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Advances in Brief

Topotecan Lactone Selectively Binds to Double- and Single-Stranded DNA in the Absence of Topoisomerase I

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Abstract

We report the first experimental observation that a clinically important camptothecin [CPT; topotecan (TPT), a water-soluble CPT] binds directly and noncovalently to double-stranded DNA and single-stranded DNA structures in the absence of topoisomerase I, but only in the lactone form. We observed clear DNA sequence specificity of the TPT lactone binding to duplex DNA, which was comprised of alternating purine-pyrimidine sequences that contained dT. These structural studies of direct TPT lactone-DNA binding support several important considerations involving possible mechanism(s) of anticancer activity of CPT-type drugs containing a 20(*S*) lactone moiety.

Introduction

During the last 30 years, the CPTs[2] (Fig. 1) have emerged as an important new class of antitumor drugs (1–5). Water-soluble CPTs, such as CPT-11 and TPT, have demonstrated clinically important anticancer activity in fluoropyrimidine-refractory colorectal and ovarian cancer, respectively (2–5). A broad spectrum of potent antitumor activity has been observed with CPT and derivatives thereof, and extensive research efforts have been made in the last 15 years to discover novel and more active CPT analogues (2–10). Despite these intensive research efforts, the precise physicochemical elements of CPT interaction that lead to tumor cell death remain poorly understood. Studies of topo I-linked DNA breaks have led to the proposal that CPT interferes with the breakage-reunion reaction of topo I by trapping a reaction intermediate known as a "cleavable complex" (11, 12). It has also been proposed that CPT binds to the DNA-topo I complex subsequent to the DNA cleavage step (13–15) and that CPT initially binds to the DNA-topo I complex noncovalently, which is then followed by alkylation of topo I or DNA at a later time (16). The current view of interactions of CPT (and derivatives thereof) in cleavable complexes is that the CPT molecule interacts with the DNA base that is situated 3′ to the topo I cleavage site.

To date, there has been very limited direct experimental evidence that CPT or derivatives thereof bind directly to DNA in the absence of topo I, although mixed observations of this possible interaction have been reported in the literature. Earlier work suggests that CPT derivatives bind to neither isolated DNA structures nor isolated topo I; they can only bind to the DNA-topo I complex (12). Other studies have suggested that CPTs may weakly bind to intact dsDNA (17, 18).

Recently, an important study by Yang *et al.* (19) showed that the presence of dsDNA resulted in increased solution concentrations of the lactone forms of TPT and CPT-11, identified and measured as unbound lactone forms by HPLC, in the absence of topo I. These investigators also reported that the presence of dsDNA promoted the conversion of carboxylate forms of TPT and CPT-11 to their respective lactone forms in solution, as measured as unbound species of the drug. They also reported NMR results, indicating that TPT binds to a short DNA oligomer at 2°C and pH 5.0 in the absence of topo I; however, a determination of any preferential TPT lactone or carboxylate DNA binding was not made. On the basis of HPLC measurements of free drug in solution in the presence of DNA, these investigators reported that TPT prefers binding to d(G-C) sequences over binding to d(A-T) sequences.

In our view, several crucial mechanistic areas regarding specific interactions of CPT class drugs remain to be explored: (*a*) quantitative characterization of direct molecular binding by CPTs to target molecules such as dsDNA, ssDNA, and topo I; (*b*) in the event that CPT binding is observed with a target molecule, quantitative characterization of the relative amounts of lactone and carboxylate species that interact with a molecular target; (*c*) in the event specific drug-DNA or drug-topo I binding interactions are identified, further characterization of any predilection for target site or sequence specificity for drug binding; and (*d*) an attempt to explain the structural and chemical mechanisms for the biological observation of lactone-mediated cytotoxicity of CPTs.

It is well known that CPTs show pH-mediated equilibration between the lactone and carboxylate form (Ref. 20; Fig. 1) in aqueous solution. The CPT lactone and carboxylate forms are two entirely different chemical entities with very different physical properties and would be expected to usually have different molecular interaction behaviors with target macromolecules, namely, DNA, topo I, and the DNA-topo I complex. Although certain types of CPTs are reported to interact in varying amounts of lactone *versus* carboxylate forms with albumin (21), no biophysical studies have been conducted to determine any differences in possible direct DNA binding between the lactone and carboxylate forms of a CPT molecule. Numerous studies have shown that the administration of the closed lactone E ring is essential for both topo I inhibition and maximal antitumor activity (22–25), although the mechanism of lactone cytotoxicity has been poorly understood from a structural and functional point of view. The research reported herein was aimed at answering some of the questions regarding the mechanism(s) of action for CPT class drugs, using TPT as a model compound that is in clinical use.

TPT (Fig. 1) is a water-soluble analogue of CPT, and it has recently been approved for the treatment of refractory recurrent ovarian cancer in the United States and, therefore, serves as a clinically relevant CPT derivative for our studies. Using TPT as a representative model compound, we initiated NMR studies of TPT interactions with dsDNA and ssDNA polymers. We focused on the identification of possible specific drug interactions of TPT with DNA. We briefly address the following objectives in this report: (*a*) to identify and

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[2] The abbreviations used are: CPT, camptothecin; TPT, topotecan; topo I, topoisomerase I; dsDNA, double-stranded DNA; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ssDNA, single-stranded DNA; CT-DNA, calf thymus DNA; D_2O, heavy water.

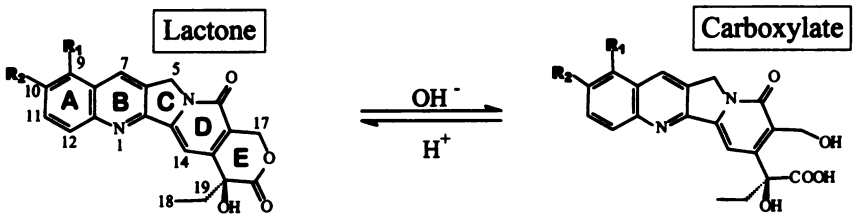

Fig. 1. The lactone and carboxylate forms of CPT and its analogue TPT.

CPT: $R_1, R_2 = H$

TPT: $R_1 = CH_2\overset{\oplus}{N}H(CH_3)_2$, $R_2 = OH$

characterize any differences between lactone and carboxylate forms regarding TPT binding to dsDNA and ssDNA and (*b*) to determine whether there is any dsDNA sequence specificity for drug binding.

Materials and Methods

Chemicals. Synthesis of 9-(dimethyl amino)methyl-10-hydroxycamptothecin (TPT; Fig. 1) acetate salt was accomplished in our laboratory using the following synthetic method. 20(*S*)-CPT was converted to the respective *N*-oxide using peracetic acid. The CPT *N*-oxide was then photoirradiated to obtain 10-hydroxycamptothecin (26). 9-(Dimethyl amino)methyl-10-hydroxycamptothecin was obtained by treatment of the crude 10-hydroxycamptothecin with aqueous dimethyl amine, aqueous formaldehyde, and acetic acid (27). The water soluble fraction containing the title compound was separated from organic soluble contaminants and purified to >97% purity for NMR studies. All synthetic DNA polymers [single strands of poly(dA) and poly(dT); double strands of poly(dA)·poly(dT), poly(dA-dT)$_2$, poly(dG)·poly(dC), poly(dG-dC)$_2$, and poly(dT-dG)·poly(dC-dA)] were purchased from Pharmacia Biotech. Sonicated CT-DNA (~200 bp) was a generous gift from Dr. W. D. Wilson (Georgia State University, Atlanta, GA).

NMR Experimental Materials and Methods. Phosphate buffer (N00) containing 7.5 mM NaH_2PO_4-0.1 mM EDTA with the final pH adjusted to 6.28 was used for all NMR studies. TPT was dissolved in N00/D_2O buffer to 0.5 mM concentration. For each NMR experiment, 0.7 ml of the 0.5 mM N00/D_2O TPT stock solution was transferred to a 5-mm NMR tube and, prior to use, was dried twice by N_2 stream and then redissolved in 100% D_2O. A trace amount of sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 was added to each NMR sample as an internal reference. A suitable quantity of DNA stock solution was added to the TPT NMR sample to achieve the desired DNA bp:TPT ratio (for duplex DNA) or base:TPT ratio (for ssDNA). NMR titration studies were carried out on the Varian GEMINI 2000 300-MHz spectrometer at 25 ± 0.5°C. 8K data points were collected for each experiment, and the raw data were processed by Felix (Biosym/MSI) software. The 8K data were zero-filled to 16K, and an exponential weighting function of 1.2 was applied prior to Fourier transformation.

Results and Discussion

The lactone-carboxylate equilibrium of TPT displayed in Fig. 1 is sensitive to the pH of the medium (20). To more fully identify and characterize the relative binding of TPT lactone *versus* carboxylate form, we found that pH 6.28 resulted in an approximate 50:50 mixture of the two TPT species; this is in agreement with reported HPLC results (20). Reasonably separated one-dimensional ^{1}H NMR resonances for the lactone and carboxylate forms of TPT were obtained and characterized at pH 6.28 and 25°C.

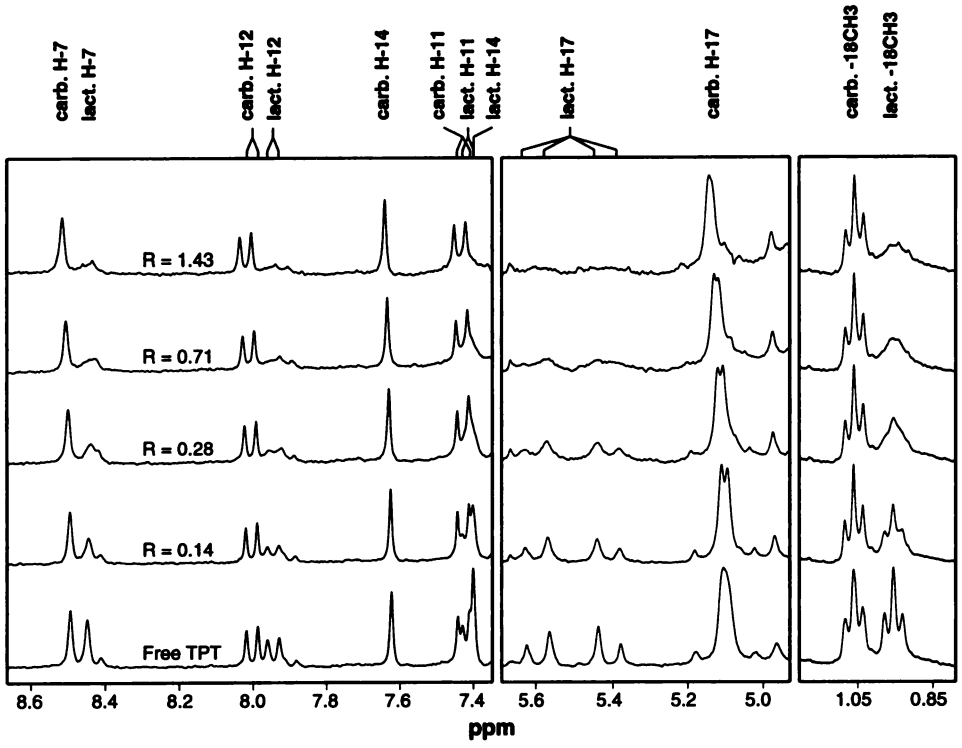

Fig. 2. Effects of titrating dsDNA poly(dG-dC)$_2$ into TPT solution on the ^{1}H NMR spectra of TPT at pH 6.28 and 25°C. *Top*, ^{1}H resonance assignments. *carb.*, carboxylate-form resonances; *lact.*, lactone-form resonances. See Fig. 1 for numbering convention. *R*, DNA bp:TPT ratio. Only the proton resonances of the lactone form TPT are affected. The presence of DNA has negligible effects on the resonances of the carboxylate form.

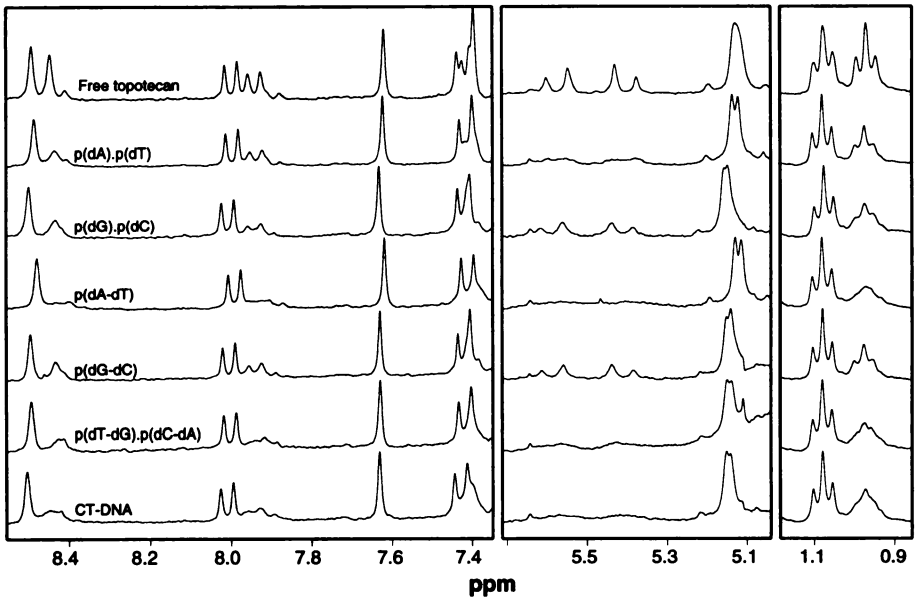

Fig. 3. Effects of dsDNA polymers with different sequences on the ^{1}H NMR spectra of TPT. See Fig. 2 for ^{1}H resonance assignments and experimental conditions. The DNA bp:TPT ratio was 0.28 in each spectrum.

These working conditions, using a 50:50 mixture of TPT lactone-carboxylate, served as a reasonable quantitative starting point to observe the effects of DNA on the ^{1}H spectrum of both species of TPT in solution. Accordingly, one-dimensional ^{1}H NMR spectra of TPT lactone and carboxylate were collected as a function of DNA concentration under these conditions. Although pH 6.28 is ~1 log less than physiological pH (*i.e.*, pH 7.35), we felt that it was important to have approximately equal concentrations of the two TPT species for these studies, for the purpose of quantifying the relative direct binding of each TPT species to DNA.

Fig. 2 shows NMR-monitored TPT ^{1}H resonances on the titration of poly(dG-dC)$_2$, with DNA bp:TPT ratios ranging from 0 to 1.43. This figure is representative of all our TPT-dsDNA titration studies; these all showed similar changes in spectral patterns with different degrees of line broadening. As shown in Fig. 2, in the presence of dsDNA, all of the resonances of the TPT lactone are broadened; ^{1}H resonance broadening is expected when a small molecule binds to dsDNA polymer (28, 29). On the other hand, resonances of the TPT carboxylate exhibited no substantive changes throughout the DNA titration process. The ^{1}H resonance broadening of only the lactone form TPT

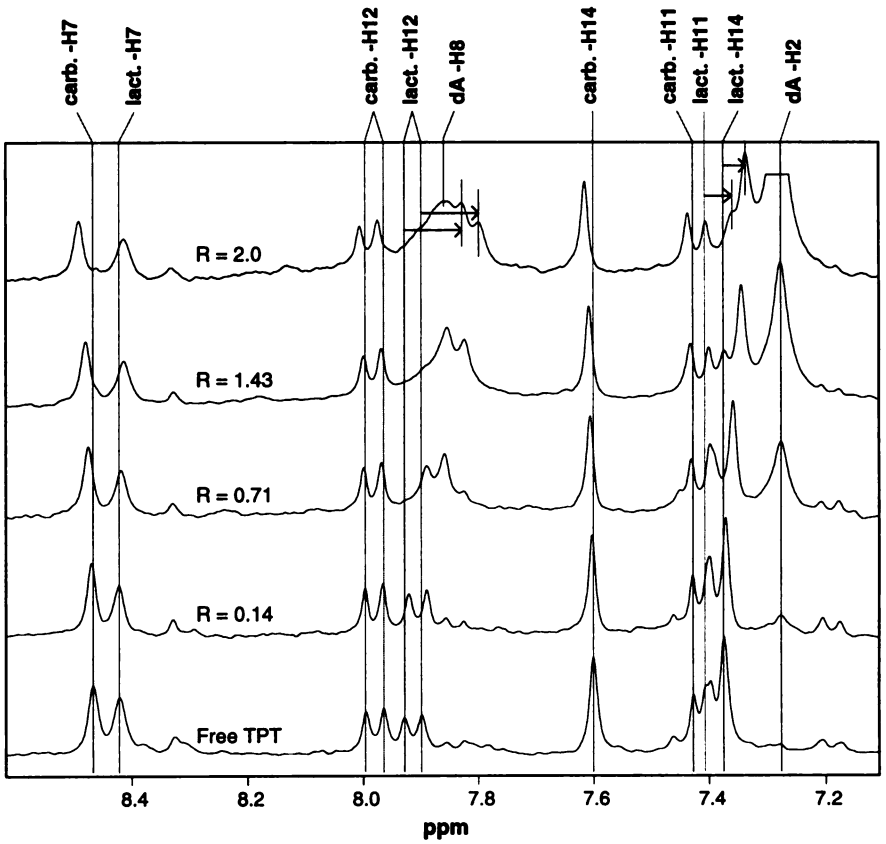

Fig. 4. The ^{1}H NMR spectra (only the aromatic ^{1}H resonance region) for the titration of ssDNA poly(dA) into TPT at pH 6.28 and 25°C. *R*, DNA base:TPT ratio. *Arrows*, amount and direction of chemical shift changes for resonances that sensitively responded to ssDNA titration.

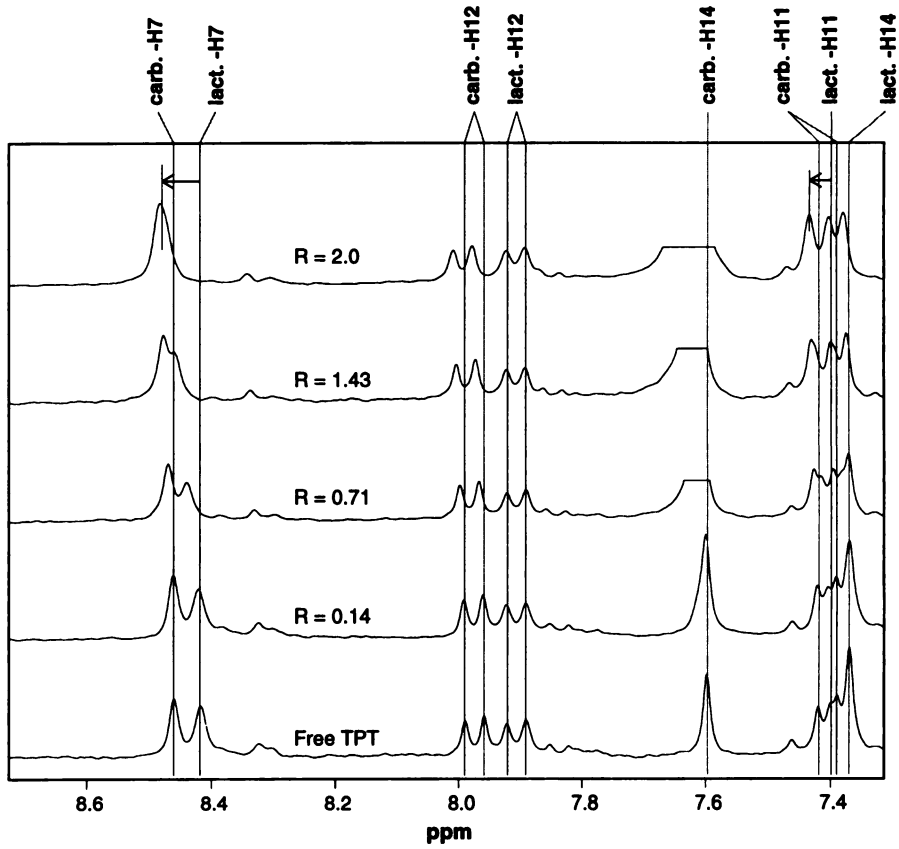

Fig. 5. The ^{1}H NMR spectra (only the aromatic ^{1}H resonance region) for the titration of ssDNA poly(dT) into TPT at pH 6.28 and 25°C. *R*, DNA base:TPT ratio. *Arrows*, amount and direction of chemical shift changes for resonances that sensitively responded to ssDNA titration.

clearly indicates that only the TPT lactone binds to the intact dsDNA structure. The TPT carboxylate form has essentially no binding interaction with dsDNA under identical experimental conditions.

The effects of different dsDNA sequences on the line broadening of TPT ^{1}H NMR resonances is depicted in Fig. 3. In all cases, the DNA bp:TPT ratio is kept constant (R = 0.28). Our first major observation is that, with a constant DNA bp:TPT ratio, the TPT lactone ^{1}H resonance line broadening is greater when the dsDNA contains dT residues, as in the cases of poly(dA)·poly(dT), poly(dA-dT)$_2$, poly(dT-dG)·poly(dC-dA), and CT-DNA. This observation is consistent with preferential TPT lactone binding to DNA sequences containing dT. Another key observation is that, of the three synthetic dsDNA polymers containing dT residues, the dsDNA polymers with alternative purine-pyrimidine sequences, poly(dA-dT)$_2$ and poly(dT-dG)·poly(dC-dA), result in much greater TPT ^{1}H resonance line broadening relative to the homopurine-homopyrimidine duplex poly(dA)·poly(dT). To confirm the possible preference for dT residues under identical pH, temperature conditions, and drug:DNA concentrations, we observe a clear reduction in TPT ^{1}H resonance line broadening in dsDNA polymers that lack dT, including poly(dG)·poly(dC) and poly(dG-dC)$_2$. Finally, we also observe that poly(dC-dG)$_2$ does not result in greater amounts of TPT ^{1}H resonance line broadening than its homopurine-homopyrimidine counterpart, poly(dG)·poly(dC). From this, it is clear that there is a molecular preference for the TPT lactone species to selectively interact with dT residues in dsDNA.

Quite interestingly, we also observe that the binding interaction of TPT lactone in poly(dT-dG)·poly(dC-dA) is equivalent to that observed in poly(dA-dT)$_2$, although the poly(dT-dG)·poly(dC-dA) polymer contains much less dT than does the poly(dA-dT)$_2$ sequence. This observation suggests that the dT-dG could be a preferred DNA sequence for TPT lactone binding to dsDNA.

Collectively, these observations provide the first direct experimental evidence that only the TPT lactone binds to dsDNA and that such dsDNA binding by TPT lactone appears to have sequence specificity. These observations suggest that dsDNA sequences containing 5′-purine-dT-3′ or 5′-dT-purine-3′ segments are preferred binding targets for the TPT lactone. The higher binding affinity of TPT lactone to DNA sequences containing dT residue also suggests that TPT could interact directly with the hydrophobic 5-methyl group of dT. The 5-methyl group of dT projects into the major groove of the DNA, and accordingly, we believe that the observed preferential binding of TPT lactone to dT residues may be mediated by hydrophobic interactions between regions of the TPT molecule and the 5-methyl group of dT in the major groove of dsDNA.

The effects of two ssDNA polymers, poly(dA) and poly(dT), on the ^{1}H NMR resonances of TPT are depicted in Figs. 4 and 5. We observed that, as in the case of poly(dA) titration studies, the H11, H12, and H14 protons of the TPT lactone form showed noticeable up-field chemical shifts. The magnitude of the up-field chemical shifts was in the relative order of H12 (>0.1ppm) $\gg$ H11 ~ H14 (~0.05 ppm), whereas the chemical shift change of the H7 proton was very small (Fig. 4). On the other hand, in the case of poly(dT) titration, the H7, H11, and H14 of lactone form TPT showed noticeable down-field shifts, whereas H12 resonance showed very small or no shifts (Fig. 5). Again, the TPT carboxylate showed only small and uniform down-field chemical shifts in both ssDNA titration studies; this observation suggests the absence of specific DNA binding by the TPT carboxylate form. These results indicate that the binding of the TPT lactone to ssDNA sequences results in unique spectral patterns as a function of DNA sequence; these differences in spectral patters indicate different modes of TPT lactone binding in a given DNA sequence. The biological significance of these differences in the mode of TPT lactone binding to poly(dA) *versus* poly(dT) and ssDNA *versus* dsDNA are unclear at this time, but we are pursuing further studies in this area.

Our results are consistent with the data published by Yang *et al.* (19), who showed that TPT can bind to a dsDNA oligomer in the absence of topo I. Additionally, we have shown that only the TPT lactone species binds to dsDNA and ssDNA. In contrast to Yang *et al.*, we did not observe any increase in the unbound TPT lactone concentration in solution in the presence of dsDNA by NMR. We have also determined that TPT lactone exhibits sequence specificity for dsDNA containing alternating pyrimidine-purine sequences when the pyrimidine residue is dT. In contrast to Yang *et al.*, we did not observe a relative sequence preference of TPT for CG sequences over TA sequences, and there is a notable difference in our methods, *i.e.*, we used NMR to identify the relative amounts of bound TPT lactone and unbound TPT carboxylate in the presence of DNA.

Recently, Chourpa *et al.* (30) published a study on DNA regulation of CPT lactone hydrolysis. They observed that the CPT lactone form was stabilized by two of three DNA oligomers, whereas no influence on CPT carboxylate forms was observed. This observation implicitly supports our conclusion here; namely, that only CPT lactone forms bind directly to DNA. Chourpa *et al.* (30) also observed, as we did, that the presence of DNA did not promote the conversion of CPT carboxylate to lactone; this observation is different from that of Yang *et al.* (19). Interestingly, Chourpa *et al.* (30) observed that the GC lacking DNA sequence containing dT residue did not stabilize the lactone form, whereas our NMR results suggested that the lactone form prefers to bind to the alternating purine-dT sequences. Considering the differences in the experimental conditions and DNA sequences applied in these studies, more investigations are clearly needed to fully understand these interactions.

In summary, we have observed, for the first time, that only TPT lactone binds directly to both dsDNA and ssDNA polymers in the absence of topo I. We have also demonstrated, for the first time, that it appears that TPT lactone exhibits sequence specificity for dT containing sequences in dsDNA and that TPT lactone exhibits a characteristic binding pattern to ssDNA polymers. It is important to note that we observed that only the TPT lactone binds to DNA. Our structural and chemical observations support the long-established biological observation that the lactone form of CPT-type drug is much more cytotoxic than the carboxylate form. Considering the fact that the lactone form of CPTs is essential for antitumor activity (22–25) and the observation that the lactone form of a clinically important CPT binds directly and selectively to dsDNA and ssDNA, one can mechanistically propose that CPT lactone binding to DNA is a general physicochemical mechanism that imparts antitumor activity to this class of compounds. Currently, we cannot clearly translate the apparent specificity of TPT lactone for dT-containing dsDNA into biological terms; however, it is possible that dT is an important molecular recognition target for TPT lactone and, possibly, similar compounds to exert cytotoxic antitumor effect in certain tumors (*e.g.*, ovarian and lung cancers). Given these considerations, it is exciting to consider the possibility of engineering new lactone-stable CPT derivatives that would more effectively exploit sequence-specific DNA binding. More detailed experimental and molecular simulation studies on the properties of CPT binding with DNA are underway.

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