DNA Interactions of Two Clinical Camptothecin Drugs Stabilize Their Active Lactone Forms

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Camptosar (CPT-11) and Hycamtin (topotecan, TPT), shown in Figure 1, are two clinically useful anticancer drugs of the camptothecin family which function by inhibiting human DNA topoisomerase I (TopoI).[1] Successful inhibition of TopoI by camptothecins is known from structure−activity studies to require an intact lactone ring (ring E) functionality.[2−4] Unfortunately, this lactone moiety is subject to hydrolysis under physiological conditions (i.e., at pH 7 and above) with each camptothecin agent existing in equilibria with its corresponding ring-opened carboxylate form. The position of the equilibria is pH-dependent, with the carboxylate form predominating at physiological pH after 1 h of incubation.[5] Previous equilibrium dialysis studies which evaluated the interactions of extensively incubated (16 h) and hydrolyzed camptothecin with sonicated calf thymus DNA and plasmid DNA (2 mM base concentrations or less) provided little to no evidence of DNA binding.[6] In this paper, we investigate the effect of synthetic duplex DNA oligonucleotides on CPT-11 and TPT stability as a function of incubation time and DNA concentration using a combination of HPLC, UV, and NMR methods. Our studies demonstrate that the positively charged and water-soluble TPT and CPT-11 congeners, as well as uncharged camptothecin, are capable of interacting directly with double-stranded DNA (dsDNA). Moreover, our results indicate that the dsDNA interactions of the camptothecin drugs of interest result in a marked stabilization of their active lactone forms.

In the absence of drug, TopoI mediates the relaxation of supercoiled DNA. TopoI first binds DNA and then nicks it on one strand, rotates the helix by one turn and finally rejoins the nicked strand.[1,7,8] The nicking of DNA by TopoI creates a covalent intermediate in which the 3′-phosphate at the nick site is attached to the phenolic hydroxyl group of a tyrosine (Tyr723 in human TopoI). Camptothecin agents are thought to interact with this covalent intermediate at the nick site, thereby preventing the religation and ultimately leading to DNA fragmentation and cell death.[1] As a result of intense study following their discovery as TopoI inhibitors,[1−4,6,9] the camptothecins are now known to act through the formation of stable ternary complexes between drug, TopoI, and DNA.[1] However, strong evidence that these agents are capable of interacting directly with DNA in the absence of TopoI has not been identified until the present study.

The impact of two different 30mer dsDNA oligonucleotides, $(dA\text{-}dT)_{15}$ and $(dG\text{-}dC)_{15}$, on drug stability in phosphate-buffered

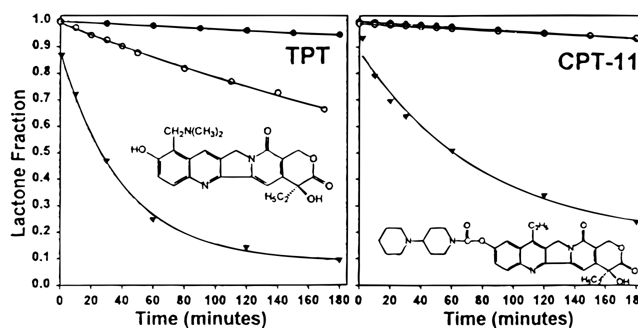

Figure 1. Kinetic evaluation of the rate of lactone ring opening for topotecan (left panel) and CPT-11 (right panel) in the presence and absence of duplex DNA oligonucleotides ($(dG\text{-}dC)_{15}$ or $(dA\text{-}dT)_{15}$). Data for drug in the absence of DNA (▼), drug in the presence of $(dG\text{-}dC)_{15}$ (●) or $(dA\text{-}dT)_{15}$ (○) are shown. All experiments were conducted in PBS (pH 7.40 ± 0.05) at room temperature. Drug and DNA concentrations of 10 μM and ∼30 mM base, respectively, were employed. Each profile represents the average of at least three independent kinetic runs with the same sampling schedules. The standard deviation of each point was typically 5% or less.

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(10) Phosphate-buffered saline (PBS) contained 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, and 3 mM KCl (pH 7.4 unless specified otherwise). The oligonucleotides d(CGTACG) were synthesized on an automated DNA synthesizer at the Genetic Facility of UIUC. $(dG\text{-}dC)_{15}$ and $(dA\text{-}dT)_{15}$ were purchased from IDT (Coralville, IA). CPT-11 was generously provided by Yakult (Tokyo), and TPT was obtained from the NCI. The drug lactone stock solutions were prepared in aqueous solution at 2 mM, pH 5. Solutions of the various drug−DNA complexes for NMR studies were prepared by mixing the appropriate amounts of drug stock solution and DNA stock solution in PBS, followed by pH adjustment to the desired value. Drug−DNA complexes solutions were vacuum-dried in a SpeedVac at room temperature and then dissolved in 0.5 mL 99.8% D_2O for 1D ^1H NMR spectra or in 0.5 mL 90%H_2O/10%D_2O for 1D H_2O spectra. The final DNA duplex concentrations ranged between 0.7 and 1 mM for all 1D NMR spectra. 1D H_2O NMR spectra were collected using the 1−1 pulse sequence (Sklenar, V.; Brooks, B. R.; Zon, G.; Bax, A. *FEBS Lett.* **1996**, *216*, 249−252). The NMR spectra were recorded on Varian VXR500 (University of Illinois, Urbana, IL) and Varian Inova 500 (University of Kentucky, Lexington, KY) 500 MHz spectrometers. The chemical shifts (in ppm) were referenced to the HDO peak which was calibrated to a sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) external standard at different temperatures. The NMR data were processed with the program FELIX v.1.1 (Hare Research, Woodinville, WA) or FELIX 95.0 (MSI) on Silicon Graphics workstations. The UV spectrum of a drug−DNA complex was obtained by subtracting the spectrum of free DNA from the spectrum of the complex. The hydrolysis kinetics of both drugs were determined by the quantitative reversed-phase high-performance liquid chromatographic (HPLC) methods as decribed previously (Mi, Z.; Malak, H.; Burke, T. G. *Biochemistry* **1995**, *34*, 13722−13728. Warner, D. L.; Burke, T. G. *J. Chromatogr. B* **1997**, *691*, 161−171). DNA stock solutions were prepared by dissolving the oligos in PBS at a concentration of ∼30 mM base concentration, with adjustment of pH to 7.4; 5 μL of 1 mM drug stock solutions (either lactone form or carboxylate form) were then added to 0.5 mL of pH 7.4 PBS or DNA stock solutions and assayed by HPLC to determine lactone stability or relactonization parameters, respectively.

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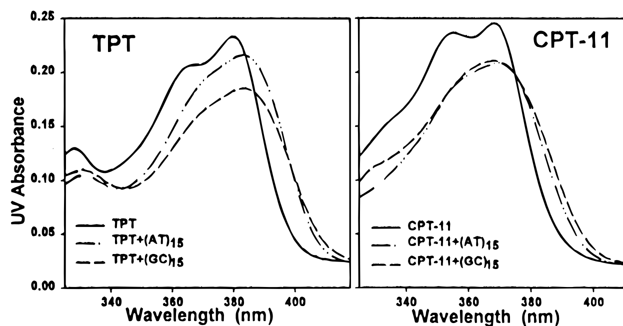

Figure 2. UV absorption spectra of topotecan lactone (left panel) and CPT-11 lactone (right panel) in the absence and presence of duplex DNA oligonucleotides ((dG-dC)$_{15}$ or (dA-dT)$_{15}$) in PBS (pH 5.00 ± 0.05 at room temperature). Drug, (dG-dC)$_{15}$, and (dA-dT)$_{15}$ concentrations of 10 μM, 16 mM base, and 24 mM base, respectively, were employed.

saline[10] (PBS) solution at pH 7.4 and room temperature is depicted in Figure 1. A reversed-phase HPLC assay was employed to assess the rate of hydrolysis and quantify the proportion of the lactone and carboxylate forms at equilibrium.[10] In the absence of DNA, 10 μM samples of CPT-11 and TPT incubated for 3 h hydrolyzed with pseudo-first-order kinetics and displayed percent lactone at equilibrium values of 24% and 12%, respectively. The extent of lactone ring opening for both agents was markedly reduced, however, in the presence of dsDNA (~30 mM base concentration). Lactone levels for CPT-11 remained at 95% following 3 h of incubation in the presence of (dA-dT)$_{15}$ or (dG-dC)$_{15}$, and at levels of 78% and 95%, respectively, for TPT. Even for time points out to 5 days, lactone levels remained high (50−60%) in the presence of DNA for both agents. Experiments conducted with 10 μM camptothecin demonstrated that the lactone form of this agent was also efficiently stabilized by the presence of ~30 mM base (dG-dC)$_{15}$ with lactone levels remaining above 80% after 3 h.

Addition of pure carboxylate forms of CPT-11 and TPT to ~30 mM base dsDNA solutions in PBS followed by incubation at room temperature was found to result in relactonization and the promotion of high levels of active lactone (~30% after 30 h). HPLC analyses have also been completed on solutions containing single-strand DNA (ssDNA) in the form of ~33 mM base (dT)$_{30}$ (see Figure S1, Supporting Information). Samples containing ssDNA showed significantly diminished effects on stabilizing both TPT and CPT-11, with dsDNA solutions some 10- to 100-fold more dilute than the (dT)$_{30}$ solutions affording approximately equal lactone stabilization. Figure 2 depicts changes in the UV spectra of the lactone forms of CPT-11 and TPT in PBS (pH 5) following addition of the (dA-dT)$_{15}$ or (dG-dC)$_{15}$ 30mers.[10] Note that a substantial hypochromic and red spectral shift is observed for both drugs in in the presence of DNA, suggestive of a possible intercalation mode of binding. Consistent with the HPLC data, the UV data also provides evidence that TPT displays a sequence preference of (dG-dC)$_{15}$ over (dA-dT)$_{15}$, while no signs of any sequence preference were observed for CPT-11.[11]

Further insight into the molecular interactions between CPT-11 and TPT with dsDNA oligonucleotide d(CGTACG) was obtained from NMR studies. Figure 3 shows that addition of

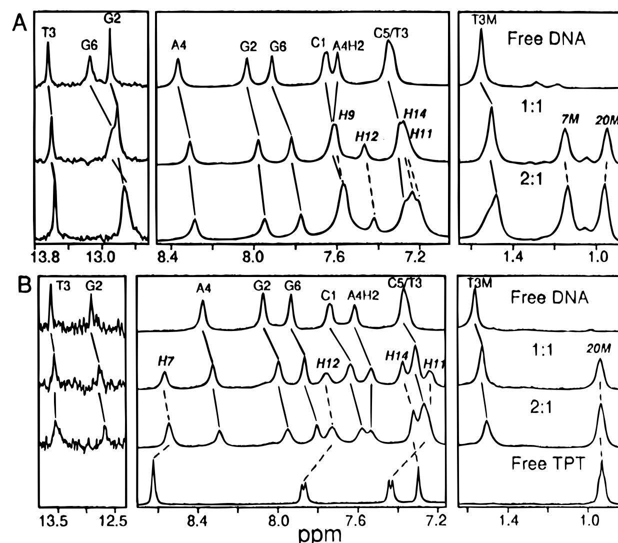

Figure 3. NMR spectra depicting the titration of (A) CPT-11 to d(CGTACG) at pH 7.0, 2 °C and (B) TPT lactone to d(CGTACG) at pH 5 at 2 °C. The ratios shown in the figure are for drug/DNA.

the drugs to DNA at increasing ratios resulted in upfield shifts of the DNA resonances, especially for the imino resonances G2 and G6. The drug resonances also shifted upfield in a correlated fashion. For TPT, the aromatic resonances of the H7, H12, and H11 protons shifted from 8.62, 7.87, and 7.43 ppm to 8.55, 7.73, and 7.24 ppm, respectively. Such upfield shifts of both DNA and drug resonances are characteristic of an intercalative mode of drug binding.[12,13] The unequivocal assignments of the resonances associated with the lactone/carboxylate forms (Figure 3) also allow us to confirm the stabilizing effect which DNA elicits on the lactone form of each agent.[14] The 20-methyl resonances of CPT-11 lactone occur at 0.95 ppm, which changes to 1.06 ppm (20M) upon the formation of opened-ring species. The NMR time course studies of CPT-11 and TPT concerning the differential rates of ring opening in the presence and absence of d(CGTACG) were consistent with determinations made using the HPLC method.

In summary, we have shown that the active lactone forms of CPT-11 and TPT are stabilized through interactions with dsDNA. The presence of dsDNA, in fact, was found to promote the conversion of inactive carboxylate to active lactone. Our results thus provide the first evidence that duplex DNA devoid of TopoI may play a functional role in the biological activities of the camptothecins through the promotion of active lactone levels within the cell nucleus. These results suggest that the agents, upon reaching chromosomal DNA, may interact directly with DNA prior to the action by TopoI (although the site of drug binding to DNA is not necessarily at the site of topoisomerase I action). The DNA-associated drugs are likely to be in their active lactone forms and ready for the subsequent drug−DNA−enzyme complex formation.

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Supporting Information Available: Figure S1 (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(11) A stronger hypochromic effect shift in the UV spectra of TPT in the presence of (dG-dC)$_{15}$ vs (dA-dT)$_{15}$ is consistent with a stronger interaction with the former, albeit differences in transition-state dipole interactions could also account for the observed effect.

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