Role of the 20-Hydroxyl Group in Camptothecin Binding by the Topoisomerase I−DNA Binary Complex[†]

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ABSTRACT: Recent findings concerning the structure of the covalent binary complex formed by DNA topisomerase I and its DNA substrate, as well as the nature of interactions with inhibitors that bind reversibly to this binary complex, have led to two proposed models for the binding of the prototype inhibitor camptothecin to the DNA−topisomerase I binary complex. While these models differ in many regards, they both suggest the involvement of the 20-OH group of camptothecin in a donor hydrogen bond with an enzyme side chain functional group. Presently, five analogues of camptothecin that differ only at C-20 have been evaluated for their ability to bind to the topoisomerase I−DNA binary complex and thereby inhibit enzyme function. Both 20-chloro- and 20-bromocamptothecin bound as well to the enzyme−DNA binary complex as 20-aminoCPT despite the absence of a substituent at C-20 capable of contributing a donor hydrogen bond.

The critical cellular processes of DNA replication and transcription are dependent on the unwinding of chromosomal DNA which provides access to the requisite DNA template. The unwinding can be carried out by either of two types of a class of enzymes called topoisomerases; these species introduce transient breaks in the DNA backbone and thereby alter the linking number of DNA (*1*−*3*).

The type II topoisomerases introduce breaks into both strands of DNA and mediate DNA relaxation in a process that requires ATP. Human topoisomerase II has been shown to constitute the critical cellular locus of a number of clinically important antitumor agents (*4*, *5*). The mechanism by which these agents kill cancer cells involves the stabilization of the intermediate topoisomerase II−DNA covalent binary complex in which both DNA strands have undergone strand scission and are covalently attached to topoisomerase II (*6*). By binding to this complex and preventing religation, the topoisomerase II inhibitors preclude DNA replication and transcription, and thereby lead to the death of cells attempting to undergo these processes.

More recently, topoisomerase I has also been shown to be the cellular target for cytotoxic agents, some of which also have useful antitumor activity. The best known agent of this type is camptothecin (CPT[1]), first shown by Hsiang et al. (*7*) to bind reversibly and noncovalently to the binary complex formed between topoisomerase I and DNA during the relaxation of DNA by this enzyme.

While the nature of topoisomerase I−DNA binding by the camptothecins is not understood at high resolution, there is an accumulating body of data relevant to the binding based on (i) structure−activity studies in which congeners of camptothecin were employed (*8*−*14*), (ii) analysis of the products formed from the topoisomerase I−DNA binary complex upon admixture of electrophilic CPT derivatives (*15*, *16*), and (iii) the identification and characterization of point mutants of topoisomerase I that are resistant to CPT (*17*−*20*). Hol and Champoux have recently succeeded in the crystallographic analysis of reconstituted and truncated human topoisomerases I in both covalent and noncovalent complexes with DNA (*21*, *22*).

On the basis of the foregoing crystallographic analysis, Hol, Champoux, and their co-workers have proposed a model for binding of CPT by the topoisomerase I−DNA binary complex (*21*). A second computationally based model has been proposed by Fan et al. (*23*), which accommodates many experimental observations concerning the behavior of CPT analogues and CPT resistant topoisomerase I mutants. While these models differ significantly, both posit the involvement of the 20-OH group of CPT as a hydrogen bond donor in stabilizing the interaction of CPT with the topoisomerase I−DNA binary complex.

By using several CPT analogues that differ structurally only at C-20, we have attempted to evaluate the role of the 20-OH group in binding to the topoisomerase I−DNA covalent binary complex. Presently, we demonstrate that 20-aminoCPT (**6**) also binds to the enzyme−DNA binary complex, but that its rate of dissociation from the binary complex upon NaCl addition is much greater than that of CPT. Critically, we have observed that the 20-chloro- and 20-bromoCPT analogues, neither of which can provide a donor H bond, stabilize the covalent enzyme−DNA binary complex fully as well as 20-aminoCPT and have off-rates following NaCl treatment from the complex that are comparable to that of CPT itself. These data suggest that that proposed models of CPT binding require further refinement

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[1] Abbreviations: CPT, camptothecin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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to accommodate the behavior of these newly characterized CPT analogues.

EXPERIMENTAL PROCEDURES

General Methods and Materials. T4 polynucleotide kinase and proteinase K were purchased from United States Biochemicals. The Klenow fragment of DNA polymerase I (lacking 5′ → 3′ exonuclease activity) and restriction endonucleases *Hin*dIII and *Pvu*II were obtained from New England Biolabs. Gentamicin, adenine, histidine, raffinose, and galactose were purchased from Sigma Chemicals. [γ-^{32}P]-ATP (7000 Ci/mmol) and [α-^{32}P]dATP (>3000 Ci/mmol) were obtained from ICN Pharmaceuticals. The medium for the growth of Sf9 cells was purchased from Life Technologies, Inc.; DNA oligonucleotides were from Cruachem Inc. pSP64 plasmid DNA was prepared as described previously (*24*). Yeast Nitrogen Base without amino acids was obtained from Difco Laboratories.

Camptothecin analogues were prepared essentially as described previously (*25*−*27*). DMSO solutions of the topoisomerase I inhibitors were used to make 5 mM stock solutions. DMSO (10%) was used to make further dilutions; the final concentration of DMSO in the incubations was 1%.

Denaturing polyacrylamide gel electrophoresis (PAGE) was carried out for 2−3 h (7 M urea, 50 W). Gels were visualized (autoradiography at −80 °C) using Kodak XAR-2 film. Quantification was carried out utilizing a Molecular Dynamics 400 E Phosphorimager, equipped with ImageQuant version 3.2 software. Distilled, deionized water (Milli-Q system) was used for all experiments.

Expression and Purification of Human DNA Topoisomerase I. Recombinant human DNA topoisomerase I was expressed in a baculovirus system and then purified as described previously (*28*). Briefly, the *htop1* gene, having its own translation initiation codon, was first subcloned into the *Bam*HI−*Eco*RI sites of baculovirus transfer plasmid pBAC-1 (Novagen, Madison, WI), affording recombinant plasmid pBAC-1/*htop1*. Linearized *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) DNA (Novagen) and pBAC-1/*htop1* were cotransfected into *Spodoptera frugiperda* (Sf9) insect cells (ATCC), producing a recombinant baculovirus (AcMNPV/*htop1*). The recombinant virus was purified by the use of plaque assays (X-Gal plates) and then identified by PCR analysis, using a standard protocol (Invitrogen, San Diego, CA). A high-titer viral stock was prepared by two rounds of amplification starting from this virus.

Sf9 cells were maintained in Sf-900 II SFM medium; the medium was supplemented with 5% fetal bovine serum, as well as 10 μg/mL gentamicin. For expression of the protein, Sf9 cells were grown to a density of 2×10^6 cells/mL and then infected with AcMNPV/*htop1* recombinant virus (multiplicity of infection of 10 plaque-forming units/cell). The cells were then harvested 72 h postinfection by centrifuging at 1000*g* for 10 min.

The baculovirus-infected insect cells were then used for the isolation of human topoisomerase I, which was carried out at 4 °C. The nuclear extract was prepared using slight modifications of a published procedure (*29*). In a typical preparation, 1×10^9 cells afforded about 12 mg of nuclear protein as well as 62 mg of cellular protein, the latter of which was discarded. The recombinant human topoisomerase I was then purified by FPLC using a heparin column. The isolated protein displayed two bands ($M_r \sim 75\,000$ and 70 000 Da) following SDS−polyacrylamide gel electrophoresis and protein visualization by Coomasie Blue staining. This is a consequence of the loss of the N-terminal domain as a result of long-term storage of the nuclear extract (*30*). The specific activity of the purified protein was 7.5×10^5 units/mg of protein. One unit is the amount of enzyme that relaxes 250 ng of pBR322 supercoiled DNA in 30 min at 37 °C.

Oligonucleotide Substrates. The oligonucleotides were purified by preparative electrophoresis on 20% denaturing polyacrylamide gels. DNA visualization was accomplished by UV shadowing (*31*); the band of interest was cut from the gel. The DNA was eluted with 2 M $LiClO_4$ (12 h at 37 °C) and recovered by precipitation with acetone (*32*).

Preparation of 5′-^{32}P End-Labeled Synthetic Oligonucleotides. Samples (1.5−10 μg) of synthetic oligonucleotides were 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. T4 polynucleotide kinase (10 units) was added to the mixture, thus initiating the reaction. The reaction mixture was incubated for 1 h at 37 °C, the reaction stopped by the addition of 12.5 μL of loading buffer (10 M urea, 1.5 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), and then the mixture applied to a 20% denaturing polyacrylamide gel. The relevant band was visualized (autoradiography) and excised from the gel. The DNA was recovered by a crush and soak procedure (*32*).

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

Oligonucleotide DNA Cleavage and Ligation by Topoisomerase I. The 5′-^{32}P end-labeled DNA duplex (3.25 fmol) was treated with 18 ng of human topoisomerase I in a 20 μL (total volume) reaction mixture 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 nicked and gapped substrates, the acceptor oligonucleotide was present in (1000-fold) excess relative to the radiolabeled oligomer. The reaction mixtures were incubated at 37 °C for the time noted, and then the reactions were stopped by treatment with proteinase K (1 mg/mL having 1% SDS at 37 °C for 60 min) prior to analysis by 20% denaturing PAGE.

To measure the time course of dissociation of individual CPTs from the ternary complexes, the ternary complexes were formed initially by incubation at 37 °C for 1 h. The solution containing the complex was then equilibrated at 23 °C, thereby lowering the eventual rate of inhibitor dissociation. Following treatment with a NaCl solution to a final concentration of 0.35 M, aliquots were removed at predetermined time intervals. After SDS−proteinase K treatment, each reaction mixture was analyzed on a 20% denaturing gel containing 7 M urea. The nucleotide at the 5′-end of the new fragment resulting from cleavage by topoisomerase I

was numbered +1; that at the 3′-end of the other fragment (attached to topoisomerase I) was numbered −1 (Figure 3A).

Recombinant Human Topoisomerase I-Mediated Cleavage of the HindIII/PvuII Restriction Fragment of pSP64 DNA. Thirty micrograms of pSP64 plasmid DNA was treated with 80 units each of restriction endonucleases *Hin*dIII and *Pvu*II at 37 °C for 2 h. [α-^{32}P]dATP (150 μCi) and 10 units of DNA polymerase I (Klenow fragment) were then added to the restriction digestion mixture to effect 3′-^{32}P end labeling of the substrate DNA on the scissile strand at the *Hin*dIII site. The reaction mixture was incubated at room temperature for 5 min and then applied to a 4% native polyacrylamide gel. The DNA was visualized by autoradiography, and the band corresponding to the 226-nucleotide fragment duplex DNA was excised from the gel, and then isolated by the crush and soak procedure as described previously (*32*). The topoisomerase I-mediated cleavage reaction was carried out at 37 °C for 30 min in a 40 μL (total volume) reaction mixture containing 20 mM Tris-HCl (pH 7.6), 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 30 μg/mL BSA, 12 fmol of labeled restriction fragment DNA, and 36 ng of human topoisomerase I. The final concentration of CPT analogues employed was 20 μM. The reactions were terminated by SDS−proteinase K treatment. Following sequential extraction with phenol and chloroform, the DNA was recovered by ethanol precipitation. The DNA was dissolved in a formamide loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, and 0.1% xylene cyanol and bromophenol blue), and analyzed on a 10% denaturing gel together with Maxam−Gilbert sequencing reactions (*33*) to determine the topoisomerase I cleavage sites.

Yeast Cytotoxicity Assay. The human topoisomerase I-specific cytotoxicity assay was carried out using yeast strain RS321Nph-TOP1 (*Mat a ade2-1 his3-1 leu3,112 trp1-1 ura3-1 can1-100 erg6 rad52::TRP1 top1-8::LEU2 phTOP1::URA*) obtained from SmithKline Beecham Pharmaceuticals. The yeast was grown to log phase (A_{595} = 2.0) at 30 °C in minimal media (0.9% Yeast Nitrogen Base without amino acids, 0.025 mg/mL adenine and histidine) containing 3% raffinose as the carbon source. The exponentially growing cells were then diluted to an absorbance of 0.015 at 595 nm with minimal media having a final concentration of 3% galactose. Ninety microliters of the diluted cell suspension was mixed with 10 μL solutions of each of the compounds to be tested in a 96-well microtiter plate. The camptothecin analogues were dissolved in DMSO to make 5 mM stock solutions and then diluted in sterile water so that the final concentration of DMSO in the yeast incubation mixture was <1%. The plates were incubated at 30 °C in a high-humidity chamber for 48 h, and the optical density of cells in each well was measured at 595 nm by using a microplate reader.

RESULTS

Synthesis of Camptothecin Analogues. As shown in Figure 1, camptothecin analogue **2** was prepared starting from (20*S*)-CPT (**1**) itself via the intermediacy of 20-chlorocamptothecin (**3**) utilizing chemical transformations reported previously (*25*, *26*, *34*). The derived 20-deoxyCPT (**2**) was purified by reverse phase HPLC immediately prior to assay and shown to be free of any contaminating CPT. Camptothecin was also converted to 20-bromocamptothecin (**4**) as shown in Figure

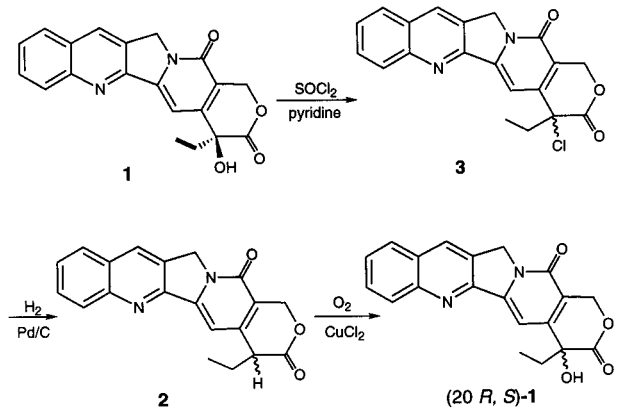

FIGURE 1: Route employed for the synthesis of 20-deoxycamptothecin (**2**) via 20-chlorocamptothecin (**3**).

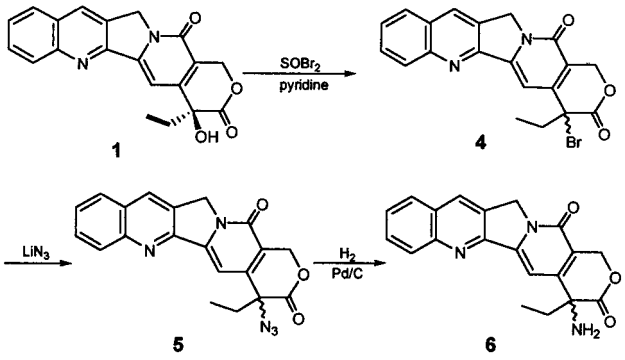

FIGURE 2: Transformations employed for the syntheses of camptothecin analogues **4**−**6**.

2 (*27*). The latter was converted successively to the 20-azidoCPT (**5**) and 20-aminoCPT (**6**) derivatives by known procedures (*27*). The analogues were all found to have low $[\alpha]_D$ values, suggesting that they may have undergone (partial) racemization. For comparative purposes, a sample of (20*R*,*S*)-camptothecin was obtained by oxidation of 20-deoxycamptothecin (**2**) by using anhydrous $CuCl_2$ and O_2 as described (*35*). In fact, admixture of Eu(hfc)$_3$ to $CDCl_3$ solutions of analogues **3**, **4**, and **6**, as well as putative (20*R*,*S*)-**1**, effected the downfield shifting of the CH_3 resonance at ∼0.1 ppm and resolution of the signals due to the constituent enantiomers, which were present in roughly comparable amounts. The effect of 2 molar equiv of Eu(hfc)$_3$ on the ^{1}H NMR spectrum of racemic **1** is shown in the Supporting Information (Figure 1); a 48:52 ratio of the two enantiomers was calculated.

Stabilization of the Human Topoisomerase I−DNA Covalent Binary Complex by CPT Analogues. The ability of the CPT analogues (Figures 1 and 2) to stabilize the topoisomerase I−DNA covalent binary complex was first investigated by using a 30-base pair synthetic DNA duplex (Figure 3A) designed to contain a single topoisomerase I cleavage site highly sensitive to camptothecin (*36*, *37*).

The DNA substrate was 5′-^{32}P end labeled on the scissile strand, and then incubated with human topoisomerase I in the presence and absence of a CPT analogue at 37 °C for 60 min. The reactions were quenched by the addition of SDS to a final concentration of 1%. Following the digestion of topoisomerase I covalently bound to DNA by proteinase K (1 mg/mL, 37 °C, 60 min), the reaction mixtures were analyzed by 20% denaturing PAGE.

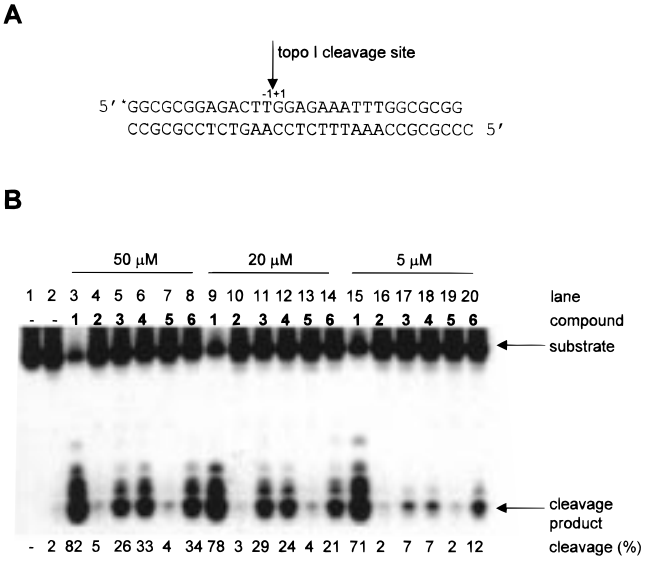

FIGURE 3: Recombinant human topoisomerase I-mediated cleavage of a full duplex DNA substrate containing a single cleavage site, in the presence and absence of CPT analogues. (A) Sequence of the DNA duplex substrate. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end labeled on the scissile strand; cleavage reactions were carried out as described in Experimental Procedures: lane 1, DNA alone; lane 2, DNA and topoisomerase I; lanes 3−8, DNA, topoisomerase I, and 50 μM CPT analogues **1**−**6**, respectively; lanes 9−14, DNA, topoisomerase I, and 20 μM CPT analogues **1**−**6**, respectively; and lanes 15−20, DNA, topoisomerase I, and 5 μM CPT analogues **1**−**6**, respectively.

As shown in Figure 3B, the extent of topoisomerase I-mediated DNA cleavage increased from 2% (Figure 3B, lane 2) to >70% in the presence of 5−50 μM CPT (**1**) (Figure 3B, lanes 3, 9, and 15). A racemic sample of CPT, prepared by oxidation of **2**, produced cleavage product to about one-half the extent of optically pure (20*S*)-CPT, when employed at concentrations of 1 μM (20 vs 42% extent of cleavage) and 0.1 μM (8 vs 19% cleavage), reflecting the known lack of activity of (20*R*)-CPT (*8*). Consistent with earlier reports (*28*, *38*), 20-deoxycamptothecin (**2**) had little if any ability to stabilize the topoisomerase I−DNA covalent binary complex. In the presence of 5−50 μM compound **2** (Figure 3B, lanes 4, 10, and 16), topoisomerase I produced essentially the same amount of DNA cleavage as in the absence of inhibitor. The introduction of an azido substituent at position 20 afforded an analogue (**5**) having essentially the same potency (Figure 3B, lanes 7, 13, and 19) as compound **2**.

Since the 20-hydroxyl group is proposed to be an important hydrogen bond donor in two different camptothecin binding models (*21*, *23*), it seemed reasonable to expect that the 20-aminocamptothecin analogue (**6**) might also exhibit reasonable activity. In fact, in the presence of this (racemic) analogue, the extent of topoisomerase I-mediated DNA cleavage increased from 12% (5 μM **6**) to 21% (20 μM **6**) and ultimately to 34% (50 μM **6**). Somewhat more surprising were the results obtained with halogenated CPT analogues **3** and **4**. As shown in Figure 3B, these compounds stabilized DNA cleavage to about the same extent as compound **6**. In fact, the 20-chloro- and 20-bromoCPT analogues (**3** and **4**) actually appeared to be more potent at 20 μM (29 and 24% extent of cleavage, respectively) than CPT analogue **6** (21% extent of cleavage).

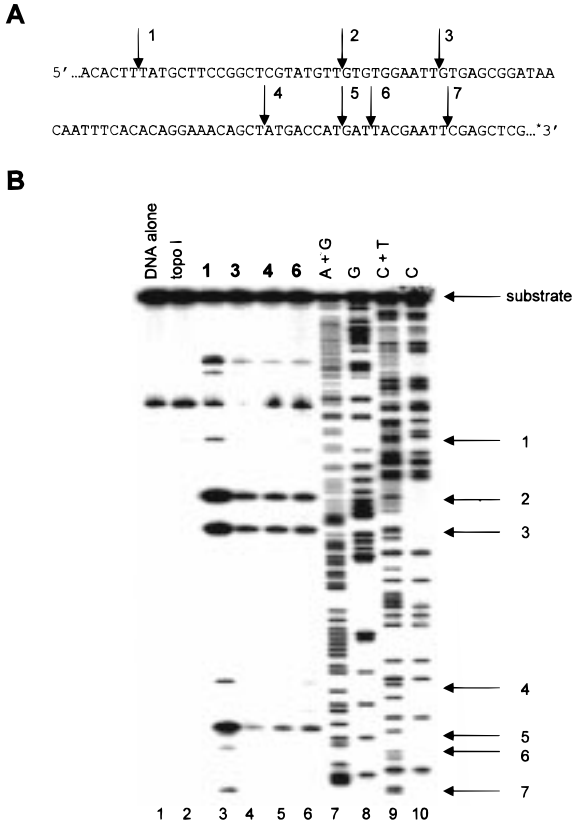

FIGURE 4: Effects of CPT analogues on human topoisomerase I-mediated cleavage of the *Hin*dIII−*Pvu*II restriction fragment of pSP64 plasmid DNA. (A) DNA sequence flanking the topoisomerase I cleavage sites. The cleavage sites are denoted by arrows. (B) Autoradiogram of a 10% denaturing polyacrylamide gel. The DNA substrate was 3′-^{32}P end labeled on the scissile strand. Human topoisomerase I-mediated cleavage reactions were carried out as described in Experimental Procedures. Cleavage sites are labeled as in panel A: lane 1, DNA alone; lane 2, DNA and topoisomerase I; lanes 3−6, topoisomerase I and 20 μM CPT (**1**), **3**, **4**, and **6**, respectively; lanes 7−10, Maxam−Gilbert sequencing reactions A + G, G, C + T, and C, respectively.

A DNA substrate containing a number of topoisomerase I cleavage sites was then used to further study the effects of CPT analogues on enzyme−DNA covalent binary complex formation (Figure 4). The *Hin*dIII−*Pvu*II restriction fragment of pSP64 plasmid DNA was prepared and 3′-^{32}P end labeled as described in Experimental Procedures. Human topoisomerase I-catalyzed cleavage reactions were carried out at 37 °C for 30 min in the presence of 20 μM compounds **1**, **3**, **4**, and **6**. As shown in Figure 4B, the 20-halo- and 20-aminoCPT analogues enhanced human topoisomerase I-mediated DNA cleavage (Figure 4B, lanes 4−6) at the same sites as CPT, but with different relative efficiencies (Figure 4B, lane 3) such that some of the bands were readily apparent only via amplification of band intensities on the phosphorimager. The topoisomerase I-mediated cleavage products, having free 5′-OH termini, migrated more slowly on the gel than the corresponding products resulting from the Maxam−Gilbert sequencing reactions. Consistent with previous observations (*38*), all cleavage sites had T at position −1, and the sites (2, 3, and 5) having a G at position +1 were the most efficient cleavage sites in the presence of each CPT analogue examined. In agreement with the results obtained using synthetic oligonucleotide DNA (Figure 3), 20-chloro- and 20-bromoCPTs produced about the same amount of

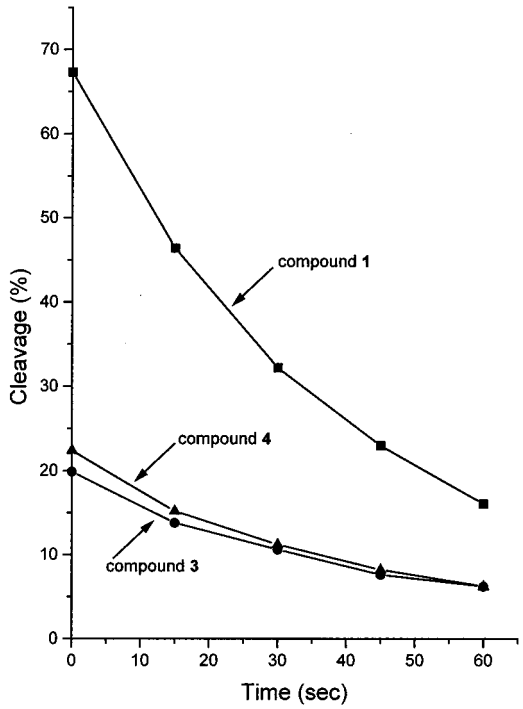

FIGURE 5: Phosphorimager analysis of the time course of religation of the DNA−topoisomerase I complexes formed in the presence of CPT analogues **1**, **3**, and **4**. The DNA substrate is shown in Figure 3A.

cleavage at the major cleavage sites as 20-aminocamptothecin (**6**).

Dissociation of CPT Analogues from the Inhibitor−Topoisomerase I−DNA Ternary Complexes following NaCl Treatment. The stability of the topoisomerase I−DNA covalent binary complexes was studied further by measuring the off-rates of the individual CPT analogues following NaCl treatment. The same oligonucleotide DNA duplex (Figure 3A) was utilized as a substrate in each case.

The DNA substrate was 5′-^{32}P end labeled on the scissile strand. The human topoisomerase I-mediated cleavage reaction was carried out at 37 °C for 60 min in the presence of 50 μM CPT analogues. Reaction mixtures were then maintained at 23 °C, and a NaCl solution was added to induce the reversal of formed ternary complexes (*38*−*40*) as described in Experimental Procedures. Aliquots were withdrawn at predetermined times. Following SDS−proteinase K treatment, the reactions were analyzed by 20% denaturing PAGE.

The time course of the dissociation of CPT and two derivatives (**3** and **4**) from the formed ternary complexes upon NaCl addition, and subsequent religation catalyzed by the covalently attached topoisomerase I, is shown in Figure 5. The dissociation of CPT analogues from the enzyme−DNA binary complexes was assumed to be rate-limiting for the overall religation reaction, as shown previously (*38*). The pseudo-first-order dissociation rate constants were obtained using the same method described previously (*38*); these are given in Table 1. Consistent with previous observations (*28*, *38*), the dissociation rates of the individual CPT analogues upon NaCl addition were not directly correlated with their efficiencies in stabilizing the topoisomerase I−DNA covalent binary complex. As can be seen from both Figure 5 and Table 1, although the 20-chloro- and 20-bromoCPTs (**3** and **4**) produced less ternary complex than CPT at a concentration of 50 μM, these CPT analogues had off-rates (18.2 and 20.0 $\times$ 10^{-3} s^{-1}) very close to that of CPT itself (23.4 $\times$ 10^{-3} s^{-1}) following NaCl treatment. In contrast, the racemic 20-aminoCPT analogue (**6**) stabilized topoisomerase I-mediated DNA cleavage to essentially the same extent as **3** and **4** (Figure 3B), but upon salt addition dissociated from the ternary complex too fast ($t_{1/2}$ < 5 s) to be measured.

Table 1: First-Order Rate Constants for Dissociation of CPT Analogues from the CPT−Topoisomerase I−DNA Ternary Complexes following NaCl Treatment

compound	rate constant k ($\times 10^{-3}$ s^{-1})[a]	compound	rate constant k ($\times 10^{-3}$ s^{-1})[a]
1	23.4	**4**	20.0
3	18.2	**6**	>139

[a] Topoisomerase inhibitors were employed at final concentrations of 50 μM. To facilitate measurement of the rate constants, the incubation mixtures were maintained at 23 °C after the addition of NaCl.

Effects of CPT Analogues on Topoisomerase I-Mediated Cleavage and Ligation of Nicked and Gapped DNA Substrates. The effects of CPT analogues on human topoisomerase I-mediated DNA structure rearrangements were examined by using nicked and gapped substrates (Figure 6) (*41*). Both substrates were 5′-^{32}P end labeled on the scissile strand. Reactions were carried out in the presence of 50 μM CPT analogues at 37 °C for 1 h (nicked substrate) or 15 min (gapped substrate). The reactions were then quenched by the addition of SDS (1% final concentration); the mixtures were treated with proteinase K (1 mg/mL, 37 °C, 60 min) and analyzed by 20% denaturing PAGE. The results are summarized in Table 2.

The ability of CPT to inhibit human topoisomerase I-mediated ligation across a three-nucleotide gap (62% extent of inhibition) was decreased significantly in the absence of the 20-hydroxyl group (compound **2**, 5% extent of inhibition), or when a 20-azido substituent was present (compound **5**, 16% extent of inhibition). Interestingly, reasonable inhibition was still noted in the presence of the halogenated analogues (21 and 37% extents of inhibition for analogues **3** and **4**, respectively) and in the presence of 20-aminoCPT (**6**, 32% extent of inhibition). As reported previously (*28*, *38*), topoisomerase I-mediated ligation across an 18-nucleotide gap was difficult to inhibit due to the putative intermediacy of a DNA hairpin that facilitates religation; in the absence of any inhibitor, religation occurred to the extent of 47% (Figure 2 of the Supporting Information). Therefore, it was not surprising that CPT analogues **3**−**6** failed to exhibit any inhibition of this process. However, consistent with the finding of differential effects of some inhibitors in individual assays (*38*), 20-deoxycamptothecin (**2**) exhibited better inhibition of religation of the gapped substrate than it did in either of the other assays employed here. Because it is known that 20-deoxyCPT can be oxidized by O_2 to afford CPT itself (*35*, *42*), all of the assays involving **2** that were carried out here employed samples of this inhibitor that had been freshly purified by HPLC and shown not to contain CPT.

Cytotoxicity of Camptothecin Analogues in the Yeast Assay. The cytotoxic effects of camptothecin analogues on yeast cells expressing human topoisomerase I were investigated using yeast strain RS321Nph-TOP1, as described in Experimental Procedures. This mutant strain of *Saccharo-*

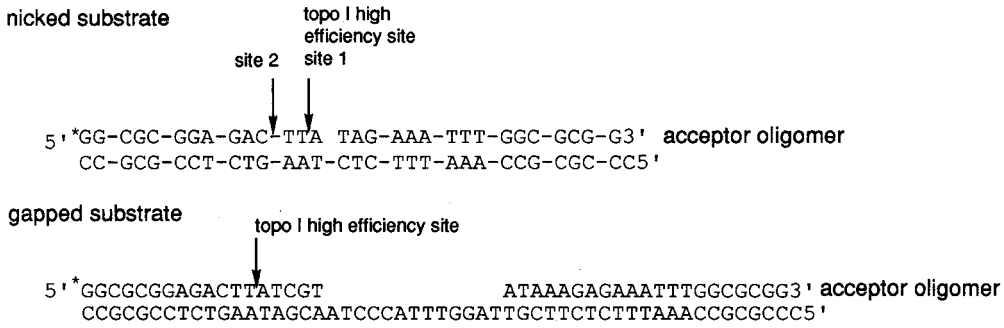

FIGURE 6: Structures of the nicked and gapped DNA substrates.

Table 2: Effects of Camptothecin Analogues on Human Topoisomerase I-Mediated Ligation of Nicked and Gapped DNA Substrates[a]

compound	extent of inhibition of ligation (%)[b]	
	nicked substrate	gapped substrate
1	62	47
2	5	13
3	21	0
4	37	0
5	16	0
6	32	0

[a] Topoisomerase inhibitors were employed at final concentrations of 50 μM. The nicked and gapped DNA substrates had the structures shown in Figure 6. [b] Inhibition of ligation after 60 min for the nicked substrate (site 2 ligation) and 15 min for the gapped substrate at 37 °C, relative to a control lacking any inhibitor.

Table 3: Human Topoisomerase I-Specific Cytotoxicity of Camptothecin Analogues

compound	IC_{50} (μM)[a]	compound	IC_{50} (μM)[a]
1	0.2	**4**	6.7
2	—[b]	**5**	—[b]
3	2.1	**6**	47

[a] Concentration that inhibited the growth of RS321Nph-TOP1 by 50% in minimal medium containing 3% galactose. [b] No cytotoxicity was observed at the highest concentration of the CPT analogue tested (100 mM).

myces cerevisiae lacks the homologous topoisomerase I, but harbors a plasmid containing the gene for human topoisomerase I under the control of a galactose promoter. DNA topoisomerase I activity and camptothecin sensitivity are detectable when the cells are grown in media containing galactose, but not glucose. Therefore, the IC_{50} values of camptothecin analogues were determined employing galactose as the carbon source for cell growth, as summarized in Table 3.

As shown in Table 3, the 20-deoxy- and 20-azidoCPTs (**2** and **5**) were not cytotoxic at the highest inhibitor concentration tested (100 μM). This is in agreement with the observation that these two CPT analogues showed very weak or no inhibition against purified human topoisomerase I in any of the cell free experiments described above. Although less cytotoxic than (20*S*)-CPT (**1**) itself (IC_{50} = 0.2 μM), the racemic 20-chloro- and 20-bromoCPTs (**3** and **4**) were still fairly active with IC_{50} values of 2.1 and 6.7 μM, respectively. However, the 20-aminocamptothecin (**6**), which stabilized the topoisomerase I−DNA covalent complex to the same extent as 20-haloCPT analogues **3** and **4** (Figures 3 and 4), had a significantly lower cytotoxicity (IC_{50} = 47 μM). While several factors could obviously contribute to the observed difference in cytotoxicity of **6**, the fact that this analogue had a greater off-rate from the formed ternary complex upon salt addition may well have contributed to the lower cytotoxcity of **6** compared to that of **3** and **4** (*43*).

DISCUSSION

DNA topoisomerase I has been identified as the major cellular target of the anticancer agent camptothecin (*5*, *7*, *44*). Camptothecin has been reported to bind neither to DNA alone nor to free topoisomerase I (*7*). A recent study has detected the binding of CPT analogues to two alternating DNA copolymers by UV and NMR spectroscopy, although the affinity constants were not reported (*45*). Binding studies with [^{3}H]camptothecin indicated that this compound binds reversibly to a DNA−topoisomerase I complex (*46*). The exact structure of the formed topoisomerase I−DNA−camptothecin complex is unknown, although two electrophilic camptothecin derivatives have been shown to alkylate the enzyme−DNA complex after CPT binding (*15*, *16*). This has provided some information about the orientation of CPT within the formed topoisomerase I−DNA−camptothecin ternary complex. The development of a plausible structural model for the ternary complex is important for understanding the nature of CPT binding, as well as for the design of novel inhibitors.

To date, most of the information pertinent to the nature of the ternary complex formed by topoisomerase I, DNA, and camptothecin has been derived from extensive structure−activity studies carried out using analogues of CPT (*8*−*14*). The characterizations of structurally altered human, yeast, and hamster topoisomerases I have also been reported (*17*−*20*); these have provided valuable insights into enzyme residues putatively involved in the binding of CPT. In the aggregate, these chemical and biochemical studies have provided a substantial body of data that help to define the nature of CPT binding by the topoisomerase I−DNA binary complex.

Recently, Hol and Champoux have reported the crystal structures of reconstituted and truncated human topoisomerases I in covalent and noncovalent complexes with a DNA substrate (*21*, *22*). Although the complexes employed for crystallographic analysis did not contain any bound inhibitor, analysis of the structures of the derived topoisomerase I−DNA complexes nonetheless permitted a camptothecin binding model to be proposed, as well as a model for DNA unwinding by topoisomerase I. Part of the camptothecin binding model is shown in Figure 7A, emphasizing the proposed stabilization of CPT E-ring interaction via

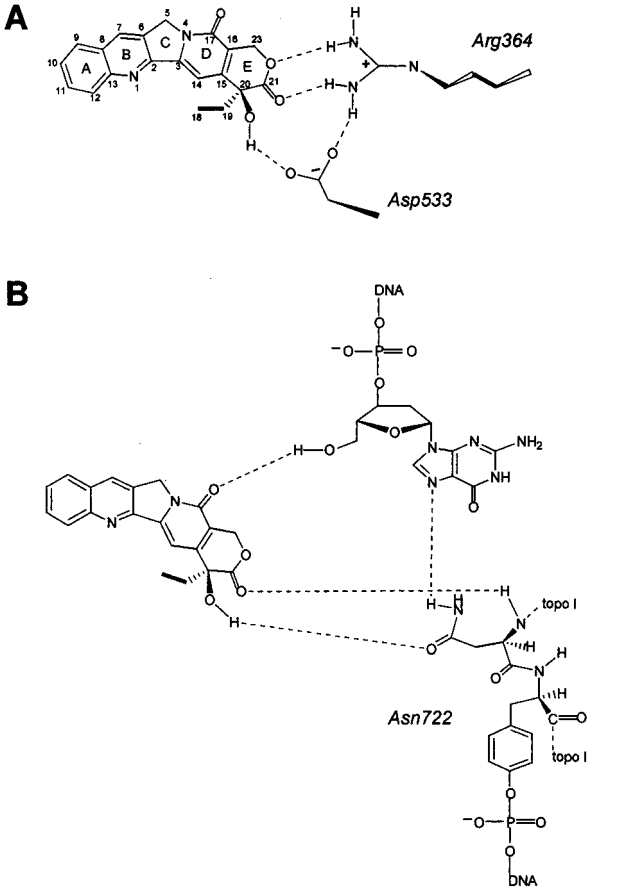

FIGURE 7: Role of the 20-OH group of CPT in the topoisomerase I−DNA binding models proposed by Redinbo et al. (*21*) (A) and Fan et al. (*23*) (B).

H-bonding interactions with Arg364 and Asp533. Particularly noteworthy is the proposal that the CPT 20-OH group participates in a donor hydrogen bond with the side chain carboxylate moiety of Asp533.

In another recent study, Fan et al. have developed a computational model for the binding of CPT to the topoisomerase I−DNA covalent binary complex (*23*). This model is a "drug stacking" model in which the CPT is pseudointercalated at the topoisomerase-linked DNA cleavage site, and is consistent with a number of experimental observations. The latter include the relative potencies of a number of CPT analogues and the fact that an Asp722Ser mutant has been found to be resistant to CPT (*47*). Eighteen different CPT derivatives with varying potencies were docked into the proposed CPT binding site to test the model and permit its refinement. The refined model also utilizes the 20-OH group of CPT as a participant in a donor hydrogen bond, in this case with the side chain carboxamide moiety of Asn722 (Figure 7B).

Since both models posit the involvement of the 20-OH group of CPT as a H bond donor, we sought to test the models through the preparation and assay of a series of 20-substituted CPT analogues (Figures 1 and 2). It may be noted that Pommier et al. (*48*) have also suggested that the CPTs may function as inhibitors by participating in topoisomerase I-mediated opening of the E-ring of CPT with concomitant covalent attachment of a nucleophilic group on the enzyme to the C-21 lactone moiety of CPT. CPT analogues that cannot undergo facile lactone ring opening, such as 20-deoxycamptothecin (**2**), are not good enzyme inhibitors. This suggests that the 20-OH group might also be participating in a hydrogen bonding interaction with the lactone carbonyl oxygen atom, thereby increasing the electrophilicity of the lactone.

The relative abilities of individual CPT derivatives **1**−**6** to enhance cleavage of the DNA duplex substrate shown in Figure 3A by human topoisomerase I are not entirely consistent with the models proposed. The 20-aminoCPT analogue (**6**) would be thought to be capable of utilizing the 20-NH_2 substituent in H bond donor interactions in a fashion analogous to that of the 20-OH group of CPT. In fact, this analogue, which is essentially a 1:1 mixture of 20*R* and 20*S* isomers, produced about one-third of the DNA cleavage obtained with (20*S*)-CPT when both were employed at a concentration of 20 or 50 μM (Figure 3). However, the off-rate of CPT analogue **6** from the formed ternary complex upon salt addition was much faster than that of CPT itself (Table 1). Significantly, 20-haloCPT analogues **3** and **4** were essentially as potent as 20-aminoCPT (**6**) at stabilizing the topoisomerase I−DNA covalent binary complex at the tested concentrations of 20 and 50 μM, although neither **3** nor **4** can utilize its 20-substituent in a donor H bonding scheme. Further, CPTs **3** and **4** actually exhibited slower off-rates from the formed enzyme−DNA ternary complexes following NaCl treatment than CPT itself (Table 1). The functional similarity of the analogues was further confirmed by investigating human topoisomerase I-mediated cleavage of a DNA restriction fragment (Figure 4). The analogues produced similar extents of cleavage at the same sites as CPT (**1**) itself. On this basis alone, it is clear that the proposed models require further refinement.

Also of interest are the results obtained with CPTs **1**−**6** in assays in which the rearrangement of nicked and gapped DNA substrates by topoisomerase I was monitored. As shown in Table 2, the 20-substituted CPTs all inhibited the conversion of the nicked substrate to a duplex product having a three-nucleotide deletion on the scissile strand. The relative potencies of the individual inhibitors were roughly the same as those noted for ternary complex stabilization (cf. Table 2 and Figure 3), although the 20-chloroCPT analogue was somewhat less potent at inhibiting the rearrangement of the nicked DNA substrate. Interestingly, only CPT itself exhibited reasonable potency in inhibiting the topoisomerase I-mediated rearrangement of DNA structure across an 18-nucleotide gap (Table 2). Apparently, the 20-OH group of CPT is very important in preventing the occurrence of this particular DNA rearrangement. The fact that the several CPT derivatives tested here affect the individual topoisomerase I-mediated processes studied in a differential fashion argues that the interaction of the 20-OH group of CPT with the enzyme−DNA covalent binary complex depends critically on the specific structure of the formed binary complex.

Aside from the implications of the present findings for the nature of CPT binding to the topoisomerase I−DNA covalent binary complex, it seems to be important to characterize the behavior of CPTs **2**−**6** as cytotoxic agents in cells having defined and controllable levels of DNA topoisomerase I. This is underscored by our determination of the cytotoxicity of CPT analogues **1**−**6** in a yeast strain lacking the homologous topoisomerase I, but containing the gene for human topoisomerase I under the control of an

inducible promoter (*49*). As can be seen from the IC_{50} values of camptothecin analogues, drug-induced cytotoxicity is not directly proportional to the ability of these compounds to stabilize the covalent binary complex. The off-rates of the analogues from the topoisomerase I−DNA−CPT ternary complex (Table 1) may play an important role, leading to significant differences in cytotoxicity between 20-amino- and 20-haloCPT analogues. Given the fact that camptothecin showed markedly different effects on topoisomerase I-mediated DNA structure rearrangements compared to other CPT analogues (*38*), the specific enzyme−DNA−inhibitor interactions characteristic of each CPT analogue may also determine the overall cytotoxicity of the compounds.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing the effect of 2 molar equiv of $Eu(hfc)_3$ on the ^1H NMR spectrum of putative (20*R*,*S*)-**1** and the effects of CPT analogues on the inhibition of religation of the gapped DNA substrate by topoisomerase I. This material is available free of charge via the Internet at http://pubs.acs.org.

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