On the Mechanism of Topoisomerase I Inhibition by Camptothecin: Evidence for Binding to an Enzyme–DNA Complex†

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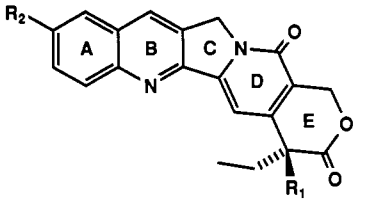
ABSTRACT: Camptothecin, a cytotoxic antitumor compound, has been shown to produce protein-linked DNA breaks mediated by mammalian topoisomerase I. We have investigated the mechanism by which camptothecin disrupts DNA processing by topoisomerase I and have examined the effect of certain structurally related compounds on the formation of a DNA–topoisomerase I covalent complex. Enzyme-mediated cleavage of supercoiled plasmid DNA in the presence of camptothecin was completely reversed upon the addition of exogenous linear DNA or upon dilution of the reaction mixture. Camptothecin and topoisomerase I produced the same amount of cleavage from supercoiled DNA or relaxed DNA. In addition, the alkaloid decreased the initial velocity of supercoiled DNA relaxation mediated by catalytic quantities of topoisomerase I. Inhibition occurred under conditions favoring processive catalysis as well as under conditions favoring distributive catalysis. By use of [^{3}H]camptothecin and an equilibrium dialysis assay, the alkaloid was shown to bind reversibly to a DNA–topoisomerase I complex, but not to isolated enzyme or isolated DNA. These results are consistent with a model in which camptothecin reversibly traps an intermediate involved in DNA unwinding by topoisomerase I and thereby perturbs a set of equilibria, resulting in increased DNA cleavage. By examining certain compounds that are structurally related to camptothecin, it was found that the 20-hydroxy group, which has been shown to be essential for antitumor activity, was also necessary for stabilization of the covalent complex between DNA and topoisomerase I. In contrast, no such correlation existed for UV-light-induced cleavage of DNA by Cu(II)–camptothecin derivatives.

Camptothecin (**1**), a cytotoxic alkaloid derived from *Camptotheca acuminata*, exhibits strong antitumor activity in several experimental tumor models including human colon, lung, and mammary tumor lines [Wall et al., 1966; reviewed in Suffness and Cordell (1985)]. Although the mechanism



1 R_1 = OH, R_2 = H
2 R_1 = H, R_2 = H (Racemic)
3 R_1 = OH, R_2 = OH

of action of this antitumor agent is not yet established, camptothecin exhibits several effects in cellular systems, including the inhibition of DNA and RNA synthesis (Abelson & Penman, 1972; Horwitz et al., 1971; Kessel et al., 1972) and the rapid and reversible breakage of cellular DNA in cultured mammalian cells (Horwitz & Horwitz, 1971; Spataro & Kessel, 1972; Mattern et al., 1987). The sites of cellular DNA cleavage have recently been localized to transcriptionally active genes (Gilmour & Elgin, 1987; Stewart & Schutz, 1987); in SV40-infected cells, DNA breakage occurred at or near the replication fork (Snapka, 1986; Avemann et al., 1988). Studies of camptothecin analogues have suggested a correlation between the ability to induce DNA breakage and antitumor activity [reviewed in Horwitz (1975)].

While camptothecin alone does not cleave purified DNA, Cu(II)–camptothecin has been reported to produce DNA breaks in purified DNA upon irradiation with 365-nm light (Kuwahara et al., 1986), and camptothecin has been reported to induce alkali-labile lesions in supercoiled closed circular DNA (Fukada, 1980, 1985). Recently, camptothecin was shown to induce protein-linked DNA breaks via mammalian DNA topoisomerase I in purified systems (Hsiang et al., 1985; Thomsen et al., 1987) and in cultured mammalian cells (Hsiang & Liu, 1988). Studies of DNA breakage in cultured mammalian cells have shown that while most of the breaks induced by camptothecin are covalently linked to topoisomerase I, a fraction of the breaks are non-protein associated (Mattern et al., 1987). It is not yet clear whether the antitumor activity of camptothecin is due to the induction of DNA breaks mediated by topoisomerase I, to DNA strand scission mediated by another (e.g., free radical) mechanism (Lown & Chen, 1980; Kuwahara et al., 1986), or to some mechanism unrelated to DNA damage. However, the recent findings that certain camptothecin-resistant cells contained an altered topoisomerase I whose ability to unwind DNA was not inhibited by camptothecin argue that topoisomerase I is the cellular target at which camptothecin exerts its antineoplastic effects (Andoh et al., 1987; Gupta et al., 1988; Kjeldsen et al., 1988).

Recent studies of the mechanism of action of several antitumor agents structurally unrelated to camptothecin have suggested that DNA topoisomerase II may be the primary locus at which they produce their antitumor effects. These agents induce reversible, protein-linked DNA fragmentation in cultured cells [reviewed in Ross (1985)]; in vitro and in vivo studies have suggested that they interrupt the normal function of topoisomerase II by stabilizing a covalent enzyme–DNA complex. Treatment of the covalent complex with a protein denaturant revealed a DNA strand break with topoisomerase II covalently linked to the 5′-phosphoryl group of DNA at the

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site of the break (Nelson et al., 1984; Tewey et al., 1984a,b; Chen et al., 1984). All but one of the antitumor agents believed to employ this mechanism interact with topoisomerase II; camptothecin is presently the only known agent that stabilizes the covalent complex between topoisomerase I and DNA.[1]

The covalent complex between eukaryotic topoisomerase I and DNA is thought to be an intermediate along the normal enzyme-mediated reaction pathway, consisting of one molecule of enzyme bound to DNA at the site of a single-stranded nick (Champoux, 1976). Topoisomerase I is covalently linked to the 3′-phosphoryl group of the DNA backbone at the nick, while the 5′-end at the break is a free hydroxy group (Champoux, 1977). During the normal conversion, it is thought that the bound enzyme first removes DNA supercoils either by strand passage through the break or by swiveling of the free DNA end, and then the phosphodiester bond is resealed concomitant with release of intact topoisomerase I [for a review, see Maxwell and Gellert (1986)]. While the covalent complex usually involves only a small percentage of the total DNA, camptothecin increased the percentage of DNA that was trapped in the cleaved form after treatment with protein denaturants (Hsiang et al., 1985). To permit better definition of the way in which camptothecin interacts with the DNA–topoisomerase I complex to form a stabilized ternary complex, we have investigated the effects of camptothecin and certain structurally related compounds on covalent complex formation and relaxation of supercoiled plasmid DNA by topoisomerase I.

Presently, we report that camptothecin bound to a complex between DNA and topoisomerase I, but did not bind to isolated enzyme or isolated DNA. This observation, which constitutes the first direct measurement of camptothecin–topoisomerase I–DNA binding, suggests that camptothecin may be more selective than many intercalative topoisomerase II inhibitors that bind to DNA. Furthermore, although camptothecin contains a reactive lactone ring, both the stabilization of DNA cleavage mediated by topoisomerase I and the enzyme–DNA binding by camptothecin were found to be reversible. In addition, kinetic experiments showed that camptothecin decreased the initial velocity of topoisomerase I catalysis in a manner consistent with reversible inhibition. Filter binding experiments designed to study the nature of the ternary complex suggested that camptothecin bound most tightly to a covalent DNA–topoisomerase I complex. The 20-hydroxy group of camptothecin, which has been shown to be essential for antitumor activity (Wall & Wani, 1977), was found to be necessary for stabilization of the covalent complex between DNA and topoisomerase I. In contrast, no such correlation existed for UV-light-induced cleavage of DNA by Cu(II)–camptothecin derivatives.

Experimental Procedures

Materials. Calf thymus topoisomerase I was purified to homogeneity by using a two-step procedure (Gupta et al., 1988). Briefly, a nuclear extract from fresh calf thymus glands was fractionated with 2 M ammonium sulfate, and the supernatant was purified by chromatography on phenyl-Sepharose (Pharmacia). The active fractions were pooled, dialyzed, and purified further by chromatography on hydroxylapatite (Bio-Rad HA). The yield of purified topoisomerase I was 2.5 mg. The molecular weight of the purified enzyme was 82 000 as determined by denaturing polyacrylamide gel electrophoresis, and its purity was >95% as judged by silver staining of the polyacrylamide gel (Laemmli, 1970). The specific activity of the purified topoisomerase was 1.3×10^8 units/mg [unit definition as described in Liu and Miller (1981)].

Plasmid pDPT2789 DNA (approximately 6400 bp;[2] >94% form I), a high copy number derivative of pDPT270, was purified by a triton cleared lysate method and CsCl/ethidium isopycnic centrifugation (Taylor & Cohen, 1979). Form I^r plasmid DNA was prepared by relaxation of form I DNA with topoisomerase I, followed by phenol extraction and ethanol precipitation. Calf thymus DNA (Sigma Chemical Co.) was sonicated, deproteinized with phenol, and dialyzed extensively against 1 mM Tris-HCl, pH 7.5, containing 5 mM NaCl. Plasmid pBR322 DNA and DNA polymerase I (Klenow fragment) were purchased from Bethesda Research Labs. All restriction endonucleases were purchased from New England Biolabs. [α-^{32}P]dTTP and [γ-^{32}P]ATP were purchased from New England Nuclear. Proteinase K was from Sigma. Camptothecin lactone (**1**, NSC 94600) and 10-hydroxycamptothecin (**3**, NSC 107124) were obtained from the National Cancer Institute. 20-Deoxycamptothecin (**2**, racemic at C-20) was prepared via total synthesis according to published procedures (Wani et al., 1980). These compounds were dissolved in DMSO at 10 mM concentrations and stored at −20 °C. [^{3}H]Camptothecin (13 Ci/mmol; labeled at C-5 and C-7) was prepared according to published procedures (Ronman et al., 1981) and dissolved in ethanol. Nitrocellulose filters were purchased from Schleicher and Schuell. Solutions of DTT (Sigma) and $CuSO_4$ (Baker) were prepared immediately before use.

Preparation of DNA Restriction Fragments. To prepare DNA for nitrocellulose filter binding studies, supercoiled pBR322 plasmid DNA was digested successively with *Bam*HI and *Eco*RI endonucleases, and the resulting DNA fragments were 3′-end-labeled with [α-^{32}P]dTTP and the Klenow fragment of DNA polymerase I (Maniatis et al., 1982). The 381-bp doubly end labeled DNA fragment was isolated from a 5% polyacrylamide gel as described (Maxam & Gilbert, 1980). To prepare 5′-end-labeled DNA for sequencing gel studies, pBR322 plasmid DNA was digested with *Bam*HI endonuclease, dephosphorylated with bacterial alkaline phosphatase, labeled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase, digested with *Sal*I endonuclease, and purified by polyacrylamide gel electrophoresis to yield a singly end labeled DNA fragment, 280 base pairs in length (Maniatis et al., 1982).

Cleavage of DNA Mediated by Topoisomerase I. The procedure was performed as described (Hsiang et al., 1985), with some modification. Each reaction mixture (20-μL total volume) contained 100 ng of pDPT2789 DNA (form I or form I^r, 1.2 nM plasmid concentration), cleavage buffer (40 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM EDTA), 0.05% DMSO, 1% methanol, 50 μg/mL BSA, 17 ng (10 nM) of topoisomerase I (when present), and camptothecin derivative (as noted). The order of addition was DNA, buffer, DTT, camptothecin/DMSO/

[1] A recent paper has suggested that actinomycin stabilizes topoisomerase I–DNA complexes, but with much lower efficiency than camptothecin (Trask & Muller, 1988).

[2] Abbreviations: bp, base pair; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; CPT, camptothecin; form I DNA, supercoiled covalently closed circular DNA; form I^r DNA, relaxed covalently closed circular DNA; form II DNA, nicked circular DNA; form III DNA, linear duplex DNA; topo I, DNA topoisomerase I.

methanol, and topoisomerase I/BSA. The reaction mixture was incubated at 37 °C for 30 min and terminated by the addition of 5 μL of a prewarmed solution containing 2.5% (w/v) SDS and 0.75 mg/mL proteinase K. The mixture was incubated at 37 °C for 60 min, treated with 3 μL of loading solution (20% Ficoll, 0.025% bromophenol blue), and analyzed by electrophoresis on 1% agarose gels in 90 mM Tris–borate and 2 mM EDTA, pH 8.0 (Maniatis et al., 1982) at 40 V for 16 h. Both the gel and running buffer contained 0.5 μg/mL ethidium bromide. After electrophoresis, the gel was subjected to photolysis by short-wave UV light (254 nm, 30 min) to nick all of the DNA samples. This was done to normalize the intensity of observed DNA bands, since covalently closed circular and open circular DNAs are stained differently with ethidium bromide. After photonicking, the gel was destained and photographed under transillumination with 300-nm UV light. The negative of the gel photograph was scanned with an LKB 2202 densitometer, and the percentage of form II DNA relative to the total DNA for each lane was quantitated with an HP-3390 integrator. The percentage of form II for each lane was corrected for the form II contaminant in the starting material (3–6%).

Cleavage of supercoiled DNA in the presence of 5 μM [^{3}H]camptothecin was carried out as above, except that 500 ng of plasmid DNA was used. Following electrophoresis, the band corresponding to form II DNA was excised and soaked in 10 mL of Econofluor/Protosol, 95:5 (New England Nuclear), and the radioactivity was determined. By use of this procedure, 97% of the radioactivity was recovered from the gel slice (as measured by using ^{3}H-labeled SV40 DNA).

Cleavage of 5′-end-labeled DNA for sequencing gel studies was carried out in reaction mixtures containing 1000–2000 cpm of ^{32}P-end-labeled DNA, 10 mM Tris-HCl (pH 9), 0.5 mM EDTA, 10 μg/mL BSA, 0.05% DMSO, 1% methanol, 17 ng of topoisomerase I, and camptothecin (as noted). After 30 min at 37 °C, the reactions were terminated with either SDS alone or SDS/proteinase K (as above) and the DNA was isolated by ethanol precipitation. The samples were analyzed on 8% polyacrylamide/50% urea gels as described (Hertzberg et al., 1988).

Reversal of Topoisomerase I Cleavage. For reversal by excess linear DNA, topoisomerase I cleavage reaction mixtures (400 μL) containing 5 μM camptothecin were prepared as above except for the enzyme concentration (50 nM). At the indicated times, aliquots were removed and either terminated directly or added to sonicated calf thymus DNA (760 μM bp; 100-fold excess over plasmid DNA) for the indicated time before termination. For reversal by dilution, a concentrated reaction mixture (6 nM plasmid, 50 nM topoisomerase I, and 5 μM camptothecin, in 40 μL of cleavage buffer) was incubated at 37 °C for 5 min. The mixture was then diluted 10-fold in cleavage buffer containing 50 μg/mL BSA, and incubation was continued. A parallel reaction mixture (0.6 nM plasmid, 5 nM topoisomerase I, and 0.5 μM camptothecin, in 400 μL of cleavage buffer) was incubated at 37 °C. The quantity of form II DNA in each reaction mixture was measured at the indicated times by terminating an aliquot (containing 100 ng of plasmid DNA) with SDS/proteinase K; electrophoresis and densitometry were carried out as described above.

Relaxation of Supercoiled DNA Mediated by Topoisomerase I. Each reaction mixture (160-μL total volume) contained 2.4 μg of supercoiled pDPT2789 DNA (3.6 nM plasmid concentration), 50 mM Tris-HCl, pH 7.5, 40 or 200 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 50 μg/mL BSA, 0.5 mM DTT, 850 pg (65 pM) of topoisomerase I, and a camptothecin derivative (as noted). The reaction mixture was incubated at 37 °C; 20-μL aliquots were removed at the indicated times and added to 5 μL of SDS/Ficoll stop mix (final concentrations 0.5% SDS, 2% Ficoll, and 0.025% bromophenol blue). Twenty-microliter aliquots (240 ng of DNA/lane) from each stopped time point were loaded onto a 0.8% agarose gel and analyzed by electrophoresis at 40 V for 16 h. The gel was stained with ethidium bromide and photographed under 300-nm UV light. The negative of the gel photograph was scanned with an LKB 2202 densitometer, and the amount of supercoiled DNA in each lane was quantitated by integration with an HP-3390 integrator. The percent DNA relaxation was calculated as $(1 - A_t/A_0) \times 100\%$, where A_t is the area under the supercoiled DNA band at time t and A_0 is the area under the supercoiled DNA band at time zero.

Cleavage of Supercoiled DNA with Camptothecin Analogues and UV Light. Each reaction mixture (30-μL total volume) contained 150 ng of pDPT2789 supercoiled DNA, 50 mM potassium phosphate, pH 7.0, 10 μM camptothecin derivative (when present), and 10 μM $CuSO_4$ (when present). All reaction mixtures, including controls, contained 0.05% DMSO and 1% methanol. The samples were exposed to 366-nm light (hand-held lamp, 0.16 A) at a distance of 1 cm for 2 h. One-tenth volume of a 20% Ficoll/0.025% bromophenol blue solution was added to each sample, followed by electrophoresis on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. DNA cleavage was quantitated by densitometry as described above.

Binding Studies with [^{3}H]Camptothecin. An eight-compartment, double-sided equilibrium dialysis cell and Spectrapor dialysis membrane (10 000–12 000 molecular weight cutoff; Spectrum Industries) were used for these studies. One side of each compartment contained 0.1 or 0.4 mL of [^{3}H]camptothecin in cleavage buffer. The other side contained an equal volume of buffer, 1.1 mM (bp) calf thymus DNA or 0.66 mM (bp) pDPT2789 supercoiled DNA, and topoisomerase I (as noted). The cell was rotated at 4 °C for 16 h, and aliquots were removed for determination of radioactivity in 10 mL of Beckman Redi-Solv scintillation fluid. Controls indicated that 16 h was sufficient time for the reaction to reach equilibrium. Reversibility of [^{3}H]camptothecin binding was examined by treating reaction mixtures with 0.5% SDS and dialyzing against a liter of 10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl for 24 h. The contents of the dialysis bag were then removed for determination of radioactivity.

Nitrocellulose Filter Binding of DNA–Topoisomerase I Complexes. Each reaction mixture (200-μL total volume) contained 7700 cpm of ^{32}P-end-labeled DNA (381 bp long; 30 pM DNA fragment concentration), 40 mM Tris-HCl, pH 7.5, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 5% glycerol, 5.2–104 nM topoisomerase I, and camptothecin (as noted). After incubation at 37 °C for 10 min, half of the reaction mixture was treated with SDS (0.5% final concentration), and each half was filtered through a nitrocellulose filter under vacuum in a Schleicher and Schuell minifold apparatus. The flow rate was approximately 1 mL/min. The filters were washed with three aliquots (100 μL) of reaction buffer, dissolved in 10 mL of Filtron X scintillation fluid (National Diagnostics), and used for determination of radioactivity. The percent DNA bound to the filter was determined for each reaction. The data were corrected for the nonspecific binding of free DNA (1% in the presence of SDS; 4% in the absence of SDS). Camptothecin had no effect on the background DNA binding.

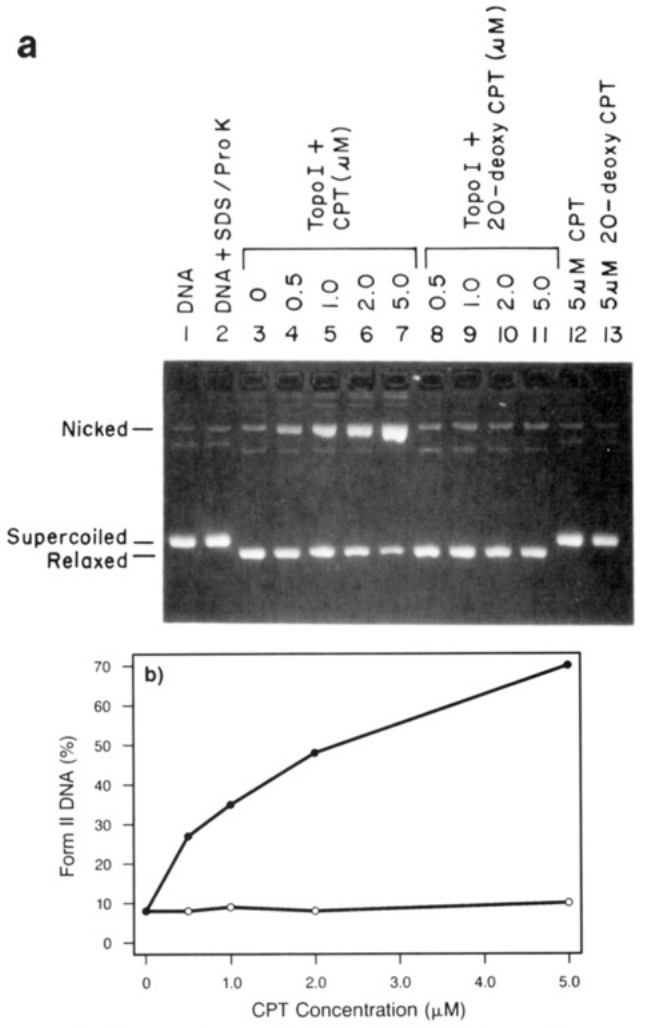


FIGURE 1: Cleavage of supercoiled pDPT2789 DNA by topoisomerase I in the presence of camptothecin congeners. In panel a, form I DNA was treated with 10 nM topoisomerase I (lanes 3–11) followed by SDS/proteinase K (lanes 2–11) and then analyzed on an agarose gel. (Lane 1) DNA alone; (lane 2) DNA alone plus SDS/proteinase K; (lane 3) topoisomerase I alone; (lane 4) 0.5 μM camptothecin (**1**); (lane 5) 1 μM camptothecin; (lane 6) 2 μM camptothecin; (lane 7) 5 μM camptothecin; (lane 8) 0.5 μM 20-deoxycamptothecin (**2**); (lane 9) 1 μM 20-deoxycamptothecin; (lane 10) 2 μM 20-deoxycamptothecin; (lane 11) 5 μM 20-deoxycamptothecin; (lane 12) 5 μM camptothecin without topoisomerase I; (lane 13) 5 μM 20-deoxycamptothecin without topoisomerase I. Panel b illustrates the formation of form II DNA as a function of camptothecin concentration on the basis of densitometric analysis of lanes 3–11 from the gel; camptothecin (**1**, ●), 20-deoxycamptothecin (**2**, ○). Assay conditions are described under Experimental Procedures.

RESULTS

DNA Cleavage and Religation Mediated by Topoisomerase I in the Presence of Camptothecin. Camptothecin has been shown to stabilize a complex between plasmid DNA and mammalian topoisomerase I in which one strand of the DNA backbone is broken and covalently linked to the enzyme (Hsiang et al., 1985; Thomsen et al., 1987). This covalent complex can be trapped by the addition of protein denaturants such as SDS. The concentration dependence of camptothecin on the formation of the covalent DNA–topoisomerase I complex was measured by using agarose gel electrophoresis and densitometry (Figure 1). The complexes were treated with proteinase K to remove the covalently bound topoisomerase I, and the resulting nicked DNA plasmids (form II) were separated from supercoiled (form I) and covalently closed circular relaxed (form I^r) DNA in the presence of ethidium bromide. When pDPT2789 plasmid DNA was incubated with 17 ng of topoisomerase I in the absence of camptothecin (Figure 1a, lane 3; Figure 1b), there was a small amount of nicked DNA produced that represented the equilibrium amount of covalent complexes in the reaction mixture (Champoux, 1976, 1977; Prell & Vosberg, 1980). Camptothecin stabilized the production of covalent complexes by topoisomerase I in a concentration-dependent manner (lanes 4–7). Also tested in comparison was 20-deoxycamptothecin, which had no effect (lanes 8–11). Neither camptothecin derivative was able to cleave DNA in the absence of topoisomerase I (lanes 12 and 13). Several other analogues of camptothecin that lacked the 20-hydroxy group also failed to stabilize the cleavage of supercoiled DNA by the enzyme (data not shown), indicating that the 20-hydroxy group of camptothecin was necessary for stabilization of the covalent complex between DNA and topoisomerase I.

DNA cleavage mediated by topoisomerase I is known to be quantitatively associated with enzyme–DNA covalent complexes (Champoux, 1976, 1977). Although camptothecin did not cleave DNA in the absence of topoisomerase I, it seemed possible that camptothecin might produce DNA breaks in the presence of topoisomerase I that were not associated with enzyme–DNA covalent complexes. One mechanism that could lead to such DNA strand breaks would involve breakage of the topoisomerase–DNA phosphotyrosine bond upon reaction with the camptothecin molecule bound to the DNA–topoisomerase I complex. Formation of an irreversible covalent linkage between camptothecin and DNA was excluded by performing the experiment shown in Figure 1 with [^{3}H]-camptothecin. No radioactivity was associated with the DNA band that migrated as form II; as little as 1 camptothecin molecule/10 DNA plasmid molecules would have been detected.

A camptothecin–DNA covalent complex, if formed during the DNA cleavage reaction, might contain a labile bond. Breakage of this bond would produce DNA fragments not covalently attached to topoisomerase I. To test for this possibility, DNA was labeled at the 5′-end with ^{32}P and subjected to topoisomerase I cleavage in the presence and absence of camptothecin. DNA cleavage was analyzed by denaturing polyacrylamide gel electrophoresis. Upon treatment of the reaction mixtures with proteinase K, sequence-specific DNA cleavage was observed (Thomsen et al., 1987; supplmentary material, Figure 1). However, when the proteinase K treatment was omitted, absolutely no DNA cleavage was observed; instead, some of the labeled DNA was caught in the well and did not enter the gel. It is likely that these DNA fragments were protein-associated. That no DNA fragments smaller than starting material were observed in the absence of proteinase K treatment implies that no enzyme-free DNA breaks were produced upon camptothecin–topoisomerase I treatment.

The effect of cytidine methylation on the sequence selectivity of camptothecin–topoisomerase I cleavage of DNA was also examined. DNA strand scission mediated by bleomycin has been shown to diminish substantially at sites in proximity of cytidines that had been methylated at the 5-position in the major groove (Hertzberg et al., 1988). In contrast, cytidine methylation had very little effect on the sequence selectivity of DNA cleavage by camptothecin–topoisomerase I (supplementary material, Figure 1).

The reversibility of DNA cleavage mediated by topoisomerase I in the presence of camptothecin was monitored by agarose gel electrophoresis and densitometry (Figure 2). The quantity of covalent complex reached a maximum very

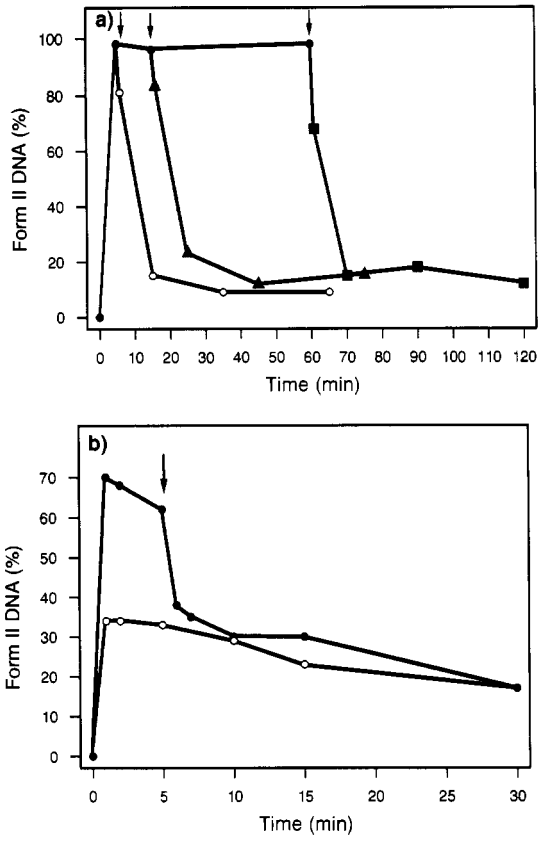


FIGURE 2: Reversal of covalent complex formation by addition of excess DNA or dilution. In panel a, form I DNA was treated with 50 nM topoisomerase I and 5 μM camptothecin for the indicated time (●). After 5 (○), 15 (▲), or 60 min (■), excess sonicated calf thymus DNA was added (arrows) and the reaction was allowed to continue. Aliquots were analyzed for covalent complex formation by measuring the appearance of form II DNA following treatment with SDS/proteinase K. Densitometric analysis was carried out as indicated in the legend to Figure 1 and under Experimental Procedures. In panel b, form I DNA was treated with 50 nM topoisomerase I and 5 μM camptothecin (●). After 5 min (arrow), the reaction was diluted 10-fold with buffer and BSA. A second reaction mixture contained 5 nM topoisomerase I and 0.5 μM camptothecin (○).

quickly and remained constant for at least 1 h.[3] The reversal of complex formation was monitored after the addition of a large excess of exogenous sonicated calf thymus DNA (Figure 2a, arrows) or by 10-fold dilution of the reaction mixture (Figure 2b, arrow). The addition of excess linear DNA to a reaction mixture containing enzyme–plasmid covalent complexes resulted in their virtual complete conversion to covalently closed relaxed plasmids. Similarly, 10-fold dilution of a reaction mixture containing preformed covalent complexes resulted in a reduction of the quantity of form II molecules to a level identical with that produced in a reaction mixture that was diluted prior to the addition of topoisomerase I. These results show that the stabilization of enzyme-mediated DNA cleavage induced by camptothecin was fully reversible. This finding is also consistent with the observation that all of the DNA cleavage induced by camptothecin is topoisomerase I associated, since enzyme-free DNA breaks would not be expected to reseal upon dilution.

Topoisomerase I can cleave different forms of DNA including linear fragments, single-stranded DNA, supercoiled plasmids, and relaxed circles (Maxwell & Gellert, 1986).

[3] We have found that DNA cleavage mediated by topoisomerase I can reverse over time when low enzyme concentrations are used, particularly in the absence of BSA. We attribute this to loss of topoisomerase I activity.

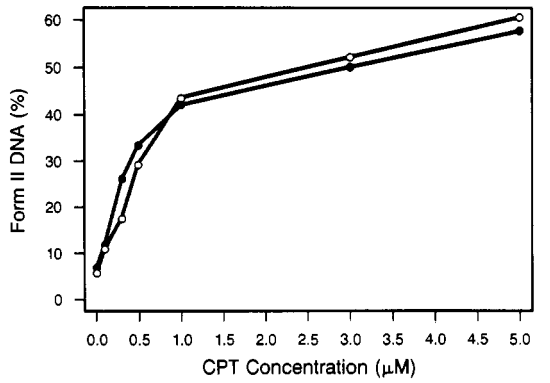


FIGURE 3: Cleavage of supercoiled and relaxed DNAs mediated by topoisomerase I. Form I (●) or form I^r (○) DNA was treated with 10 nM topoisomerase I and camptothecin. The assay was run and DNA cleavage measured as described under Experimental Procedures.

Supercoiled and relaxed plasmids were compared as starting materials for topoisomerase I cleavage in the presence of varying concentrations of camptothecin. As shown in Figure 3, there was no difference in the amount of cleavage that was produced from these two substrates. In the case of supercoiled DNA, there was no starting material left in any of the reaction mixtures; the products were form II and form I^r DNA molecules. In addition, a time course of DNA cleavage showed that maximum DNA cleavage was observed within 15 s when either supercoiled or relaxed closed circular DNA was used as the starting material (data not shown).

Effect of Camptothecin on the Rate of DNA Relaxation Mediated by Topoisomerase I. The ratio of topoisomerase I molecules to DNA plasmid molecules used for the measurement of covalent complex formation was >8:1. To investigate the effect of camptothecin on the rate of DNA relaxation mediated by topoisomerase I, the topoisomerase I:DNA plasmid ratio was lowered to 1:55. The time course for the relaxation of form I DNA (3.6 nM) to form I^r DNA by topoisomerase I (65 pM) is shown in Figure 4. DNA relaxation was measured at two different KCl concentrations. When the KCl concentration was relatively low (40 mM), the enzyme behaved in a processive manner and very few DNA molecules with intermediate linking numbers were observed. The velocity was linear for the first 5 min of the reaction, at which time more than half of the plasmid molecules had been relaxed. The presence of 5 μM camptothecin decreased the initial velocity of the relaxation reaction significantly. Consistent with reversible inhibition of DNA relaxation, almost all of the DNA had been relaxed after 1 h, even in the presence of camptothecin. Very little change in the apparent processive behavior of topoisomerase I was observed. When the KCl concentration was relatively high (200 mM), the rate of relaxation was lower and the enzyme behaved in a more distributive manner as DNA molecules with intermediate linking numbers were observed. The presence of 5 μM camptothecin decreased the initial velocity of DNA relaxation at the higher salt concentration, and the apparent distributive behavior of the enzyme was maintained. The relaxation of DNA by topoisomerase I in the presence of 20-deoxycamptothecin was unchanged relative to the control lacking this analogue, consistent with the inability of this compound to stabilize the covalent complex.

Binding of Camptothecin to a DNA–Topoisomerase I Complex. The binding of [^{3}H]camptothecin to a DNA–topoisomerase I complex was measured by equilibrium dialysis. As shown in Table I, the binding of camptothecin to isolated calf thymus DNA or supercoiled DNA was negligible. Sim-

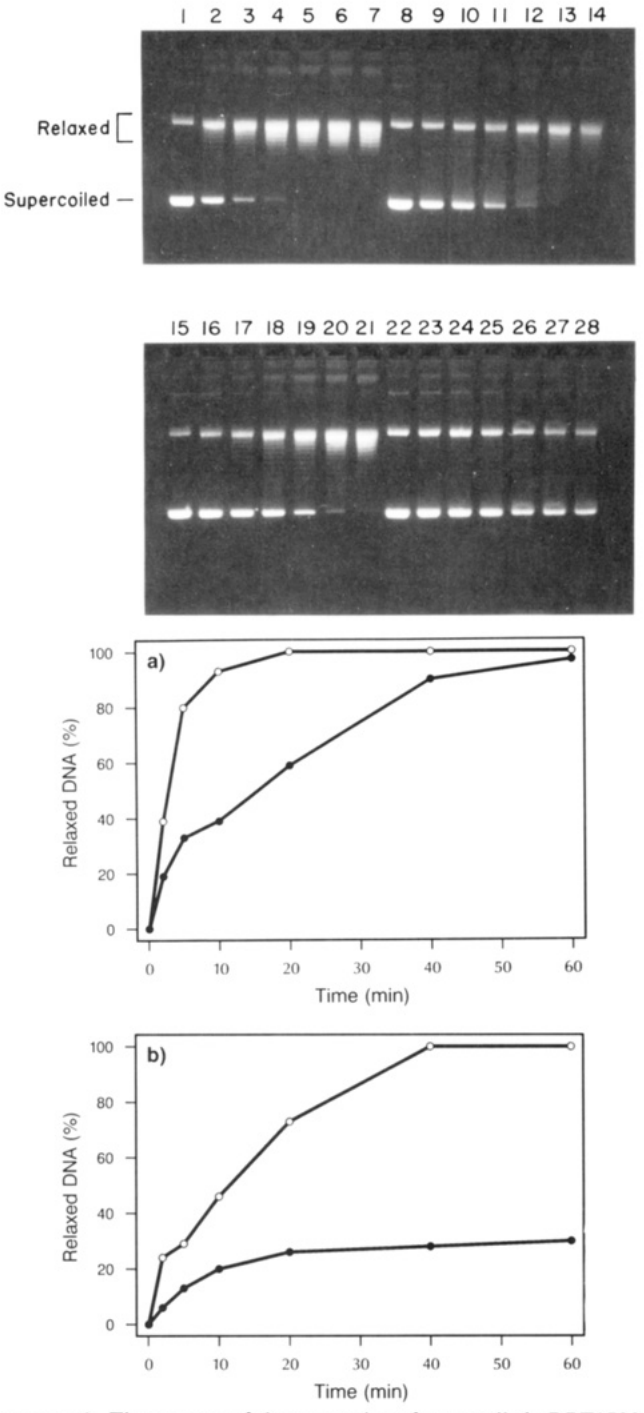


FIGURE 4: Time course of the conversion of supercoiled pDPT2789 DNA to relaxed covalently closed circular DNA. Form I DNA was treated with 65 pM topoisomerase I and then analyzed on an agarose gel. Lanes 1–14 contained no camptothecin; lanes 15–28 contained 5 μM camptothecin; lanes 1–7 and lanes 15–21 contained 40 mM KCl; lanes 8–14 and lanes 22–28 contained 200 mM KCl. Sets of lanes represent increasing reaction times before termination with 0.5% SDS: 0 min (lanes 1, 8, 15, 22), 2 min (lanes 2, 9, 16, 23), 5 min (lanes 3, 10, 17, 24), 10 min (lanes 4, 11, 18, 25), 20 min (lanes 5, 12, 19, 26), 40 min (lanes 6, 13, 20, 27), 60 min (lanes 7, 14, 21, 28). The time courses of DNA relaxation based on densitometric analysis of the gel are illustrated in panel a (40 mM KCl) and panel b (200 mM KCl), in the absence (○) or presence (●) of 5 μM camptothecin. The assay conditions are given under Experimental Procedures. Densitometer tracings for lanes 4, 12, 20, and 28 are in supplementary material, Figure 3.

ilarly, camptothecin was found not to bind to isolated topoisomerase I. Under the same experimental conditions, however, the retention of camptothecin was increased in a dialysis chamber containing both topoisomerase I and calf thymus

Table I: Binding of [^{3}H]Camptothecin to a DNA–Topoisomerase I Complex As Determined by Equilibrium Dialysis[a]

sample	bound CPT[d] (nM)	free CPT (nM)
linear DNA[b] (1.1 mM, bp)	0.1	22.3
supercoiled DNA[c] (0.66 mM, bp)	0	21.6
topoisomerase I (2.1 μM)	0	22.9
topoisomerase I (2.1 μM) with linear DNA (1.1 mM)	4.0	19.9

[a] The binding assay was carried out and analyzed as described under Experimental Procedures. The volume was 0.4 mL. [b] Sonicated calf thymus DNA. [c] pDPT2789 plasmid DNA. [d] Precision of detection was ± 0.3.

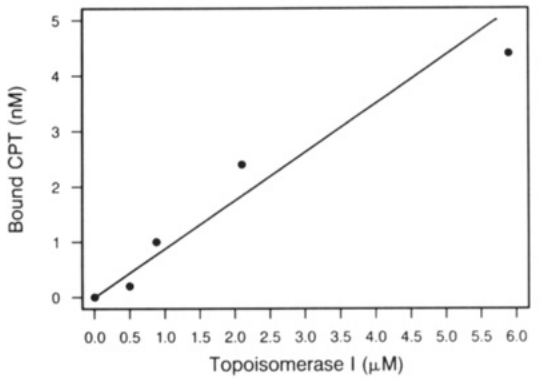


FIGURE 5: Dependence of DNA–topoisomerase I binding of [^{3}H]-camptothecin on the concentration of topoisomerase I. The binding was determined by equilibrium dialysis as described under Experimental Procedures. Each reaction (half-cell volume 0.1 mL) contained 1.1 mM (bp) sonicated calf thymus DNA and the indicated amount of topoisomerase I. The initial camptothecin concentration was 15 nM.

DNA. Further, the amount of bound camptothecin increased linearly with increasing enzyme concentration (Figure 5). The DNA concentration used in this titration (1.1 mM, bp) was presumably high enough to ensure that most or all of the added enzyme was complexed to a DNA binding site. These equilibrium dialysis experiments were performed at 4 °C in the presence of 25% glycerol to minimize the loss of enzyme activity during the course of the equilibration. The amount of camptothecin binding remained constant for up to 24 h, suggesting that the binary complex was stable under these conditions. The binding was reversible, since dialysis of the putative ternary complex for 24 h versus a large quantity of buffer resulted in the complete loss of labeled camptothecin.

The chemical nature of the ternary complex was also studied. [^{3}H]Camptothecin (25 nM) was incubated with either topoisomerase I (2.1 μM), calf thymus DNA (1.1 mM, bp), or a mixture of enzyme and DNA. The incubation mixtures were maintained at 37 °C for 30 min and then dialyzed to remove unbound camptothecin and analyzed for irreversible binding of camptothecin to either of the macromolecules. No significant amount of [^{3}H]camptothecin remained associated with DNA or topoisomerase I (data not shown). Another experiment, using supercoiled DNA, topoisomerase I, and labeled camptothecin under conditions identical with those employed for the DNA cleavage studies, demonstrated that no labeled camptothecin remained associated with plasmid DNA or topoisomerase I after SDS treatment and dialysis (data not shown).

Analysis of DNA–Topoisomerase I Complexes by Filter Binding. Topoisomerase I and DNA form both covalent and noncovalent binary complexes. The covalent complex contains a bond between a phosphate ester moiety at the 3′-end of DNA

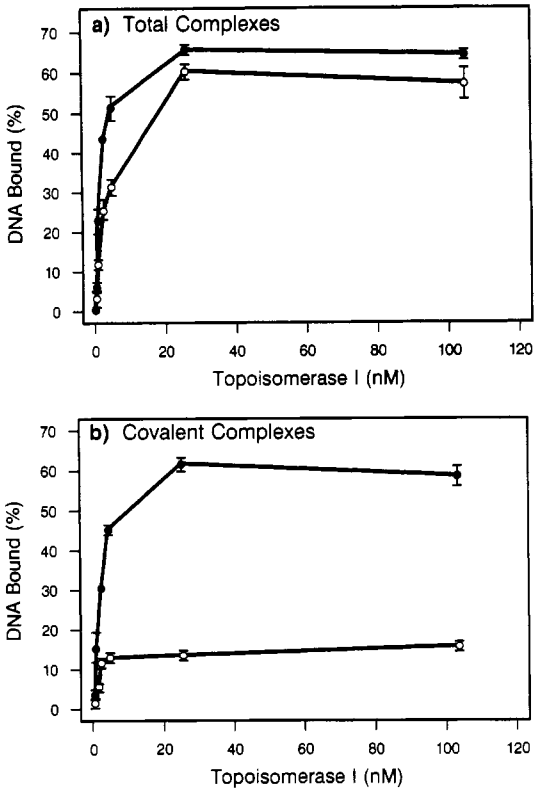


FIGURE 6: Quantitation of the binding of topoisomerase I to a 381-bp fragment of pBR322 DNA as analyzed by nitrocellulose filter binding. Reaction mixtures contained ^{32}P-labeled DNA, topoisomerase I, and 5 μM camptothecin (●) or no camptothecin (○). In panel a, reaction mixtures were filtered before the addition of SDS. In panel b, reaction mixtures were filtered after the addition of SDS. Values represent the mean ± SD (n = 2). The assay conditions are given under Experimental Procedures.

and a tyrosine residue on the enzyme and is generally assayed following the addition of a protein denaturant such as SDS to the reaction mixture (Champoux, 1976; Prell & Vosberg, 1980). The noncovalent interaction between topoisomerase I and DNA can be measured by using a nitrocellulose filter binding assay, which has been employed extensively for the study of protein–DNA interactions (Riggs et al., 1970; Melancon et al., 1982; Woodbury & von Hippel, 1983). Double-stranded DNA binds very poorly to nitrocellulose, while many proteins bind quite strongly. The basis of the assay is the retention on the nitrocellulose filter of topoisomerase I–DNA complexes. In the present study, the filter binding assay has been used to quantitate both noncovalent and covalent binding of topoisomerase I to DNA, in the presence and in the absence of camptothecin (Figure 6). The DNA used for this study was a 381-bp ^{32}P-end-labeled fragment derived from pBR322.

Filtration of the reaction mixtures was carried out both without and after SDS addition. The former gave a measure of the percentage of DNA molecules bound to topoisomerase I both covalently and noncovalently, while the latter provided a measure of the percentage of DNA molecules bound to topoisomerase I covalently. As indicated in Figure 6a, the percentage of enzyme-bound DNA fragments was found to increase as the topoisomerase I concentration was raised. Although there was a greater quantity of enzyme-bound DNA in the presence of camptothecin than in its absence, particularly at low and intermediate enzyme concentrations, significant quantities of enzyme-bound DNA were observed when camptothecin was omitted. As indicated in Figure 6b, very few DNA molecules were covalently bound to topoisomerase

Table II: DNA Cleavage Mediated by Camptothecin Analogues in the Presence of Topoisomerase I or Long-Wavelength UV Light

camptothecin derivative	% DNA cleavage with topoisomerase I[a]	% DNA cleavage with light[b]	
		−Cu(II)	+Cu(II)
none	5.4 ± 1.4	2.5	2.6
camptothecin (**1**)	66 ± 5.1	40	51
20-deoxycamptothecin (**2**)	4.7 ± 1.5	26	28
10-hydroxycamptothecin (**3**)	64 ± 6.0	4.2	1.7

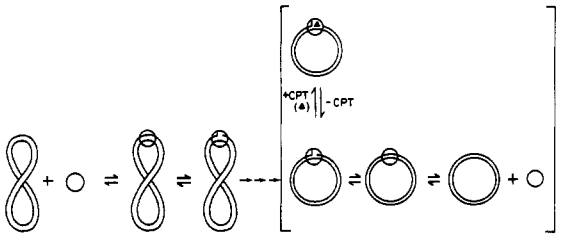
[a] Topoisomerase I mediated cleavage was determined as described under Experimental Procedures; reaction mixtures contained 5 μM camptothecin derivative. Values represent the mean ± SD (n = 3). [b] Light-dependent cleavage was determined as described under Experimental Procedures; reaction mixtures contained 10 μM camptothecin derivative and 10 μM $CuSO_4$ (when present). In the absence of light, no DNA cleavage was produced.

in the absence of camptothecin, even at relatively high topoisomerase I concentrations. In contrast, in the presence of 5 μM camptothecin the percentage of DNA fragments retained on the filter after SDS treatment was similar to the percentage retained without SDS treatment. These results imply that the majority of DNA molecules were noncovalently bound to at least one topoisomerase I molecule in the absence of camptothecin. When camptothecin was added, the majority of DNA molecules became covalently attached to at least one enzyme molecule.

Comparison of Light-Dependent and Topoisomerase I Mediated DNA Cleavage with Camptothecin Derivatives. Camptothecin has recently been shown to cleave DNA in the presence of Cu(II) and long-wavelength UV light (Kuwahara et al., 1986; Lown & Chen, 1980). Using three different camptothecin analogues, we investigated whether the same structural features that were important for DNA–topoisomerase I complex formation were also necessary for light-dependent DNA cleavage (Table II). While 10-hydroxycamptothecin (**3**) and camptothecin (**1**) were both able to promote DNA cleavage by topoisomerase I, 20-deoxycamptothecin (**2**) did not increase the amount of enzyme-mediated DNA cleavage (Table II and Figure 1). Similarly, **1** and **3** inhibited DNA relaxation by catalytic amounts of topoisomerase I, while **2** did not. In contrast, light-dependent DNA cleavage was observed with **2**, but not with **3**. The addition of Cu(II) had only a slight effect, increasing the amount of cleavage produced by camptothecin. It is likely, however, that the reaction mixtures to which Cu(II) was not added were contaminated with trace amounts of Cu(II) that may have contributed to the light-dependent DNA cleavage. In the absence of light, none of these compounds cleaved DNA in the absence or presence of Cu(II). In addition, light alone did not produce DNA cleavage under these conditions, although some light-induced strand scission was observed in the absence of DMSO (data not shown).

The effect of Cu(II) on DNA cleavage mediated by topoisomerase I in the presence of camptothecin was also examined. The addition of Cu(II) (equimolar to camptothecin) to a standard topoisomerase I DNA cleavage assay had no effect on the ability of camptothecin to stabilize covalent complex formation (supplementary material, Figure 2). Since the standard buffer contained EDTA, the majority of added Cu(II) was probably sequestered; this was probably also true for trace metal ions present in the standard reaction mixture. When EDTA was omitted from the reaction buffer, the addition of equimolar Cu(II) inhibited the ability of camptothecin to stabilize topoisomerase I cleavage of DNA. This may be the result of an interaction between Cu(II) and a putative

Scheme I: Hypothetical Model of the Interaction of Camptothecin with Topoisomerase I and Plasmid DNA

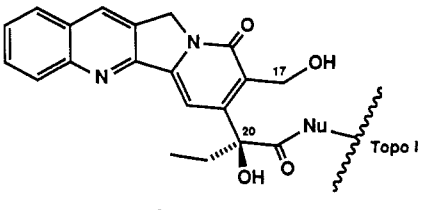


divalent metal ion site on the enzyme, since Cu(II) alone inhibited DNA relaxation by topoisomerase I. Alternatively, it could reflect binding of the DNA substrate by Cu(II). When both Mg(II) and EDTA were omitted from the reaction mixture, Cu(II) alone was even more effective at inhibiting topoisomerase I relaxation of DNA (supplementary material, Figure 2). Mg(II) has been shown to stimulate the activity of topoisomerase I but is not absolutely required (Liu & Miller, 1981).

Discussion

Several antitumor agents have been shown to increase DNA cleavage mediated by topoisomerase II (Nelson et al., 1984; Tewey et al., 1984a,b; Chen et al., 1984; Ross, 1985). Although a number of these molecules are known to bind to DNA by an intercalative mechanism, the nature of the interactions between the antitumor agents, DNA, and enzyme that lead to the observed effects are understood poorly. Similarly, the mechanism of topoisomerase I inhibition by camptothecin is not well understood. The observation that camptothecin does not bind to DNA at physiological salt concentrations (Fukada, 1980, 1985; Table I) but stabilizes DNA cleavage mediated by mammalian topoisomerase I (Hsiang et al., 1985; Thomsen et al., 1987; Figure 1) suggests that camptothecin binds to an enzyme–DNA complex. We have investigated the effects of camptothecin and structurally related compounds on DNA binding, cleavage, and relaxation mediated by topoisomerase I. In addition, direct binding assays were carried out to determine the nature of the macromolecular complex(es) with which camptothecin interacts.

The results presented here demonstrate that the interaction of camptothecin with topoisomerase I is reversible. The addition of excess linear DNA to a reaction mixture containing camptothecin-stabilized DNA–topoisomerase I covalent complexes resulted in the virtual disappearance of these covalent complexes (Figure 2a). Similarly, dilution of a reaction mixture containing preformed covalent complexes reduced their amount to a level that was present in a prediluted reaction (Figure 2b). These results suggest that camptothecin reversibly perturbed a set of equilibria between DNA and topoisomerase I; the accumulated data are accommodated by the equilibria outlined in Scheme I.



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Regarding Scheme I, it may be noted that the only products detected in the reaction mixture following SDS/proteinase K treatment were form I^r and form II DNAs. Since the ratio of products did not change with time, it seems likely that the equilibria shown in brackets were established quickly. Those experiments involving addition of excess linear DNA to effect reversal of covalent complex formation between topoisomerase I and plasmid DNA presumably involved displacement of the equilibrium to the right via binding of free topoisomerase I to the linear DNA. Likewise, dilution of the reaction mixture established a new equilibrium that reflected the lower concentrations of reactants.

This scheme is also consistent with the reversal of covalent complex formation that has been observed as a result of the addition of high concentrations of salt (Hsiang et al., 1985; data not shown). Since the apparent binding affinity of topoisomerase I for DNA is probably diminished at high ionic strength (Record et al., 1978), salt should effect dissociation of noncovalently bound enzyme from the complex and shift the equilibrium toward free enzyme and free relaxed DNA (Scheme I). That no supercoiled DNA was detected after such reversal implies that the equilibria inside the brackets had been established at the time of salt addition.

The equilibrium model shown in Scheme I predicts that the ratio of reaction products should not depend on the conformation of the DNA initially bound by topoisomerase I; the same equilibrium mixture should be established starting with form I or form I^r DNA. Consistent with this model, cleavage of form I^r DNA mediated by topoisomerase I was enhanced by camptothecin (Figure 3). In addition, the concentration dependence of cleavage was similar for mixtures starting with either form I or form I^r DNA. It is likely that in reaction mixtures starting with form I DNA all of the DNA was quickly relaxed by topoisomerase I, and the interaction of camptothecin with a complex between form I^r DNA and enzyme was measured.

When supercoiled DNA is relaxed by catalytic amounts of topoisomerase I, the reaction pathway shown in Scheme I must be repeated over several cycles. The enzyme presumably binds to the supercoiled substrate, creates a single-stranded nick, unwinds the supercoils, reseals the DNA backbone, and then dissociates from the DNA (Maxwell & Gellert, 1986). The free enzyme can then bind to another supercoiled DNA substrate for another cycle or to a relaxed DNA molecule, leading to product inhibition (Pulleyblank & Ellison, 1982). Camptothecin decreased the initial velocity of the relaxation reaction (Figure 4) but did not inhibit the enzyme irreversibly since all of the DNA was eventually relaxed in its presence. This was consistent with the reversible binding of camptothecin to an enzyme–DNA complex (Scheme I). However, these results alone did not rule out the possibility that camptothecin can also interact with free DNA or free topoisomerase I.

Several groups have reported that the binding of camptothecin to DNA was very weak or nonexistent (Horwitz et al., 1971; Fukada, 1985; Hsiang et al., 1985). Fukada (1980) reported that camptothecin induced alkali-labile sites in DNA after incubation at 1 M NaCl, but not at lower salt concentrations. In addition, two groups have reported that camptothecin cleaved DNA in a light-dependent reaction (Lown & Chen, 1980; Kuwahara et al., 1986). Clearly, the latter observations do not necessarily imply that camptothecin binds to DNA, especially under physiologically relevant conditions (Peak et al., 1984). Using [^{3}H]camptothecin in an equilibrium dialysis assay, we found that retention of [^{3}H]camptothecin was proportional to the concentration of topoisomerase I when DNA was present; no binding was observed for topoisomerase I alone. It seems logical to conclude that camptothecin interacts with both enzyme and nucleic acid within a binding site on the binary complex. However, we cannot exclude the

possibility that a camptothecin binding site is produced entirely within the DNA helix or topoisomerase I molecule as the covalent complex between the two macromolecules is formed.

The observation that camptothecin binds to an enzyme–DNA complex raises the question of which enzyme–DNA complex is bound. At least two complexes are possible: a noncovalent complex and a covalent complex (Scheme I). The results obtained by using the nitrocellulose filter binding assay (Figure 6) demonstrated that in the absence of camptothecin a large proportion of the DNA molecules had at least one enzyme molecule bound noncovalently. Camptothecin markedly increased the proportion of DNA molecules containing a covalently bound enzyme. This observation suggests that camptothecin is bound most tightly to a covalent DNA–topoisomerase I complex. The formation of such a ternary complex would perturb a set of equilibria and result in a greater proportion of DNA molecules covalently linked to enzyme (Scheme I, Figure 6b). The possibility that camptothecin also binds to the noncovalent complex cannot be excluded; further, there could be more than one type of noncovalent complex (e.g., at different DNA sequences).

The unusual electrophilicity of the lactone carbonyl moiety of camptothecin (Hutchinson, 1981) is consistent with the possibility that a nucleophilic group on topoisomerase I is acylated by this compound during covalent complex formation (**4**). This linkage would possibly be reversible due to the intramolecular attack of the C-17 hydroxy group on the carbonyl carbon, regenerating camptothecin and intact enzyme. An example of this type of reaction, the formation of camptothecin-21-isopropylamide and its conversion back to camptothecin, was described by Adamovics and Hutchinson (1979). Accordingly, we prepared [^{3}H]camptothecin to carry out binding studies with DNA, topoisomerase I, and the enzyme–DNA complex and to test the possibility of covalent binding. We found that the binding of camptothecin was completely reversible, since extensive dialysis of the camptothecin–enzyme–DNA ternary complex resulted in complete loss of the radiolabeled compound. If camptothecin was covalently bound to topoisomerase I or DNA, the reaction appears to have been completely reversible under the experimental conditions employed for this study. These results imply that the mechanism of enzyme inhibition for this antitumor agent could be more selective than for some of the agents shown to bind to the topoisomerase II–DNA complex, since a number of these are also known to bind to DNA alone (Nelson et al., 1984; Tewey et al., 1984a,b; Pommier et al., 1987).

It is interesting to note that the camptothecin derivative that lacked an α-hydroxy lactone ring failed to inhibit DNA relaxation by topoisomerase I and was incapable of stabilizing a covalent complex between topoisomerase I and DNA. This is consistent with the suggestion that functional groups within the α-hydroxy lactone moiety may interact directly with some portion of the topoisomerase I–DNA complex. At present, it is unclear whether these putative interactions involve topoisomerase I, DNA, or both. Several analogues of camptothecin have been tested for antitumor activity, and it has been fairly well established that the α-hydroxy lactone ring is the most critical structural feature for activity in vitro, as well as in vivo (Wall & Wani, 1977; Suffness & Cordell, 1985; Wani et al., 1987). For example, 20-deoxycamptothecin, 20-chlorocamptothecin, and camptothecin hemilactol were completely inactive as antitumor agents (Wall & Wani, 1977). Each of these compounds was also ineffective when tested for the ability to stabilize the topoisomerase I–DNA covalent complex (Table II; data not shown).

The structural features in camptothecin that are important for DNA cleavage in the presence of long-wavelength UV light do not parallel those necessary for topoisomerase I inhibition (Table II). Camptothecin and 20-deoxycamptothecin both effected DNA cleavage in the presence of light, but only camptothecin exhibited antitumor activity (Wall & Wani, 1977) and inhibited topoisomerase I (Figure 1). While 10-hydroxycamptothecin was inefficient at DNA cleavage in the presence of light (Table II), it has been shown to have good antitumor activity and inhibits topoisomerase I. Lown and Chen (1980) also found that the structural elements of camptothecin essential for DNA strand scission in the presence of 360-nm light consisted only of intact rings A–D. It would seem that topoisomerase I–DNA interaction is a much better predictor of antitumor activity for these camptothecin analogues than is light-dependent DNA cleavage.

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Supplementary Material Available

Polyacrylamide gel analysis of the cleavage of a DNA fragment with topoisomerase I and camptothecin, both without and after proteinase K treatment; agarose gel analysis of the effect of Cu(II) on the cleavage of supercoiled DNA by topoisomerase I and camptothecin; densitometer tracings of lanes from Figure 4 (5 pages). Ordering information is given on any current masthead page.

Registry No. **1**, 7689-03-4; **2**, 34141-35-0; **3**, 67656-30-8; Topo I, 80449-01-0.

References

Abelson, H. T., & Penman, S. (1972) *Nature (London), New Biol. 237*, 144–146.

Adamovics, J. A., & Hutchinson, C. R. (1979) *J. Med. Chem. 22*, 310–314.

Andoh, T., Ishii, K., Suzuki, Y., Ikegami, Y., Kusunoki, Y., Takemoto, Y., & Okada, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5565–5569.

Avemann, K., Knippers, R., Koller, T., & Sogo, J. M. (1988) *Mol. Cell. Biol. 8*, 3026–3034.

Champoux, J. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3488–3491.

Champoux, J. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3800–3804.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 13560–13566.

Fukada, M. (1980) *J. Biochem. 87*, 1089–1096.

Fukada, M. (1985) *Biochem. Pharmacol. 34*, 1225–1230.

Gilmour, D. S., & Elgin, S. C. R. (1987) *Mol. Cell. Biol. 7*, 141–148.

Gupta, R. S., Gupta, R., Eng, B., Lock, R. B., Ross, W. E., Hertzberg, R. P., Caranfa, M. J., & Johnson, R. K. (1988) *Cancer Res. 48*, 6404–6410.

Hertzberg, R. P., Caranfa, M. J., & Hecht, S. M. (1988) *Biochemistry 27*, 3164–3174.

Horwitz, M. S., & Horwitz, S. B. (1971) *Biochem. Biophys. Res. Commun. 45*, 723–727.

Horwitz, S. B. (1975) in *Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents* (Corcoran, J. W., & Hahn, F. E., Eds.) pp 48–57, Springer, New York.
Horwitz, S. B., Chang, C., & Grollman, A. P. (1971) *Mol. Pharmacol. 7*, 632–644.
Hsiang, Y.-H., & Liu, L. F. (1988) *Cancer Res. 48*, 1722–1726.
Hsiang, Y.-H., Hertzberg, R., Hecht, S., & Liu, L. F. (1985) *J. Biol. Chem. 260*, 14873–14878.
Hutchinson, C. R. (1981) *Tetrahedron 37*, 1047–1065.
Kessel, D., Bosmann, H. B., & Lohr, K. (1972) *Biochim. Biophys. Acta 269*, 210–216.
Kjeldsen, E., Bonven, B., Andoh, T., Ishii, K., Okada, K., Bolund, L., & Westergaard, O. (1988) *J. Biol. Chem. 263*, 3912–3916.
Kuwahara, J., Suzuki, T., Funakoshi, K., & Sugiura, Y. (1986) *Biochemistry 25*, 1216–1221.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Liu, L. F., & Miller, K. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3487–3491.
Lown, J. W., & Chen, H.-H. (1980) *Biochem. Pharmacol. 29*, 905–915.
Maniatis, T., Fritsch, E., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Mattern, M. R., Mong, S.-M., Bartus, H. F., Mirabelli, C. K., Crooke, S. T., & Johnson, R. K. (1987) *Cancer Res. 47*, 1793–1798.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem. 38*, 69–107.
Melancon, P., Burgess, R. R., & Record, M. T. (1982) *Biochemistry 21*, 4318–4331.
Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361–1365.
Peak, M. J., Peak, J. G., Foote, C. S., & Krinsky, N. I. (1984) *J. Photochem. 25*, 309–315.
Pommier, Y., Covey, J., Kerrigan, D., Mattes, W., Markovits, J., & Kohn, K. W. (1987) *Biochem. Pharmacol. 36*, 3477–3486.
Prell, B., & Vosberg, H.-P. (1980) *Eur. J. Biochem. 108*, 389–398.
Pulleyblank, D. E., & Ellison, M. J. (1982) *Biochemistry 21*, 1155–1161.
Record, M. T., Anderson, L. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Biol. Chem. 48*, 67–83.
Ronman, P. E., Wani, M. C., & Wall, M. E. (1981) *J. Labelled Compd. Radiopharm. 18*, 319–329.
Ross, W. E. (1985) *Biochem. Pharmacol. 34*, 4191–4195.
Snapka, R. M. (1986) *Mol. Cell. Biol. 6*, 4221–4227.
Spataro, A., & Kessel, D. (1972) *Biochem. Biophys. Res. Commun. 48*, 643–648.
Stewart, A. F., & Schutz, G. (1987) *Cell 50*, 1109–1117.
Suffness, M., & Cordell, G. A. (1985) in *The Alkaloids: Chemistry and Pharmacology* (Brossi, A., Ed.) Vol. 25, pp 73–89, Academic Press, Orlando, FL.
Taylor, D. P., & Cohen, S. N. (1979) *J. Bacteriol. 137*, 92–104.
Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984a) *J. Biol. Chem. 259*, 9182–9187.
Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984b) *Science 226*, 466–468.
Thomsen, B., Mollerup, S., Bonven, B. J., Frank, R., Blocker, H., Nielson, O. F., & Westergaard, O. (1987) *EMBO J. 6*, 1817–1823.
Trask, D. K., & Muller, M. T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1417–1421.
Wall, M. E., & Wani, M. C. (1977) *Annu. Rev. Pharmacol. Toxicol. 17*, 117–132.
Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. T., & Sim, G. A. (1966) *J. Am. Chem. Soc. 88*, 3888–3890.
Wani, M. C., Ronman, P. E., Lindley, J. T., & Wall, M. E. (1980) *J. Med. Chem. 23*, 554–560.
Wani, M. C., Nicholas, A. W., & Wall, M. E. (1987) *J. Med. Chem. 30*, 2317–2319.
Woodbury, C. P., & von Hippel, P. H. (1983) *Biochemistry 22*, 4737–4745.