2 Crystallographic Insight Into the Mechanism of Drug-Induced Topoisomerase I DNA Damage

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1. BACKGROUND

Topoisomerase I (TOP-I) is an essential eukaryotic enzyme that acts to remove supercoils generated during transcription and DNA replication *(1)*. Because of the size of the eukaryotic chromosome, removal of these supercoils can only be accomplished locally by introducing breaks into the DNA helix. Being a type 1 enzyme, TOP-I mediates DNA relaxation by creating a transient, single-strand break in one strand of the DNA duplex. This tran-

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sient nicking allows the broken strand to rotate around its intact complement, effectively removing local supercoils. Strand nicking results from the transesterification of an active-site tyrosine (Tyr723 in the human TOP-I) at a DNA phosphodiester bond forming a 3'-phosphotyrosine covalent enzyme–DNA complex. The covalent intermediate is reversed when the released 5'-OH of the broken strand reattacks the phosphotyrosine intermediate in a second transesterification reaction *(1)*. The rate of relegation is normally much faster than is the rate of cleavage *(2)*. This ensures that the steady state concentration of the covalent 3'-phosphotyrosyl TOP-I–DNA complex is extremely low. Several DNA lesions and drugs, however, have been shown to stabilize the covalent 3'-phosphotyrosyl intermediate *(3)*. For example, camptothecin (CPT) is a natural product that was originally discovered because of its antitumor activity *(4)* and was later demonstrated to promote the accumulation of TOP-I-DNA adducts in vitro and in vivo *(5,6)*. It is generally believed that CPTs act to convert TOP-I into a DNA-damaging agent by binding the covalent 3'-phosphotyrosyl intermediate and, specifically, blocking DNA relegation *(7,8)*. Topo I is the sole intramolecular target of CPT and the cytotoxic effects of CPT poisoning are S-phase-specific *(9)*. Both in vitro and in vivo data support the idea that during DNA replication, the replication complex can collide with the "trapped" TOP-I-DNA complex, resulting in a double-strand break and subsequent apoptotic cell death *(10)*. Presumably, these compounds have anticancer activity because rapidly dividing cells (e.g., cancer cells) enter S-phase more frequently than do normal cells.

2. THE TERNARY TOP-I–DNA–DRUG COMPLEX

It has been extremely difficult to study the mechanism of CPT activity because the drug acts as an uncompetitive inhibitor and binds only to the transient enzyme-substrate complex *(7,11)*. There is no reported equilibrium binding constant for any TOP-I poison. To overcome this fundamental problem, we have used DNA substrates containing a 5'-bridging phosphorothioate linkage to covalently trap the enzyme-substrate complex *(12)*. TOP-I-mediated cleavage of these substrates generates a 5'-sulfhydryl, instead of a 5'-hydroxyl, which is inert in subsequent ligation reactions. These substrates have allowed the crystallization of human TOP-I covalently joined to duplex DNA in the absence (3.2 Å) and presence (2.1 Å) of topotecan, a clinically approved CPT analog (Hycamtin®) *(13)*.

A comparative analysis of these structures demonstrates that topotecan intercalates at the site of DNA cleavage, forming base-stacking interactions with both the –1 (upstream) and +1 (downstream) base pairs. The planar five-membered ring system of topotecan mimics a DNA base pair in the DNA duplex and occupies the same space as the +1 base pair in the structure without drug bound (Figs. 1,2). The intercalation binding site is created by conformational changes at the phosphodiester bond between the +1 and –

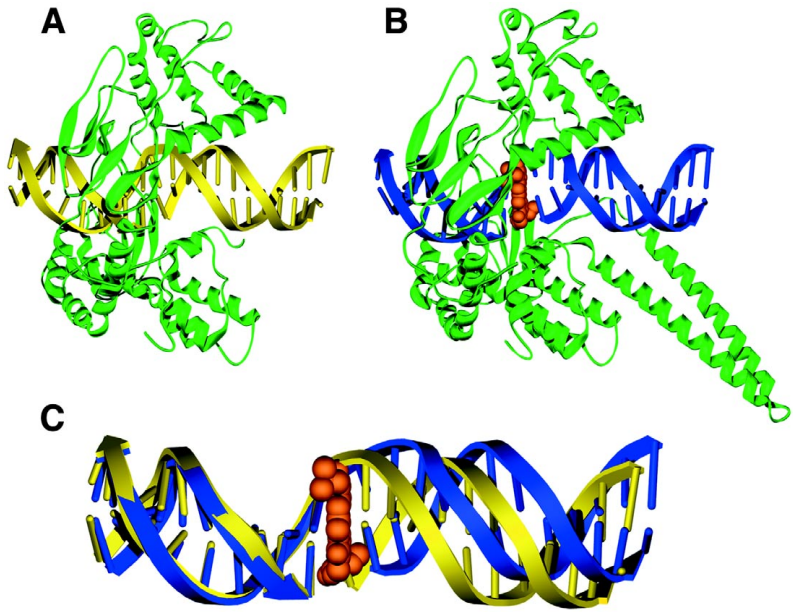

Fig. 1. Model of topoisomerase I (TOP-I)–DNA complex without **(A)** and with bound topotecan **(B)** is diagrammed with protein (green), DNA unbound (**A**: yellow), DNA-topotecan bound (**B**: blue), and topotecan (orange, CPK representation). A comparison of the topotecan ternary complex structure to the non-topotecan structure reveals minor differences in the overall C-α backbone trace with an RMSD = 1.33 Å, excluding C-terminal linker residues Gln633-Gln697, which were not visible in the electron density of the non–drug-bound protein. The 2.1 Å drug-bound structure represents the most complete TOP-I structure reported to date, providing visible electron density from Gln201 to the COOH-terminal Phe765. The specific activity and sensitivity to camptothecin of topo70 is indistinguishable from that of the full-length native human TOP-I *(34,35)*. Previously reported crystal structures of human TOP-I include the inactive Tyr723Phe versions of topo70 and topo58/6.3 (a reconstituted linkerless enzyme) in noncovalent complex with DNA, and topo58/6.3 in covalent complex with DNA *(29,36)*. Each of these structures contained unresolvable portions of the protein in the connector region (Pro635–Phe640). Moreover, the reconstituted enzyme has altered kinetics and is not sensitive to camptothecin in a plasmid relaxation assay *(35)*. Hence, the structures reported here are the first structures of a fully active human TOP-I in covalent complex with DNA in the absence and presence of bound drug. **(C)** Comparison of the 22mer duplex oligonucleotides of the drug-bound (blue) and nondrug-bound (yellow). Topotecan (orange, CPK) binds to the enzyme-substrate complex by intercalating in the DNA. Intercalation is accommodated by unwinding of the DNA and translation downstream of the cleavage site. The binding pocket is stabilized primarily by contacts between the enzyme and DNA substrate. A detailed analysis of the contacts between the ternary complex of TOP-I and DNA reveals a total of 36 direct protein-DNA contacts and 6 additional water-mediated protein-DNA contacts.

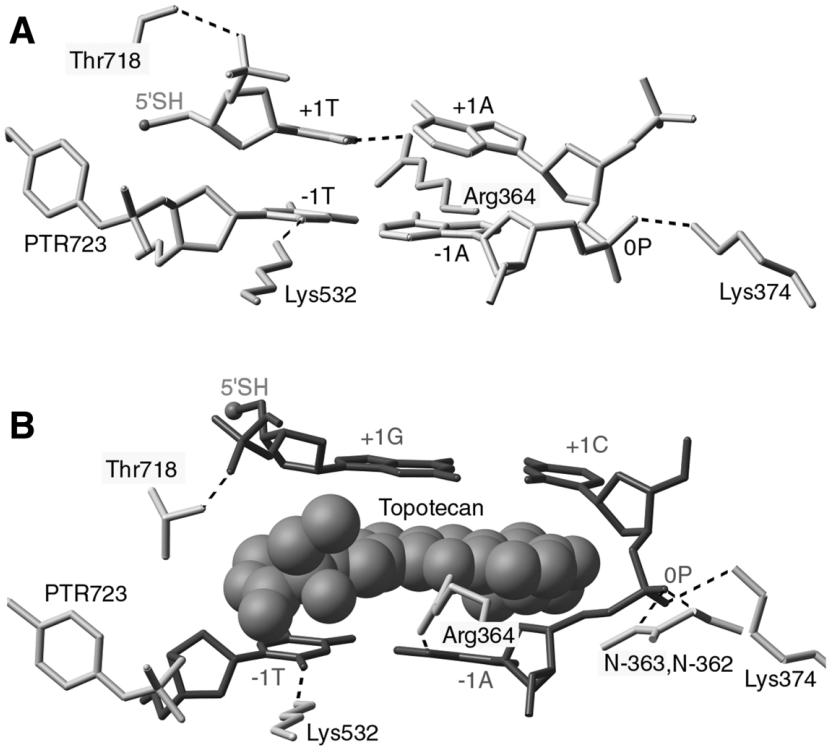

Fig. 2. Topotecan binding pocket. **(A)** Molecular diagram showing the nondrug-bound topoisomerase I–DNA complex. The +1 and –1 bases of the duplex DNA are shown making four contacts to the surrounding protein to stabilize the protein-DNA complex. **(B)** Topotecan (CPK) intercalates between the +1 and –1 bases of the duplex DNA (stick). Six protein contacts stabilize the open form of the DNA. Topo70 residues, whose mutation leads to drug resistance, are highlighted with gray boxes. 5'SH of the +1 G is indicated and the covalent phosphotyrosine attachment to DNA is shown between PTR723 and the –1 T of the cleaved strand. Mobile phosphodiester of the intact DNA strand is labeled 0P.

1 base pairs of the uncleaved strand (0P). This conformational change in the DNA requires only a minimal change within the protein (Fig. 1). The intercalation pocket, however, is stabilized by several protein-DNA interactions (Fig. 2). The hydroxyl of Thr718 makes a hydrogen bond contact with the nonbridging phosphodiester oxygen of guanosine at position +1 of the cleaved strand (+1G). Arg364 makes a hydrogen bond contact with N3 of adenosine at position –1 of the uncleaved strand (–1A). Lys532 makes a hydrogen bond with the oxygen of thymidine at position –1 (–1T). Lys374 and the main chain nitrogens of 362 and 363 make hydrogen bond contacts

with the nonbridging phosphodiester of the uncleaved strand (0P). Consistent with the observed drug-binding mode, mutations at position Arg364 *(14)* would be expected to destabilize the topotecan binding site and are known to result in camptothecin resistance. Lys532 acts as the general acid during strand cleavage *(15)*, and mutations could not result in camptothecin resistance because these mutations produce an inactive enzyme. Interestingly, substitution of alanine for threonine at position 718 stabilizes the enzyme-DNA complex even in the absence of camptothecin *(16)*.

The intercalation binding mode explains how CPTs specifically block DNA relegation, because the binding/intercalation results in a 3.4 Å shift of the downstream duplex and displaces the reactive 5'-OH of the cleaved strand 10 Å away from the phosphotyrosine (Fig. 2). For a relegation event to occur, the topotecan molecule must be released from the nicked DNA and diffuse out of the complex. The results also explain why CPTs bind the enzyme-substrate complex, but do not bind the enzyme or substrate alone; the topotecan-binding pocket is located within the DNA substrate, but this binding site can only form after TOP-I-mediated cleavage. Approximately 380 $Å^2$ of the total 626 $Å^2$ solvent-accessible portion of topotecan, or 61% of the drug surface, is covered by base stacking interactions with DNA. An additional 27% of the solvent-accessible region of topotecan is covered by protein side chains; the remaining 11% is solvent accessible. The single direct-protein contact mediated by Asp533 that hydrogen bonds to the 20(S) hydroxyl represents only 5% of the total solvent accessible surface of topotecan.

The E-ring is known to be in equilibrium between the closed lactone form and a hydrolyzed open carboxylate form *(4)* (Fig. 3A). It is therefore not surprising that difference Fourier maps of the ternary TOP-I–DNA-topotecan structure demonstrate the presence of both the open and closed E-ring conformers of topotecan (Fig. 3B). An unrestrained full matrix refinement of occupancy factors *(17)* (with all positional and thermal parameters fixed) for the closed lactone and open carboxylate versions of topotecan converge to an occupancy of 63% (standard uncertainty 7%) closed lactone and 37% (standard uncertainty 7%) open carboxylate conformers.

It is not possible to determine the relative affinities of open (carboxylate) versus closed (lactone) forms of topotecan based on a crystal structure; however, the model demonstrates that both conformers can bind within the same intercalation pocket (Fig. 3C) and presumably inhibit relegation. There may be differences in the binding affinities of the lactone and carboxylate forms; however, it is unlikely that this difference would be apparent in the crystals because the crystallizations were performed at extremely high topotecan concentrations (0.1 m*M*). This concentration is probably well above the K_D of both the lactone and the carboxylate, therefore both would be expected to bind. In addition, it is not possible to determine if there

A
B
C
OH⁻
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is preferential binding of the carboxylate or lactone forms because it is not possible to determine the true equilibrium ratio within the micro-environment of the active site.

3. DRUG INTERACTIONS

Stacking interactions with the +1 and –1 base pairs are one the primary forces stabilizing topotecan in the ternary complex. This feature may explain the preference for a G:C base pair at the +1 position of sites stabilized by camptothecins and may also explain why modifications that disrupt the planar ring system eliminate drug binding. There are relatively few hydrogen bonds stabilizing the drug (Fig. 4). Two water-mediated hydrogen bonds assist in coordinating topotecan: the oxygen of the D-ring pyridone makes a water mediated contact to Asn722, and the C-21 oxygen of the E-ring is bridged by a water molecule to the phosphotyrosine (Tyr723). In the carboxylate model, the 22-hydroxyl is 2.7 Å from the R-group of Asn722, and the 21-carboxylate oxygen is 2.8 Å from Lys532, a known catalytic residue *(15)*. The 20(S)-hydroxyl can still coordinate Asp533, and can make an additional hydrogen bond contact (3.1 Å) to the ε-nitrogen of Arg364 (Fig. 4). Consistent with the proposed structural model, mutations at residues Asp533, Arg364, and Asn722 would be expected to destabilize the bound drug and enzymes with mutations at these positions are resistant to camptothecin *(14,18,19)*.

The observed Asp533:20-(OH) interaction is the only hydrogen bond contact made in both the lactone and carboxylate models and emphasizes the importance of the 20-(OH) for CPT activity. Many studies demonstrate

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Fig. 3. *(opposite page)* Topotecan electron density. **(A)** Topotecan with reversible hydrolysis of the base-labile E-ring from the closed lactone conformation to the open carboxylate form is diagrammed. **(B)** Left panel: 3.0 σ $|F_o|–|F_c|$ electron density map calculated with the lactone form of topotecan (100% closed E-ring) is diagrammed. Negative electron density (indicated by contours) is seen in the vicinity of the lactone oxygen, and positive (indicated by contours) electron density peaks are located nearby. **(B)** Right panel: 3.0 σ $|F_o|–|F_c|$ electron density calculated with the carboxylate form of topotecan (100% open E-ring) is diagrammed. Negative electron density (contoured) surrounds the terminal hydroxyl and carboxylic acid moieties, whereas a positive (contoured) electron density peak is in the location of what would be the lactone oxygen in the closed E-ring conformation. **(C)** 3.0 σ $|F_o|–|F_c|$ omit map of electron density for topotecan is illustrated. The electron density map reveals that both the lactone (left panel) and carboxylate (right panel) forms of the E-ring are present in the crystal structure. The E-ring of topotecan is oriented toward the phosphotyrosine. The c-9-dimethyl-amine group of topotecan projects into the major groove of the B-form DNA duplex. The c-20-ethylene group of the E-ring faces into the minor groove.

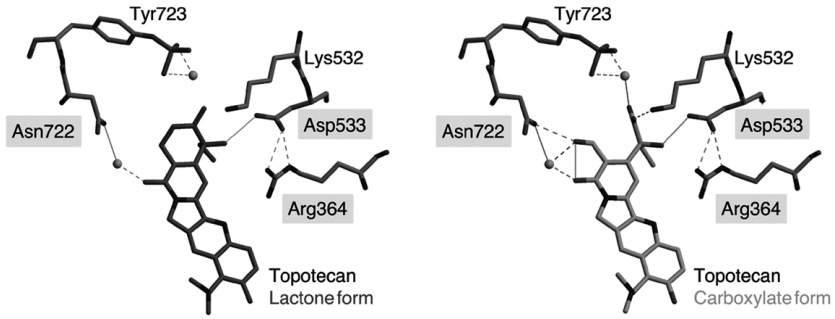


Fig. 4. Mode of topotecan binding. Topotecan interactions with protein side chains for the lactone (panel A) and carboxylate (panel B) forms of the drug. Hydrogen bonds (predicted by contact distance and geometry of the refined atomic positions) are shown as solid lines. Contacts less than 3.0 Å between polar atoms are shown as dashed lines. Labels for residues that, if mutated, produce a camptothecin resistant enzyme are highlighted in gray boxes. The oxygen atoms of water molecules are depicted as spheres. The left and right panels are oriented as a stereo pair so that a pseudostereo overlap of the lactone and carboxylate forms of topotecan can be achieved.

the importance of the 20-(OH) for CPT activity. For example, the 20(R) stereoisomer of CPT is inactive *(20,21)*. This is explained by the crystal structures that predict that the 20(R) ethyl group would sterically clash with the side chains of Asp533 and Lys532 (Fig. 5), and that the 20(R)-OH would also not be able to hydrogen bond with Asp533. Hertzberg et al. reported that if 20(S)-OH is substituted for hydrogen or acetate, no covalent complex accumulated in vitro *(22)*. The acetate modification would sterically clash with Asp533 and both modifications would eliminate the observed hydrogen bond contact. Similar observations were made by Wang et al., who have shown that conversion of the 20(S)-OH to 20(S/R)-H eliminates the accumulation of TOP-I–DNA covalent complex in vitro *(23)*. The difficulty in interpreting these data is that the 20-H modification would also be expected to prevent or minimize E-ring opening. These modifications would therefore be expected to simultaneously eliminate the 20-hydroxyl:Asp533, the 21-keto:Lys532, and the 22-hydroxyl:Asn722 hydrogen bond interactions of the carboxylate form of topotecan. Fortunately, Wang et al. have also synthesized analogs in which the 20-hydroxyl was replaced with chlorine or bromine. The advantage of these substrates is that they would be expected to eliminate the 20-hydroxyl:Asp533 interaction; however, these analogs should still allow E-ring opening and therefore should still allow the 21-keto:Lys532 and the 22-hydroxyl:Asn722 hydrogen bond interactions. Indeed, the 20-Cl and 20-Br have intermediate effects on in vitro stabilization

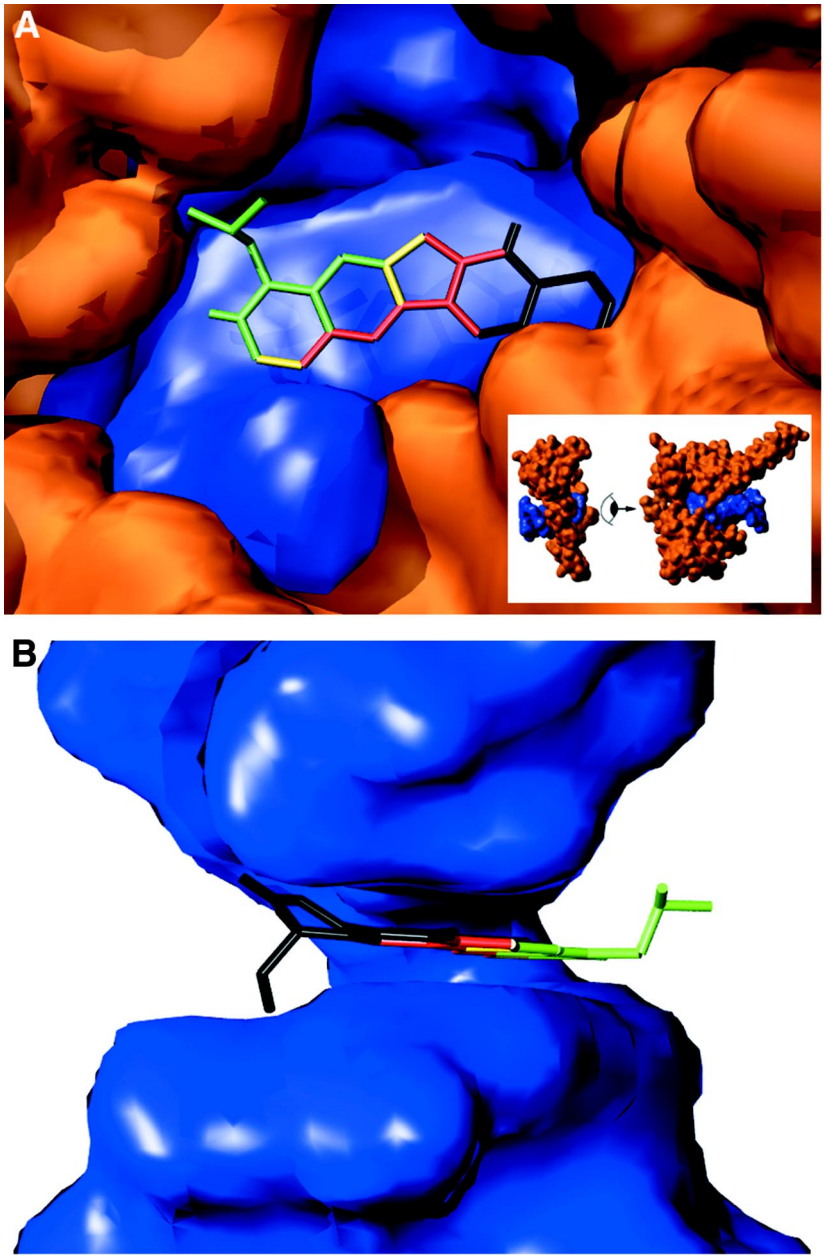
of the covalent complex *(23)*, and provide circumstantial evidence that E-ring opening is important for CPT binding and activity.

It is widely believed that the closed lactone E-ring is essential for inhibition of TOP-I *(24)*, and several analogs have been synthesized that stabilize the lactone form. For example, homocamptothecin is a seven-member E-ring analog that has in vitro and in vivo activity *(25)*. This analog still contains an appropriately positioned 20-(OH) group, and the slightly larger ring can still fit within the intercalation binding pocket. This E-ring modification results in a slower rate of hydrolysis (formation of carboxylate); however, the equilibrium is actually shifted in favor of formation of the carboxylate over the lactone *(26)*. In addition, conversion of the E-ring lactone to a lactam prevents E-ring opening and simultaneously destroys in vitro activity of the drug *(22)*. In light of the structural model, these results suggest that opening of the E-ring is actually necessary for activity. There is experimental evidence for E-ring opening on formation of the ternary protein–DNA–drug complex *(27)*; in vitro activity *(21)* and in vivo activity *(28)* of the carboxylate form have been previously reported.

The structures also provide an explanation for the observed structure-activity relationships that improve in vivo efficacy. For example, previous functional analyses have demonstrated that a large number of modifications can be placed at positions 7, 9, and 10 of CPT *(24)*, and in some cases these modifications can increase in vivo activity. The structural model demonstrates that these positions face into the major groove of the DNA and modifications that improve solubility or stability would not sterically interfere with drug binding (Fig. 5).

4. POISON EFFECTS ON DNA RELAXATION

TOP-I has been proposed to relax DNA via a mechanism of "controlled rotation" in which the DNA duplex located downstream of the cleavage site rotates around the phosphodiester bond (0P) between the +1 and –1 base pairs of the uncleaved strand, effectively passing the unbroken strand through the single-strand break with each complete rotation event *(29)*. The rotation event is thought to occur in a controlled manner because the simplified models for the trajectory of the DNA during rotation predict that the rotating DNA, located downstream of the cleavage site, will experience transient electrostatic interactions with positively charged regions of the TOP-I enzyme that are in close proximity to the downstream DNA *(29)*. The protein encircles the DNA, and the linker (residues Gln633 to Gln697) and nose cone (residues Thr303 to Glu337) domains of TOP-I contain a variety of positively charged residues that are likely to contact the DNA during rotation. Within the framework of the controlled rotation model for DNA relaxation, intercalative camptothecin binding would not necessarily be expected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to have any affect on the speed with which DNA is relaxed. In fact, one might predict that, by inhibiting relegation, TOP-I poisons might be expected to enhance the rate of supercoil release. On the contrary however, it has been shown that TOP-I poisons such as camptothecin actually inhibit the rotation/relaxation process in vitro *(30)*.

It is also known that the inhibitory effects of camptothecin on DNA relaxation can only be observed with relatively high micromolar inhibitor concentrations, as compared with the much lower nanomolar concentrations of CPT needed to observe stabilization of the covalent complex in a typical detergent-mediated DNA breakage assay *(31,32)*. This observation is often interpreted as being a nonspecific inhibitory effect of large concentrations of inhibitor molecules binding nonspecifically to TOP-I. However, our structural observations suggest an alternative explanation, wherein the intercalative binding of inhibitor to the TOP-I–DNA covalent complex places constraints on DNA rotation that would otherwise not be present. That large concentrations of inhibitor are required to observe an inhibitory effect on plasmid relaxation is actually anticipated because the plasmid molecules (approximately 1–2 Kb supercoiled circles) used in the assay can be fully relaxed by a single TOP-I molecule, and there are thousands of possible TOP-I binding sites on a plasmid. As such, for an inhibitory effect on relaxation to be observed, a substantial proportion of all TOP-I–DNA

Fig. 5. *(opposite page)* Model of topotecan binding pocket. **(A)** Molecular surface diagram of both the protein (orange) and DNA (blue) of the topotecan binding pocket. The protein–DNA complex has been separated to show a view of the topotecan binding pocket (*see* inset). The convex surface of the topotecan faces into a pocket within the protein–DNA complex. The bonds of the topotecan molecule have been color-coded to represent the structure activity relationship (SAR) of chemical derivatives of the camptothecin scaffold. Bonds that are absolutely essential, such as the E-ring, and D-ring pyridole are colored black. Bonds in which additive moieties decrease inhibitory activity are colored red. Bonds in which additive moieties have a mixed effect on inhibitory activity (sometimes increasing, sometimes decreasing) are colored yellow. Bonds in which additive moieties can increase inhibitory activity are colored green. Although position 5 of the camptothecin scaffold is positioned toward the open pocket, substituents at this position do not increase inhibitory activity. It is likely that this is due to the sp3 stereochemistry at this position. Rather than planar extensions, as at sp2 positions 7 and 9, substituents at position 5 would project up or down into the surrounding DNA and thus disrupt the base stacking interactions that topotecan makes with both upstream and downstream DNA, shown in **(B)**. Molecular surface of DNA shown with protein removed. Topotecan intercalates the cleaved DNA and is tightly sandwiched between the upstream and downstream base pairs.

binding events would have to be rendered ineffective before a significant effect on relaxation could be observed. It logically follows that large concentrations of inhibitors will be required to observe an effect on plasmid relaxation.

Thus it has been a mystery how camptothecins stabilize the nicked complex but prevent DNA relaxation, because nicked DNA should be able to rotate and allow DNA relaxation *(30)*. To explain how intercalative binding of a TOP-I could inhibit both relaxation and relegation, one could invoke the idea that the rotating DNA may somehow clash with the bound poison. Alternatively, the intercalative binding of TOP-I poison may place constraints on rotatable bonds within the phosphodiester backbone of the uncleaved strand within the vicinity of drug binding. Indeed, a comparison of the unbound and CPT-bound structures shows that topotecan displaces the critical 0P phosphodiester bond and results in several interactions that could inhibit rotation (Fig. 6). In the drug-bound structure, Phe361 is positioned closely underneath the +1 phosphodiester and would be expected to sterically hinder rotation at 0P. Phe361Ser mutants are resistant to camptothecin poisoning of DNA relaxation *(14)*. In addition, drug binding and displacement of the +1 phosphodiester bond causes the nonbridging oxygens to form hydrogen bonds with the main chain nitrogens of residues Gly363 and Arg364. A hydrogen bond contact to Lys374 is present in both structures. The tight positioning of 0P against the peptide backbone of the protein effectively restrains three (α, β, γ) of the five potentially rotatable backbone bonds *(33)*. This tight packing arrangement would be expected to prevent the downstream DNA from rotating about 0P. This packing arrangement would not eliminate all possible DNA rotation; for example, rotation could still occur at the +2 (or +3, and so on) phosphodiester. However, additional base-pair hydrogen bond interactions would have to be broken to allow this rotation. Alternatively, rotation could still occur at +1 because two rotatable bonds are not hindered. However, in both cases, the trajectory of the rotating DNA would be significantly altered, and this would require significant conformational flexibility that is not likely to be present in the protein.

It is also important to note that the DNA must unwind or open through conformational changes at 0P to create the topotecan binding site. This suggests that the partially unwound DNA conformation observed in the ternary complex may represent a conformational intermediate that normally forms during the unpoisoned catalytic reaction. For example, the open conformation may be the first step of DNA relaxation; after DNA cleavage, conformational changes at 0P would break the stacking interactions between the +1 base pair and the –1 base pair and could facilitate unwinding. It is important to note that this opening is clearly stabilized by several protein–DNA interactions (Fig. 6).

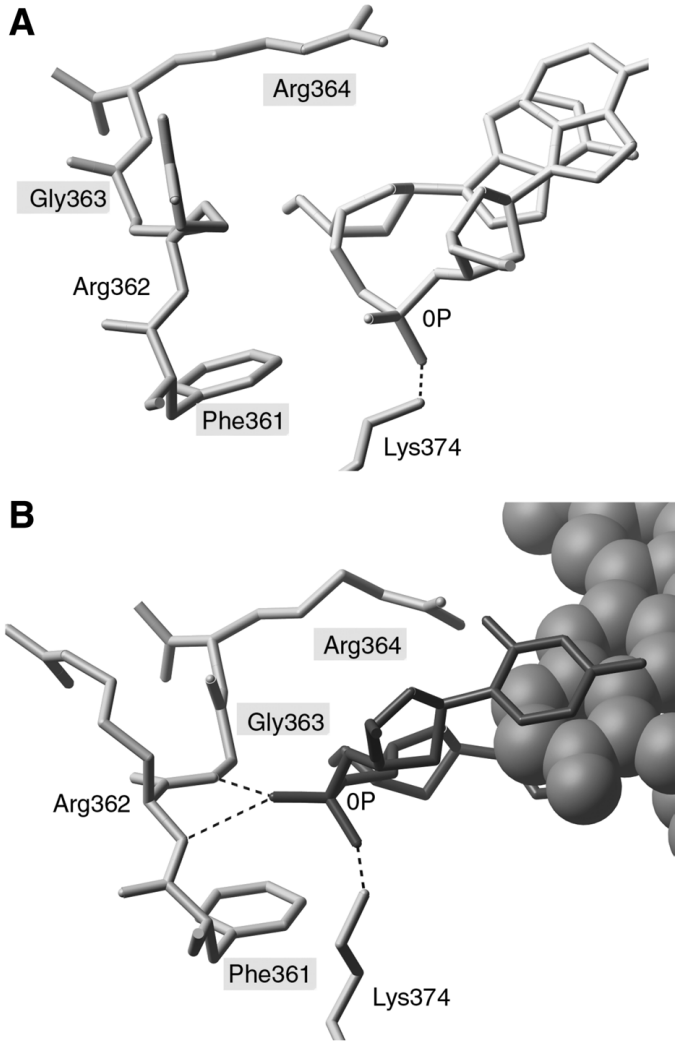

Fig. 6. Topotecan inhibits DNA relaxation. **(A)** Molecular diagram showing the nondrug-bound topoisomerase I–DNA complex. The +1 and –1 bases of the intact DNA strand are shown making a contact to Lys374 of the surrounding protein. **(B)** Topotecan (CPK) intercalates between the +1 and –1 bases of the duplex DNA (stick). Three protein contacts stabilize the open form of the intact DNA strand. Topo70 residues, whose mutation leads to drug resistance, are highlighted with gray boxes. Mobile phosphodiester of the intact DNA strand is labeled 0P.

5. CONCLUSIONS

In conclusion, the 2.1 Å structure of topotecan bound to the TOP-I–DNA covalent complex solves a 40-year mystery of how the camptothecins bind to their molecular target. The structures explain why the drugs only bind the enzyme-substrate complex and specifically block both DNA relegation and relaxation. The drug binds to the complex by intercalating between DNA bases of both strands at the enzyme-induced nick and makes specific hydrogen bond contacts with both the DNA and the enzyme. The ternary structure demonstrates that topotecan is tightly wedged against the protein and phosphodiester backbone that could prevent DNA rotation. Close examination of the ternary complex also indicates that the bound drug exists in both the closed lactone and open carboxylate forms. This result is important because it has been generally agreed that the E-ring open carboxylate form is inactive in vivo. This result demonstrates that the E-ring is in equilibrium between lactone and carboxylate forms when bound to the TOP-I–DNA complex and raises the possibility that both forms can poison the reaction.

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