Differential Effects of Camptothecin Derivatives on Topoisomerase I-Mediated DNA Structure Modification†

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Received February 25, 1998; Revised Manuscript Received April 29, 1998

ABSTRACT: The effects of eleven camptothecin derivatives on calf thymus topoisomerase I-mediated cleavage of synthetic DNA duplex have revealed that the A ring of camptothecin is very important for its biochemical activity. Depending on the type, number, and location of substituents, highly active or inactive analogues were obtained. The persistence of CPT-induced topoisomerase I−DNA covalent binary complexes was investigated by using as substrates DNA containing several good topoisomerase I cleavage sites, or else a synthetic DNA duplex of defined structure with a single high-efficiency cleavage site. The ligation kinetics at a given topoisomerase I cleavage site were sometimes quite different in the presence of CPT derivatives whose structures were closely related. Even in the presence of a single CPT analogue, topoisomerase I−DNA covalent binary complexes underwent ligation with different kinetics, presumably reflecting a dependence on DNA sequences flanking the individual topoisomerase I cleavage sites. Individual camptothecin derivatives also exhibited a spectrum of inhibitory potentials in blocking the topoisomerase I-mediated rearrangement of branched, nicked, and gapped DNA duplex substrates; in some cases the potencies of inhibition observed in these assays for individual camptothecin analogues were quite different than those determined for stabilization of the unmodified DNA−topoisomerase I binary complex.

Topoisomerases are essential nuclear enzymes that modify the topological state of DNA through the introduction of transient breaks in the phosphodiester backbone of DNA (*1*, *2*). They can relax torsional stress in supercoiled DNAs and resolve topologically complex DNA molecules via unknotting and decatenation (*3*−*6*). The topoisomerases have essential roles in the key cellular processes of replication and transcription (*3*−*6*).

Eukaryotic type I topoisomerase nicks one strand of double-stranded DNA via nucleophilic attack of an active site tyrosine OH group and becomes covalently bound to the 3′-phosphate of DNA at the site of the nick as illustrated in the crystal structure of a human topoisomerase I construct in a covalent complex with a DNA oligonucleotide (*7*). Usually, the break is resealed in a process that may be regarded superficially as reversal of the forward reaction, restoring the continuity of DNA backbone (*8*) (Scheme 1). However, the uncoupling of the cleavage and ligation reactions has been demonstrated in a cell free system (*9*); at least for vaccinia topoisomerase I, it has been shown convincingly that the ligation reaction has a different rate limiting step than the forward reaction (*10*, *11*). Providing that there are regions of complementarity, topoisomerase I has been shown to undergo ligation involving nonhomologous acceptors, resulting in products containing DNA mismatches, deletions or insertions (*12*, *13*). The involvement of topoisomerase I in illegitimate recombination is of great interest due to the fairly limited understanding of the molecular mechanism(s) of nonhomologous recombination in mammalian cells.

Since its isolation by Wall and co-workers in 1966 (*14*), 20-*S*-camptothecin (CPT)[1] has been the lead compound for the development of several anticancer agents (*15*). Two of these are currently marketed. The cytotoxic effects of CPT are believed to be a consequence of the inhibition of eukaryotic DNA topoisomerase I function (*16*). CPT inhibits topoisomerase I by stabilizing covalent topoisomerase I−DNA binary complexes (*16*−*24*), apparently via the formation of a ternary complex containing noncovalently bound CPT (Scheme 1). Upon removal of CPT, the stabilized covalent binary complexes are generally rapidly reversible. Reversal can be achieved by lowering drug concentration through dilution, increased salt (NaCl) concentration, or the addition of competitor DNA (*16*, *23*, *25*). The actual mechanism of cell killing by CPT has not been established firmly, but may be due to encounter of the stabilized topoisomerase I−DNA binary complex by the replication complex in actively

† This work was supported at the University of Virginia by Research Grant CA53913 from the National Cancer Institute.

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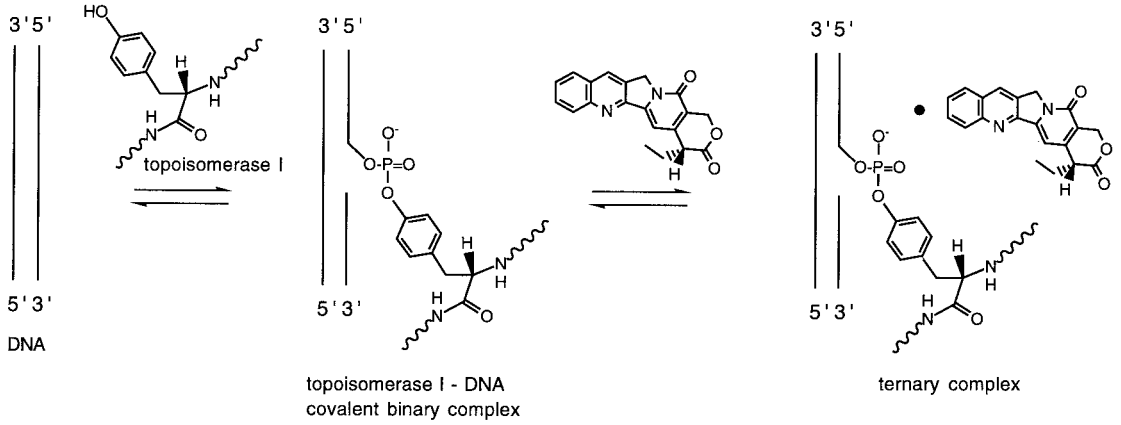
[1] Abbreviations: CPT, camptothecin; 7-CM-EDO-CPT, 7-chloromethyl-10,11-ethylenedioxy-CPT; 7-CM-MDO-CPT, 7-chloromethyl-10,11-methylenedioxy-CPT; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; BSA, bovine serum albumin.

S0006-2960(98)00451-6 CCC: $15.00
Published on Web 06/12/1998

Scheme 1: Equilibria between DNA and the DNA−Topoisomerase I Covalent Binary Complex, and between the Enzyme−DNA Binary Complex and a Ternary Complex Formed with Camptothecin



replicating cells. This could lead to dislodging of the 5′-terminus of the cleaved strand, such that continued replication could lead to a potentially lethal double-strand break (*26*).

In the past several years, many CPT derivatives have been synthesized with the goals of optimizing key physicochemical properties such as water solubility, and enhancing the therapeutic efficacy of the CPTs, as well as an understanding of the inhibition of topoisomerase I function by this structural class of compounds (*27*−*31*). However, the molecular interactions between the camptothecins and the topoisomerase I−DNA binary complexes have not been well established, due in part to the lack of structural information about the nature of the formed complexes. The recent findings that topoisomerase I can mediate insertions and deletions of nucleotides in model systems reminiscent of illegitimate recombination (*13*) suggests that the camptothecins may also exert their antitumor effects via processes other than those involving stabilization of the intermediate involved in DNA relaxation.

Presently, we document the differential effects of CPT derivatives in promoting the cleavage of a DNA duplex and inhibiting the ligation of homologous acceptor oligonucleotides to form an intact duplex versus their ability to inhibit the topoisomerase I-mediated deletion of nucleotides from branched, nicked, and gapped substrates. Also described are assays that employ DNA's with one or several high efficiency topoisomerase I cleavage sites to characterize the rates of dissociation of individual CPT analogues from the ternary complexes that they form with the covalent enzyme−DNA binary complexes.

EXPERIMENTAL PROCEDURES

General Methods and Materials. Camptothecin analogues were prepared in analogy with published methods (*29*, *31*). The topoisomerase I inhibitors were dissolved in 100% DMSO to make 5 mM stock solutions. Further dilutions were made in 10% DMSO before use so that the final concentration of DMSO in the incubation mixtures was 1%. T4 polynucleotide kinase and proteinase K were purchased from United States Biochemicals; trypsin was from Sigma Chemicals. The Klenow fragment of DNA polymerase I (lacking 5′→3′ exonuclease activity) and restriction endonucleases *Hin*dIII and *Pvu*II were from New England Biolabs. [γ-^{32}P]ATP (7000 Ci/mmol) and [α-^{32}P]dATP (>3000 Ci/mmol) were obtained from ICN Pharmaceuticals. DNA oligonucleotides were purchased from Cruachem Inc. pSP64 plasmid DNA was prepared as described (*32*).

Denaturing polyacrylamide gel electrophoresis was carried out (8M urea, 50 W) for 2−3 h. Gels were visualized by autoradiography at −80 °C using Kodak XAR-2 film and quantified utilizing a Molecular Dynamics 400 E Phosphorimager with ImageQuant version 3.2 software. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

Enzyme Purification. Calf thymus DNA topoisomerase I was purified by slight modification of a published procedure (*32*). The isolated protein exhibited two major bands (M_r ~ 96 000 and 82 000) when analyzed by SDS−polyacrylamide gel electrophoresis and visualization of the protein by silver staining. The heterogeneity of the isolated topoisomerase I can be attributed to loss of the N-terminal domain as a consequence of proteolysis during the isolation procedures (*33*). This domain contains the nuclear localization signal, but does not otherwise contribute to topoisomerase I function (*33*). The purified protein had a specific activity of 1.4×10^7 units/mg of protein. One unit is the amount of enzyme that relaxes 250 ng of pB*R*322 supercoiled DNA in 30 min at 37 °C.

Oligonucleotide Substrates. All oligonucleotides were purified on preparative 20% denaturing polyacrylamide gels. The DNA was visualized by UV shadowing (*34*) and the band of interest was excised from the gel. The DNA was eluted (2M $LiClO_4$) for 12 h at 37 °C and recovered by precipitation with acetone as described (*35*).

Preparation of 5′-^{32}P End Labeled Synthetic Oligonucleotides. The synthetic oligonucleotide (1.5−10 μg) was added to 25 μL (total volume) of 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.16 mCi of [γ-^{32}P]ATP. Ten units of T4 polynucleotide kinase was added to the mixture to initiate the reaction. The reaction mixture was incubated for 1−2 h at 37 °C and terminated by the addition of 12.5 μL of loading buffer (10 M urea, 1.5 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and applied directly to a 20% denaturing polyacrylamide gel. The band of interest was visualized by autoradiography and excised from the gel. The DNA was recovered by crush and soak and precipitation as described (*35*).

Hybridization of Substrates. Oligonucleotides were hybridized in a solution (50 μL total volume) containing 10 mM Tris-HCl, pH 7.6, 40 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. The solution was heated to 80 °C for 5 min and cooled slowly to room temperature under ambient conditions (~3 h). Due to the low DNA strand concentrations, hybridization mixtures contained 65 fmol of the labeled strand and a 100-fold excess of the unlabeled (noncleaved) strand to ensure complete hybridization of the labeled DNA.

Oligonucleotide DNA Cleavage and Ligation by Topoisomerase I. The 5′-^{32}P end labeled DNA duplex (6.5 fmol) was treated with 25 ng of calf thymus topoisomerase I in a reaction mixture (20 μL total volume) containing 10 mM Tris-HCl, pH 7.6, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM DTT. For the branched, nicked, and gapped substrates, the acceptor oligomer was present in 1000-fold excess relative to the radiolabeled oligonucleotide. The reaction mixtures were incubated at 37 °C for the time indicated, and then quenched by treatment either with trypsin (1 mg/mL containing 1% SDS, 37 °C, 30 min) or proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) prior to analysis by 20% denaturing PAGE.

Topoisomerase I-Mediated Cleavage of HindIII/PvuII Restriction Fragment of pSP64 DNA. A DNA duplex 226 nt in length was obtained by the treatment of 45 μg of pSP64 plasmid DNA with 140 units each of restriction endonucleases *Hin*dIII and *Pvu*II. The DNA was then 3′-^{32}P end labeled at the *Hin*dIII site by using 150 μCi of [α-^{32}P]dATP and 10 units of the Klenow fragment of DNA polymerase I. The reaction mixture was incubated at room temperature for 5 min, then subjected to electrophoresis on a 4% native polyacrylamide gel. The band corresponding to the smaller DNA fragment was excised from the gel and soaked with 0.5 M ammonium acetate overnight at 37 °C; DNA was then precipitated from solution by the addition of two volumes of cold ethanol. The topoisomerase I-mediated cleavage reaction was carried out in incubation mixtures (50 μL total volume) containing 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 30 μg/mL of BSA, and 4 fmol of the 3′-^{32}P end labeled *Hin*dIII/*Pvu*II restriction fragment. The final concentration of the CPT analogue in each reaction mixture was 50 μM. Reactions were initiated by the addition of 125 ng of topoisomerase I and incubated at 37 °C for 30 min. To investigate the time course of dissociation of CPT derivatives from the inhibitor−topoisomerase I−DNA ternary complexes, the ternary complexes were initially formed by incubation at 37 °C. The solution containing the complex was then equilibrated at 23 °C (to diminish the eventual rate of dissociation of the inhibitors) and treated with NaCl solution to a final concentration of 0.35 M and aliquots were removed at predetermined time intervals. Following SDS-proteinase K treatment, each aliquot was extracted successively with phenol and chloroform, and the DNA was recovered by ethanol precipitation. The recovered DNA was dissolved in a formamide loading solution (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and bromophenol blue) for analysis on 8% denaturing gels containing 7 M urea. Cleavage sites were determined by comparison with Maxam−Gilbert sequencing reactions (*36*). The nucleotide at the 5′-end of the fragment resulting from topoisomerase I-mediated cleavage was numbered +1; that at the 3′-end of the other fragment (attached to topoisomerase I) was numbered −1 (Figure 2).

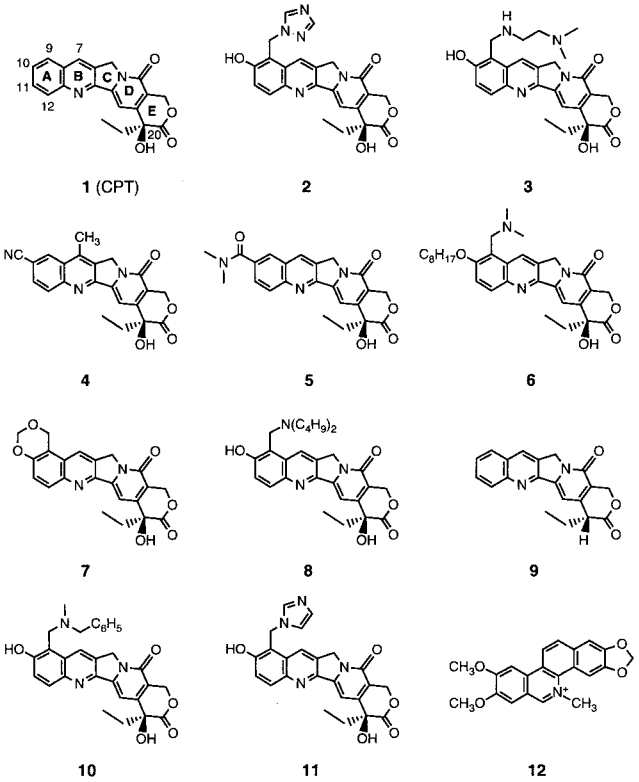


FIGURE 1: Structures of the camptothecin analogues studied.

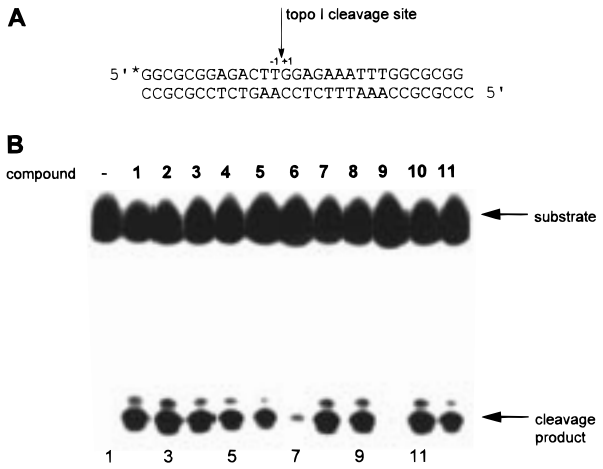


FIGURE 2: The effects of CPT derivatives on topoisomerase I-mediated cleavage of an oligonucleotide containing one topoisomerase I high-efficiency cleavage site. (A) Sequence of the DNA duplex substrate. (B) Autoradiogram of a 16% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end labeled on the scissile strand; cleavage reactions were carried out as described in Experimental Procedures. Lane 1, DNA + topoisomerase I; lanes 2−12, DNA + topoisomerase I + CPT analogues **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, and **11**, respectively.

RESULTS

Effects of CPT Derivatives on Calf Thymus Topoisomerase I-Mediated Cleavage of a DNA Duplex Containing One High-Efficiency Cleavage Site. The ability of CPT derivatives (Figure 1) to induce topoisomerase I−DNA covalent binary complex formation was examined by using a synthetic DNA duplex substrate containing one topoisomerase I high-

efficiency site (Figure 2A). It is derived from the sequence motif in the rDNA spacers of *Tetrahymena* which is tightly associated with topoisomerase I in vivo (*37*). However, the base at position +1 was changed from adenosine to guanosine to increase the sensitivity to camptothecin derivatives (*38*).

The DNA substrate was 5′-^{32}P end labeled on the scissile strand, incubated in the presence of topoisomerase I and a CPT derivative (4 μM) at 37 °C for 60 min, then quenched by the addition of SDS to 1% final concentration. Following proteolysis of topoisomerase I covalently bound to DNA with proteinase K, the reactions were analyzed by 16% PAGE.

In the presence of CPT (**1**), topoisomerase I-mediated DNA cleavage increased from essentially zero (Figure 2B, lane 1) to 35% (Figure 2B, lane 2). The absence of the 20-hydroxyl group (**9**) nearly abolished stabilization of the covalent binary complex. There was little difference in the potencies of most of the 9-substituted CPT derivatives. In the presence of compound **10**, topoisomerase I produced 39% cleavage of the DNA duplex substrate; comparable numbers were observed for analogues **2** (36%), **3** (34%), **8** (31%), and **11** (28%). In contrast, while small substituents such as CN (**4**, 28% cleavage) at the 10-position were well tolerated, the lower potency of **5** (22% cleavage) and **6** (9% cleavage) indicated that larger groups at the 10-position diminished the ability of CPT to promote DNA duplex cleavage. For analogue **7**, in which the 9- and 10-substituents were connected as a cyclic formacetal derivative, topoisomerase I-mediated cleavage was enhanced (34% cleavage) to essentially the same extent as for CPT.

DNA Religation Following NaCl Treatment of the Putative CPT−Topoisomerase I−DNA Ternary Complexes. The dissociation of CPT derivatives from the formed ternary complexes, and subsequent religation of DNAs from the topoisomerase I−DNA binary complexes, was studied initially by using a DNA substrate containing a number of topoisomerase I cleavage sites (Figure 3). This permitted the effects of DNA sequences flanking the topoisomerase I cleavage sites to be assessed.

The DNA substrate was obtained by treatment of pSP64 plasmid DNA with restriction endonucleases *Hin*dIII and *Pvu*II. The resulting restriction fragment was then 3′-^{32}P end labeled and purified as described in Experimental Procedures. DNA−topoisomerase I covalent binary complexes were allowed to form for 30 min at 37 °C in the presence of 50 μM CPT derivatives, leading to ternary complex formation. Samples were then maintained at 23 °C and treated with 5 M NaCl solution to a final concentration of 0.35 M to effect the reversal of complex formation (*16*, *21*, *23*, *25*); aliquots were removed at the indicated times. Reactions were quenched by the addition of SDS and processed as described in Experimental Procedures before analysis by PAGE. Quantification of radioactivity associated with each cleavage product as well as the total radioactivity in each lane was accomplished using a phosphorimager. The sequence at each cleavage site was determined by comparison to Maxam−Gilbert sequencing reactions. As anticipated, because the cleavage products resulting from topoisomerase I-mediated cleavage had 5′-OH termini, they migrated more slowly on polyacrylamide gels than the corresponding products of the Maxam−Gilbert sequencing reactions (Figure 3B).

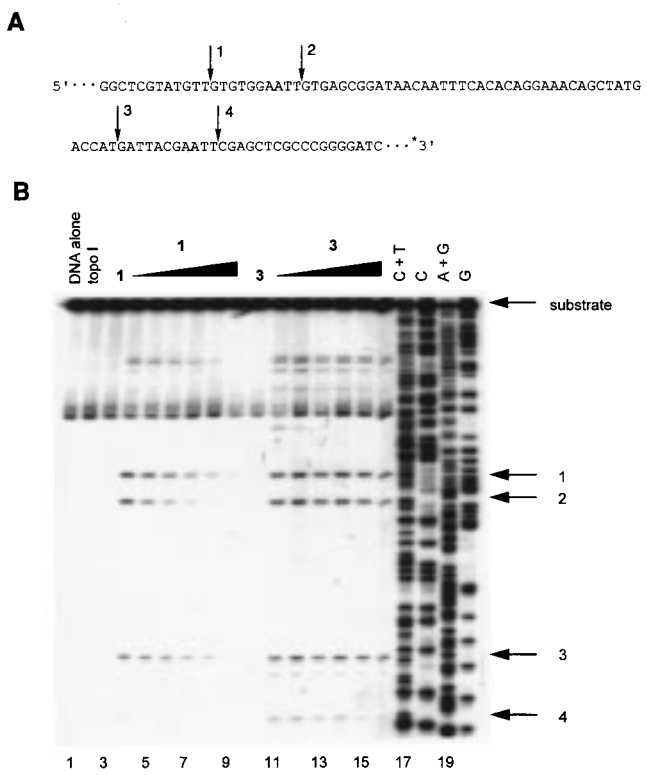


FIGURE 3: Differential reversibilities of topoisomerase I-induced covalent complexes formed from the *Hin*dIII/*Pvu*II restriction fragment of pSP64 plasmid DNA in the presence of CPT (**1**) and **3**. (A) Nucleotide sequence flanking the topoisomerase I cleavage sites of the substrate DNA. The cleavage sites are denoted by arrows. (B) Autoradiogram of an 8% denaturing polyacrylamide gel. The substrate was 3′-^{32}P end labeled on the scissile strand; cleavage and NaCl reversal reactions were carried out as described in Experimental Procedures. Maxam−Gilbert sequencing reactions were included on the same gel. Cleavage sites are numbered sequentially as in panel A. Lane 1, DNA alone; lane 2, DNA + topoisomerase I; lane 3, 50 μM CPT (**1**); lanes 4−9, topoisomerase I + CPT treated with 0.35 M NaCl for 0, 30, 60, 120, 180, and 300 s, respectively; lane 10, 50 μM **3**; lanes 11−16, topoisomerase I + **3** treated with 0.35 M NaCl for 0, 30, 60, 120, 180, and 300 s, respectively; lanes 17−20, Maxam−Gilbert sequencing reactions C + T, C, A + G, and G, respectively.

Shown in Figure 3B as an example is the time course of DNA religation following treatment of the ternary complexes containing **1** and **3** with NaCl. All of the topoisomerase I cleavage sites had T at position −1 and three of the four sites had G at position +1 (Figure 3A). In agreement with previous observations (38), the sites having G at position +1 gave the most intense cleavage bands (Figure 3, cleavage sites 1, 2, and 3 vs cleavage site 4). The complexes containing compound **3** were more persistent at each cleavage site than those containing CPT (**1**). This argues that dissociation of the CPT derivative is likely the rate-limiting step leading to DNA religation (Scheme 1). It is interesting to note that the rate of religation at site 2 in the presence of CPT was significantly faster than that at sites 1 or 3, presumably reflecting differences in the rates of CPT dissociation from the individual ternary complexes.

Further evidence concerning the rate-limiting step in the process leading from the ternary complex to religated DNA was accessible by the use of nitidine (**12**), a member of another structural class of topoisomerase I inhibitor that functions in the same fashion as the CPTs mechanistically (*39*). When employed with the same DNA substrate shown

Table 1: First-Order Rate Constant for Dissociation of CPT Derivatives-Induced Topoisomerase I−DNA Binary Complexes

compound	cleavage (%)[a]	rate contant k ($\times 10^{-3}$ s^{-1})[b]
1	35	28.06
2	36	5.29
3	34	5.64
4	28	2.98
5	22	7.82
6	9	--
7	34	8.51
8	31	3.45
9	6	--
10	39	4.14
11	28	2.76
12	18	>139

[a] Topoisomerase I inhibitors were employed at 4 μM final concentration. [b] Topoisomerase I inhibitors were employed at 50 μM final concentration. The incubation mixtures were maintained at 23 °C.

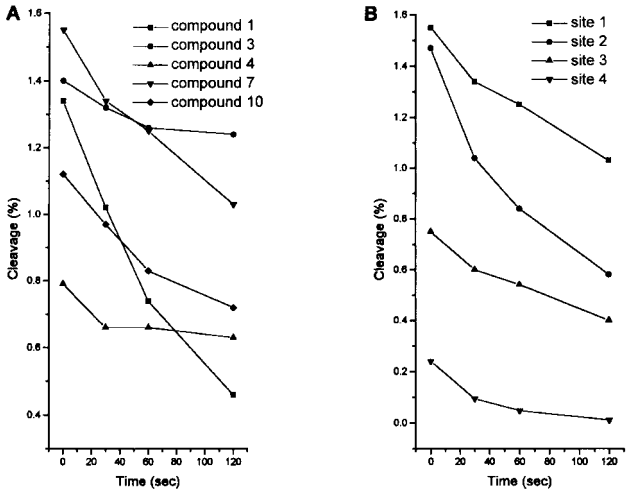


FIGURE 4: Time course of religation of the complexes formed in the presence of various CPT analogues; the DNA substrate is shown in Figure 3A. (A) Phosphorimager analysis of the time course of ligation at cleavage site 1 of complexes formed in the presence of CPT (**1**), **3**, **4**, **7**, and **10**. (B) Phosphorimager analysis of the time course of religation and complexes induced at four sites in the presence of **7**, following treatment with NaCl.

in Figure 3A, **12** produced lesions at the same four sites noted for the CPTs. However, admixture of NaCl caused religation of all four sites that was too fast ($t_{1/2} < 5$ s) to be quantified. This observation strongly supports the thesis that inhibitor dissociation from the individual ternary complexes must be rate-limiting in the overall religation process (Scheme 1) and that the ligation reaction per se is at least 5-fold faster than that determined in the presence of any of the CPT derivatives that afforded a measurable overall rate of religation (Table 1). Thus the actual rate of ligation is likely too fast to be measured by the technique employed here. The rates of religation in the presence of CPTs **6** and **9** could not be measured due to the limited amount of complex produced. All of the other CPT derivatives produced ternary complexes that exhibited somewhat slower ligation kinetics than CPT itself. The phosphorimager analysis of the ligation kinetics at cleavage site 1 for CPT and four CPT derivatives is shown in Figure 4A. The observed rate of cleavage band disappearance upon salt addition should represent the overall rate of inhibitor dissociation and subsequent religation to produce the intact DNA duplexes. The differential rates of ligation at a single site for the various CPT derivatives again reflect a rate-limiting dissociation of the CPT derivatives followed by a faster ligation step.

Shown in Figure 4B is the religation of DNA from the complexes formed in the presence of CPT **7** at the four different DNA cleavage sites. This figure illustrates that the topoisomerase I−DNA complexes underwent religation with different kinetics, presumably reflecting differences in the off-rates of **7** from the individual ternary complexes as a consequence of differences in the microenvironments surrounding the individual topoisomerase I cleavage sites. The site (cleavage site 2) at which cleavage was reversed most quickly had a G at position +1 and participated in ternary complex formation quite efficiently. This indicates that the persistence of topoisomerase I-mediated DNA cleavage in the presence of CPT derivatives need not be related to the extent of enzyme−DNA binary complex stabilization, nor to the identity of the nucleobase at position +1 (Figure 3). The equilibrium concentration of the CPT−topoisomerase I−DNA ternary complex can be described as the ratio of the on- and off-rates of CPT from the ternary complex, since ligation (and presumably also cleavage) is fast (vide supra). It is clear from Figure 4B that DNA sequence can significantly alter the off-rate of a CPT from the ternary complex. Also apparent from a comparison of DNA sequences at the four sites are that the equilibrium concentration of the ternary complex and off-rates of CPT analogues are not defined simply by the ...TG... sequence common to sites 1−3.

Since the topoisomerase I recognition domain on DNA extends over several nucleotides, when using a DNA substrate having several closely spaced topoisomerase I cleavage sites, it is possible that cleavage at a given site could be affected by topoisomerase interaction at adjacent sites. To avoid such effects, the kinetics of dissociation of CPT analogues from the formed ternary complexes was studied using an oligonucleotide containing a single high-efficiency topoisomerase I cleavage site (Figure 5). The DNA substrate was 5′-^{32}P end labeled on the scissile strand. Topoisomerase I-mediated cleavage and NaCl-induced religation were carried out as described above. CPT derivatives such as **2** and **3** clearly dissociated from their ternary complex with DNA and topoisomerase less quickly than CPT itself (Figure 5A).

The dissociation of CPT derivatives from the enzyme−DNA binary complexes was assumed to be rate limiting for the overall religation reaction and to proceed with first-order kinetics. In fact, a plot of $\ln(A_o/A)$ vs time after the addition of NaCl yielded a straight line having a slope equal to the pseudo first-order dissociation constant k (Figure 5B). A_o and A were the concentrations of cleavage products at time zero and time t, respectively, after the addition of NaCl. The pseudo first-order dissociation rate constants so obtained are given in Table 1. The extent of topoisomerase I-mediated cleavage of the same oligonucleotide in the presence of the various CPT derivatives (Figure 2) was quantified using a phosphorimager and listed in Table 1 as well. The k value for CPT (28.1×10^{-3} s^{-1}; $t_{1/2} = 0.4$ min) was comparable with the reported reversal half-time (0.3 min) using a 33-mer oligonucleotide with one topoisomerase I cleavage site under similar religation conditions (0.35 M NaCl, 25 °C) (*40*). Generally consistent with the data obtained with the *Hin*dIII/*Pvu*II restriction fragment of pSP64 plasmid DNA, the dissociation rates of the individual CPT derivatives were not directly correlated with their efficiencies in stabilizing

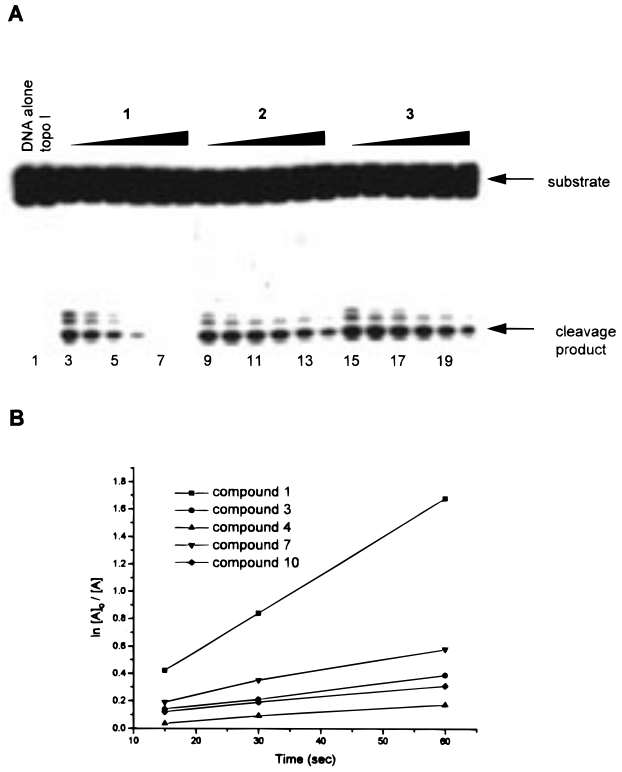


FIGURE 5: Time course of religation of the complexes formed in the presence of various CPT analogues; the DNA substrate containing a single topoisomerase I cleavage site is shown in Figure 2A. (A) Autoradiogram of a 16% denaturing polyacrylamide gel. The full duplex was 5′-^{32}P end labeled on the scissile strand; cleavage and NaCl-induced religation were carried out as described in Experimental Procedures. Lane 1, DNA alone; lane 2, DNA + topoisomerase I; lanes 3−8, topoisomerase I + CPT (**1**) treated with 0.35 M NaCl for 0, 15, 30, 60, 120, and 300 s, respectively; lanes 9−14, topoisomerase I + **2** treated with 0.35 M NaCl for 0, 15, 30, 60, 120, and 300 s, respectively; lanes 15−20, topoisomerase I + **3** treated with 0.35 M NaCl for 0, 15, 30, 60, 120, and 300 s, respectively; (B) A plot of $\ln(A_o/A)$ versus time after the addition of NaCl for five different CPT−topoisomerase I−DNA ternary complexes.

topoisomerase I−DNA covalent binary complex formation. For example, although **11** did not produce ternary complex to the same extent as CPT (28% vs 35% cleavage), this CPT analogue had an off-rate from the ternary complex 10 times slower than that of CPT itself. In addition, CPTs **2** and **3** stabilized topoisomerase I-mediated DNA cleavage to the same extent as CPT, but upon salt addition dissociated from the ternary complex about 5-fold less quickly than CPT.

Inhibition of Topoisomerase I-Mediated Cleavage/Ligation of a Branched Substrate by CPT Derivatives. The inhibition of a topoisomerase I-mediated strand transfer reaction by CPT derivatives was investigated using a synthetic DNA substrate containing a branch on the scissile strand (Figure 6A). The acceptor oligomer with free 5′-OH groups was fully complementary to the noncleaved strand up to the topoisomerase I high-efficiency cleavage site.

The DNA substrate was 5′-^{32}P end labeled on the scissile strand, incubated with 25 ng of topoisomerase I in the presence of a 1000-fold excess of acceptor oligonucleotide and individual CPT derivatives (at 20 μM concentration) at 37 °C for 5 min. The incubation mixtures were then quenched by the addition of SDS, treated with proteinase K, and analyzed by 20% denaturing PAGE.

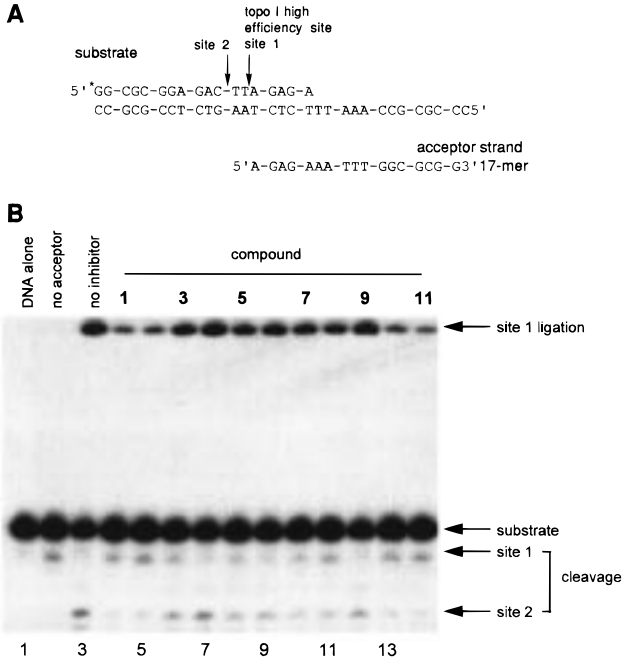


FIGURE 6: Inhibition of topoisomerase I-mediated cleavage/ligation of a branched substrate by CPT analogues. (A) Sequence of the branched DNA substrate. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end labeled on the scissile strand; reactions were carried out as described in Experimental Procedures. Lane 1, branched substrate containing the 17-mer acceptor strand; lane 2, branched substrate lacking the acceptor strand + topoisomerase I; lane 3, branched substrate containing the 17-mer acceptor strand + topoisomerase I; lanes 4−14, branched substrate containing the 17-mer acceptor strand + topoisomerase I + 20 μM **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, and **11**, respectively.

Individual CPT derivatives exhibited quite different levels of inhibition of the topoisomerase I-mediated strand transfer reactions (Figure 6B). CPTs **1**, **2**, **10**, and **11** inhibited the cleavage/ligation to an extent greater than 70% (Figure 6B, lanes 4, 5, 13, and 14) while **6** and **9** exhibited low (22%) and no (0%) inhibition (Figure 6B), respectively. Unexpectedly, while it efficiently enhanced the cleavage of a DNA duplex (Figure 2B, lane 5), **4** failed to inhibit the resealing reaction (Figure 6B, Lane 7).

Inhibition of Topoisomerase I-Mediated Cleavage/Ligation of Nicked and Gapped DNA Duplexes by CPT Derivatives. The effects of CPT derivatives on topoisomerase I-mediated ligation across 3- and 18-nucleotide gaps were examined by using nicked and gapped substrates (Figures 7 and 8) (*13*). Both substrates were 5′-^{32}P end labeled on the scissile strand. Following incubation with topoisomerase I in the presence of 20 μM CPT analogues at 37 °C for 1 h (nicked substrate) or 5 min (gapped substrate), respectively, the reactions were quenched by the addition of SDS to 1% final concentration, treated with either trypsin (nicked substrates, 1 mg/mL, 37 °C, 30 min) or proteinase K (gapped substrates, 1 mg/mL, 37 °C, 60 min) and analyzed by 20% denaturing PAGE.

As reported previously (*13*), the action of topoisomerase I near a preexisting nick resulted in deletions of one or three nucleotides (Figure 7B, lane 3), depending on the sequence of the acceptor nucleotide. This recombination reaction was inhibited strongly (~70% inhibition efficiency) by most of the CPT derivatives, including **6** (Figure 7B). Analogue **6** had neither blocked the topoisomerase I-mediated ligation of a branched substrate (Figure 6B) nor enhanced the

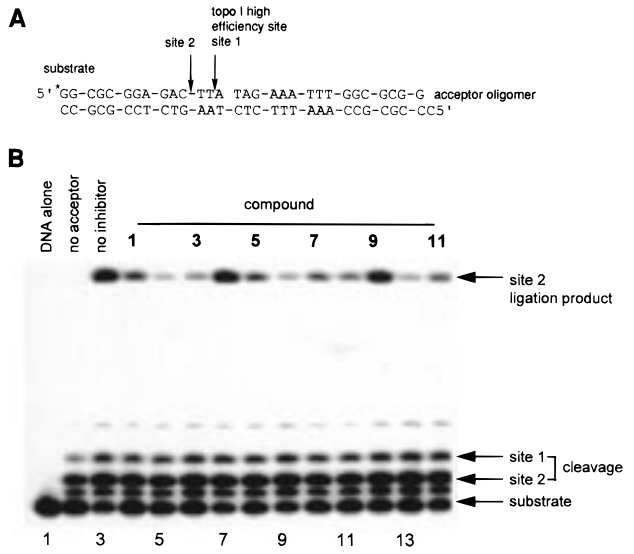


FIGURE 7: Inhibition of topoisomerase I-mediated ligation across a three-nucleotide gap by CPT analogues. (A) Sequence of the nicked DNA substrate. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end labeled on the scissile strand; reactions were carried out as described in Experimental Procedures. Lane 1, nicked substrate containing the acceptor strand; lane 2, nicked substrate lacking the acceptor strand + topoisomerase I; lane 3, nicked substrate containing the acceptor strand + topoisomerase I; lanes 4−14, nicked substrate containing the acceptor strand + topoisomerase I + 20 μM **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, and **11**, respectively.

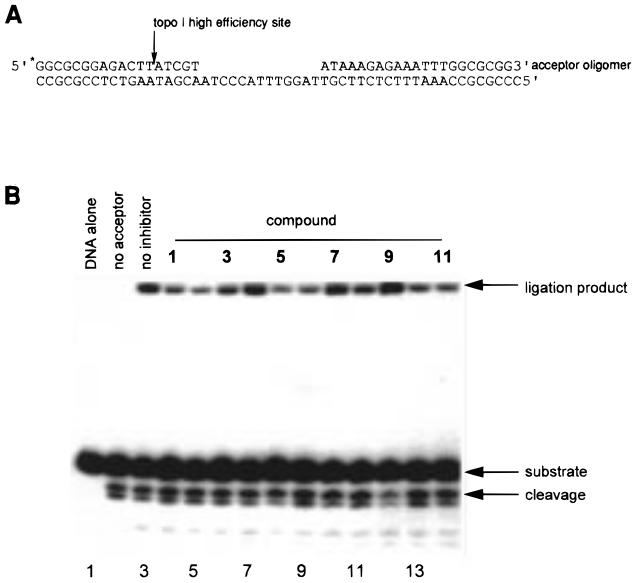


FIGURE 8: Inhibition of topoisomerase I-mediated ligation across an 18-nucleotide gap by CPT analogues. (A) Sequence of the gapped DNA substrate. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end labeled on the scissile strand; reactions were carried out as described in Experimental Procedures. Lane 1, gapped substrate containing the acceptor strand; lane 2, gapped substrate lacking the acceptor strand + topoisomerase I; lane 3, gapped substrate containing the acceptor strand + topoisomerase I; lanes 4−14, gapped substrate containing the acceptor strand + topoisomerase I + 20 μM **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, and **11**, respectively.

cleavage of a full DNA duplex (Figure 2B). Likewise, analogue **9** inhibited ligation across the three-nucleotide gap (∼26% inhibition) (Figure 7B, lane 12) although it had been found to stabilize the enzyme−DNA binary complex poorly (Figure 2) and failed to inhibit the strand transfer reaction. Only CPT analogue **4** failed to inhibit ligation across the three-nucleotide gap.

The ability of topoisomerase I to delete larger segments of DNA has been demonstrated by the ligation of two DNA strands across an 18-nt gap (Figure 8B, lane 3) (*13*). This topoisomerase I-mediated process, which is promoted by hairpin formation, could occur so efficiently that after a 1-h incubation, 80% of the substrate was converted to ligation product and none of the compounds showed any inhibition (data not shown). However, after a short incubation period (37 °C, 5 min), CPT and six different derivatives (Figure 8B, lanes 4−6, 8, 9, 13, and 14) inhibited the topoisomerase I-mediated ligation reaction across the 18-nucleotide gap by ∼50%. Once again **4** had no effect on this topoisomerase I-mediated process. An actual increase in topoisomerase I-mediated ligation by **9** (Figure 8B, lane 12) was observed reproducibly in several independent experiments (data not shown). It may be noted that **7** and **8** exerted completely different effects on topoisomerase I-mediated ligation across the three and 18-nucleotide gaps (Figures 7 and 8, lanes 10 and 11). Both were strong inhibitors of the cleavage/ligation reaction on the nicked substrates; in comparison, **8** was weakly (∼30%) inhibitory toward the ligation across an 18-nucleotide gap, while **7** had no effect at all.

DISCUSSION

In recent years, the importance of DNA topoisomerases as molecular targets in cancer chemotherapy has become firmly established (*41*, *42*). DNA topoisomerase I is concentrated in cell nuclei, which is the site of DNA replication and transcription (*43*−*45*). Topoisomerase I is expressed continuously during the cell cycle and in quiescent cells, whereas topoisomerase II expression increases during the S phase of the cell cycle and is almost absent in quiescent cells (*46*, *47*). Since the sensitivity of cells to topoisomerase inhibitors is directly proportional to the concentrations of the enzymes present (*48*), this predicts that topoisomerase I could potentially be used to treat slowly growing tumors that are usually resistant to chemotherapy. Recent evidence suggests that topoisomerase I may also be involved in nonhomologous DNA recombination (*49*) as well as genomic rearrangements of eukaryotic viral DNA (*50*, *51*). This suggests that appropriate topoisomerase inhibitors could potentially find utility as antiviral agents (*52*, *53*). Extensive structural, functional, and mechanistic studies of DNA topoisomerases I are, therefore, important in that they may facilitate the design of new types of therapeutic agents that target this enzyme.

DNA topoisomerase I inhibitors can also be valuable tools for dissecting the mechanistic behavior and roles of the target enzyme. The relative abilities of the individual CPT analogues **1**−**11** to stabilize topoisomerase I−DNA covalent complex formation (Figure 2) provides detailed information about the structural elements in CPT essential for interaction with the enzyme−DNA binary complex. Assuming that calf thymus and human topoisomerases I have similar binding pockets, these results are in general agreement with the hypothetical CPT binding model proposed by Redinbo et al. (*7*). First, the lack of activity of CPT derivative **9** indicated that the hydrogen bond between the 20(*S*)−hydroxyl group of the CPTs and the hydrogen bond acceptor

of the enzyme (i.e., Asp533 in human topoisomerase I) is a key element for ternary complex stabilization. Second, substituents at position 9 of CPT are probably oriented in a relatively open space with a consequently large degree of bulk tolerance for substituents, as suggested by the activities of compounds **2**, **3**, **8**, and **11** in stabilizing the covalent binary complex. In the proposed CPT binding model (*7*), C-7 and C-9 of CPT are in close proximity to N-3 and N-7 of guanosine +1, respectively. The benzene ring at the C-9 position of compound **10** could plausibly improve the stacking interaction between this CPT analogue and the terminal guanosine on the scissile strand, thus better stabilizing the covalent binary complex. On the other hand, the bulk tolerance at position 10 seems very limited (**5** and **6**). This is consistent with the interpretation that the bulky group could perturb the interaction between the A-ring of CPT and Asn722 on human topoisomerase I (*7*) or a corresponding (nucleophilic) site on the calf thymus enzyme (*54*). Recently, it has been shown that an Asn722Ser mutation which removed the planar conjugated side chain at position 722 of human topoisomerase I produced resistance to both CPT and saintopin (*55*). While a small CN group (**4**) at C-10 diminished CPT activity slightly, an OH group was fairly well tolerated (**2**, **3**, **8**, and **10**), possibly due to its potential hydrogen bonding with nearby functional groups on the enzyme. Finally, the nature and location of multiple substituents attached to the A-ring appear to be critical for stabilization of the covalent binary complex. 10,11-(Methylenedioxy)CPT is one of the most potent topoisomerase I inhibitors, while the 10,11-(ethylenedioxy) analogue is only about one-seventh as potent (*30*). 9,10-(Methylenedioxy)-(20 *R,S*)-CPT has been shown to have one-half and one-fifth the potential for binary complex stabilization as (20 *R,S*)-CPT and 10,11-(methylenedioxy)-(20 *R,S*)-CPT, respectively (*30*). Nonetheless, as shown in Figure 2B, lane 8, CPT analogue **7**, which can be regarded as a close structural variant of 9,10-(ethylenedioxy) CPT, was as active as CPT in enhancing topoisomerase I-mediated cleavage of the DNA duplex studied here. Thus, the region involving 9, 10, and 11-positions in ring A of CPT appears to interact with the nucleotide at position +1 of the DNA scissile strand and amino acid residues close to the active site tyrosine on the enzyme. This region is, therefore, very important for the biochemical activity of CPT. Depending on the type, number, and location of substituents, highly active or inactive analogues can be obtained.

It is also instructive to consider the biological activities of the CPT analogues studied here as topoisomerase I inhibitors. As noted previously (*29*) for 9-(dimethylaminomethyl)-10-hydroxycamptothecin (topotecan) and CPT analogue **3**, both of which were cytotoxic toward cultured L1210 cells in vitro and in mice bearing L1210 leukemia, 10-hydroxy-9-substituted CPT analogues **8** and **10** were also found to be cytotoxic in vitro. In comparison CPT analogues **2** and **11** were not toxic (data not shown), although they acted as inhibitors of topoisomerase I function in all of the assays employed in this study and had off-rates from the formed ternary complex slower than that of CPT itself. While this may simply reflect alterations in cell permeability or susceptibility to cellular efflux mechanisms for **2** and **11**, it could plausibly indicate that the expression of cytotoxicity by CPT and its analogues depends on their ability to function at some cellular locus in addition to the topoisomerase I−DNA binary complex. The latter possibility is underscored by the finding that CPT **6** was cytotoxic despite its inability to stabilize the topoisomerase I−DNA covalent binary complex.

As envisioned by the replication fork collision model (*26, 41, 56*), the trapping of the topoisomerase I−DNA covalent binary complex by CPT is not a cytotoxic event per se. Only those camptothecin-induced topoisomerase I-linked DNA breaks occurring within genomic regions being replicated would be lethal, as a consequence of replication of the strand released concomitant with covalent binary complex formation. The requirement of DNA synthesis and replication fork arrest for cell killing is entirely consistent with the observed dependence of camptothecin cytotoxicity on exposure time noted previously in several cell lines (*57*). Although a DNA replication-independent cytotoxicity has recently been reported in neuronal cells (*58*), the persistence of the CPT induced DNA−enzyme covalent binary complexes should still increase the likelihood of collision with the transcription machinery. To determine the probable persistence of the CPT derivatives being evaluated in the present study, the reversibility of the ternary complexes formed in the presence of each CPT analogue was investigated following NaCl treatment (Figures 3−5, Table 1). The religation kinetics at a single site in the *Hin*dIII/*Pvu*II restriction fragment of pSP64 plasmid DNA, and also for the oligonucleotide DNA duplex containing only one topoisomerase I cleavage site, clearly reflected significant differences in the rate-limiting dissociation of CPT analogues that were closely related structurally. As noted in Table 1, CPT has a relatively rapid off-rate compared with those analogues bearing substituents at positions 9 or 10. The off-rate was also dependent on DNA sequence; the presence of a guanosine at position +1 did not necessarily result in a slow off-rate (Figure 4). For the design of new generations of CPT derivatives, it may well be of interest to have species with slower off-rates from the ternary complex. In fact, the better salt resistance of ternary complexes containing SN-38 (i.e., the active metabolite of CPT-11) has been found to be consistent with the slower reversibility of single-strand breaks induced by this agent in isolated human colon carcinoma cell nuclei and the consequent higher cytotoxicity of this agent as compared with CPT (*15*). Additionally, alkylating derivatives of CPT that induced persistent ternary complexes also exhibited significantly enhanced cytotoxicity (*59*).

Using oligonucleotide substrates, Westergaard and coworkers have demonstrated that CPT inhibits topoisomerase I-induced DNA cleavage and more efficiently blocks the topoisomerase I−DNA religation reaction (*60*). Similar conclusions were also reached by Porter and Champoux (*61*). In the proposed CPT binding model (*7*), topoisomerase I is trapped in its covalent complex with DNA in the presence of CPT due to the >4.5 Å distance between the 5′- hydroxyl group and 3′-phosphate group on the cleaved DNA strand. Most of the CPT analogues investigated in the present study exhibited behavior in the cleavage and religation assays qualitatively similar to CPT itself. However, one of the analogues modified within the A-ring (**4**) produced cleavage of the duplex substrate shown in Figure 2, although it was not found to have substantial inhibitory activity in the religation assay (Figure 6). These results suggest that

inhibition of the religation reaction may not be the only way for CPT analogues to effect stabilization of the topoisomerase I−DNA binary complex.

Mammalian cells possess the ability to catalyze genetic recombination, leading to the rearrangement of genetic material. Illegitimate recombination, i.e., the joining of nonhomologous DNA sequences, is potentially the most disruptive reaction of all recombination mechanisms as it can lead to deletions, amplifications, insertions, and translocations, as well as the integration of foreign DNA (*62*). Illegitimate recombination is believed to be implicated in mechanisms of carcinogenesis, inherited diseases, and genome evolution (*63*). However, the actual molecular mechanism(s) of such recombination events is poorly understood.

To the extent that DNA topoisomerases I actually do participate in illegitimate recombination, it seems likely that the separation of strand scission and religation processes noted in cell-free systems may provide the mechanistic wherewithal for such structural alterations. In fact, there are already several lines of evidence to suggest that topoisomerase I activities may promote illegitimate recombination between nonhomologous DNAs. Vaccinia topoisomerase I has been shown to promote sequence specific illegitimate recombination in *Escherichia coli* (*50*). In *Saccharomyces cerevisiae*, the combined action of DNA topoisomerases I and II suppresses mitotic recombination specifically within the rDNA cluster (*64*). Also in *S. cerevisiae*, Schiestl and co-workers found that overexpression of the yeast *TOP1* gene increased the frequency of illegitimate integration 6- to 12-fold and that it also increased the position of events of integration into sites identical in sequence with preferred topoisomerase I strand scission sites (*65*). In higher eukaryotes, the role of DNA topoisomerases in recombination is less clearly understood. Calf thymus topoisomerase I is implicated in the illegitimate integration of hepadnavirus DNA into the host chromosome (*51*). In addition to these integration events, calf thymus topoisomerase I has also been shown to mediate ligations across DNA gaps as large as 18 nucleotides, using suicide substrates and nonhomologous acceptor strands in a cell-free system (*13*).

In the present report, we have described the effects of a number of different CPT derivatives on the ability of topoisomerase I to mediate a strand exchange reaction, as well as the deletion of nucleotides from DNA via strand scission and religation of nicked and gapped DNA substrates (Figures 6−8). In general, the more efficient topoisomerase I-mediated ligation across a formed 18-nt gap containing a defined secondary structure was less readily inhibited than the ligation across a three-nucleotide gap. It is noteworthy that CPT **6** strongly inhibited the topoisomerase I-mediated ligation reactions across a 3-nt (84% inhibition) and an 18-nt (64% inhibition) gap, while it poorly stabilized the covalent binary complex (9% cleavage). It seems possible that the initial noncovalent binding of topoisomerase I to an intact DNA duplex occurs in a fashion that prevents CPT from binding to the enzyme−DNA complex. Following topoisomerase I-mediated cleavage, the nucleotide in position +1 on the scissile strand is repositioned, allowing CPT to occupy the vacated space (*7*). Conceivably, the large lipophilic group at C-10 of the compound precludes binding to this narrow pocket. In contrast, topoisomerase I-mediated cleavage of nicked and gapped substrates leaves a large single-stranded region downstream from the cleavage site. There may be enough room for CPT analogues such as **6** to bind to these enzyme−DNA complexes, thereby inhibiting the religation reaction from the complexes. Also interesting is the selective inhibition of topoisomerase I−mediated 3-nt deletion, but not the 18-nt deletion, by CPT **7**. Since the cleavage of the DNA substrates leading to the intermediate gapped binary complexes was not affected, it is logical to conclude that the structures of the formed topoisomerase I−DNA covalent binary complexes having a bulge or a hairpin on the nonscissile strand are sufficiently different to permit their discrimination by appropriately constituted molecular probes such as **7**. A similar observation may be made for a number of derivatives as regards their effects on cell-free models of homologous vs nonhomologous recombination. It has been reported that in monkey COS cells, VM-26 (teniposide), a topoisomerase II inhibitor, stimulates deletions of nucleotides mediated by illegitimate recombination (*66*). In another report, the effects of DNA topoisomerase inhibitors on recombination events were studied using gene transfer assays with an APRT$^-$ CHO cell mutant (*67*). These workers found that treatment of DNA-transfected cells with the DNA topoisomerase II inhibitors VP-16 (etoposide) and VP-26 stimulated nonhomologous recombination significantly but homologous recombination only slightly, and that the DNA topoisomerase I inhibitor CPT had no effect. However, the effects of CPT derivatives on topoisomerase I-mediated illegitimate recombination may be quite different, depending on the specific events examined. In addition, the final inhibitor concentration can also be important, as reflected in the recent report that in an in vitro system camptothecin did not reduce recombination (intermolecular religation) at the concentrations that strongly enhanced inhibition of intramolecular religation (*68*).

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BI980451K