Structures of Three Classes of Anticancer Agents Bound to the Human Topoisomerase I−DNA Covalent Complex

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Human topoisomerase I (top1) is the molecular target of a diverse set of anticancer compounds, including the camptothecins, indolocarbazoles, and indenoisoquinolines. These compounds bind to a transient top1−DNA covalent complex and inhibit the resealing of a single-strand nick that the enzyme creates to relieve superhelical tension in duplex DNA. (Hertzberg, R. P.; et al. *Biochem.* **1989**, *28*, 4629−4638. Hsiang, Y. H.; et al. *J. Biol. Chem* **1985**, *260*, 14873−14878. Champoux, J. J. *Annu. Rev. Biochem.* **2001**, *70*, 369−413. Stewart, L.; et al. *Science* **1998**, *729*, 1534−1541.) We report the X-ray crystal structures of the human top1−DNA complex bound with camptothecin and representative members of the indenoisoquinoline and indolocarbazole classes of top1 poisons. The planar nature of all three structurally diverse classes allows them to intercalate between DNA base pairs at the site of single-strand cleavage. All three classes of compounds have a free electron pair near Arg364, a residue that if mutated confers resistance to all three classes of drugs. The common intercalative binding mode is augmented by unexpected chemotype-specific contacts with amino acid residues Asn352 and Glu356, which adopt alternative side-chain conformations to accommodate the bound compounds. These new X-ray structures explain how very different molecules can stabilize top1−DNA covalent complexes and will aid the rational design of completely novel structural classes of anticancer drugs.

Introduction

Topoisomerases are ubiquitous enzymes that relieve the torsional stress in the DNA helix that is generated as a result of replication, transcription, and other nuclear processes.[5] All topoisomerases act through a conserved active-site tyrosine residue to cleave the phosphodiester backbone and form a covalent phosphotyrosine intermediate with the DNA.[3] Human topoisomerase I (top1) cleaves a single DNA strand through transesterification of Tyr723 and forms a 3′-phosphotyrosine linkage to the DNA. After cleavage, the broken (scissile) DNA strand can rotate around the unbroken (nonscissile) strand and remove DNA supercoils.[4] The enzyme allows both the rewinding of underwound negatively supercoiled DNA and the unwinding of overwound positively supercoiled DNA.[6] The DNA phosphodiester backbone is restored in a second transesterification reaction when the 5′-OH of the broken DNA strand attacks the 3′-phosphotyrosine bond. This religation reaction therefore liberates top1 for subsequent cleavage/unwinding reactions.

The rate of DNA religation by top1 is normally much faster than the rate of cleavage and this ensures that the steady-state concentration of the covalent 3′-phosphotyrosyl top1−DNA complex remains low.[3] This is important to maintain the integrity of the genome; however, top1−DNA adducts can accumulate in the presence of naturally occurring DNA damage, such as nicks,[7] abasic sites,[8] modified bases,[9] modified sugars,[10] or as a result of exposure to a variety of anticancer compounds.[11,12] For example, the natural product camptothecin (CPT), initially discovered because of its potent antitumor activity,[13] has been shown to target top1 by binding to the covalent top1−DNA complex.[2,14,15] CPT specifically inhibits religation and causes the reversible accumulation of top1−DNA adducts in vitro and in vivo.[2,16] Top1 inhibitors that bind to the covalent complex are termed "poisons", since they convert an essential enzyme into a DNA-damaging agent.[17] The cytotoxic effects of top1 poisons are S-phase specific,[14,18,19] and are roughly proportional to their capacity to stabilize the covalent enzyme−DNA complex.[20,21] In rapidly dividing cells, the DNA replication fork is thought to collide with the "trapped" top1−DNA complex, resulting in double strand breaks and ultimately apoptotic cell death.[22,23]

There are currently two camptothecin derivatives in clinical use, topotecan (Hycamptin) and irinotecan (Camptosar). These analogues (Figure 1) have shown tremendous promise as solid tumor drugs, but they still suffer from low tumor response rates and dose-limiting toxicity.[24−27] Because of these problems and the early promise of top1 poisons for treating human cancer, significant efforts have been made to identify noncamptothecin top1 poisons. The availability of biochemical

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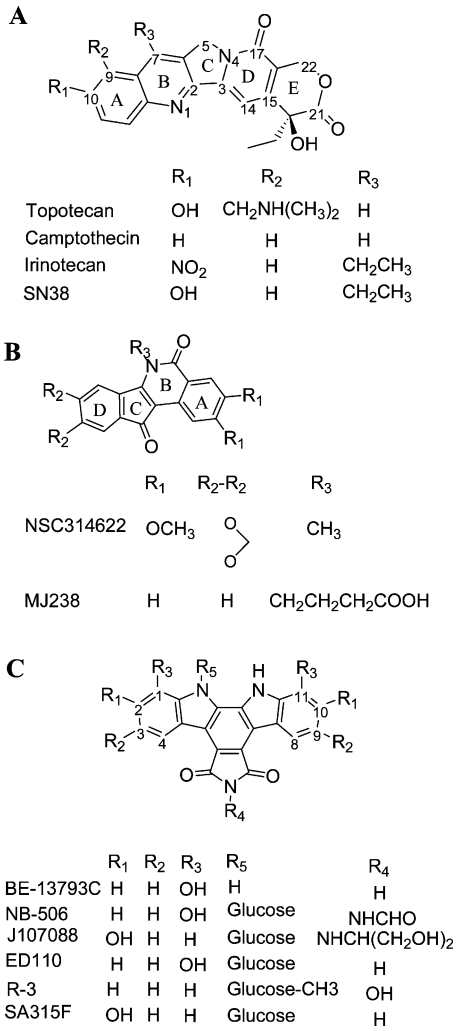

Figure 1. Chemical schematics of topoisomerase inhibitor families. The structures of camptothecin, indenoisoquinoline, and indolocarbazoles are shown in panels a, b, and c.

and cellular assays for testing topoisomerase I activity has resulted in the discovery of two additional classes of top1 poisons, the indolocarbazoles and indenoisoquinolines.[11]

NSC314622 was the first indenoisoquinoline compound found to have antitumor activity,[28] and subsequent studies have shown that a large number of indenoisoquinoline derivatives are top1 poisons.[29−31] The natural product BE−13793c[32] was the first indolocarbazole compound reported to poison top1, and there are at least two indolocarbazole compounds currently undergoing clinical trials, NB506 and J107088.[11] The development of new anticancer drugs has been hindered by a lack of understanding how these compounds bind the topoisomerase I−DNA complex. We have recently published an X-ray crystal structure model of topotecan bound to the topoisomerase I−DNA complex.[33] This model has been extremely useful for understanding structure−activity relationships among the camptothecins; however, it has not been possible to explain how other top1 poisons function; although all three classes of compounds are planar, they have very different shapes and contain a surprisingly diverse set of chemical functionalities. Several models have been proposed for how the indolocarbazoles and indenoisoquinolines would bind the top1−DNA complex,[11,34] but these

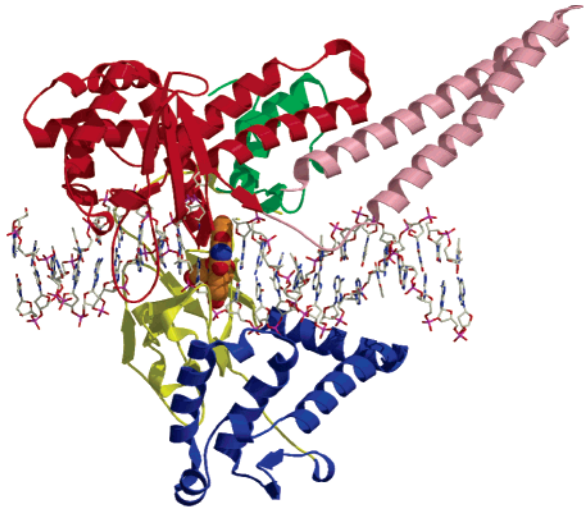

Figure 2. Ribbon diagram of the top70−DNA covalent complex bound with indolocarbazole SA315F. The indolocarbazole compound is diagrammed in space-filling mode (CPK) bound into the intercalation binding pocket at the center of the protein (ribbon) and DNA (stick) ternary complex.

models cannot explain all known structure−activity relationships. Here we report the X-ray crystal structures of the human top1−DNA complex bound with camptothecin, the parent drug for the top1 poisons used clinically, and representative members of the indenoisoquinoline and indolocarbazole classes of top1 poisons. The results show that all three classes share the same intercalative binding mode and mimic a DNA base pair at the site of top1-mediated cleavage. The three classes of molecules make contacts with both the DNA and protein (Figure 2) but exploit different contacts to stabilize intercalation. Surprisingly, Asn352 and Glu356 can adopt alternative side-chain conformations that also result in chemotype-specific contacts. The results provide a structural explanation for the activity relationships of known top1 poisons and should allow the rational design of new structural classes of anticancer drugs targeting top1.

Results and Discussion

We have previously reported the crystal structure of human top1 in complex with the anticancer drug topotecan,[33] a semisynthetic derivative of the natural product camptothecin. In this paper, we extend our understanding of the structural mechanisms of top1 poisoning by reporting the X-ray crystal structures of three additional top1 poisons; an indolocarbazole (SA315F), an indenoisoquinoline (MJ238), and the natural product camptothecin (CPT).

Crystallization and Structure Determinations of the Ternary Complexes. In contrast to our earlier report of topotecan ternary complex crystals which grew readily within 2−3 weeks,[33] crystals of the ternary complex with camptothecin, indolocarbazole, or indenoisoquinoline compounds were difficult to obtain. For each compound, crystallization trials were set up with wild-type top70 or top70N722S. This asparagine to serine mutation confers resistance to camptothecin and some, but not all, other top1 poisons.[35] Each compound was screened in crystallization trials with four different suicide dsDNA oligomers containing a 5′-bridging phos-

phorothiolate at the preferred site of top1 cleavage and one of the four different base pairs at the +1 position immediately downstream of the cleavage site. Crystallization of the ternary complex was attempted with several camptothecin compounds including 9-aminocamptothecin, 10,11-dimethoxycamptothecin, and camptothecin. Likewise, we tested for crystallization in the presence of two regioisomeric analogues of indolocarbazole, ED110[36] and SA315F (Figure 1). Over 40 different indenoisoquinoline and related compounds were screened for ternary complex crystallization, with only the compound MJ238 producing crystals that diffracted X-rays to a resolution of 3.5 Å or better.[29−31] Both MJ238 and SA315F crystallizations produced diffracting crystals in the presence of wild-type and mutant top70 enzyme. However, the most complete and highest resolution diffraction data set for SA315F was obtained with the Asn722Ser Cpt^r version of the enzyme. Poor compound solubility was a significant problem in ternary complex crystallization. Compounds that were soluble in DMSO or partially soluble in water tended to precipitate out of solution when added to the crystallization solution of 10% w/v PEG 8000. Those compounds soluble in water (5 mM or greater) were more likely to produce crystals, though many such crystals did not diffract X-rays to sufficient resolution for structure determination.

Complex with Camptothecin. The 3.0 Å X-ray crystal structure of the ternary campthothecin top1 DNA complex reveals that camptothecin intercalates at the site of DNA cleavage and its binding mode is similar to the binding mode of topotecan.[33] Camptothecin (CPT) was placed unambiguously into the SigmaA-weighted $|F_o| - |F_c|$ maps of the ternary complex (Figure 3). An analysis of the TPT ternary complex[33] demonstrated that both carboxylate and lactone forms of the drug were present in the crystal. Electron density maps of the CPT ternary crystals allowed modeling of both the open carboxylate and closed lactone E-rings; however, an analysis of difference densities could not be used to unambiguously model the partial occupancies of alternative conformations. Because the observed SigmaA-weighted $|F_o| - |F_c|$ maps could not accurately distinguish these alternative conformers, the coordinate model of CPT is modeled only in the closed lactone E-ring conformation. The limited resolution of 3.0 Å also did not allow for the placement of water molecules in this structure.

The E-ring of CPT is positioned closely toward the active site of top1. The C21 lactone oxygen is 4.0 Å away from the bridging phosphodiester oxygen between Tyr723 and thymidine-10, as well as 3.8 Å from the ϵ nitrogen of Lys532. The pyridone ring oxygen is 4.0 Å from the side chain nitrogen of Asn722. The C20 hydroxyl is 3.4 Å from the Oδ1 side chain atom of Asp533, a residue known to be required for enzyme sensitivity to CPT.[37] The closest protein−drug interaction in the CPT ternary complex structure is 2.9 Å from an Nϵ of Arg364 to a free electron pair of the B-ring N1 of CPT (Figure 4).

A comparison between the ternary complex structures of CPT and TPT reveals only minor differences in the overall position of the heterocyclic ring structures (Figures 5 and 6A). There is an 11° twist in the orientation of CPT relative to TPT along the vertical

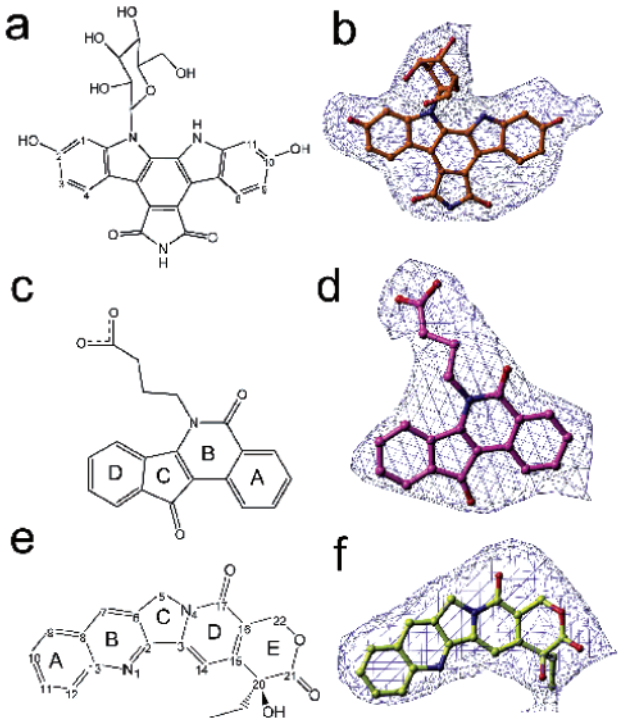

Figure 3. Chemical diagrams and electron density for topoisomerase I poison compounds bound in ternary complex. The chemical structures of indolocarbazole SA315F (a), indenoisoquinoline MJ238 (c), and camptothecin (e) are shown. These structures modeled into the observed 2.6σ $|F_o| - |F_c|$ electron density difference maps are shown in panels b (SA3a5F), d (MJ238), and f (CPT).

axis of the duplex DNA, suggesting some flexibility in how camptothecins can fit into the intercalation binding pocket (Figure 5). Also, the heterocyclic ring structure of CPT is slightly closer to the minor groove side of the intercalation binding site. The relative change in placement and orientation of TPT relative to CPT may result from steric interactions of the C7 substituent of TPT with N6 of the upstream (position −1) adenosine base (intact strand). This translation and rotation movement of the planar CPT ring induces movement of the Arg364 side chain of the CPT ternary complex and decreases the distance between the N1 of CPT and Arg364 to 2.9 Å (vs 3.8 Å in TPT structure), thus forming a hydrogen bond.[33] In order for Arg364 to hydrogen bond to CPT in the ternary complex, it must alter a coordinating hydrogen bond with Asp533. Arg364 probably exists in equilibrium between the Asp533 coordinating state and the N1−CPT/TPT coordinating state. Slight changes in the position of the intercalating drug molecule, caused by movements of the DNA or sequence variations at the intercalation binding site, may draw the Arg364 side chain toward one side of the equilibrium or the other. This may also be a contributing factor for why CPT's preferentially poison top1 at particular nucleotide sequences.[38,39]

Complex with Indenoisoquinoline. The X-ray crystal structure of the indenoisoquinoline bound to top70wt/DNA in ternary complex shows that, like TPT and CPT, MJ238 intercalates at the site of DNA cleavage, between the +1 and −1 base pairs. Rings C and D stack with the noncleaved strand bases, while rings A and B stack with the scissile strand bases. The

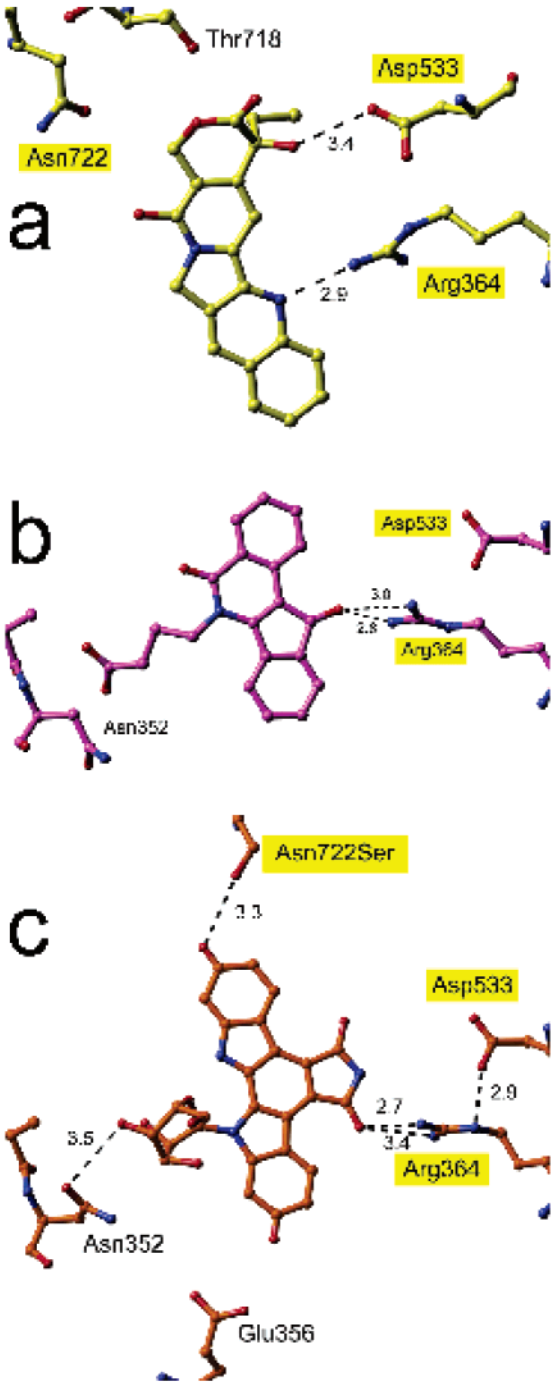

Figure 4. Comparison of the protein and drug interactions. Amino acid side chains near the top1 poison are diagrammed. Positions that are known to confer camptothecin resistance to top1 when mutated are highlighted with yellow boxes. The distance (Å) between proposed hydrogen-bond contacts are indicated. Oxygen atoms are highlighted in red, nitrogen atoms are highlighted in blue. Carbon atoms in the camptothecin (a), MJ238 (b), and SA315F structures (c) are highlighted in yellow, purple, and orange, respectively.

C-ring carbonyl is on the minor groove side of the indenoisoquinoline ring and forms a bidentate interaction with the two nitrogens of the Arg364 side chain at distances of 2.8 and 3.0 Å. The butyl-carboxylic acid substituent of the B-ring nitrogen projects in the major groove toward Asn352 and Ala351. Placement of MJ238 into the $|F_o| - |F_c|$ electron density maps was complicated by the lack of strong electron density for the B-ring

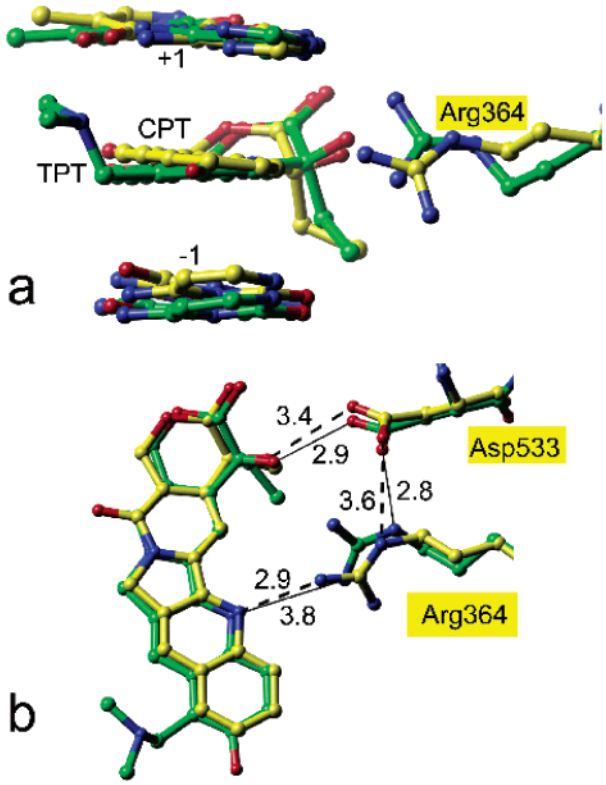

Figure 5. Movement of protein and DNA in TPT and CPT structures. Side view of CPT ternary complex (yellow) and TPT ternary complex (green) is diagrammed in panel a. Camptothecin intercalates into the DNA helix with a slight 11° twist relative to the vertical axis of the duplex DNA in the TPT structure model. The position of Arg364 is shown overlayed between the two structures. The top view of CPT and TPT models are shown in panel b. The side chains of Arg364 and Asp533 are visualized in two alternative conformations in the two different ternary complex structures.

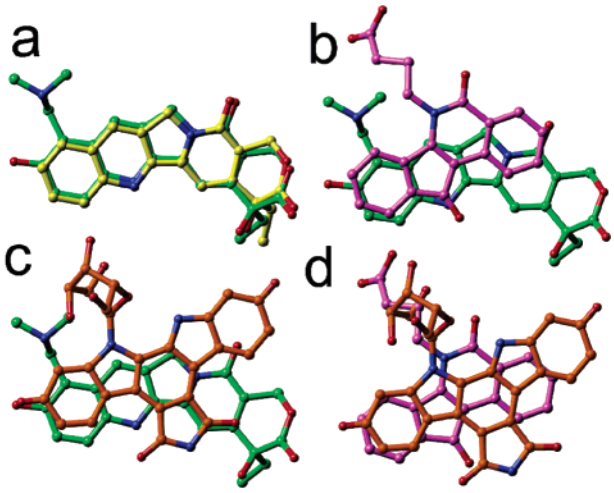

Figure 6. Superpositions of top1 poisons bound in ternary complex. Superposition of protein residues using all residues between 210 and 610 for each ternary complex were completed using XtalView.[57] Oxygen atoms (red), nitrogen atoms (blue), carbon atoms of topotecan (green), carbon atoms of camptothecin (yellow), carbon atoms of MJ238 (magenta), and carbon atoms of SA315F (orange) are diagrammed. (a) Camptothecin vs topotecan, (b) MJ238 vs topotecan, (c) SA315F vs topotecan, and (d) MJ238 vs SA315F.

nitrogen substituent. However, the presence of electron density in the vicinity of Asn352 and the asymmetric

Table 1. Refinement Statistics[a]

inhibitor bound	camptothecin (CPT)	indolocarbazole (SA315F)	indinoisoquinoline (MJ238)
resolution (Å)	20−3.0 (3.16−3.00)	20−3.0 (3.16−3.00)	20−3.0 (3.08−3.00)
no. reflections	16492 (1427)	14673 (973)	18923 (1224)
R_{sym}[b]	8.0 (42.0)	10.7 (32.5)	9.6 (32.7)
completeness	91% (47%)	80.5% (38.1%)	90.1% (63.8%)
$I/\sigma(I)$	13.7 (1.8)	15.4 (2.5)	15.1 (2.3)
space group	*P*21	*P*21	*C*2
a (Å)	57.363	57.382	260.940
b (Å)	114.423	115.965	74.659
c (Å)	74.118	73.547	57.494
b	93.49	93.71	96.94
reflections used in RFREE	5%, 900	5%, 803	5%, 1029
no. of protein atoms	4687	4685	4681
no. of DNA atoms	892	892	892
no. of inhibitor atoms	26	38	25
no. of solvent atoms	0	0	0
RFACTOR	24.1 (37.5)	24.0 (32.3)	23.8 (35.7)
RFREE	29.1 (44.0)	28.3 (32.2)	28.5 (49.9)
rms deviations from ideal stereochemistry			
bond lengths (Å)	0.015	0.016	0.016
bond angles (deg)	1.858	1.765	1.731
impropers (deg)	3.447	3.308	3.121
dihedrals (deg)	22.292	21.442	19.044
mean *B*-factor, all atoms (Å^3)	72.25	73.4	49.44
PDB ID	1T81	1SEU	1SC7

[a] Numbers in parentheses represent the final shell of data. [b] $R_{sym} = \Sigma|I_i - I_m|/\Sigma I_m$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections.

nature of the heterocyclic ring electron density allowed unambiguous placement of the compound (Figure 3).

MJ238 was first described in 1998 as being one of the most cytotoxic indenoisoquinoline compounds reported at that time.[31] Since then, Cushman and co-workers have expanded on their series of indenoisoquinoline compounds and developed several more highly active cytotoxic compounds that poison top1.[29,30,34,40,41] An analysis of the indenoisoquinoline structure−activity relationships when compared to the ternary crystal structure of MJ238 reveals that several generalizations can be made concerning indenoisoquinolines. First, the C- and D-rings intercalate DNA by stacking on the intact strand of the DNA duplex similar in position to the A- and B-rings of TPT/CPT. Second, almost all indenoisoquinoline compounds contain a putative electron pair donor for making a hydrogen bond with Arg364. Third, substituents of the N6 nitrogen are predicted to project into the Asn352 major groove binding site.[34]

Many active indenoisoquinoline compounds contain the addition of a methylenedioxy substituent on the D-ring. Several potent camptothecin derivatives also contain a 10,11-methylenedioxy substituent[42] on the CPT A ring. Both the D-ring of MJ238 and the A-ring of CPT overlap on the ternary complex comparison and are positioned on the intact strand side of the intercalation binding site. A model of methylenedioxy on both CPT and MJ238 reveals sufficient space for the nonplanar substituent (data not shown). A possible clash with c′5 of the +2 phosphodiester may require movement of the planar ring structure toward the major groove. Clearly, a complete understanding of the effects of methylenedioxy substituents at the intact strand intercalation site awaits the determination of a structure of either a camptothecin or indenoisoquinoline compound with this substituent. Many indenoisoquinoline compounds also contain the addition of two methoxy groups on the A-ring side of the tetracyclic scaffold. The methoxy groups could potentially pose a problem by disruption of the planar intercalation of the smaller tetracyclic scaffold. However, a comparison with the TPT/CPT structures reveals that the methoxy groups of an indenoisoquinoline would be positioned similarly to the nonplanar E-ring of TPT/CPT and are thus predicted to occupy a portion of the E-ring binding site in the ternary complex.

Complex with Indolocarbazole. The 3.0 Å X-ray crystal structure of indolocarbazole SA315F bound to the top70n722s/DNA covalent complex shows that SA315F also intercalates DNA at the site of top1-mediated cleavage. Hence the intercalative binding mode is a common feature for CPT's, indolocarbazoles, and indenoisoquinoline compounds. Unambiguous placement of SA315F into $|F_o| - |F_c|$ difference electron density maps was facilitated by the prominent globular density for the pyranosyl substituent on the major groove side of the intercalation binding pocket. The compound SA315F is a symmetrical molecule except for the placement of the pyranosyl moiety linked to an indole nitrogen. The glycosylated indole ring (IG) stacks with bases on the intact strand side of the duplex DNA similar to the A- and B-rings of CPT as well as the C- and D-rings of MJ238. The nonglycosylated indole ring (I) stacks with bases on the cleaved strand side of the duplex DNA. The maleimide ring is on the minor groove side of the intercalation binding pocket and one of its two carbonyls is 2.7 Å from Arg364 (Figure 3). The hydroxyl of the glycosylated indole ring is 4.0 Å from Glu356, whereas the hydroxyl on the nonglycosylated indole ring is 3.3 Å from Ser722. A model of Asn722 at this position would result in a 2.7 Å distance between this hydroxyl and Asn722.

The structure−activity relationships of indolocarbazole compounds have recently been reviewed,[11] and as noted above, the glucose moiety seems to be important for binding in the major groove of the ternary complex near Asn352. The most highly active indolocarbazole

Table 2. Summary of Drug Binding Features

	camptothecin (CPT)	indolocarbazole (SA315F)	indenoisoquinoline (MJ238)	topotecan (TPT)
MW	348.35	519.46	333.34	422.45
(1) SAA[a] covered by DNA (Å^3)	378 (70%)	383 (57%)	317 (57%)	390 (60%)
(2) electron pair near Arg364	yes	yes	yes	yes
(3) major groove pocket	no	yes	yes	yes
(4) hydroxyl near Glu356	no	yes	no	yes
(5) active site contacts	yes	yes	no	yes
IC_{50} (μM) (covalent trapping)[b]	0.30 ± 0.05	0.03 ± 0.02	4.9 ± 1.8	0.05 ± 0.01
PDB ID	1T81	1SEU	1SC7	1K4T

[a] SAA, solvent accessible area. Calculated by subtracting the SAA of the drug compound in the presence of protein or DNA from the SAA of the drug alone. Calculations made with the program EDPDB[59] using a solvent radius of 1.4 Å. [b] IC_{50} values were determined from a drug-dependent covalent trapping assay. Values indicate the μM concentration of compound needed to trap 50% of topoisomerase I on duplex DNA (no phosphorothiolate linkages present). Details of the assay are supplied in the Supporting Information.

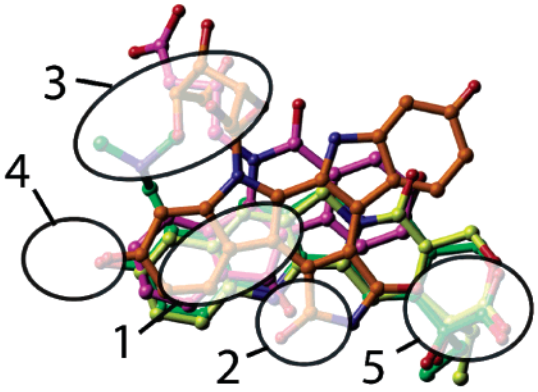

Figure 7. Composite of top1 poisons. Camptothecin (yellow), SA315F (orange), MJ238 (magenta), and topotecan (green) are overlayed. Features or regions of the molecules that are shared among all four structures are highlighted and numbered: (1) flat planar ring structure on intact strand side of DNA; (2) free electron pair on minor groove side of intercalation binding pocket; (3) major groove substituents; (4) hydroxyls near Glu356, intact strand side of DNA; (5) E-ring, cleaved strand side of DNA. These features/regions are summarized in Table 2.

compounds, and those that are currently undergoing clinical trials, contain substituents on the N6 position that is on the minor groove side of the intercalation binding site adjacent to Arg364. NB506 and J-107088 (Figure 1) contain either an *N*-formyl group or dihydroxymethyl groups, respectively. In the ternary complex structure of SA315F, this substituted nitrogen is presumably protonated and positioned approximately 4 Å from Arg364. The E-ring binding pocket of camptothecin derivative compounds is adjacent to this position. A structural model of N6-substituted compounds suggests that the substituent groups on NB506 and J107088 could occupy a portion of the E-ring binding site (not shown). A translational movement of less than 1 Å of the indolocarbazole scaffold or a conformational movement of Arg364 is required in order to optimize this positioning.

Comparative Analysis of Ternary Complexes. A comparative analysis of the four different ternary structures reveals some striking similarities between the top1 poisons (Table 2 and Figures 6 and 7). An obvious characteristic of top1 poisons is their planar heterocyclic ring structure, which allows the poisons to mimic a DNA base pair and thereby form stacking interactions with both upstream and downstream base-pairs. A superposition of the four ternary complexes (Figure 7) reveals that the only portion of the poisons that are conserved are those that stack with the bases of the intact DNA strand (Figure 7, region 1). For CPT and TPT, rings A, B, and C bind in a similar manner as the four-ring indenoisoquinoline as well as one-half of the indolocarbazole compound. Although there are base-stacking interactions between drug molecules and the cleaved strand side of the DNA duplex, these interactions are not spatially conserved. The E- and D-rings of CPT/TPT and the nonglycosylated indole half of SA315F only partially stack with the downstream base on the cleaved strand side of the duplex DNA.

A second conserved feature among the three classes of poison compounds is the presence of a hydrogen-bond acceptor (free electron pair) on the minor groove side of the drug molecules (Figure 7, region 2). This hydrogen-bond acceptor is positioned to form a hydrogen bond with Arg364 in the ternary complexes of CPT, SA315F, and MJ238. The interaction between this free electron pair of each drug molecule and the Nϵ of Arg364 is the sole conserved protein−drug interaction between all three different classes of top1 poisons.

A third common feature of the three different classes of inhibitor compounds is the presence of a substituent on the major groove side of some of the compounds that project near to the side chain of Asn352 (Figure 7, region 3). The nature of the substituent is quite diverse among the three compounds. In SA315F, it is the pyranose moiety that projects into the major groove cavity toward the upstream DNA. The butylcarboxy substituent of indenoisoquinoline MJ238 projects into the major groove cavity; however, it turns toward the downstream DNA in contrast to SA315F. For TPT, the dimethylamine substituent on C7 projects into the major groove turning upstream. However, the C7 substituent is spatially displaced by 3.3 Å relative to the pyranose moiety of SA315F. The diversity of these substituents represents a shared method of optimized binding to the covalent complex. Removal of the glucose moiety decreases top1 inhibition and DNA intercalation by indolocarbazoles.[43] Likewise, indenoisoquinolines with hydroxyalkyl or alkyl halide side chains are more cytotoxic than with those without a side chain at the nitrogen.[29] Camptothecin derivatives that contain substituents on the major groove side of the scaffold are also more highly active than CPT.[44] In the chemoevolution of top1 poison design, addition of a substituent into the major groove represents a pathway of convergent evolution towards optimized binding for each family of compounds.

A summary of the contacts between the three classes of top1 poisons and the top1−DNA complex is described

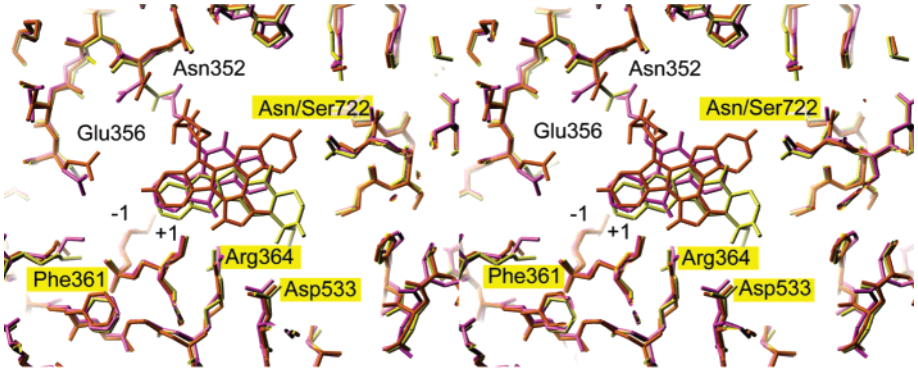

Figure 8. Stereoview of intercalation binding site showing superposition of three ternary complexes. Atoms of MJ238, CPT, and SA315F are shown in magenta, yellow, and orange, respectively. Selected top1 protein side chains are labeled. Amino acids that result in resistance to camptothecin when mutated are highlighted with a yellow box. The −1 and +1 basepairs have been removed for clarity.

in Table 2. It is not surprising that the intercalation binding mode results in a majority of the solvent accessible area of each compound being covered by DNA (57−70%). It has been shown that many top1 poisons can intercalate into DNA at high concentrations, and stacking interactions may be the primary binding interaction for these top1 poisons. However these compounds have been isolated because they specifically poison the top1−DNA complex. Consistent with this result, all of the compounds make specific contacts to top1 (see Table 2).

Comparison of Protein Substructures. The major groove cavity is bounded by protein residues Glu356, Asn352, Pro431, Lys751, and Asn722 (Figure 8). A comparison of the different ternary complex structures reveals conformational mobility in several of the major groove residues. Asn352 and Glu356 each change their side chain conformations by 180° rotations in different structures. The movement of the Asn352 side chain can be correlated to the positioning of the major groove substituent of the various inhibitor molecules. In the case of SA315F, the glucose moiety is extended into the major groove pocket and oriented toward the upstream DNA. The side chain of Asn352 is also turned toward the upstream DNA. The Asn352 side chain is turned toward downstream DNA in the ternary complex of MJ238, whose substituent is also pointed downstream. Molecular dynamics simulations of the top1−DNA complex have also predicted a high mobility for Asn352, and our observations support the hypothesis that Asn352 plays a key role in modulating drug binding.[45]

Glu356 is present in multiple conformations in the various ternary complex structures. One possible explanation for the conformational flexibility of Glu356 is that the negatively charged side chain is repulsed by changes in the position of the nearby negatively charged phosphodiester between the +2 and +3 bases of the intact strand (Figure 9). A comparison of the TPT and SA315F ternary complex structures shows that the +2/+3 phosphodiester has shifted 3.7 Å between the two structures, and this change in position accompanies the conformational change in the Glu356 side chain (Figure 9). Thus conformational movement of Glu356 may be a factor that is mediated through inhibitor−DNA interactions as well as through direct inhibitor−protein interactions.

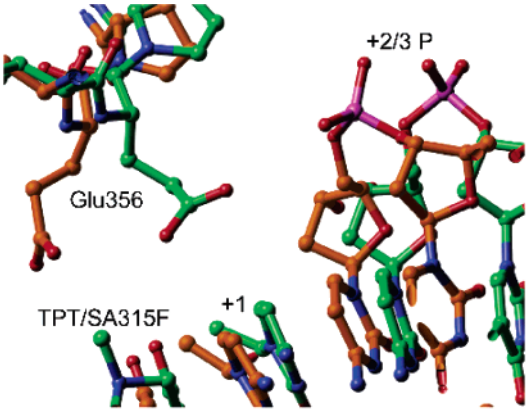

Figure 9. Comparison of topotecan and SA315F ternary complex structures in the vicinity of Asp356. The structure models of the TPT (carbon atoms in green) and SA315F (carbon atoms in orange) ternary complexes are overlayed.

Camptothecin Resistant Mutations. Several recent studies comparing camptothecin-resistant mutations (CPT^r) and their effects on indolocarbazole compounds were conducted that revealed that four catalytically active CPT^r mutants also contributed to indolocarbazole resistance of top1. These residues were Phe361Ser,[46] Arg364His, Gly503Ser, and Asn722Ser.[47] The importance of Arg364 in binding top1 poisons is discussed above. This contact is the sole protein interaction on the minor groove side of the intercalation binding site as well as the sole consistent protein−drug interaction visualized between all ternary complexes. The genetic and structural data clearly argue that removal mutation of Arg364 to His decreases the affinity of indolocarbazoles and camptothecins for the top1−DNA complex by eliminating an essential contact. The other three mutations cannot be explained by direct contacts between the ligand and the protein. Residue Phe361 is adjacent to −1/+1 intact phosphodiester in the ternary complex crystal structures (Figure 8). Phe361 has been proposed to form a steric pocket for DNA involved in creating the intercalation binding site of top1−DNA, and thus mutation of this residue has been proposed to disrupt the intercalation binding site of top1 poisons.[33] Analysis of the four ternary structures supports this hypothesis, since the −1/+1 phosphodiester is positioned similarly in all four structures. Additionally we propose that CPT^r mutant Gly363Cys[48]

would disrupt the binding site and lead to resistance of top1 to indolocarbazoles as well as other top1 poisons that act through intercalation of the covalent complex at −1/+1.

A similar analysis of the four available ternary structures reveals that Gly503 sits behind the side chain of active site residue Arg488, relative to the intercalation binding site. Addition of a side chain at Gly503 would disrupt the conformation of Arg488. A possible explanation for TPT/CPT resistance is that disruption of the TPT/CPT binding site is caused by disruption of the water-mediated contact from Arg488 to the E-ring of TPT/CPT. Unfortunately, an explanation for indolocarbazole resistance is less apparent, as the E-ring binding pocket is empty in the ternary complex of SA315F. However, the E-ring binding pocket may be occupied for indolocarbazoles containing an N6 substituent, such as NB506 and J-107088. It is possible that mutation at Gly503 produces resistance to topoisomerase poisons whose binding requires the use of the E-ring contacts. Alternatively, instability of the top1 binding site caused by the disruption of a Gly503 side chain may have a more profound effect upon the conformation of residues in this region. A complete explanation of the effects of Gly503 mutation on the structure of top1 awaits further structure determinations of this mutant.

It is important to emphasize that because topI poisons bind a transient intermediate in the reaction pathway, there are multiple mechanisms to confer drug resistance. First, a mutation within the vicinity of the drug binding site can inhibit binding by removing or modifying a specific drug−protein contact. A second less appreciated mechanism is that mutations that decrease the steady-state concentration of the binding target or modify the structure of the binding pocket can also result in resistance to various top1 poisons.[33,49] If a mutation allows a complete catalytic cycle, but lowers the steady-state concentration of the appropriate top1−DNA complex, then the mutant top1 will be resistant to the poison. In some cases, a single point mutation may result in camptothecin resistance by altering a drug−protein contact and the steady-state concentration of the top1−DNA complex. Unfortunately, top1−DNA crystal structures can only be obtained using 5′-bridging phsophorothiolate linkages, since this modification prevents religation and irreversibly traps the top1−DNA complex, resulting in a homogeneous macromolecule solution.

We have solved the crystal structure of indolocarbazole SA315F in the presence of the Asn722Ser mutant top70, a mutation known to cause camptothecin resistance as well as resistance to other top1 poisons, such as indolocarbazole NB506.[47] Recent studies have shown that a variety of mutations of the homologous residue in yeast (Asn726) can alter the cleavage/religation equilibrium.[50,51] This complicates the analysis of top1 structures containing the Asn722Ser mutation; however, a previous study demonstrated that topotecan−top1−DNA ternary complexes could be obtained with this mutant, and both the wild-type and drug resistant Asn722Ser top70 revealed no significant differences in the drug binding mode.[49] An analysis of the four ternary complex structures available reveals that two poison compounds, TPT and SA315F, contain interactions with residue 722. TPT interacts through a water-mediated hydrogen bond to Asn722, whereas SA315F interacts with Ser722 at a distance of 3.3 Å. A model of 722 as Asn in the SA315F structure predicts a distance of 2.7 Å from the N of Asn722 to a hydroxyl of SA315F. We have previously predicted that Asn722 is important for drug binding because of its role in contacting the drug molecule via water-mediated interactions,[33,49] and the structures reported here support this hypothesis. However, as evidenced by the ternary complex of SA315F, the mutation of Asn to Ser does not abolish the potential of 722 to form direct or water-mediated interactions with some bound drug molecules. Finally, it remains possible that the topI poison resistant phenotype of Asn722Ser may also result from an alteration in the steady-state concentration of the top1−DNA covalent complex in vivo.

Conclusions

We have solved the ternary complex crystal structures of three topoisomerase poisons: camptothecin, an indenoisoquinoline, and an indolocarbazole. A comparative analysis of three different chemical classes of top1 inhibitors reveals some general principles of top1 poison binding that should be emphasized when designing new top1 posions. (1) The flat planar ring structures intercalate between the −1/+1 basepairs at the site of enzyme-mediated DNA cleavage. (2) Base stacking interactions are only spatially conserved on the intact strand side of the duplex DNA. Variable ring structures and base-stacking interactions are allowed on cleaved strand side of duplex DNA. (3) A free electron pair for hydrogen bonding on the minor groove side of the intercalation binding pocket near Arg364 is observed in all complexes, although the exact chemical nature of this hydrogen-bond acceptor can be very different. (4) Three distinct binding pockets for additional substituents exist, consisting of (A) a major groove in the vicinity of Asn352, (B) an intact strand side in the vicinity of Glu356, and (C) an "E-ring binding pocket" in the vicinity of the phosphotyrosine linkage to DNA and active site residues. These results explain how very diverse chemotypes can have the same binding mode but exploit different features of the protein DNA complex to poison top1. It may therefore be possible to combine different features of each chemotype to develop entirely new top1 poisons.

Experimental Methods

Protein Purification and Crystallization. A 70 kDa construct of human topoisomerase I (top70), residues Lys175−Phe765, was expressed and purified from baculovirus-insect cells (SF9) as described previously.[52] A top70 construct with the Asn722Ser (top70n722s) mutation was expressed and purified following the same procedure as the wild-type top70 construct (top70wt).

The covalent complex of top70−DNA was prepared using a 5′-bridging phosphorothiolate duplex oligonucleotide previously described.[53] The oligonucleotide sequence of the cleavable strand of the duplex oligomer was 5′-AAAAAGACTTsX-GAAAAATTTTT-3′, where "s" represents the 5′-bridging phosphorothiolate of the cleaved strand and "X" represents any of the four bases A, G, C, or T. All ternary complex crystals were grown as described previously.[33] Crystallization trials were set up with either the top70wt protein or top70n722s. Crystals

were grown by sitting drop vapor diffusion by preparing drops containing 2.0 μL of precipitant, 1.5 μL of 50 μM duplex DNA, 0.5 μL of compound, and 1.5 μL of 4.2 mg/mL protein. Precipitant was 10−12% PEG 8000, 100 mM MES pH 6.4, 200 mM lithium sulfate. The compound solutions used were 1−5 mM ligand dissolved in 0−10% DMSO v/v in water. All structures solved contained phosphorothiolate DNA containing guanine in the 5′-bridging phosphorthiolate position. Camptothecin and indenoisoquinoline MJ238 structures were determined with the top70 Asn722 protein. Indolocarbazole SA315F was determined bound to Ser722 top70 protein.

Crystals were cryoprotected for data collection by passing them through precipitant plus 30% v/v PEG 400. Data were collected at 100 K at the COM-CAT, Sector 32 beamline of the Advanced Photon Source, Argonne National Laboratory, and the National Synchrotron Light Source, Brookhaven National Laboratory beamline X25. Structures were solved by molecular replacement using the program AMORE[54] with protein coordinates from the top70 ternary complex structure with topotecan.[33] Refinements were conducted using CNX[55] and REFMAC[56] and iterative model adjustments with XtalView[57] (see Table 1). DNA's were placed into the $|F_o| - |F_c|$ electron density and refined. Drug molecules were then placed into the to $|F_o| - |F_c|$ electron density using the QUANTA module of X-LIGAND.[58]

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Supporting Information Available: IC_{50} values of camptothecin, SA315F, MJ238, and topotecan obtained from the covalent trapping assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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