Differential Stabilization of Eukaryotic DNA Topoisomerase I Cleavable Complexes by Camptothecin Derivatives

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ABSTRACT: Camptothecins belong to a group of anticancer agents with a specific mechanism of action: stabilization and trapping of eukaryotic DNA topoisomerase I (top1) cleavable complexes. Two water-soluble camptothecin derivatives are in clinical trial, and their anticancer activity appears promising: topotecan and CPT-11. The latter is hydrolyzed to its active metabolite, SN-38. We have previously reported that SN-38 is among the most cytotoxic camptothecin derivatives and that the cleavable complexes induced by SN-38 are more stable than those induced by CPT in human colon carcinoma cells [Tanizawa et al. (1994) *J. Natl. Cancer Inst. 86*, 836−842]. Top1 inhibition was further investigated by determining the salt-induced religation rates of top1-cleavable complexes in fragments from the top1 cDNA. Religation depended on both the local DNA base sequence and the drug structure. Cleavable complexes induced by SN-38 and 10,11-methylenedioxycamptothecin were markedly more stable (less rapidly reversible) than those induced by CPT, topotecan, and 9-aminocamptothecin. The stability of 10-hydroxycamptothecin-induced cleavable complexes was intermediate to those of CPT and SN-38, indicating that both the 10-hydroxy and the 7-ethyl group of SN-38 probably interact with the drug binding site of top1-cleavable complexes. A DNA oligonucleotide containing a single top1 cleavage site was also used to compare the camptothecin derivatives. The salt stability of drug-induced cleavable complexes in the top1 oligonucleotide was correlated with the drug potencies to induce top1 cleavage. Cell killing requires that trapped cleavable complexes be converted to DNA damage as a result of replication fork collision. Therefore, the persistence of cleavable complexes may have important implications for anticancer drug efficacy.

DNA topoisomerase I (top1) has become an important target for cancer chemotherapy since the discovery that this enzyme is inhibited by camptothecin (CPT) [for reviews, see Chen and Liu (1994), Pommier (1993), and Pommier and Tanizawa (1993)]. Top1 is expressed in both proliferative and quiescent cells. It participates in many vital cellular functions, such as RNA transcription, DNA replication, and recombination (Champoux, 1990; Chen & Liu, 1994; Pommier and Tanizawa, 1993; Wang, 1991).

CPT (Figure 1) was isolated in the 1960's from the bark of the Chinese tree *Camptotheca acuminata* and shown to exhibit potent antitumor activity (Wall et al., 1966). Clinical trials with the sodium salt of camptothecin in the 1970's were rapidly discontinued because of severe adverse effects [for reviews, see Potmesil (1994) and Slichenmyer et al. (1993)]. Two water-soluble camptothecin derivatives, CPT-11 (Kunimoto et al., 1987) and topotecan (Johnson et al., 1989), have recently been reported to have promising clinical activity (Potmesil, 1994; Slichenmyer et al., 1993). CPT-11 is poorly active against top1, but its metabolite SN-38 [7-ethyl-10-hydroxycamptothecin] (Kaneda et al., 1990; Kawato et al., 1991) is among the most potent top1 inhibitors (Tanizawa et al., 1994) (Figure 1). A water-insoluble analog, 9-aminocamptothecin (NSC603071) (9-AC), has recently been introduced in the clinic because of its strong antitumor activity against human colon cancer xenografts (Giovanella et al., 1989).

CPT inhibits top1 by stabilizing cleavable complexes, which are enzyme-catalyzed DNA single-strand breaks with the enzyme linked to the 3′-DNA termini of the breaks [for reviews, see Champoux (1990), Chen and Liu (1994), and Pommier (1993)]. CPT probably forms a drug−top1−DNA ternary complex and binds only weakly to either the DNA or top1 alone (Hertzberg et al., 1989; Hsiang et al., 1985; Leteurtre et al., 1993). Using oligonucleotides, Westergaard and co-workers have demonstrated that CPT inhibits top1-induced DNA cleavage and more efficiently blocks the top1 DNA religation reaction (Kjeldsen et al., 1992; Svejstrup et al., 1991). Similar conclusions had been drawn by Porter and Champoux (Porter & Champoux, 1989) who found that CPT enhances top1-mediated DNA cleavage the most at those sites where religation of the cleaved strand is the slowest.

CPT-stabilized cleavable complexes generally are rapidly reversible upon lowering of the drug concentration by dilution. Reversal can also be accomplished by increasing

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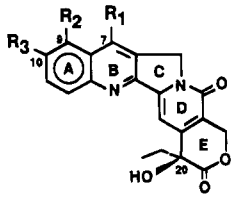
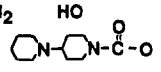
[1] Abbreviations: 9-AC, 9-amino-20-*S*-camptothecin; CPT, 20-*S*-camptothecin; CPT-11, 7-ethyl-10-[[[4-(1-piperidino)-1-piperidino]carbonyl]oxy]-20-*S*-camptothecin; MDO-CPT, 10,11-methylenedioxy-20-*RS*-camptothecin; 10-OH-CPT, 10-hydroxy-20-*S*-camptothecin; SDS, sodium dodecyl sulfate; SN-38, 7-ethyl-10-hydroxy-20-*S*-camptothecin; SSB, DNA single-strand breaks; top1, DNA topoisomerase I; TPT (topotecan), 9-[(dimethylamino)methyl]-10-hydroxy-20-*S*-camptothecin.



	R_1	R_2	R_3
Camptothecin (CPT)	H	H	H
9-amino-CPT (9-AC)	H	NH_2	H
Topotecan (TPT)	H	$(CH_3)_2NCH_2$	HO
CPT-11	CH_3CH_2	H	
SN-38	CH_3CH_2	H	HO
10-hydroxy-CPT (10-OH-CPT)	H	H	HO

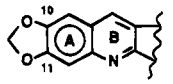
10,11-methylenedioxy-CPT (MDO-CPT) 

FIGURE 1: Chemical structures of camptothecin derivatives used in the present study.

salt (NaCl) concentration or by adding competitor DNA (Champoux & Aronoff, 1989; Covey et al., 1989; Hertzberg et al., 1989; Hsiang et al., 1985; Kjeldsen et al., 1988b; Tanizawa et al., 1994). In the cell, however, a fraction of the cleavable complexes are converted into DNA double-strand breaks upon collision with replication forks (Snapka & Permana, 1993; Tsao et al., 1993). Persistent DNA double-strand breaks have been hypothesized to trigger the drug-induced cytotoxic cascade (Holm et al., 1989; Hsiang et al., 1989a; Ryan et al., 1991). Persistence of cleavable complexes therefore should increase the likelihood of collision of replication forks with drug-stabilized cleavable complexes and consequent cytotoxicity.

We had previously found that SN-38 was more potent than CPT, TPT, and 9-AC in producing cleavable complexes in isolated nuclei, and that the cleavable complexes induced by SN-38 were less reversible upon drug removal than those induced by CPT (Tanizawa et al., 1994). The molecular basis for these differences was further examined by studying the religation rates of drug-induced cleavable complexes using purified DNA and topl.

EXPERIMENTAL PROCEDURES

Drugs. CPT, 9-AC, TPT, and CPT-11 were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) (Figure 1). CPT-11 and SN-38 were generously provided by Dr. Kiyoshi Terada (Yakult Honsha Co., Tokyo, Japan). 10-Hydroxycamptothecin (10-OH-CPT) was a gift from Dr. Randall K. Johnson (SmithKline Beecham, King of Prussia, PA). 10,11-Methylenedioxy-20-(*RS*)-camptothecin (MDO-CPT), 9-amino-10,11-methylenedioxy-20-(*RS*)-camptothecin (9-A-MDO), and 9-chloro-10,11-methylenedioxy-20-(*RS*)-camptothecin (9-C-MDO) were synthesized and kindly provided by Drs. Monroe E. Wall and Mansukh R. Wani (Research Triangle Institute, Research Triangle Park, NC). Drugs were dissolved in 10 mM dimethyl sulfoxide, except for MDO-CPT, which was dissolved in 1 mM dimethyl sulfoxide. Drug aliquots were kept for no more than 2 weeks at −20 °C. Further dilutions were made in water immediately before use.

DNA Substrates. Human topl cDNA (T1B) was kindly provided by Dr. William C. Earnshaw (Johns Hopkins University, Baltimore, MD) (D'Arpa et al., 1988). Cleavage reactions were performed with the *Ppu*MI/AvrII (from genomic position 1832 to 2391) and *Eco*RI/*Avr*II (from genomic position 2388 to 3644) fragments of T1B that had been labeled at the 3′-ends of antisense and sense strands with [α-^{32}P]dATP (New England Nuclear, Boston, MA) using Klenow fragment of DNA polymerase I (Stratagene, La Jolla, CA) (Tanizawa et al., 1994).

The following double-stranded 32mer oligonucleotide containing a single topl recognition site (caret) (Bonven et al., 1985; Tanizawa et al., 1993a) was purchased from the Midland Reagent Co. (Midland, TX):

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5'-GATCTAAAAGACTT^GGAAAAATTTTTAAAAAA
        ATTTTCTGAA CCTTTTTAAAAATTTTTTCTAG-5'
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The scissile (upper) strand was 3′-end-labeled with [α-^{32}P]-cordycepin (New England Nuclear, Boston, MA) using terminal deoxynucleotidyltransferase (Stratagene, La Jolla, CA). After removal of unincorporated cordycepin, 33mer ^{32}P-labeled single-stranded DNA was annealed with the same amount of the unlabeled complementary strand as described previously (Jaxel et al., 1991) (see Figure 5).

Topl Reactions. In order to form cleavable complex, substrate DNA was incubated with drugs in standard reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/mL bovine serum albumin) at 25 °C in the presence of calf thymus topl (GIBCO BRL, Gaithersburg, MD) for the indicated times. Reactions were stopped by adding sodium dodecyl sulfate (SDS) and proteinase K (final concentrations, 1% and 0.1 mg/mL, respectively), and reaction mixtures were further incubated at 37 °C for 30 min. In reactions with topl cDNA, DNA was precipitated with ethanol, rinsed twice with 70% ethanol, and resuspended in 2.5 μL of Maxam−Gilbert loading buffer. In the case of the oligonucleotide, 3.3 vol of loading buffer was added directly to the samples without ethanol precipitation. The treated topl cDNA and oligonucleotide were analyzed by 6% and 16% denaturing polyacrylamide sequencing gel electrophoresis, respectively (Jaxel et al., 1991). Cleavage sites were determined by comparison with Maxam−Gilbert sequencing markers (Jaxel et al., 1991). Radioactivity counting at drug-induced cleavage products and total radioactivity in each lane were quantified using a Betascope 603 blot analyzer (Betagen Inc.) or a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Drug-induced DNA cleavage was expressed as

$$\text{percent cleavage} = 100 \times \frac{\text{radioactivity in cleavage product}}{\text{total radioactivity}}$$

To investigate time course of reversal of drug-induced cleavable complexes, cleavable complexes were allowed to form at 25 °C for 15 min. Samples were then either kept at 25 °C or transferred to a 10 °C bath. Concentrated NaCl stock solution (7.5% of total volume) was then added to the reaction mixtures. Aliquots were taken at indicated times, and reactions were stopped by adding SDS. The samples were processed as described above. The bases at the 3′-

and 5′-termini of the analyzed cleavage sites were numbered −1 and +1, respectively (Jaxel et al., 1991; Tanizawa et al., 1993a,b).

RESULTS

Differential Reversal of Top1-Cleavable Complexes Induced by CPT and SN-38: Effects of Local DNA Sequence. The substrate DNA that was used for these experiments is the top1 cDNA that contains a number of top1 cleavage sites (Tanizawa et al., 1993b). In a first set of experiments, cleavable complexes were induced for 15 min at 25 °C. After taking an aliquot at time 0, NaCl solution was added (final concentration, 0.35 M) and aliquots were taken at various times. Figure 2 shows that top1 cleavage sites reversed with different kinetics depending on the local DNA sequence. Most of the CPT-induced top1 sites had a T at the 3′-terminus of the break (position −1), and the most intense sites (3422, 3489) had a G at the 5′-terminus (+1 position), which is consistent with previous observations (Been et al., 1984; Jaxel et al., 1991; Kjeldsen et al., 1988a,b; Porter & Champoux, 1989; Tanizawa et al., 1993b). Cleavage site 3489 was one of the strongest sites with a local base sequence around the break 5′-TAATT∧G-3′ [cleavage induced between T and G (caret)]. This result is consistent with the strong preference of CPT for TT∧G sequences (Jaxel et al., 1991; Tanizawa et al., 1993a,b). However, TT∧G is probably not sufficient for intense cleavage since site 3572 has the same local DNA sequence (5′-TAATT∧G) that site 3489 has but is weakly enhanced by camptothecin. For instance, we have recently found that the presence of a methyl base (5-methylcytosine or -thymine) at position −3 prevents top1 cleavage (Leteurtre et al., 1994b). Therefore, more distal DNA sequence elements probably modulate the intensity of top1 cleavage (Camilloni et al., 1991; Tanizawa et al., 1993b).

The slowly reversible sites generally had G^{+1} sites, while the rapidly reversible sites generally had a different base at position +1. It should also be noted that cleavage intensity was not always correlated with slow reversal kinetics (for instance, the rapidly reversible sites in panel B of Figure 2 were more intense than two of the slowly reversible sites (3530 and 3572). Hence, the steady-state number of cleavable complexes depends on both the formation and the reversal rate, which in turn depend on the local base sequence.

In both groups of cleavage sites (Figure 2, panel B)—slowly and rapidly reversible sites—SN-38-induced cleavable complexes reversed more slowly than CPT-induced complexes. These results demonstrate that SN-38-induced cleavable complexes are less reversible, and therefore appear more stable, than CPT-induced complexes.

Role of the 7-Ethyl and 9-Hydroxyl Groups of SN-38 in the Stabilization of Top1-Cleavable Complexes. SN-38 and CPT differ in side-chain moieties at positions 7 and 10. SN-38 has a 7-ethyl group and a 10-OH instead of hydrogens as in the case of CPT. In order to determine the origin of the differences between SN-38 and CPT, we compared the kinetics of formation and reversal of cleavable complexes induced by 10-OH-CPT with those induced by SN-38 and CPT (Figure 3). The reversal kinetics of 10-OH-CPT-induced cleavable complexes at three sites (3489, 3575, and 3579) was intermediate between those of SN-38 and CPT

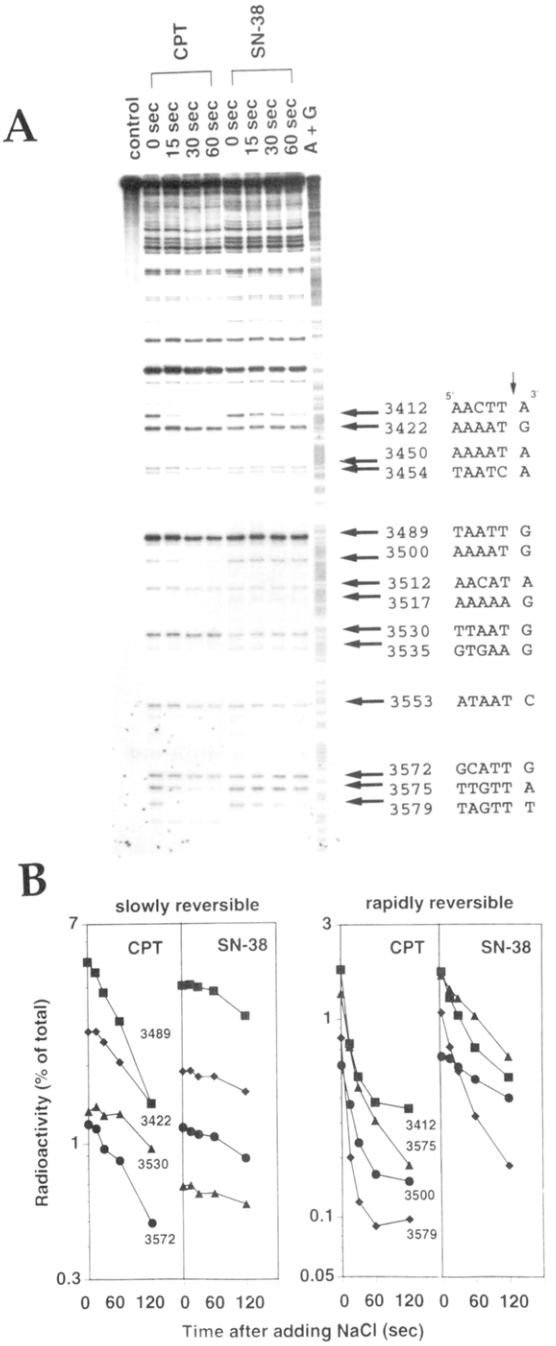

FIGURE 2: Differential reversibility of CPT- and SN-38-induced cleavable complexes in the presence of 0.35 M NaCl. The *Eco*RI/*Avr*II fragment of human top1 cDNA, which had been labeled at the 3′-end of the sense strand, was incubated with either 1 μM CPT or SN-38 in the presence of calf thymus top1 for 15 min at 25 °C. After an aliquot was taken at time 0, NaCl stock solution was added to 0.35 M (final concentration). Samples were taken at 15, 30, 60, and 120 s (samples at 120 s are not shown in panel A). Panel A: Autoradiography. Arrows and numbers indicate genomic positions of bases at the 3′-termini of the breaks (position −1). Base sequences from −5 to +1 are shown to the right. Untreated DNA is shown as control. DNA fragments after formic acid reactions (A+G) were electrophoresed in the same gel. Panel B: Cleavage sites classified into two groups according to their reversal kinetics. Four representative sites (see panel A) are shown for each group.

(Figure 3). Similar relationships among these derivatives were observed at all other sites examined (data not shown). These results indicate that both the 10-hydroxyl and the 7-ethyl group of SN-38 contribute to the stability of SN-38-induced top1-cleavable complexes.

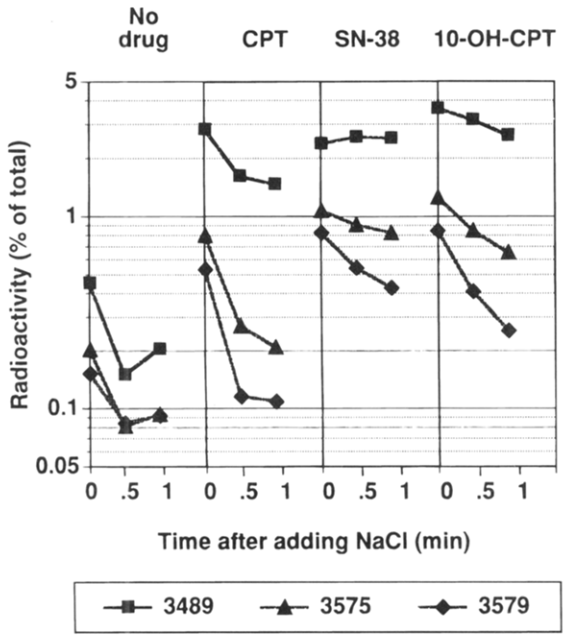

FIGURE 3: Time course of salt-induced reversal of top1-cleavable complexes induced in the presence and absence of camptothecin (CPT), SN-38, and 10-hydroxycamptothecin (10-OH-CPT). Sequencing gels as shown in Figure 2 were used to monitor cleavage at three top1 sites (at positions 3489, 3575, and 3579). Drug concentrations were 1 μM. Reversal was induced by adding 0.35 M NaCl (see Figure 2).

Salt Dependence and DNA Sequence Dependence for Reversal of Cleavable Complexes Induced by SN-38, 10-OH-CPT, CPT, TPT, and 9-AC. The stability of the drug-induced cleavable complexes was compared as a function of the NaCl concentration added during the reversal phase. Figure 4 shows that addition of 0.09 M NaCl was sufficient to reverse the CPT-, TPT-, and 9-AC-induced cleavable complexes, while the cleavable complexes induced by SN-38 and 10-OH-CPT were more resistant to NaCl. Increasing the NaCl concentration from 0.18 to 0.35 M NaCl did not reverse the cleavable complexes further for any of the drugs. These results are consistent with the reversal rates described above and indicate that the cleavable complexes induced by 9-AC and TPT are less stable than those induced by SN-38 and 10-OH-CPT.

We have previously shown that most top1 cleavage sites are induced by camptothecin derivatives, but that the relative cleavage enhancement at different sites is different among drugs (Tanizawa et al., 1994). Figures 2 and 4 are also consistent with DNA sequence selectivity differences among CPT derivatives. For example, relative cleavage intensities at positions 3530 and 3535 (Figure 2) were different in the presence of CPT or SN-38. CPT was approximately 4-fold more potent than 9-AC at position 2284, while the difference between CPT and 9-AC was less pronounced at the other three sites (Figure 4).

Differential Stability of Drug-Induced Cleavable Complexes in an Oligonucleotide Containing a Single Top1 Cleavage Site. When substrate DNA is cleaved at several sites, the amount of cleavage at a given site can be affected by the other sites, especially in the case of cleavages far from the 3'-labeled end. To avoid such masking effects and to confirm the above results obtained using top1 cDNA as a substrate, we analyzed the kinetics of dissociation of drug-induced cleavable complex using a 33mer oligonucleotide

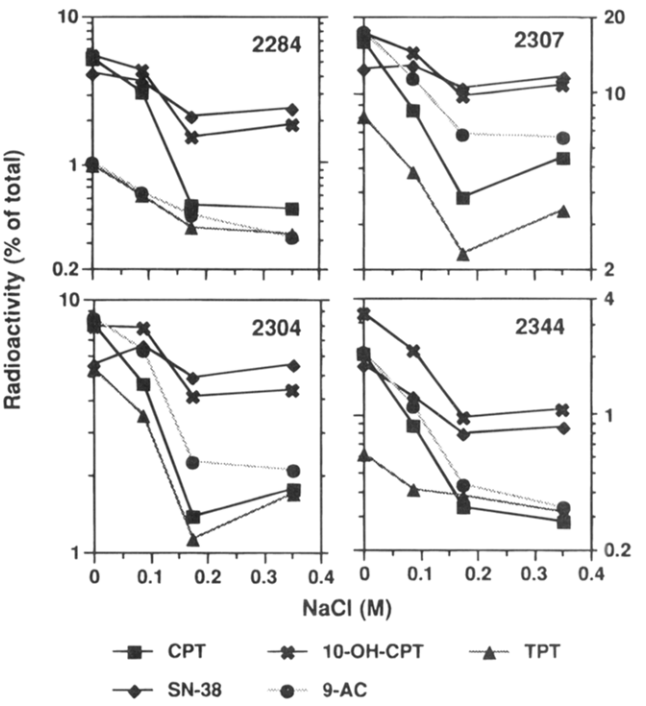

FIGURE 4: Differential stability of top1-cleavable complexes in the presence of camptothecin and derivatives under various NaCl concentrations. The *Ppu*MI/*Avr*II fragment of human top1 cDNA, which had been 3'-^{32}P-end-labeled on the antisense strand, was used for reactions. Cleavable complexes were formed at 25 °C for 15 min in the presence of 1 μM drug concentrations. NaCl stock solution or water [7.5% (v/v)] was added to reaction mixtures that were then incubated for an additional 5 min. Cleavage was monitored at four selected sites detected on the autoradiography (not shown). CPT-, SN-38-, 10-OH-CPT-, TPT-, and 9-AC-induced cleavages at positions 2284, 2304, 2307, and 2344 were analyzed by Betascope. Top1-mediated cleavages without drug and NaCl are less than 0.4, 0.3, 0.4, and 0.5% for the sites at positions 2284, 2304, 2307, and 2344, respectively.

with a single top1 recognition site (Tanizawa et al., 1993a) (Figure 5). Again SN-38 produced very stable cleavable complexes. In these experiments, we also examined 10,11-methylenedioxycamptothecin (MDO-CPT) since it is one of the most potent CPT derivatives (Hsiang et al., 1989b; Jaxel et al., 1989; O'Connor et al., 1990, 1991; Pommier et al., 1991). Figure 5 shows that MDO-CPT induced more intense and more stable top1 cleavage than SN-38. Half-time reversals (minutes at 25 °C) were 1.3 for MDO-CPT, 0.9 for SN-38, 0.6 for 10-OH-CPT, 0.5 for TPT, 0.4 for 9-AC, and 0.3 for CPT. Therefore, the results obtained with the oligonucleotide are generally consistent with the data obtained in the cDNA fragments. CPT derivatives exhibit marked differences in the reversibility and stability of top1-cleavable complexes.

The oligonucleotide was used further to investigate the time course of salt-induced reversal as a function of temperature, with lower drug concentrations (0.1 instead of 1 μM), and with other top1 inhibitors unrelated to CPT: saintopin (STP) and bulgarein (BLG) (Fujii et al., 1993; Leteurtre et al., 1994a; Yamashita et al., 1991). Figure 6 shows the result of a typical experiment using 0.25 M NaCl for reversal. In panel A, reversals were performed at 25 °C. Saintopin and bulgarein top1-cleavable complexes reversed very rapidly (half-time reversal, approximately 0.3 min; Figure 6A). In panel B, reversals were performed at 10 °C. CPT-induced top1-cleavable complexes reversed approximately 10-fold more slowly than at 25 °C (half-time

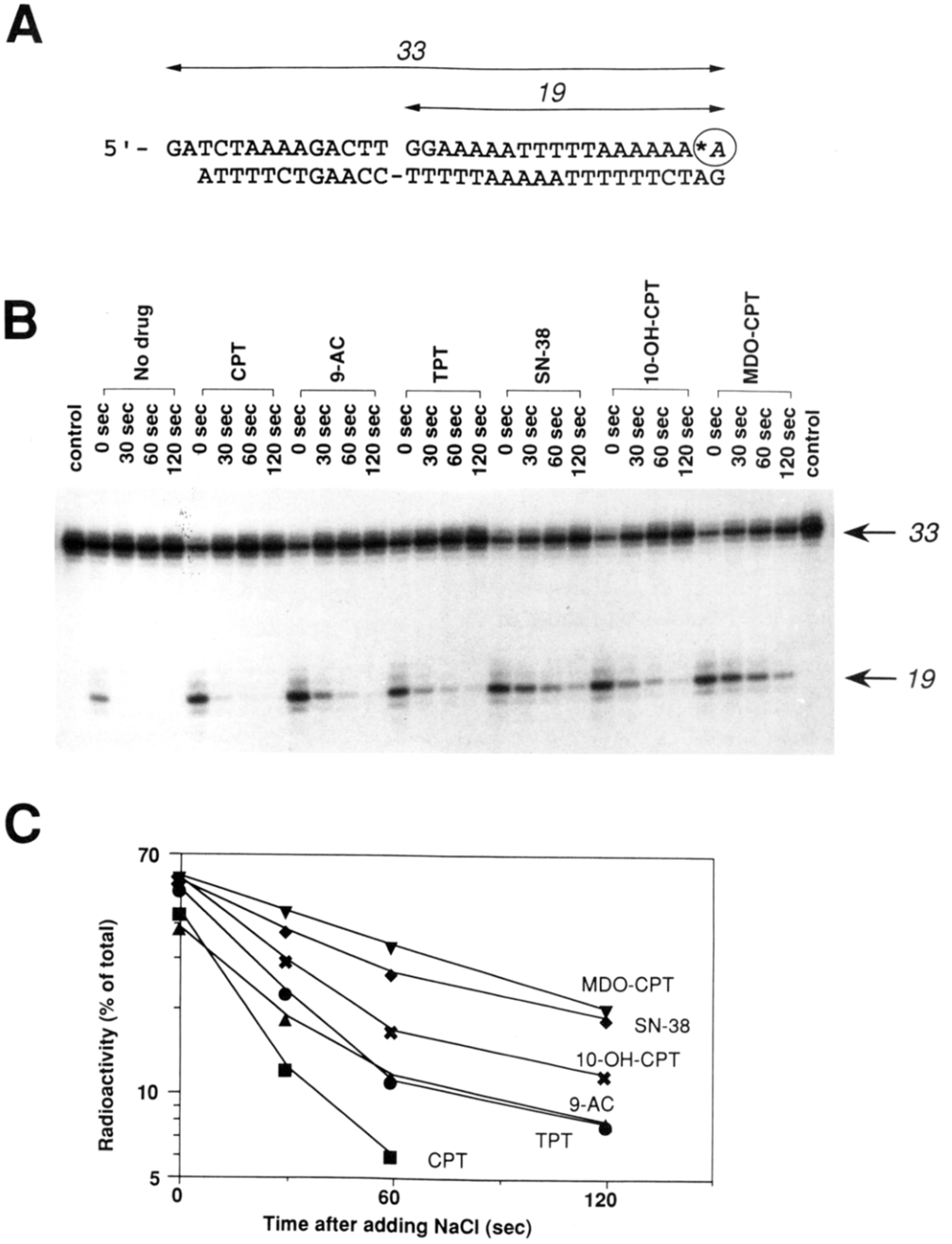

FIGURE 5: Differential reversal of drug-induced cleavable complex in an oligonucleotide containing a single top1 cleavage site. Salt-induced reversal of drug-induced top1-cleavable complexes which had been formed at 25 °C for 15 min in the presence of 1 μM drug concentrations was investigated under 0.35 M NaCl. Panel A: Oligonucleotide used. Labeling was performed with [^{32}P]cordycepin (*A) at the 3′-terminus of the scissile (upper) strand. Upon cleavage between T and G, the 33-mer is converted to a 19-mer. Panel B: Autoradiography. Panel C: Cleavage quantified using a Betascope and plotted as a function of time after addition of 0.35 M NaCl. Symbols are the same as in Figure 4.

= 2.9 vs 0.35 min). Similar differences were observed for 9-AC (half-time = 4.1 at 10 °C vs 0.42 min at 25 °C) and MDO-CPT (half-times = 12 vs 1.2 min). 9-Amino-MDO-CPT and 9-chloro-MDO-CPT exhibited even slower reversal time courses than MDO-CPT.

Drug-induced cleavage immediately before reversal (time 0 in Figure 6) was also markedly different between drugs at the lower concentration used (0.1 μM). A direct relationship was observed between intensity of cleavage before reversal and half-life of salt-induced reversal (Figure 6B). This observation is consistent with the fact that CPT (and probably its derivatives as well) enhance top1-cleavable complexes mainly by inhibiting DNA religation (Svejstrup et al., 1991).

DISCUSSION

The present study indicates that the local DNA sequence immediately around the top1 cleavage sites influences the intensity and stability of drug-induced top1-cleavable complexes. As reported previously, the most intense cleavage occurred at sites with T^{-1} and G^{+1} (Jaxel et al., 1991; Tanizawa et al., 1993b). Also, these sites tended to be the most slowly reversible, consistent with the notion that intense cleavage is primarily due to slow religation (Porter & Champoux, 1989). Inhibition of top1 by CPT has been postulated to result from inhibition of the religation step (Kjeldsen et al., 1988a,b) and from the drug preferentially binding into the top1 DNA cleavage sites bearing a guanine

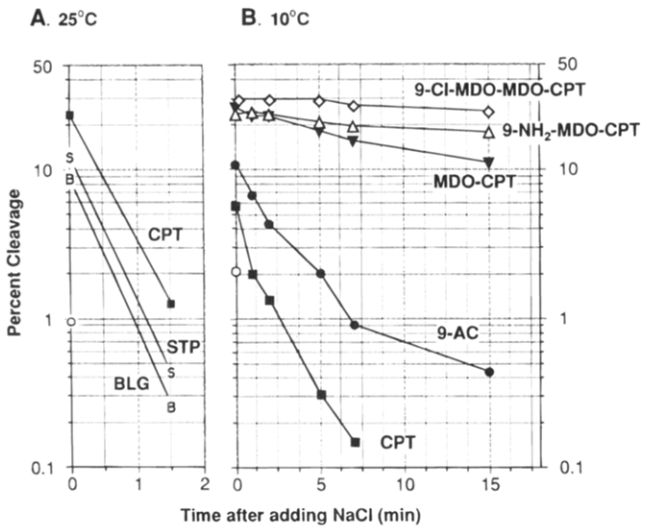

FIGURE 6: Effects of drugs and temperature on salt-induced reversal of top1-cleavable complexes in the top1 oligonucleotide. The oligonucleotide used is shown in Figure 5A. Top1-cleavable complexes were induced at 25 °C for 15 min in the presence of 0.1 μM drugs (as indicated). Reaction mixtures were then either kept at 25 °C (panel A) or transferred to 10 °C (panel B) for 5 min before addition of NaCl (0.25 M final concentration). Aliquots were taken at the indicated times. Cleavage was quantified using a PhosphorImager. Top1-induced cleavage shown in the *Y* abscisses (○) reversed below detectable levels at the first time points.

at the 5′-DNA terminus (G^{+1}) (Jaxel et al., 1991). UV activation experiments have confirmed the affinity of CPT for guanine residues (Leteurtre et al., 1993). The present study also demonstrates that, for some cleavage sites, slow reversal is not always associated with strong intensity (see Figure 2). Hence, stability of a CPT-induced top1−DNA complex is DNA sequence dependent, and both the formation ("on-rate") and the reversal ("off-rate") rate determine cleavage intensity (Leteurtre et al., 1994b).

This study focussed on CPT derivatives that are presently used in the clinic and demonstrates mechanistic differences among them. The derivatives were found to differ with respect to (1) potency of induction of cleavable complexes, (2) stability of cleavable complexes in the presence of high NaCl concentration, and (3) DNA sequence preferences for complex formation (Tanizawa et al., 1994). These results are in agreement with, and further document at the molecular level, the differences observed in human colon carcinoma HT-29 cells and isolated nuclei for SN-38 and CPT (Tanizawa et al., 1994). Other differences have also been observed among camptothecin derivatives with respect to their binding to human serum albumin (Burke & Mi, 1994) and the stability of the E-ring (see Figure 1) that needs to be in the lactone form for the drug to be active (Burke et al., 1993; Hertzberg et al., 1989; Hsiang et al., 1989b; Jaxel et al., 1989).

Comparison of stability among CPT-, SN-38-, and 10-OH-CPT-induced cleavable complexes reveals that the OH group at position 10 enhances stability of cleavable complexes. The difference between SN-38- and 10-OH-CPT-induced cleavable complexes demonstrates that the ethyl group at position 7 is also important for stabilizing the interaction between CPT derivatives and top1−DNA complex. In animal models, Kunimoto et al. showed that SN-22 (7-ethylcamptothecin) was more potent than CPT (Kunimoto et al., 1987). The weaker activity of TPT compared to 10-OH-CPT shows that the side chain added at position 9 for water solubility decreases top1 inhibition. This finding is consistent with Kingsbury's report in which drug concentrations that produced 50% DNA cleavage in the presence of top1 were compared (Kingsbury et al., 1991). Altogether, these results are consistent with the binding of CPT to a selective receptor site, probably at the enzyme−DNA interface. Addition of both the hydrophobic 7-ethyl group and the 10-hydroxy (or 10,11-methylenedioxy) group may increase the drug receptor site interactions and affinity. At the present time it is not possible to test this possibility further due to lack of top1 structural information.

MDO-CPT induced salt-resistant cleavable complexes. MDO-CPT is also among the most cytotoxic camptothecins toward several established cancer cell lines (Hsiang et al., 1989b; Jaxel et al., 1989; O'Connor et al., 1990, 1991; Pommier et al., 1991). In the case of MDO-CPT, as previously proposed (Pommier et al., 1991), the additional ring system opposite the B ring may stabilize the CPT molecule through hydrophobic interaction with top1−DNA complexes, hence forming stronger interactions with top1−DNA cleavable complexes. Further stabilization was observed with amino and chloro substitutions at position 9.

The salt resistance of SN-38-cleavable complexes is consistent with the slow reversibility of cleavable complexes upon drug removal in isolated cell nuclei (Tanizawa et al., 1994). We also found that the most potent inducers of cleavable complexes (SN-38 and MDO-CPT and its two derivatives, 9-NH_2-MDO-CPT and 9-Cl-MDO-CPT) (Hsiang et al., 1989b; Jaxel et al., 1989; Tanizawa et al., 1994; Wall et al., 1993) exhibited the slowest salt-induced reversal. 9-AC and 10-OH-CPT produced top1-cleavable complexes that were slightly more salt resistant than those induced by CPT, and both are also slightly more potent inducers of top1-cleavable complexes than camptothecin (Jaxel et al., 1989). These observations suggest that resistance to salt-induced reversal may be relevant to the stability of drug-induced top1-cleavable complexes under physiological conditions.

The stability of the SN-38- and MDO-CPT-induced cleavable complexes may be important for drug activity. CPT and its derivatives, TPT, CPT-11 (SN-38 being the active metabolite), and 9-AC exhibit promising activity in early clinical trials. The cytotoxicity of CPT and its derivatives is strongly time dependent probably because of the need for the drug-induced cleavable complexes to be converted into DNA double-strand breaks upon collision with replication forks (Holm et al., 1989; Hsiang et al., 1989a; Pommier et al., 1994). Hence, persistence of cleavable complexes is probably essential for optimum cytoxicity. Stable cleavable complexes, such as those observed with SN-38, probably explain the slow reversal and the high frequency of drug-induced protein-linked DNA breaks in cells and are probably a key element in the drug cytotoxic potency (Tanizawa et al., 1994). Development of camptothecin derivatives with even slower reversal kinetics may provide new ways to obtain more active anticancer agents.

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