Articles

The Camptothecin-Resistant Topoisomerase I Mutant F361S Is Cross-Resistant to Antitumor Rebeccamycin Derivatives. A Model for Topoisomerase I Inhibition by Indolocarbazoles†

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ABSTRACT: DNA topoisomerase I is a major cellular target for antitumor indolocarbazole derivatives (IND) such as the antibiotic rebeccamycin and the synthetic analogue NB-506 which is undergoing phase I clinical trials. We have investigated the mechanism of topoisomerase I inhibition by a rebeccamycin analogue, R-**3**, using the wild-type human topoisomerase I and a well-characterized recombinant enzyme, F361S. The catalytic activity of this mutant remains fully intact, but the enzyme is resistant to inhibition by camptothecin (CPT). Here we show that the mutated enzyme is cross-resistant to the rebeccamycin analogue. Despite their profound structural differences, CPT and R-**3** interfere similarly with the activity of the wild-type and mutant topoisomerase I enzymes, and the drug-induced cleavable complexes are equally sensitive to the NaCl concentration. CPT and IND likely recognize identical structural elements of the topoisomerase I−DNA covalent complex; however, differences do exist in terms of sequence-specificity of topoisomerase I-mediated DNA cleavage. For the first time, a molecular model showing that CPT and IND share common steric and electronic features is proposed. The model helps to identify a specific pharmacophore for topoisomerase I inhibitors.

The DNA−topoisomerase I covalent complex is the nuclear target for a number of anticancer agents derived from the plant alkaloid camptothecin (CPT)[1] (*1*). These agents include topotecan (Hycamtin) and the prodrug irinotecan (CPT-11, Campto) that are approved for the treatment of ovarian and colorectal cancers, respectively (*2*). Several other synthetic analogues such as 9-aminoCPT, 9-nitroCPT, lurtotecan (GI147211 or GG211), and the analogue DX-8951 are currently under (pre)clinical investigation (*3*).

Many topoisomerase I poisons that differ from the camptothecins have been identified recently (*1*, *4*, *5*). Certain DNA minor groove binders including Hoechst 33342 (*6*−*8*), a few plant alkaloids such as bulgarein and coralyne (*9*−*11*), and the antitumor agent intoplicine (*12*) as well as saintopins and related naphthacene-dione antibiotics (*13*, *14*) can bind and stabilize the topoisomerase I−DNA cleavable complex. Actinomycin D (*15*), NSC314622 (*16*), and aclarubicin (*17*) are also known to inhibit topoisomerase I (see *18* for a recent review). This enzyme also represents a privileged cellular target for indolocarbazoles (IND) including the promising antitumor agent NB-506 which is undergoing phase I clinical trials (*19*−*23*). Over the last 2 years, we have synthesized different series of IND structurally related to NB-506 and the antibiotic rebeccamycin (Figure 1) with the aim of elucidating the structure−activity relationships (*24*−*27*). Among the molecules developped thus far, the analogue R-**3** shown in Figure 1 was found to be a potent DNA-intercalating topoisomerase I inhibitor (*28*).

Despite profound structural differences, CPT and IND seem to interact similarly with the topoisomerase I−DNA complex. We have previously shown that CPT and R-**3** preferentially stabilize topoisomerase I-mediated cleavage of T−G linkages (*28*). Topoisomerase I-deficient P388 leukemia cells resistant to CPT are cross-resistant to R-**3** (*29*). Different biochemical and cellular information suggests that rebecca-

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[1] Abbreviations: IND, indolocarbazole; CPT, camptothecin.

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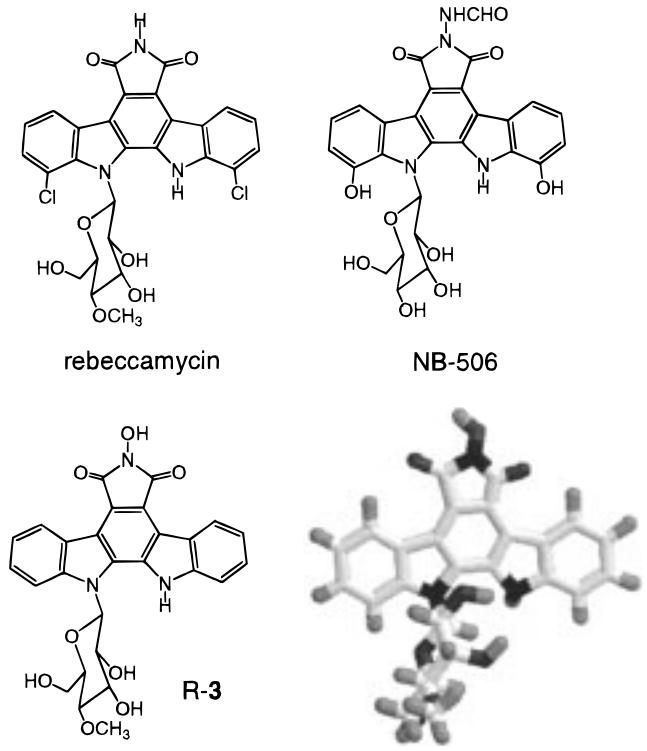

FIGURE 1: Chemical structures of rebeccamycin, NB-506, and the analogue R-**3** used in this study. An energy-minimized structure of R-**3** is shown. The softwares HyperChem 5.01 and Alchemy 2000 were used to construct the structures.

mycin-type inhibitors use the same molecular determinants as CPT to freeze the topoisomerase I−DNA covalent complex. However, the reasons why structurally different drugs such as CPT and IND are equally potent at inhibiting topoisomerase I remain largely unknown. This issue is addressed here.

Several key amino acid residues of topoisomerase I that are essential for poisoning by CPT have been identified. Point mutations at residues 363 (Gly→Cys) (*30*), 503 (Gly→Ser) (*31*), 533 (Asp→Gly) (*32*), 583 (D→Gly) (*33*), 717 (Gly→Val) (*34*), 722 (Asn→Ser) (*35*), and 729 (Thr→Ala) (*36*) all result in the production of a CPT-resistant enzyme (reviewed in ref *1*). The phenylalanine residue at position 361 is also essential. Serine substitution (F361S) produces a topoisomerase I mutant with intact catalytic activity but resistant to CPT (*37*, *38*). This phenylalanine residue, which is located near the active site of human topoisomerase I (*39*), may participate in the anchoring of indolocarbazoles within the catalytic site of the enzyme. To explore this issue and to comprehend further the mechanism of action of IND, we compared the effects of R-**3** on the cleavage activity of the wild-type and F361S mutant enzymes. The study demonstrates that the F→S mutation confers resistance to R-**3** and therefore reinforces the idea that CPT and IND are functionally similar.

MATERIALS AND METHODS

Drugs. Camptothecin was purchased from Sigma Chemical Co. (La Verpillière, France). The synthesis of the indolocarbazole R-**3** has been reported (*24*). Drugs were dissolved in dimethyl sulfoxide (DMSO) at 3 mg/mL and then further diluted with water. Fresh dilutions were prepared immediately prior to use. The final DMSO concentration never exceeded 0.3% (v/v), conditions under which DMSO (also present in the controls) is known not to affect the topoisomerase I activity (*28*).

Chemicals and Biochemicals. Nucleoside triphosphates labeled with [^{32}P] (α-dATP and γ-ATP) were obtained from Amersham. Restriction endonucleases *Ava*I and *Eco*RI, alkaline phosphatase, T4 polynucleotide kinase, and AMV reverse transcriptase were purchased from Boehringer (Mannheim, Germany) and used according to the supplier's recommended protocol in the activity buffer provided. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore filtered water.

DNA Purification and Labeling. The 160 base pair *tyr*T DNA fragment was prepared by 3′-[^{32}P]-end labeling of the *Eco*RI−*Ava*I double digest of plasmid pKMp27 (*40*) using [α-^{32}P]dATP (6000 Ci/mmol) and AMV reverse transcriptase or by 5′-[^{32}P]-end labeling of the *Eco*RI/alkaline phosphatase treated plasmid using γ-[^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase followed by treatment with *Ava*I. The digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE buffer (89 mM Tris−borate, pH 8.3, 1 mM EDTA). After autoradiography, the band of DNA was excised, crushed, and soaked in water overnight at 4 °C. This suspension was filtered through a Millipore 0.22 μm filter, and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum-drying of the precipitate, the labeled DNA was resuspended in 10 mM Tris adjusted to pH 7.0 containing 10 mM NaCl.

Proteins. Experiments were performed either with human topoisomerase I from TopoGen Inc. (Columbus, OH) or with recombinant topoisomerase I proteins expressed in *E. coli*: The mutants F361S (substitution of a serine for a phenylalanine at position 361), R362L (substitution of a serine for an arginine at position 362), and R364G (substitution of a glycine for an arginine at position 364) were expressed and purified as previously described (*37*, *38*).

DNA Relaxation Experiments. Supercoiled pKMp27 DNA (0.5 μg) was incubated with 6 units of human topoisomerase I at 37 °C for 1 h in relaxation buffer (50 mM Tris, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in a 1% agarose gel at room temperature for 2 h at 120 V. Gels were stained with ethidium bromide (1 mg/mL), washed, and photographed under UV light. Similar experiments were performed using ethidium-containing agarose gels ([ethidium] = 1 μg/mL).

Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites. Each reaction mixture contained 2 μL of 3′- [^{32}P]-end-labeled DNA (~1 μM), 5 μL of water, 2 μL of 10× topoisomerase I buffer, and 10 μL of drug solution at the desired concentration (5−25 μg/mL). After 10 min incubation at 37 °C to ensure equilibration, the reaction was initiated by the addition of 2 μL (40 units) of purified topoisomerase I. Samples were incubated for 60 min at 37 °C prior to adding SDS to 0.25% and proteinase K to 250 μg/mL to dissociate the drug−DNA−topoisomerase I cleavable complexes. The DNA was precipitated with ethanol and then resuspended

in 5 μL of formamide−TBE loading buffer, denatured at 90 °C for 4 min, and then chilled on ice for 4 min prior to loading on to the sequencing gel. DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea). After electrophoresis (about 2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer Model S2), gels were soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, and dried under vacuum at 80 °C. A Molecular Dynamics 425E PhosphorImager was used to collect data from the storage screens exposed to dried gels overnight at room temperature. Base-line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the DNA fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with dimethyl sulfate (G) and/or formic acid (G+A) followed by piperidine-induced cleavage at the modified bases in DNA.

Salt Dependence for Reversal of Cleavable Complexes. The procedure described by Tanizawa et al. (*41*) was followed. Briefly, the cleavable complexes were induced for 15 min at 37 °C prior to adding NaCl up to 0.5 M, and the reaction was continued for 15 min prior to the SDS−proteinase K treatment as described above. For the kinetic experiments, the cleavable complexes were induced for 30 min at 37 °C, an aliquot was taken out (time 0) and NaCl was added (0.1 M final concentration), and aliquots were then taken at various times. The DNA samples were precipitated with ethanol and then analyzed on a sequencing gel.

Molecular Modeling. A recently described computational procedure for the alignment of the drug structures was followed (*42*). Briefly, ab initio calculations were performed at the Hartree−Fock level with Gaussian basis set 3-21G(*) (*43*) using the software package Spartan 5.0. Drug geometries were fully optimized without any constraints. After calculations of the optimized geometries and the atomic charge properties, steric and electrostatic analyses were performed using the Superimposition/Similarity facility to Spartan 4.0 (Wave function Inc., Irvine, CA). The superimposition was computed by maximizing the electrostatic potential field and steric overlaps of the 3D structures. All molecular modeling calculations were performed on a Silicon Graphics 02 Unix workstation.

RESULTS

Complementary biochemical assays using a series of previously characterized recombinant enzymes were performed to determine whether the indolocarbazole compound R-**3** inhibited topoisomerase I in the same manner as camptothecin. First we studied DNA relaxation in the absence and presence of the drugs. Negatively supercoiled plasmid pKMp27 was incubated with wild-type or mutant topoisomerase I and increasing concentrations of R-**3**, ranging from 0.1 to 10 μg/mL. The DNA samples were treated with SDS and proteinase K to remove any covalently bound protein and resolved in a 1% agarose gel. The gel shown in Figure 2 indicates that indolocarbazole inhibits the relaxation of DNA mediated by either the wild type or the mutant enzymes. In agreement with a recent study (*38*), we found

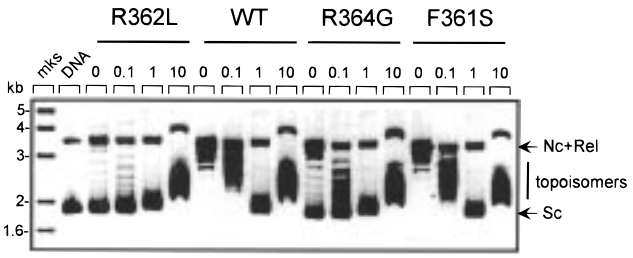

FIGURE 2: Inhibition of topoisomerase I-mediated DNA relaxation by R-**3**. Native supercoiled pKMp27 DNA (0.5 μg) (lane DNA) was incubated for 30 min at 37 °C with 6 units of topoisomerase I in the absence or presence of drug at the indicated concentration (μg/mL). The drug inhibits the activity of the wild-type enzyme (WT) and the three mutant enzymes F361S, R362L, and R364G. In each case, the reaction was stopped with sodium dodecyl sulfate and treatment with proteinase K. The DNA was analyzed by native agarose gel electrophoresis. Nc, nicked; Rel, relaxed; Sc, supercoiled. The gel was stained with ethidium bromide and photographed under UV light. The size of the molecular weight markers (in kb) is indicated.

that the replacement of the highly conserved arginine residues at positions 362 and 364 with leucine and glycine, respectively, reduced significantly the catalytic activity compared to the wild-type enzyme whereas the substitution of phenylalanine at residue 361 with a serine has little effect on the relaxation activity. In every case, the relaxation of DNA by topoisomerase I is inhibited by R-**3**. For drug concentrations ≥ 1 μg/mL, the relaxation of plasmid substrate is completely abolished, using either wild type or mutants R362L, F361S, or R364G.

Under the standard conditions used in Figure 2, nicked DNA and relaxed (closed circular) DNA comigrate in the agarose gel matrix, but the two forms can be distinguished on an ethidium-containing agarose gel. With ethidium bromide, the electrophoretic mobility of relaxed DNA, but not that of nicked DNA, is markedly changed because of DNA unwinding effects. Accordingly, we repeated the experiments with the wild-type and mutant enzymes using ethidium-prestained gels. The results shown in Figure 3 confirm that R-**3** is a specific topoisomerase I inhibitor. With the wild-type enzyme, the level of nicked DNA is considerably increased, just as observed with camptothecin. In contrast, the failure of R-**3** to produce nicked DNA species with the F361S enzyme indicates that this topoisomerase I mutant, which is resistant to camptothecin (see lane Cpt) and to 9-nitrocamptothecin (*37*), is cross-resistant to the indolocarbazole compound. Identical results were obtained with the mutant R362L which is resistant to both compounds. Therefore, we must conclude that the inhibition of the mutant enzymes observed under standard (ethidium-free) conditions (Figure 2) is nonspecific and arises solely from DNA binding rather than from interaction between the drug and covalent enzyme−DNA species. The shift in the mobility of supercoiled plasmid (form I) observed with increasing concentrations of R-**3** is attributed to an inhibition of the catalytic activity of the enzyme rather than to a decrease in plasmid DNA linking number due to intercalation because no such effect is observed in the absence of the enzyme. In other words, R-**3** can behave both as a specific topoisomerase I inhibitor, trapping the cleavable complexes, and as a nonspecific inhibitor of a DNA-processing enzyme, acting via DNA binding.

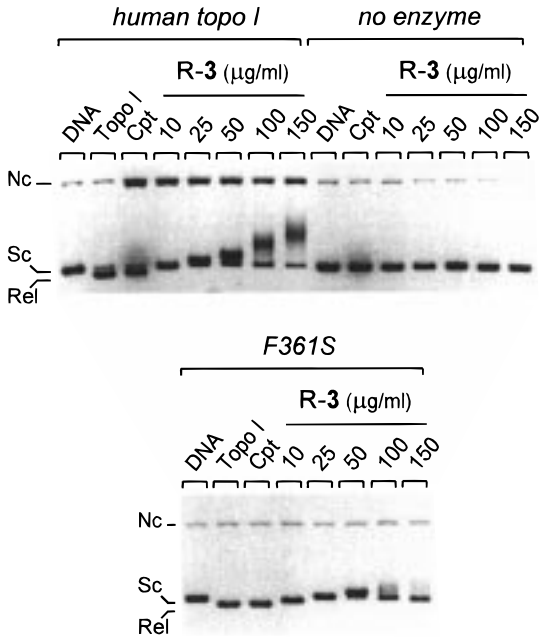

FIGURE 3: Effect of increasing concentrations of R-**3** on the relaxation of plasmid DNA by topoisomerase I. Supercoiled DNA (lane DNA) was incubated with or without human topoisomerase I or the mutant F361S enzyme. DNA samples were separated by electrophoresis on an agarose gel containing ethidium bromide. Nc, nicked; Rel, relaxed; Sc, supercoiled. Other details as for Figure 2.

R-**3** is a sequence-selective intercalating agent whereas camptothecin does not appreciably bind to DNA in the absence of topoisomerase I (*28*, *44*). However, the two drugs apparently interfere similarly with the activity of the wild-type and mutant topoisomerase I enzymes. It is therefore likely that despite their profound structural differences and their distinct DNA binding properties, CPT and IND interact with topoisomerase I in a similar fashion or, at least, recognize similar structural elements of the topoisomerase I−DNA covalent complex.

The compound R-**3** is not simply a mimic of camptothecin. Differences do exist in terms of sequence-specificity of topoisomerase I-mediated DNA cleavage. To examine further the activity of the compound on the wild-type and mutant F361S enzymes, the topoisomerase cleavage sites induced by R-**3** and camptothecin were mapped using a 160 base pair DNA fragment. The *Eco*RI−*Ava*I restriction fragment from plasmid pKMp27 was uniquely end-labeled at the 3′-end of the lower strand at the *Eco*RI site and used as a substrate for the topoisomerase I cleavage reaction. The cleavage products were analyzed on a sequencing polyacrylamide gel (Figure 4). Here again it can be seen unambiguously (i) that R-**3** and CPT strongly promote DNA cleavage by human topoisomerase I and (ii) that the mutant F361S enzyme is resistant to both R-**3** and CPT. The recombinant protein may be slightly more resistant to the indolocarbazole than to the plant alkaloid since one cleavage site at position 61 (arrow on the left side of the gel in Figure 4) is detected with camptothecin whereas there is no band with R-**3**. Densitometric analysis of the cleavage sites detected with R-**3** and CPT reveals interesting differences between the two drugs. Although in both cases the cleavage occurs principally at sites having a T and a G on the 5′ and 3′ sides of the cleaved bond, respectively (see ref *22* for a statistical analysis of the cleavage specificity), it is clear that the cutting

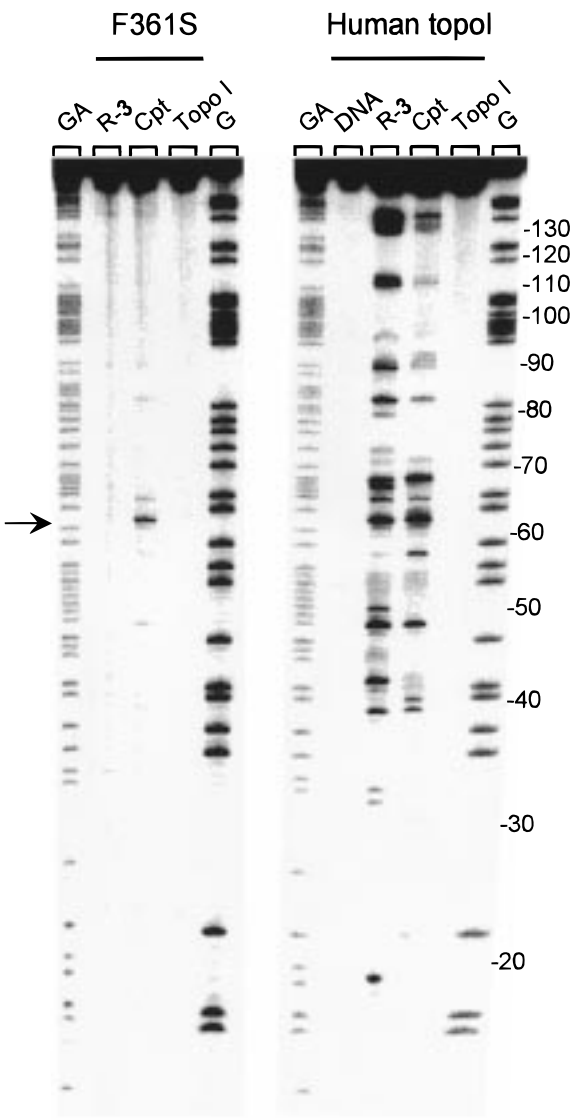

FIGURE 4: Phosphorimage comparing the susceptibility of the 160 bp *tyr*T fragment to cutting by human topoisomerase I and the mutant F361S enzyme in the presence of camptothecin and R-**3**. The 3′-end-labeled fragment (lane DNA) was incubated in the absence (lane Topo I) or presence of the indolocarbazole derivative at 25 μg/mL or camptothecin at 5 μg/mL (lanes R-**3** and Cpt). Topoisomerase I cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel as described under Materials and Methods. Numbers at the right of the gel show the nucleotide position, determined with reference to the guanine and purine nucleotide tracks labeled G and G+A, respectively.

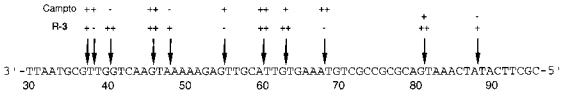

FIGURE 5: Nucleotide positions of the topoisomerase I cleavage sites stimulated by R-**3** and camptothecin.

can sometime occur at different nucleotide positions (Figure 5). For example, R-**3** but not CPT promotes the cleavage at positions 41 and 49 corresponding to the sequences ACTG↓GTTG and AAAA↓ATGA, respectively. Conversely, cleavage at position 56 (CGTT↓GAGA) is specific to camptothecin. In several cases, but not all, the drug-stimulated cleavage sites on DNA in the presence of topoisomerase I coincide with the preferred drug binding sites inferred from footprinting studies (*44*). For example, the

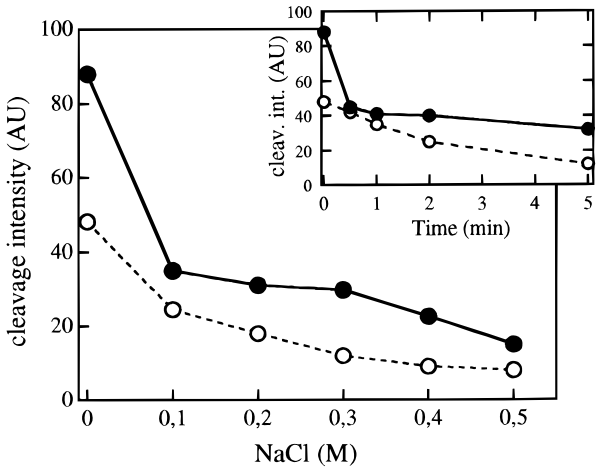

FIGURE 6: Salt-induced reversibility of (●) camptothecin- and (○) R-**3**-induced cleavable complexes. The 3′-end-labeled *Eco*RI−*Ava*I restriction fragment was incubated with human topoisomerase I for 15 min in the presence of the drug prior to adding NaCl. Samples were incubated for a further 15 min at 37 °C and then treated with SDS−proteinase K and precipitated. A sequencing gel was used to monitor cleavage at position 47 (AAAT$^{\downarrow}$GTAA). For the kinetic experiments (inset), the cleavable complexes were induced for 30 min at 37 °C, an aliquot was taken out (time 0) and NaCl was added (0.1 M final concentration), and aliquots were then taken at various times.

prominent cleavages sites between positions 60−70 correspond to a preferred binding region for R-**3**. Therefore, it is possible that the sequence selectivity of the drug plays a role in determining its effect on topoisomerase I.

Another important aspect of the topoisomerase I inhibition is the persistence of the cleavable complexes. CPT-stabilized cleavable complexes are rapidly reversible upon lowering of the drug concentration or adding competitor DNA. The reversal can also be accomplished by increasing the salt concentration (*41*). This prompted us to compare the salt-dependent reversibility of R-**3** and camptothecin (Figure 6). The extent of cleavage at site 47 (one of the strongest G^{+1} cleavage sites which is common to the two drugs) was measured as a function of NaCl concentration (up to 0.5 M) and as a function of the time after adding 0.1 M NaCl. With both drugs, the cleavage intensity decreased markedly upon addition of 0.1 M NaCl, and the effect occurs within less than 1 min. At low NaCl concentrations (≤0.1 M), the effect is more pronounced with CPT than with R-**3**, but at higher salt concentrations, the cleavage activity is markedly decreased for both drugs. The results suggest that the cleavage complexes induced by R-**3** are not more stable than those induced by CPT.

DISCUSSION

The results presented above confirm that the indolocarbazole compound R-**3** is a specific inhibitor of topoisomerase I capable of promoting the formation of DNA−enzyme covalent complexes and reveal that this intercalating compound can also behave as a nonspecific inhibitor preventing the enzyme from binding to DNA. The action of the drug is similar to that of CPT in terms of the stability of the drug-induced cleavable complexes and in terms of sequence selectivity, even though minor differences do exist with regard to the nucleotide position of certain cleavage sites. Both CPT and R-**3** seem to interact with the 361−364 region

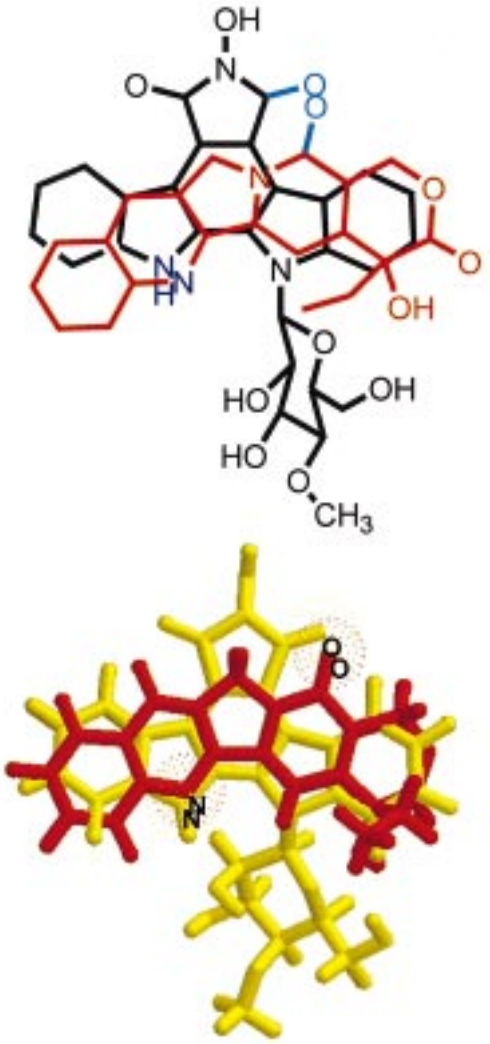

FIGURE 7: Superimposition of the CPT and R-**3** chemical structures. The CPT chromophore is shown in red with the N1 nitrogen in blue and the CO16 carbonyl group in cyan. The indolocarbazole chomophore of R-**3** (in black) was overlapped with the CPT structure so as to place the nitrogen indole and the CO group on top of the same groups in CPT. The lower part of the figure shows the conformational analogy between the two drugs.

of topoisomerase I which is essential for the DNA cleavage/ligation activity and is known to be part of a "lip" that interacts with DNA in both covalent and noncovalent enzyme−DNA complexes (*39*).

This study indicates that CPT and IND interfere similarly with topoisomerase I−DNA covalent complexes, despite differences in chemical structures. However, the superimposition of the two structures reveals strong similarities. The molecular model in Figure 7 shows that the indolocarbazole chromophore can be superimposed with the CPT structure. The optimized geometries and atomic charges of R-**3** and CPT were obtained from ab initio calculations. The steric and electrostatic aligment of the two drugs revealed a high degree of similarity with a correlation coefficient of 0.74. Three overlapping sites can be distinguished: (i) the two aromatic structures are extended; (ii) the position of the N1 nitrogen atom of CPT coincides with the position of one of the nitrogen indoles of R-**3**; and (iii) one of the carbonyl groups of the naphthalimide moiety of R-**3** is positioned like the CPT carbonyl group at position 16. We infer from the

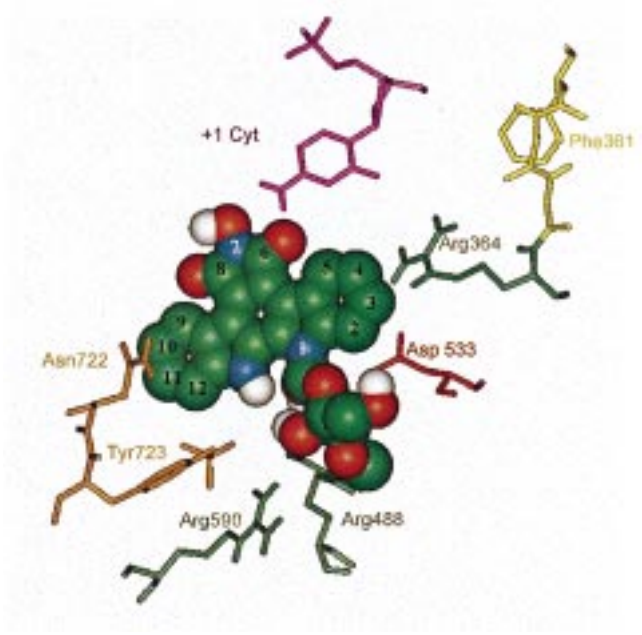

FIGURE 8: Molecular model illustrating the insertion of the planar indolocarbazole chromophore within the active site of topoisomerase I. The amino acid residues at the topoisomerase I catalytic site that are essential for the poisoning effect of CPT are indicated (adapted from ref *39*). The cytosine residue on the noncleaved strand of DNA is positioned. From this view, the proximity between the drug carbohydrate residue and the Arg 488 and Asp 533 residues of the enzyme can be clearly observed. It is also worth noting the short distance between the CO group at position 6 on the drug naphthalimide ring and the exocyclic amino group of the +1 cytosine residue.

model that these overlapping sites could be the simplest explanation for the similar effects of the drugs on topoisomerase I. It is important to mention that the similarity between the two structures is even more important with the clinically active IND compound NB-506 than with R-**3**. Unlike R-**3**, NB-506 bears an OH group at position 11 (Figure 1). According to the molecular arrangement presented in Figure 7, the 11-OH group of NB-506 would be positioned just like the OH group of CPT at position 20. Thus, based on the superimposition of the two drug structures, one can now better understand why the two drug families produce very similar effects on topoisomerase I, despite their apparent chemical differences.

Recently, on the basis of the crystal structure of the complex formed between a 22 bp DNA duplex and a large fragment of human topoisomerase I, a model for the positioning of CPT within the enzyme active site was proposed (*39*). In this model, the CPT molecule interacts directly with the +1 cytosine residue on the nonscissile strand and with diverse key amino acid residues of the enzyme, including Asn 722, Arg 364, and Asp 533. Given the above-mentioned similarities between CPT and IND, it is plausible that the CO group of R-**3** engages in contact with the exocyclic amino group of the C+1 residue and the Arg 488 residue interacts with the carbohydrate substituent attached to the indole nitrogen, as schematized in Figure 8. The terminal CO group of the Asp 533 residue may also engage in direct contact with the 5′-hydroxyl group of the glucose residue. Stacking interactions with the Tyr 723 residue and the G+1 would further stabilize the drug−enzyme−DNA ternary complex. This model agrees satisfactorily with the structure−activity relationships previously reported (*24*−*27*). In particular, it supports the essential role played by the carbohydrate substituent attached to the IND chromophore (*28*).

We also considered the alternative "drug-stacking" model for the CPT−topoisomerase I−DNA ternary complex in which the CPT molecule is intercalated (*45*). According to this molecular model, the carbonyl group of CPT at position 16 engages in a hydrogen bond with the 5′ DNA terminal hydroxyl of the topoisomerase break. By analogy, we can postulate that the same hydrogen bond would form with the equivalent carbonyl group of IND at position 7. The stacking model is particularly well suited to rationalize the topoisomerase I poisoning activity of IND because R-**3** and related compounds have been shown to intercalate into DNA (*28*, *44*).

The Phe361 residue is apparently not in direct interaction with CPT, but it is in close proximity to the essential Arg 364 residue (*39*). So far, no molecular explanation has yet been offered to explain why the Phe361→Ser mutation results in the production of a catalytically active but CPT-resistant enzyme. Here we would like to suggest that in the resistant enzyme, the newly created Ser 361 residue attracts the Asp 533 residue which becomes inaccessible to CPT or R-**3**, rendering the drug inefficient toward topoisomerase I. However, this speculative explanation that would account satisfactorily with the experimental data is open to experimental probing.

In some way, the situation described here with topoisomerase I and its inhibitors CPT and IND is reminiscent of that known for the type 2 enzyme. Mutations in topoisomerase II can confer similar resistance to drugs of diverse structures. For example, the topoisomerase II inhibitors amsacrine and bisantrene present very distinct chemical structures but exert similar effects on topoisomerase II. Modeling studies have revealed that these two drugs share common steric and electronic features that constitute a specific pharmacophore (*42*, *46*). In the future, topoisomerase I and II inhibitors may be rationally designed by comparing the geometric and electronic properties of the drug−enzyme−DNA ternary complexes. The structural diversity between topoisomerase I/II poisons should be exploited to define the functional drug interaction domains.

This study has important implications in terms of drug design. Indeed, the work raises the concept that mechanistic differences between series of topoisomerase I mutant enzymes can be exploited by medicinal chemists to identify a specific pharmacophore for topoisomerase I inhibitors. There is no doubt that the enzymological approach will be increasingly exploited to elucidate the SAR of antitumor agents targeting topoisomerase I and hopefully to shape inhibitors endowed with novel pharmacological profiles.

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