In contrast, increasing concentrations of berberine caused only a slight increase in DNA breaks, above the background level produced by the topoisomerase II alone (Figure 3).

Effect of Berberrubine on the Religation Activity of Topoisomerase II. The formation of a cleavable complex

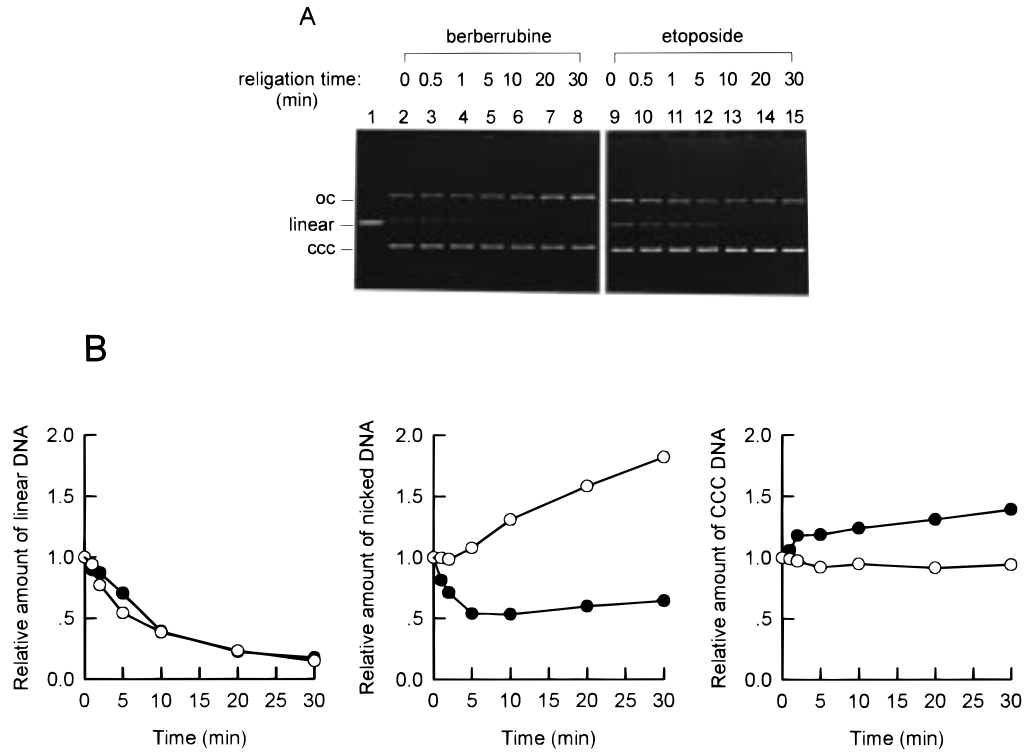

FIGURE 4: Effects of berberrubine or etoposide on the ability of topoisomerase II to mediate DNA religation. (A) A large reaction mixture (140 μL) containing 100 μM either berberrubine (lanes 2−8) or etoposide (lanes 9−15) was incubated with 56 units of topoisomerase II at 37 °C for 30 min. The reaction mixture was then heated to 65 °C, and aliquots (20 μL) were withdrawn at various times after heat treatment. The reactions were terminated by the addition of SDS and proteinase K as described in the Experimental Procedures. The reaction products were analyzed on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide: lane 1, linear DNA. The positions of open circular (oc), linear, and covalently closed circular (ccc) DNA are indicated. (B) The amount of linear, nicked, or covalently closed circular DNA was quantified by densitometric analysis of lanes 2−8 for berberrubine (open circles) and lanes 9−15 for etoposide (closed circles) from the gel, and was expressed as a function of religation time. The amount of linear, nicked, or covalently closed circular DNA for each assay was arbitrarily set to 1 at time 0.

can be readily reversed by adding EDTA or salt to the reaction mixture or by elevating the temperature prior to the addition of SDS (*32*−*37*). The mechanism by which berberrubine enhances DNA breakage was determined by investigating its effect on topoisomerase II-mediated DNA religation using a heat-induced religation assay. DNA religation induced by temperature shift relies on the fact that religation of topoisomerase II remains less sensitive to variations in temperature than DNA cleavage activity (*34*−*36*). Shifting the temperature from 37 to 65 °C before termination with SDS and proteinase K reconverted linear DNA molecules generated by topoisomerase II-mediated DNA cleavage to covalently closed circular DNA in a time-dependent manner (Figure 4). Previous studies have shown that antitumor drugs can stabilize DNA cleavable complex either by increasing the enzyme's rate of cleavage or by decreasing its rate of religation (*7*, *36*, *38*, *39*). It is also known that etoposide induces enzyme-mediated DNA breaks primarily by inhibiting the religation activity of topoisomerase II (*40*, *41*). Similarly, berberrubine inhibited the enzyme's ability to religate linear DNA to the extent comparable to that of etoposide (Figure 4). Thus, it can be concluded that berberrubine induces formation of DNA cleavable complex primarily by inhibiting DNA religation like etoposide. Consistent with previous findings, densitometric scanning of the gel shown in Figure 4A reveals that topoisomerase II religates double-stranded breaks one strand at a time; first, enzyme converts linear DNA to nicked DNA and, second, enzyme converts nicked DNA to covalently closed circular DNA (*40*). The step at which berberrubine induces cleavable complex may differ from that of etoposide as revealed by the difference in the formation of nicked DNA (Figure 4B). Contrary to etoposide, berberrubine increasingly produced nicked DNA in a time-dependent manner. This could imply that berberrubine's primary mode of linear formation may involve trapping nicked molecules, formed at a transition from linear to covalently closed DNA.

DNA Unwinding by Berberrubine. Some of the antitumor drugs which can induce topoisomerase II-mediated cleavable complex are known to be DNA intercalators (*1*, *42*). To investigate the ability of berberrubine to intercalate into DNA, the effect of the drug on the average linking number of topoisomers was examined using DNA unwinding assay (*28*, *43*, *44*). In this assay, drug-induced alterations in DNA linking number are measured by alterations in the Gaussian distribution of topoisomers generated by topoisomerase I action in the presence of increasing ligand. The DNA unwinding assay was performed with a mixture of relaxed and supercoiled DNA to properly assess the influence of berberrubine-induced inhibition of topoisomerase I catalytic activity on the relaxation. The DNA samples were analyzed on agarose gel in the presence of chloroquine to resolve maximally the topoisomer distribution. Close inspection of the gel in Figure 5 reveals that berberrubine unwinds DNA in a concentration-dependent fashion starting at 30 μM and continuing up to the maximum tested concentration of 240 μM. This is consistent with an intercalative binding mode for berberrubine. Note that, even at the highest concentra-

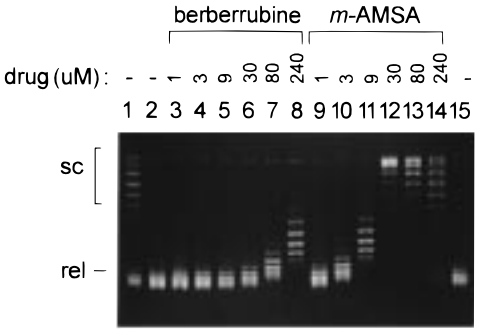

FIGURE 5: Unwinding of DNA by berberrubine or *m*-AMSA. DNA unwinding reactions were performed using a mixture of supercoiled (0.15 μg) and relaxed DNA (0.15 μg) and 8 units of topoisomerase I in the presence of increasing concentrations of berberrubine (lanes 3−8) or *m*-AMSA (lanes 9−14): lane 1, supercoiled and relaxed input DNA; lane 2, relaxed DNA. Lane 15 contained topoisomerase I and DMSO as a control. The DNA samples were analyzed on a 1.2% agarose gel in the presence of 0.94 μg/mL chloroquine. sc and rel denote supercoiled and relaxed DNA, respectively.

tion, unwinding did not occur to the extent seen with the control drug, *m*-AMSA (another topoisomerase II poison). The unwinding by *m*-AMSA at 9 μM was about the same as that seen with 240 μM berberrubine. Thus, the intercalative action of a berberrubine is roughly 30 times less than the prototypic topoisomerase II poison, *m*-AMSA.

Inhibiton of the Catalytic Activity of DNA Topoisomerase II by Berberrubine. In addition to topoisomerase II cleavage reactions, drug titration experiments revealed that, at higher concentrations of berberrubine, the relaxation activity of topoisomerase II was inhibited, whereas etoposide did not display this effect at the same concentrations. To further investigate inhibition of catalytic activity of topoisomerase II by berberrubine, we employed a decatenation reaction using k-DNA (*44*, *45*). Each reaction contained 0.13 μg of catenated k-DNA and 1 unit of topoisomerase II in the presence of increasing concentrations of berberrubine. Berberrubine detectably inhibited decatenation activity by topoisomerase II at 30 μM and strongly inhibited at 80 μM and at higher concentrations under the conditions employed in this assay (Figure 6A, lanes 3−8), whereas etoposide negligibly inhibited decatenation at concentrations up to 80 μM and detectably inhibited at 240 μM (lanes 9−14). These observations can be explained in either of two ways. One possibility is that berberrubine interacts with the DNA substrates, precluding topoisomerase II activity (like other DNA-binding drugs such as doxorubucin). Another possibility is that the drug interacts with the enzyme at a step prior to the formation of the topoisomerase II-mediated cleavable complex (like catalytic inhibitors such as ICRF-193 and merbarone).

This was examined by adding either the DNA substrate or topoisomerase II in excess to the reaction mixture to overcome the inhibition (Figure 6B). Increasing amounts of k-DNA (0.13, 0.26, and 0.52 μg) were incubated with 1 unit of topoisomerase II in the absence of berberrubine (Figure 6B, lanes 2−4). In all cases, catenated k-DNA was completely converted to decatenated products. The identical reactions were performed except that the drug was present at 80 μM. Since strong inhibition by berberrubine was detected at 80 μM under the conditions employed (Figure 6A, lane 7), this drug concentration was selected. In the

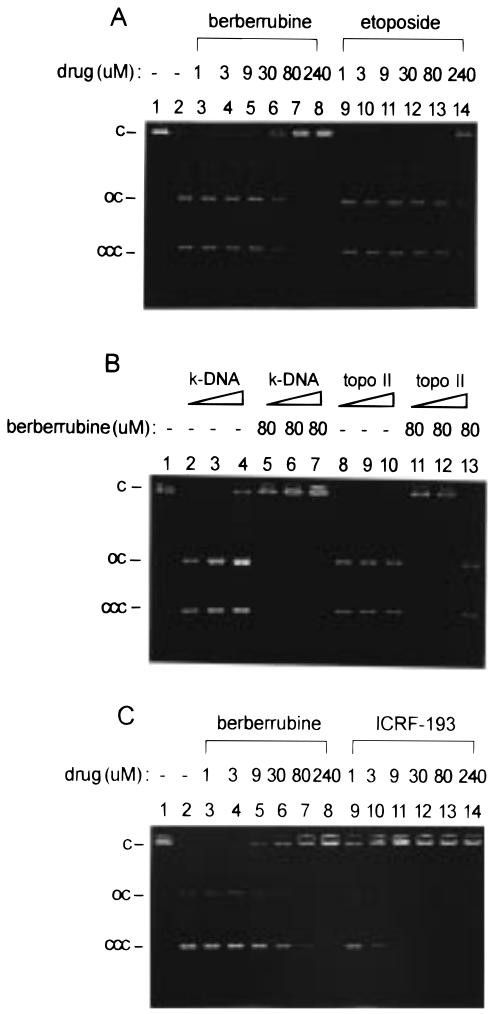

FIGURE 6: Inhibition of topoisomerase II catalytic activity by berberrubine or etoposide. Topoisomerase II decatenation reactions were performed and analyzed on agarose gel containing 0.5 μg/mL ethidium bromide, as described in the Experimental Procedures. The positions of catenated k-DNA (c), decatenated open circular (oc), and decatenated covalently closed circular DNA (ccc) are indicated. (A) Lane 1, catenated k-DNA; lane 2, decatenated k-DNA products. Decatenation reactions containing 1 unit of topoisomerase II and 0.13 μg of k-DNA were performed in the presence of increasing amounts of either berberrubine (lanes 3−8) or etoposide (lanes 9−14). (B) Lane 1, catenated k-DNA. Decatenation reactions contained 1 unit of topoisomerase II and either no drug (lanes 2−4) or 80 μM berberrubine (lanes 5−7), with k-DNA amounts of 0.13 μg (lanes 2 and 5), 0.26 μg (lanes 3 and 6), or 0.52 μg (lanes 4 and 7). Reactions contained 0.13 μg of k-DNA and either no drug (lanes 8−10) or 80 μM berberrubine (lanes 11−13), with topoisomerase II amounts of 1 unit (lanes 8 and 11), 2 units (lanes 9 and 12), or 4 units (lanes 10 and 13). (C) Lane 1, catenated k-DNA; lane 2, decatenated k-DNA products. Decatenation reactions were performed in the presence of increasing amounts of either berberrubine (lanes 3−8) or ICRF-193 (lanes 9−14).

presence of berberrubine, additional quantities of k-DNA up to 0.52 μg did not increase the level of decatenation activity of topoisomerase II and only increased the amount of trapped catenated k-DNA in the wells (Figure 6B, lanes 5−7). Next, we investigated the effects of enzyme titration, in the absence (lanes 8−10) or presence (lanes 11−13) of 80 μM berberrubine, on a decatenation reaction by topoisomerase II. In the absence of berberrubine, 1 unit of enzyme was sufficient for complete decatenation of 0.13 μg of k-DNA (lane 8). In the presence of berberrubine, no decatenated product was

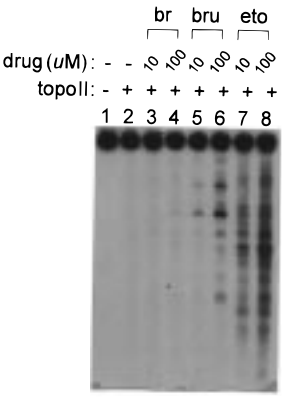

FIGURE 7: DNA cleavage patterns induced by berberrubine, berberine, and etoposide. DNA cleavage reactions were performed on a whole pBS DNA in the presence of berberrubine (lanes 5 and 6) as well as with no drug (lane 2), etoposide (lanes 7 and 8), or berberine (lanes 3 and 4). Lane 1 contained fragment alone. The cleavage products were linearized and analyzed on a 1.2% agarose gel containing 0.5 μg/mL ethidium bromide. The DNA was transferred onto a nylon membrane and hybridized with radiolabeled probe as described in the Experimental Procedures: br, berberine; bru, berberrubine; eto, etoposide.

visible with 1 or 2 units of topoisomerase II (lanes 11 and 12). However with 4 units of enzyme, decatenation of k-DNA was nearly complete (lane 13). Thus, additional amounts of the enzyme were able to overcome the catalytic inhibition by berberrubine. As revealed by Figure 5, berberrubine intercalates DNA. However, under the solution conditions employed in the assays, the binding affinity for DNA is weak, with high concentrations of drug being required for the observation of significant DNA unwinding activity. Thus, pronounced berberrubine-induced inhibition of topoisomerase II-mediated DNA decatenation requires high drug concentrations at which DNA binding increases. Under such conditions, the drug can compete with the enzyme for DNA binding. Thus, more enzyme is required to reverse the inhibitory effects of the drug on decatenation of the k-DNA.

To compare potency of catalytic inhibition of berberrubine with that of a known catalytic inhibitor, ICRF-193, we carried out decatenation reactions in the presence of increasing concentrations of these compounds. Densitometric comparison of the band intensities revealed that the inhibitory effect of berberrubine on topoisomerase II is about 20 times less than that of ICRF-193 (Figure 6C).

Mapping of Berberrubine-Induced Topoisomerase II Cleavage Sites and Comparison of Cleavage with Etoposide. Comparison of the DNA cleavage sites induced by the topoisomerase II poisons with different chemical structures has revealed different cleavage patterns (*44, 46*−*52*). Since berberrubine is a new class of topoisomerase II poison, we characterized topoisomerase II-mediated cleavage sites induced by this drug using an indirect end-labeling method (*30*). Cleavage reactions were performed on a whole plasmid pBS DNA in the presence of berberrubine as well as with no drug, etoposide, and berberine. Little or no cleavage was detected in the absence of the drug (Figure 7, lane 2); however, cleavage sites induced by berberrubine were observed in a concentration-dependent manner, indicative of trapping of cleavable complexes (lanes 5 and 6). Berberrubine-induced topoisomerase II sites exhibited a specific intensity DNA cleavage pattern that differed from those induced by etoposide; two prominent cleavage bands were observed at a final concentration of 10 μM and additional minor cleavage bands appeared at a higher drug concentration (100 μM). Etoposide induced more sites of DNA cleavage by topoisomerase II than did berberrubine (lanes 7 and 8). However, most topoisomerase II cleavage sites induced by berberrubine coincided closely with etoposide. In contrast, berberine induced a rather weak topoisomerase II cleavage site even at a final concentration of 100 μM (lane 4).

To investigate more precisely the differences in the cleavage patterns between berberrubine and etoposide, cleavage reactions were performed on a linear, end-labeled DNA substrate with topoisomerase II and various concentrations of berberrubine or etoposide. The cleavage products were analyzed on a sequencing gel. Consistent with the results shown in Figure 7, berberrubine exhibited a concentration-dependent DNA cleavage pattern (Figure 8, lanes 9−11), and all of topoisomerase II cleavage sites observed with berberrubine appear to coincide with etoposide-induced sites (lane 12). In other words, berberrubine induced no novel topoisomerase II cleavage site in the DNA substrate used because all nine berberrubine sites shown on the DNA substrate colocalized with etoposide. In the absence of drug, two cleavage sites were produced by topoisomerase II alone (lane 4). Berberine, despite its similar chemical structure, stimulated enzyme cutting about 2−3-fold at the same sites detected in the absence of drug (compare lanes 4 with 5−8).

DISCUSSION

DNA topoisomerase II is one of the most important molecular targets currently used in clinical cancer treatment (*1, 7*−*9*). Despite the availability of a wide range antitumor drugs, a recurrent problem of resistance and secondary complication hampers the efficacy in cancer chemotherapy (*16*). The need for the development of new drugs or treatment strategies to overcome such hindrance increases with time. In this study, we present data showing that berberrubine has a potential activity as a new class of antitumor drug, which selectively stimulates topoisomerase II-mediated DNA cleavage. Clearly manifested in DNA cleavage assays, berberrubine has an ability to induce formation of covalent cleavable complex, an important characteristic of topoisomerase targeting drug (Figure 2). Furthermore, cleavable complex formation is concentration-dependent (Figure 3). Proteinase K treatment indicates that berberrubine-induced cleavable complex was mediated solely by topoisomerase II. Despite much resemblance in chemical structure, berberrubine is a much more potent poison of topoisomerase II than either berberine or palmatine. This result is consistent with previous reports that berberine had no antitumor activity but berberrubine had a strong antitumor activity (*26, 27*). Berberrubine has a hydroxyl group at the 9-position, while both berberine and palmatine have a methoxy group at this position. Such a structural difference is essential for the antitumor activity of berberrubine. Protoberberine alkaloids including coralyne and other analogues are known to act as topoisomerase I poisons by stabilizing the topoisomerase I-DNA cleavable complex formation (*28, 29, 53*−*55*). Recently, Pilch and co-workers have shown that protoberberine analogues exhibit both intercalative and minor groove-directed interactions with the

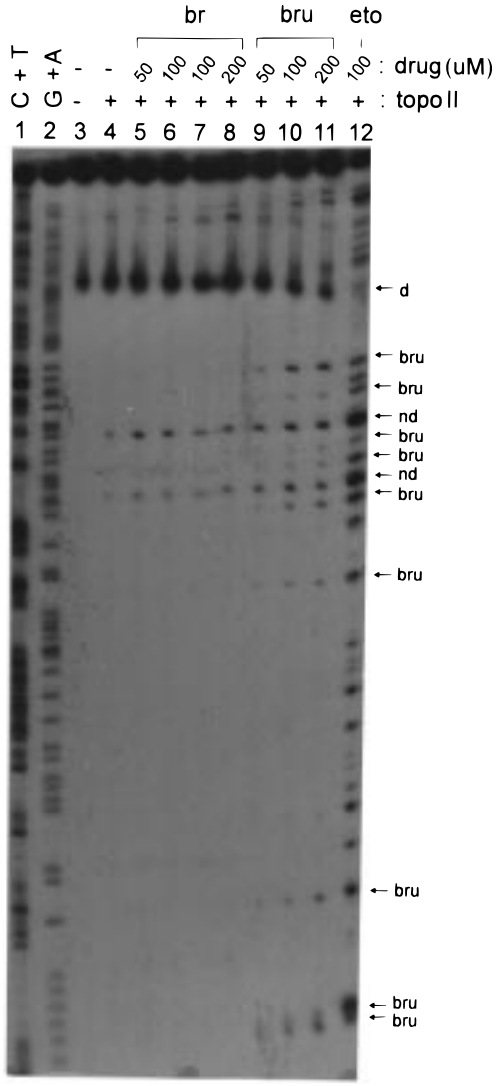

FIGURE 8: Sequencing of drug-induced topoisomerase II cleavage sites. Topoisomerase II cleavage reactions were performed on a *Hin*dIII/*Pvu*II end-labeled DNA fragment (^{32}P-labeled at the *Hin*dIII site) from pBS in the presence of berberine (lanes 5−8), berberrubine (lanes 9−11), or etoposide (lane 12) as described in the Experimental Procedures. Lanes 1 and 2 contained pyrimidine (C + T) and purine (G + A) chemical sequencing reactions of the DNA fragment, respectively. Samples were run on a 8% denaturing polyacrylamide gel. d, nd, and bru arrows denote degraded fragment, no drug site, and berberrubine-induced cleavage site, respectively: br, berberine; bru, berberrubine; eto, etoposide.

DNA duplex, which is consistent with a "mixed-mode" DNA binding model (*28*). Overall DNA binding strength of protoberberine analogues closely correlates with topoisomerase I poisoning property. The bulky methoxy group at the 9-position of protoberberines significantly diminishes their DNA binding affinity, thereby also reducing their ability to poison topoisomerase I (*28*). In a similar mode, the enhanced topoisomerase II poisoning activity of berberrubine relative to that exhibited by berberine or palmatine may be due to enhanced DNA binding strength. However, additional studies will be required to clarify the relationship between the chemical structure and the topoisomerase II poisoning property of protoberberines. Some of protoberberine derivatives are also shown to poison both topoisomerase I and II (*56*); however, neither berberrubine nor berberine had any effect on topoisomerase I activity even at the highest concentration tested (Figure 5).

Although many topoisomerase II-directed drugs exert their effects through a common cellular target, the mechanisms by which structurally dissimilar drugs enhance topoisomerase II-mediated DNA cleavage are distinct. Some drugs such as genistein, CP-115,953, and Ro 15-0216 stabilize cleavable complex primarily by stimulating the formation of cleavable complex (*36, 57*), whereas etoposide and amsacrine stabilize linear product primarily by inhibiting religation (*35, 40*). To elucidate the mechanism by which berberrubine stabilizes a linear product, a heat-induced religation assay was conducted. The results revealed that berberrubine induced the formation of a cleavable complex primarily by inhibiting the religation activity of topoisomerase II in a fashion similar to that of etoposide (Figure 4). However, the increase in nicked product in berberrubine religation possibly suggests that the mechanism of the cleavable complex formation by berberrubine differs from that of etoposide and involves a critical step of capturing nicked products during the transition from linear to covalently closed circular product.

During the drug titration experiments, we observed that high concentrations of berberrubine inhibited the relaxation activity by topoisomerase II. To elucidate the mechanisms of this catalytic inhibition, we performed decatenation reactions in which the amount of either the k-DNA substrate or the enzyme was titrated in the presence of a constant amount of drug. While addition of excess k-DNA substrate up to 0.52 μg had no effect, addition of excess topoisomerase II overcame the catalytic inhibition by berberrubine (Figure 6). DNA unwinding experiments showed that berberrubine is a much weaker intercalator than *m*-AMSA (Figure 5). Under the solution conditions employed in the decatenation assays, high concentrations of berberrubine are required for the observation of significant DNA unwinding activity as well as for the inhibition of topoisomerase II-mediated decatenation activity. These results suggest that catalytic inhibition of topoisomerase II is correlated with DNA intercalating activity of berberrubine. Under such conditions, berberrubine can compete with the enzyme for DNA binding, and thus more enzyme is required to reverse the inhibitory effects of berberrubine on enzyme-mediated decatenation activity. It seems possible that increasing the DNA concentration by a factor of only four in the presence of 80 μM beberrubine simply does not introduce enough drug-free DNA to yield enzyme-mediated decatenated products. Thus, it is likely that DNA intercalation by berberrubine is critical to the inhibition of topoisomerase II catalytic activity as well as to the topoisomerase II poisoning by berberrubine.

The determination of the sequence specificity of drug stimulation on topoisomerase II-mediated DNA cleavage may be of great value to identify the mechanism of drug action. Previous studies have demonstrated that topoisomerase II poisons with different chemical structures show different DNA cleavage patterns. For example, *m*-AMSA and epipodophyllotoxin enhance topoisomerase II-mediated DNA cleavage at multiple DNA sites (*44, 52, 58*), whereas amnonafide and RO 15-0216 stimulate DNA cleavage at few specific sites (*57, 59*). Indirect end-labeling and sequencing cleavage reactions were performed to determine the sequence specificity of berberrubine-induced DNA cleavage. Both data showed that berberrubine induces DNA cleavage in a

site-specific and concentration-dependent manner, deducible from consistency and increasing intensity of the bands at cleavage sites over varying concentrations (Figures 7 and 8). Comparison of the cleavage pattern of berberrubine with that of etoposide shows that berberrubine shares many common sites of cleavage with that of the etoposide. It is of interest to note that, despite the structural diversity, berberrubine and etoposide recognize many common cleavage sites.

In conclusion, stabilization of a cleavable complex mediated by topoisomerase II is inducible by berberrubine, signifying its potential use as antitumor agent. A possible difference in the mechanism of inhibition of religation activity from that of etoposide and other drugs increases its efficacy as a new class of drug against resistance developed by repetitive drug treatments. Nonetheless, further experiments with drug mechanisms and clinical tests are required to consolidate its novelty; however, the in vitro results suggest that it is indeed a worthwhile and promising new class of antitumor drug.

ACKNOWLEDGMENT

We thank S. H. Kim for his technical assistance and Dr. R. Ishida for providing ICRF-193.

REFERENCES

1. Chen, A. Y., and Liu, L. F. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 191−218.
2. Osheroff, N. (1989) *Pharmacol. Ther. 41*, 223−241.
3. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
4. Watt, P. M., and Hickson, I. D. (1994) *Biochem. J. 303*, 681−695.
5. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) *Nature 379*, 225−232.
6. Roca, J., and Wang, J. C. (1994) *Cell 77*, 609−616.
7. Corbett, A. H., and Osheroff, N. (1993) *Chem. Res. Toxicol. 6*, 585−597.
8. Froelich-Ammon, S. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21429−21432.
9. Pommier, Y., Leteurtre, F., Fesen, M. R., Fujimori, A., Bertrand, R., Solary, E., Kohlhagen, G., and Kohn, K. W. (1994) *Cancer Invest. 12*, 530−542.
10. Beck, W. T., Kim, R., and Chen, M. (1994) *Cancer Chemother. Pharmacol. 34* (Suppl.), S14−18.
11. Ishida, R., Miki, T., Narita, T., Yui, R., Sato, M., Utsumi, K. R., Tanabe, K., and Andoh, T. (1991) *Cancer Res. 51*, 4909−4916.
12. Roca, J., Ishida, R., Berger, J. M., Andoh, T., and Wang, J. C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1781−1785.
13. Tanabe, K., Ikwgami, Y., Ishida, R., and Andoh, T. (1991) *Cancer Res. 51*, 4903−4908.
14. Drake, F. H., Hofmann, G. A., Mong, S.-M., Bartus, J. O., Hertzberg, R. P., Johnson, R. K., Mattern, M. R., and Mirebelli, C. K. (1989) *Cancer Res. 49*, 2578−2583.
15. Permana, P. A., Snapka, R. M., Shen, L. L., Chu, D. T. W., Clement, J. J., and Plattner, J. J. (1994) *Biochemistry 33*, 11333−11339.
16. Sobulo, O. M., Borrow, J., Tomek, R., Reshmi, S., Harden, A., Schlegelberger, B., Housman, D., Doggett, N. A., Rowley, J. D., and Zeleznik-Le, N. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8732−8737.
17. Gros, P., Ben Neriah, Y., Croop, J. M., and Housman, D. E. (1986) *Nature 323*, 728−731.
18. Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. C., and Deeley, R. G. (1994) *Cancer Res. 54*, 357−361.
19. Zaman, G. J. R., Flens, M. J., Van Leusden, M. R., De Haas, M., Muller, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J., and Borst, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8822−8826.
20. de Jong, S., Zijlstra, J. G., de Vies, E. G. E., and Mulder, N. H. (1990) *Cancer Res 50*, 304−309.
21. Giaccone, G., Gazdar, A. F., Beck, H., Zunino, F., and Capranico, G. (1992) *Cancer Res. 52*, 1666−1674.
22. Mirski, S. E. L., Evans, C. D., Almquist, K. C., Slovak, M. L., and Cole, S. P. C. (1993) *Cancer Res. 53*, 4866−4873.
23. Takano, H., Kohno, K., Ono, M., Uchida, Y., and Kuwano, M. (1991) *Cancer Res. 51*, 3951−3957.
24. Son, Y. S., Suh, J. M., Ahn, S. H., Kim, J. C., Yi, J. Y., Hur, K. C., Hong, W.-S., Muller, M. T., and Chung, I. K. (1998) *Cancer Chemother. Pharmacol. 41*, 353−360.
25. Spath, E., and Polgar, N. (1929) *Monatsh. Chem. 52*, 117−128.
26. Hoshi, A., Ikekawa, T., Ikeda, Y., Shirakawa, S., Iigo, M., Kuretani, K., and Fukuoka, F. (1976) *Gann 67*, 321−325.
27. Ikekawa, T., and Ikeda, Y. (1982) *J. Pharmacobio-Dyn. 5*, 469−474.
28. Pilch, D. S., Yu, C., Makhey, D., LaVoie, E. J., Srinivasan, A. R., Olsen, W. K., Sauers, R. R., Breslauer, K. J., Geacintov, N. E., and Liu, L. F. (1997) *Biochemistry 36*, 12542−12553.
29. Kobayashi, Y., Yamashita, Y., Fujii, N., Takaboshi, K., Kawakami, T., Kawamura, M., Mizukami, T., and Nakano, H. (1995) *Planta Med. 61*, 414−418.
30. Choi, I. Y., Chung, I. K., and Muller, M. T. (1995) *Biochim. Biophys. Acta 1264*, 209−214.
31. Spitzner, J. R., Chung, I. K., and Muller, M. T. (1990) *Nucleic Acids Res. 18*, 1−11.
32. Anderson, A. H., Sorensen, B. S., Christiansen, K., Svejstrup, J. Q., Lund, K., and Westergaard, O. (1991) *J. Biol. Chem. 266*, 9203−9210.
33. Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., and Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560−13566.
34. Hsiang, Y.-H., and Liu, L. F. (1989) *J. Biol. Chem. 264*, 9713−9715.
35. Robinson, M. J., and Osheroff, N. (1990) *Biochemistry 29*, 2511−2525.
36. Robinson, M. J., Martin, S. A., Gootz, T. D., McGuirk, P. R., Moynihan, M., Sutcliffe, J. A., and Osheroff, N. (1991) *J. Biol. Chem. 266*, 14585−14592.
37. Tewey, K. M., Chen, G. L., Nelson, E. M., and Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182−9187.
38. Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351−375.
39. Pommier, Y. (1993) *Cancer Chemother. Pharmacol. 32*, 103−108.
40. Robinson, M. J., and Osheroff, N. (1991) *Biochemistry 30*, 1807−1813.
41. Osheroff, N. (1989) *Biochemistry 28*, 6157−6160.
42. D'Arpa, P., and Liu, L. F. (1989) *Biochim. Biophys. Acta 989*, 163−177.
43. Pommier, Y., Civey, J. M., Kerrigan, D., Markovits, J., and Pham, R. (1987) *Nucleic Acids Res. 15*, 6713−6731.
44. Spitzner, J. R., Chung, I. K., Gootz, T. D., McGuirk, P. R., and Muller, M. T. (1995) *Mol. Pharmacol. 48*, 238−249.
45. Chung, I. K., and Muller, M. T. (1991) *J. Biol. Chem. 266*, 9508−9514.
46. Capranico, G., Zunino, F., Kohn, K. W., and Pommier, Y. (1990) *Biochemistry 29*, 562−569.
47. Capranico, G., Kohn, K. W., and Pommier, Y. (1990) *Nucleic Acids Res. 18*, 6611−6619.
48. Cornarotti, M., Tinelli, S., Willmore, E., Zunino, F., Fisher, L. M., Austin, C., and Capranica, G. (1996) *Mol. Pharmacol. 50*, 1463−1471.
49. De Isabella, P., Zunino, F., and Capranico, G. (1995) *Nucleic Acids Res. 23*, 223−229.
50. Fosse, P., Brigitte, R., Le Bret, M., Paoletti, C., and Saucier, J.-M. (1991) *Nucleic Acids Res. 19*, 2861−2868.
51. Leteurtre, F., Fujimori, A., Tanizawa, A., Chhabra, A., Mazumder, A., Kohlhagen, G., Nakano, H., and Pommier, Y. (1994) *J. Biol. Chem. 269*, 28702−28707.
52. Spitzner, J. R., and Muller, M. T. (1988) *Nucleic Acids Res. 16*, 5533−5556.

53. Gatto, B., Sanders, M. M., Yu, C., Wu, H.-Y., Makhey, D., LaVoie, E. J., and Liu, L. F. (1996) *Cancer Res. 56*, 2795−2800.
54. Wang, L.-K., Johnson, R. K., and Hecht, S. M. (1993) *Chem. Res. Toxicol. 6*, 813−818.
55. Wang, L.-K., Rogers, B. D., and Hecht, S. M. (1996) *Chem. Res. Toxicol. 9*, 75−83.
56. Makhey, D., Gatto, B., Yu, C., Liu, A., Liu, L. F., and LaVoie, E. J. (1995) *Med. Chem. Res. 5*, 1−12.
57. Sorensen, B. S., Jensen, P. S., Anderson, A. H., Christiansen, K., Alsner, J., Thomsen, B., and Westergaard, O. (1990) *Biochemistry 29*, 9507−9515.
58. Pommier, Y., Capranico, G., Orr, A., and Kohn, K. W. (1991) *Nucleic Acids Res. 19*, 5973−5980.
59. Hsiang, Y.-H., Jiang, J. B., and Liu, L. F. (1989) *Mol. Pharmacol. 36*, 371−376.

BI9810961