Homocamptothecin, an E-Ring-Modified Camptothecin Analogue, Generates New Topoisomerase I-Mediated DNA Breaks †

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Homocamptothecin, an E-Ring-Modified Camptothecin Analogue, Generates New Topoisomerase I-Mediated DNA Breaks†

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ABSTRACT: Homocamptothecin (hCPT) contains a seven-membered β-hydroxylactone in place of the conventional six-membered α-hydroxylactone ring found in camptothecin and its tumor active analogues, including topotecan and irinotecan. The homologation of the lactone E-ring reinforces the stability of the lactone, thus reducing considerably its conversion into a carboxylate form which is inactive. We have recently shown that hCPT is much more active than the parent compound against a variety of tumor cells in vitro and in xenograft models, suggesting that a highly reactive lactone is not essential for topoisomerase I-mediated anticancer activity [Lesueur-Ginot et al. (1999) *Cancer Res. 59*, 2939−2943]. In the present study, we provide further evidence that hCPT has superior topoisomerase I inhibition capacities to CPT. In particular, we show that replacement of the camptothecin lactone E-ring with a homologous seven-membered lactone ring changes the sequence-specificity of the drug-induced DNA cleavage by topoisomerase I. Both CPT and hCPT stimulate the cleavage by topoisomerase I at T↓G sites, but in addition, hCPT stabilizes cleavage at specific sites containing the sequence AAC↓G. At low drug concentrations, the cleavage at the T↓G sites and at the hCPT-specific C↓G sites is more pronounced and more stable with hCPT than with CPT. The in vitro data were confirmed in cells. Higher levels of protein−DNA complexes were detected in P388 leukemia cells treated with hCPT than those treated with CPT. Immunoblotting experiments revealed that endogenous topoisomerase I was efficiently trapped onto DNA by hCPT in cells. Finally, the use of a leukemia cell line resistant to CPT provided evidence that topoisomerase I is involved in the cytotoxicity of hCPT. Altogether, the results show that the β-hydroxylactone ring of hCPT plays an important and positive role in the poisoning of topoisomerase I. An explanation is proposed to account for such remarkable changes in the sequence specificity of topoisomerase I cleavage consequent to the modification of the lactone. The study sheds new light on the importance of the lactone ring of camptothecins for the stabilization of topoisomerase I−DNA complexes.

The discovery in 1985 that the nuclear enzyme DNA topoisomerase I (topoI)[1] is inhibited by camptothecin (CPT) (*1*), a potent antitumor alkaloid present in extracts from the Chinese tree Xi Shu (*Camptotheca acuminata*, Decaisne, Nyssaceae) (*2*), initiated the development of anticancer drugs targeting topoI−DNA complexes. CPT itself was tested clinically in the form of a water-soluble sodium carboxylate, but the clinical development was discontinued in the early 1970s due to the appearance of unacceptable side effects such as hemorrhagic cystitis and severe myelosuppression (*3*). Over the last 10 years, a large number of CPT analogues have been developped (*4*, *5*, and references cited therein), and two of them, topotecan (TPT, Hycamtin) and irinotecan (CPT-11, Campto), are now approved for the treatment of refractory ovarian and colorectal cancer, respectively (*6*). Other analogues such as 9-amino-CPT, 9-nitro-CPT, DX-8951f, and GG-211 are currently undergoing clinical trials (*6*−*8*).

CPT and the aforementioned tumor active analogues all possess a pentacyclic core that can be divided into three parts: (i) the quinoline moiety (A- and B-rings) which can be extensively substituted with various functional groups without loss of activity; (ii) the indolizine moiety (C- and D-rings) containing the 4-keto group essential for the topoI poisoning effect and the biological activity; and (iii) the lactone E-ring which is viewed as the active center of the CPT molecule.

The lactone E-ring represents the *Achilles-heel* of the CPT molecule. An intact lactone form is required for topoI inhibition, but this lactone, which is activated by the chiral C-20 hydroxy group, is highly susceptible to hydrolysis under physiological conditions (at pH ≥ 7.0). In solution, CPT, TPT, and SN-38 (the active principle of CPT-11) all exist in equilibria between the ring-closed lactone form and the ring-

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[1] Abbreviations: topoI, DNA topoisomerase I; CPT, camptothecin; hCPT, homocamptothecin.

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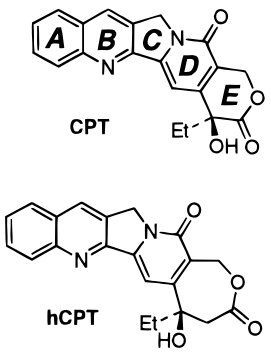

FIGURE 1: Structures of camptothecin (CPT) and homocamptothecin (hCPT).

opened carboxylate form which is essentially inactive. The pH of the solution, and also the presence of topoI and specific DNA sequences, can shift the position of the equilibria and thereby modulate the biological activity (*9*, *10*). Various attempts have been made to stabilize the α-hydroxylactone in order to limit the hydrolysis responsible for the inactivity.

Several nonhydrolyzable derivatives of CPT including the isosteric lactam derivative (21-lactam-20-S-CPT) have been synthesized, but they were much less active than the parent compound, if not totally inactive. Reduction of the lactone under mild conditions to give the lactol or removal of the 20-α-hydroxy group (20-desoxy-CPT) results in complete loss of activity (*11*). However, it was shown recently that replacement of the 20-α-hydroxy group with a chlorine or a bromine atom or an amino group reduces the cytotoxicity but does not prevent the drug from binding to the topoI−DNA binary complex (*12*).

In fact, a highly reactive lactone is not essential for topoI-mediated anticancer activity (*13*). Very recently, we reported the first successful attempt to drastically reduce the reactivity of the lactone E-ring without loss of activity. The replacement of the six-membered α-hydroxylactone with a seven-membered β-hydroxylactone reduces considerably the electrophilicity of the lactone and hence decreases the rate of lactone hydrolysis. The homoCPT analogue thus designed, henceforth referred to as hCPT (Figure 1), was found to display enhanced stability in buffer solutions. hCPT is also considerably more stable than CPT in plasma, and, most importantly, it proves to be much more cytotoxic (up to 10 times) than the parent compound against a variety of tumor cells. Furthermore, in vivo evaluation showed that hCPT exhibits superior antitumor activities compared to CPT in the HT-29 xenograft model (*13*).

We have recently reported that hCPT remains a potent topoisomerase I inhibitor despite the reduced reactivity of the lactone ring (*13*). Here we present further evidence that hCPT has superior topoI inhibition capacities to CPT. In addition, we show that replacement of the CPT lactone E-ring by a homologous seven-membered β-hydroxylactone ring changes the sequence-specificity of the drug-induced DNA cleavage by topoI and reinforces the stability of the drug-stabilized covalent complexes. The discovery of hCPT-specific DNA cleavage sites sheds light on the importance of the lactone ring of CPTs for the stabilization of topoI−DNA complexes.

MATERIALS AND METHODS

Drugs and Chemicals. Camptothecin was purchased from Sigma Chemical Co. (La Verpillière, France). The preparations of the enantiopure homocamptothecin derivative hCPT used in this study and related compounds have been described recently (*14*, *15*). The drugs were dissolved in dimethylacetamide (DMA) at 5 mM and then further diluted with water. The final DMA concentration never exceeded 0.3% (v/v) in the cleavage reactions. Under these conditions, DMA, which is also used in the controls, does not affect the topoisomerase activity. The stock solutions of drugs were kept at −20 °C and freshly diluted to the desired concentration immediately prior to use. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionized, Millipore filtered water.

Biochemicals. Restriction endonucleases *Ava*I, *Eco*RI, *Hin*dIII, and *Pvu*II, 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. Calf thymus topoI was from Life Science (Cergy-Pontoise, France).

DNA Relaxation Experiments. Supercoiled pKMp27 DNA (0.5 μg) was incubated with 6 units of topoI at 37 °C for 45 min 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 3 h. Gels were stained with ethidium bromide (1 mg/mL), washed, and photographed under UV light. Similar experiments were performed using ethidium-containing agarose gels.

Purification and Radiolabeling of DNA Restriction Fragments. Plasmids pBS and pKS (Stratagene, La Jolla, CA) were isolated from *E. coli* by a standard SDS−NaOH lysis procedure and purified by banding in CsCl−ethidium bromide gradients. Ethidium was removed by several 2-propanol extractions followed by exhaustive dialysis against Tris−EDTA buffered solution. The purified plasmid was then precipitated and resuspended in appropriate buffered medium prior to digestion by the restriction enzymes. The 117 and 176 base pair DNA fragments were prepared by 3′-^{32}P-end labeling of the *Eco*RI−*Pvu*II double digest of plasmid pBS and pKS, respectively, using [α-^{32}P]dATP (3000 Ci/mmol; Amersham, Buckinghamshire, England) and AMV reverse transcriptase. In both cases, the digestion products were separated on a 6% polyacrylamide gel under native conditions in TBE buffered solution (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 37 °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.

Sequencing of Topoisomerase I-Mediated DNA Cleavage Sites. Each reaction mixture contained 2 μL of 3′-end ^{32}P-labeled DNA (∼1 μM), 5 μL of water, 2 μL of 10× topoI buffer, and 10 μL of drug solution at the desired concentra-

tion (1−100 μM). After 10 min incubation to ensure equilibration, the reaction was initiated by addition of 2 μL (20 units) of calf thymus topoI. Samples were incubated for 45 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 in ice for 4 min prior to loading onto 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 followed by piperidine-induced cleavage at the modified guanine residues.

Salt Dependence for Reversal of Cleavable Complexes. The procedure described by Tanizawa et al. (*16*) was followed. Briefly, the 3′-end-labeled 117-mer pBS fragment was incubated with 20 μM drug and 20 units of calf thymus topoisomerase I (Life Science, Cergy-Pontoise, France) in a 50 mM Tris buffer containing 50 mM KCl and 10 mM $MgCl_2$. The cleavable complexes were induced for 15 min at 37 °C prior to adding NaCl up to 25 mM (final concentration), and the reaction was continued for 15 min prior to the SDS−proteinase K treatment as described above. The DNA samples were precipitated with ethanol and then analyzed on a sequencing gel.

Cell Cultures and Cell Survival Assay. P388 and P388CPT5 murine leukemia cell lines sensitive and resistant to CPT, respectively, were kindly provided by Dr. J.-F. Riou (Rhône-Poulenc Rorer, France). The P388CPT5 cell line resistant to campthotecin was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage (*17*). Both cell lines were grown in RPMI 1640 medium containing 0.01 mM 2-mercaptoethanol, 10 mM L-glutamine, 10% (v/v) fetal calf serum, 100 IU/mL penicillin, 2 μg/mL streptomycin, 50 μg/mL gentamycin, and 50 μg/mL nystatin at 37 °C in a humidified atmosphere containing 5% CO_2. Inhibition of cell proliferation was determined by WST1 colorimetric assay. Cells were seeded from 1000 to 4000 cells/80 μL of DMEM/well on a microtiter plate (tissue culture grade, 96 wells, flat bottom) 24 h prior to treatment. For dose-responsive studies, cells were incubated with 20 μL of each drug for 72 h over final concentrations ranging from 5.12×10^{-13} to 10^{-6} M. All drugs were dissolved just before use in their appropriate solvent. Further drug dilutions were made in culture medium. The final DMA concentration, when this solvent was used, never exceeded 1/1000 (v/v). As a control, drugs were replaced by a solvent which was diluted successively in the same way as the drugs. After the incubation period, 10 μL of WST1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate) labeling reagent was added to each well. Cells were incubated for 2 h at 37 °C in a humidified atmosphere. The tetrazolium salt was reduced to formazan by the succinate−tetrazolium reductase system which belongs to the respiratory chain of the mitochondria and is active only in viable cells. The formazan dye produced by metabolically active cells was quantified by a scanning multiwell spectrometer by measuring the absorbance of the dye solution at 450 and 620 nm. Experiments were carried out at least 3 times, each experiment representing 8 determinations. For each drug, the values included in the linear part of each experiment's sigmoid were retained in a linear regression analysis and were used to estimate the 50% inhibitory concentration (IC_{50}).

Formation of Protein−DNA Complexes in Intact Cells. DNA−protein complexes were quantified by the KCl/SDS coprecipitation assay (*18*). DNA and proteins of P388 cells (5×10^5 cells/ mL) were respectively radiolabeled with 0.6 μCi/mL [^{3}H]thymidine and 0.1 μCi/mL [^{14}C]leucine for 18 h (Amersham, Buckinghamshire, England). After incubation for 1 h at 37 °C with various concentrations of the drugs, cells were lysed in a 1 mL solution of 1.25% SDS, 5 mM EDTA, pH 8, and 0.4 mg/mL salmon testes DNA (Sigma Chemical Co., La Verpillère, France). Five hundred microliters of lysates was passed through a 22 gauge needle 5 times and incubated at 37 °C for 15 min. The lysates were then adjusted to 65 mM KCl, vortexed for 10 s, and placed on ice for 15 min, and the precipitates were collected by centrifugation. The pellets were washed 3 times in 10 mM Tris-HCl, pH 8, 100 mM KCl, 1 mM EDTA, and 0.1 mg/mL salmon testes DNA at 65 °C, before being dissolved in 0.5 mL of 65 °C water and mixed with 5 mL of scintillation fluid (Instagel plus, Packard) for determination of radioactivity. Data were expressed as the ratio of [^{3}H]DNA to [^{14}C]-protein, with the amount of protein being an internal standard for the number of cells used. All experiments were done at least 3 times with each point in duplicate.

Immunoblot Assay of Topoisomerase I−DNA Complexes in Cells. The in vivo topoisomerase I link kit of TopoGEN, Inc. (Columbus, OH), was used, and the recommended protocol was followed with a few modifications. Briefly, 10^7 exponentially growing P388 cells in 5 mL of serum-free RPMI 1640 medium were treated with the test drug at 50 μM for 1 h at 37 °C. Cells were pelleted by centrifugation (1000 rpm, 5 min) and rapidly resuspended in 0.8 mL of the lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% sarkosyl). The lysed cell mixture was then overlaid onto a CsCl density gradient containing four different density steps (0.8 mL of CsCl at 1.82, 1.72, 1.50, and 1.37 g/mL). The tubes were centrifuged in a Beckman SW60 rotor at 31 000 rpm (13000*g*) for 15 h at 25 °C. From the top of the gradient, 12 fractions of 330 μL were collected. The DNA content in each fraction was estimated by absorbance measurement at 260 nm. For the immunoblot analysis, 50 μL of each fraction was diluted with 100 μL of 25 mM sodium phosphate buffer (PBS, pH 6.5) prior to applying the diluted solution into the slot blot unit under a mild vacuum. PBS-washed Hybond-C nitrocellulose membranes (Amersham) cut to fit the vaccum slot-blot device (Life Science, Cergy-Pontoise, France) were loaded with the diluted samples, washed briefly with PBS, and then soaked for 2 h in TBSTB (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, 1% bovine serum

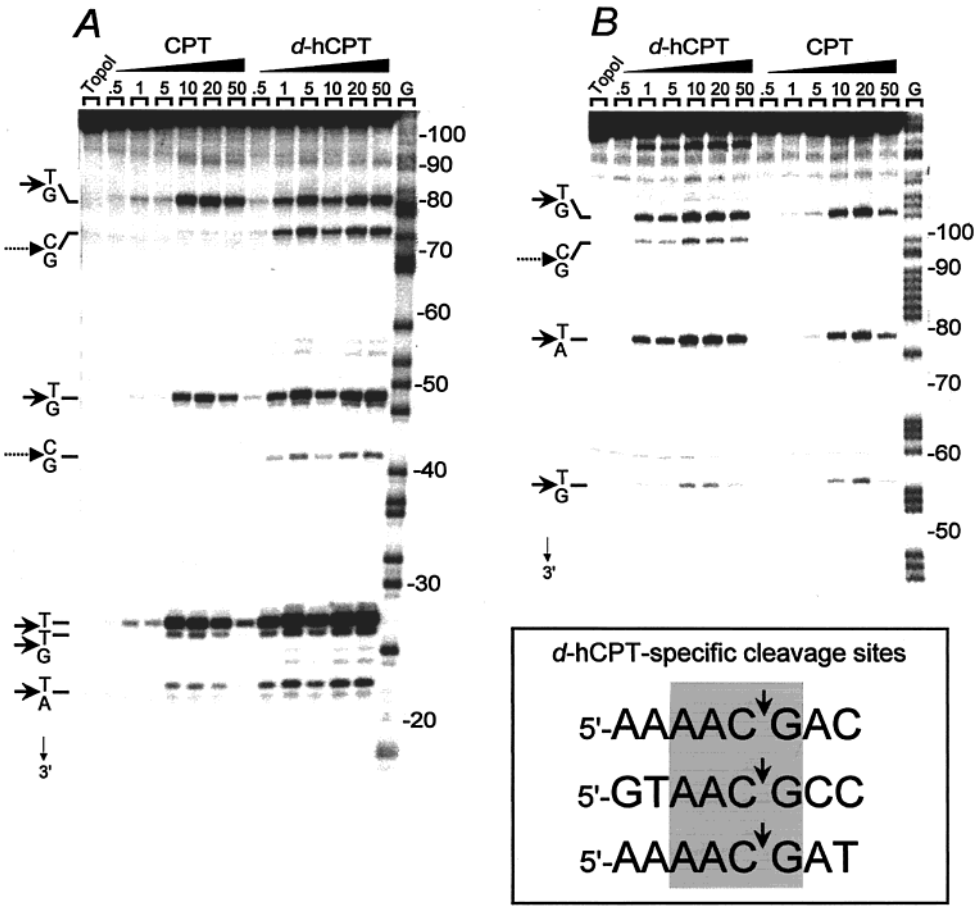

FIGURE 2: Phosphorimages comparing the susceptibility of (A) the 117 bp and (B) the 176 bp restriction fragments to cutting by human topoisomerase I in the presence of CPT and hCPT. In both cases, the 3′-end-labeled fragment was incubated in the absence (lane TopoI) or presence of the test drug at the indicated micromolar concentration. Topoisomerase I cleavage reactions were analyzed on 8% denaturing polyacrylamide gels. Numbers at the right of the gels show the nucleotide positions, determined with reference to the guanine tracks labeled G. Short arrows point to the cleavage sites common to CPT and hCPT. Dotted arrows indicate the hCPT-specific cleavage sites at sequences containing the AAC↓G element.

albumin) supplemented with 5% nonfat dried milk. The membranes were washed 3 times (10 min per wash) with TBST prior to incubation for 1 h at room temperature with the anti-topoI antibody (1/10 000 dilution in 25 mL of TBST). After 3 successive washes (10 min with TBST), the membranes were incubated with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Life-Sciences, 1/1000 dilution in 25 mL of TBST) for 30 min. After 4 successives washes (10 min each with TBST), the Western blot chemiluminescence reagent from NEN (Boston, MA) was used for the detection. Bands were vizualized by autoradiography.

RESULTS

In a recent study, we showed that hCPT was more efficient than CPT in inhibiting topoI-mediated relaxation of supercoiled DNA (*13*). To investigate further the topoI poisoning activity of drugs, DNA cleavage experiments were performed using radiolabeled DNA subtrates. Two DNA fragments of 117 and 176 bp were used to map the topoI cleavage sites induced by hCPT and CPT. In both cases, the *Eco*RI−*Pvu*II double-digest of the corresponding plasmid (pBS and pKS) was uniquely end-labeled at the 3′-end at the *Eco*RI site and used as a substrate for the topoI cleavage reactions. The cleavage products were analyzed on sequencing polyacrylamide gels (Figure 2). The results fully confirm that hCPT strongly promotes the cleavage of DNA by topoisomerase I. Moreover, they attest unambiguously that hCPT has superior topoisomerase I inhibition properties to CPT. Visual inspection of the gels suffices to demonstrate that the cleavage of DNA by the enzyme is weak in the presence of CPT at 1 and 5 μM whereas it is already very pronounced with hCPT at these low concentrations. Densitometric analysis of the gels (Figure 3) reveals that at all cutting sites cleavage in the presence of low concentrations of hCPT is higher than with CPT. In the 10−50 μM range, either the two drugs behave similarly (e.g., see cleavage sites TG81 and TG107) or the cleavage is more pronounced with hCPT than with CPT (e.g., sites TG26 and TA80 in Figure 3).

It is remarkable to observe that three sites of cleavage (pointed out by dotted arrows on the gels in Figure 2) are detected with hCPT but not with CPT. On the 117-mer fragment, the strong cutting sites at positions 26, 48, and 81 are common to CPT and hCPT and reflect cleavage at a 5′-T↓G site which is known to be the most preferred recognition site for CPT-promoted DNA cleavage by topoisomerase I in vitro (*19*−*21*). Both CPT and hCPT stabilize only a subset of sites cleaved by topoI. The two sites at positions 41 and 73 which are detected only with hCPT correspond to cleavage at a 5′-C↓G step. The situation is identical with the 176-mer fragment. Here again, one T↓A site and two T↓G sites are common to CPT and hCPT whereas an additional

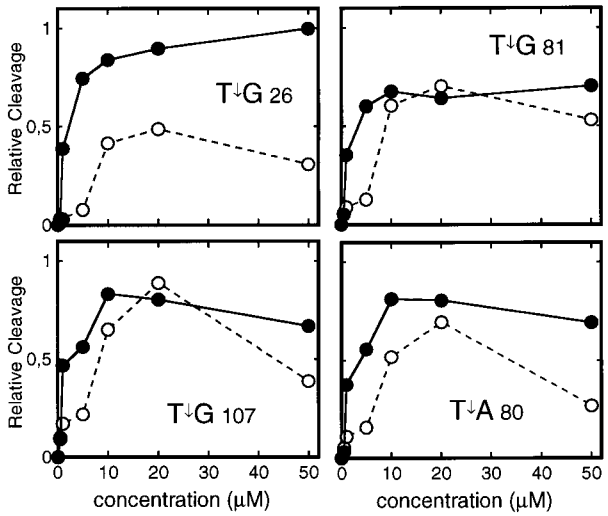

FIGURE 3: Cleavage plots comparing the relative cutting intensity at specific sites within the 117-mer and 176-mer fragments in the presence of increasing concentrations of (●) hCPT or (○) CPT.

site observed with hCPT coincides with a C↓G sequence. In fact, as pointed out in Figure 2, all three hCPT-specific sites encompass the recognition sequence 5′-AAC↓G. Altogether these experiments leave no room for doubt that hCPT is a potent topoI inhibitor. Despite the structural difference of the lactone ring, CPT and hCPT interact with topoI in a similar fashion or recognize identical structural elements of the topoI−DNA covalent complex.

Next we investigated the persistence of the covalent complexes. CPT-stabilized cleavable complexes are rapidly reversible upon increasing the salt concentration (*16*). This prompted us to compare the salt-dependent reversibility of hCPT and CPT (Figure 4). The 117-mer fragment was incubated with the drug at 20 μM in the presence of topoI in order to form the covalent complexes. After 15 min incubation, NaCl was added to each tube, and the samples were incubated for a further 15 min prior to the SDS−proteinase K treatment. DNA cleavage products were then resolved on a denaturing polyacrylamide gel (Figure 4). With CPT, the intensities of the three T↓G cleavage sites at positions 26, 48, and 81 rapidly decrease in the presence of increasing amounts of NaCl. No further cleavage was detected with NaCl concentrations ≥15 mM. In contrast, the same three bands in the samples treated with hCPT remain clearly visible even with 25 mM NaCl. The hCPT-specific C↓G site at position 73 is equally stable compared to the T↓G sites. With hCPT, the band intensities at each site decrease by about 50% with 25 mM NaCl. The extent of cleavage at sites 26 and 48 (two of the strongest TG cleavage sites common to the two drugs) was quantified (Figure 5). The cleavage intensity decreases much more rapidly with CPT than with hCPT. The topoI−DNA covalent complexes formed with hCPT, be it the TG or the specific CG sites, are more stable than those produced in the presence of CPT under identical conditions.

The above experiments indicate that hCPT has superior topoI poisoning activity to CPT in vitro. We were interested to determine whether the same differences can be found in cells. For this reason, we measured the extent of protein−DNA complexes formed in cells treated with either CPT or hCPT. The results in Figure 6 show that more protein−DNA

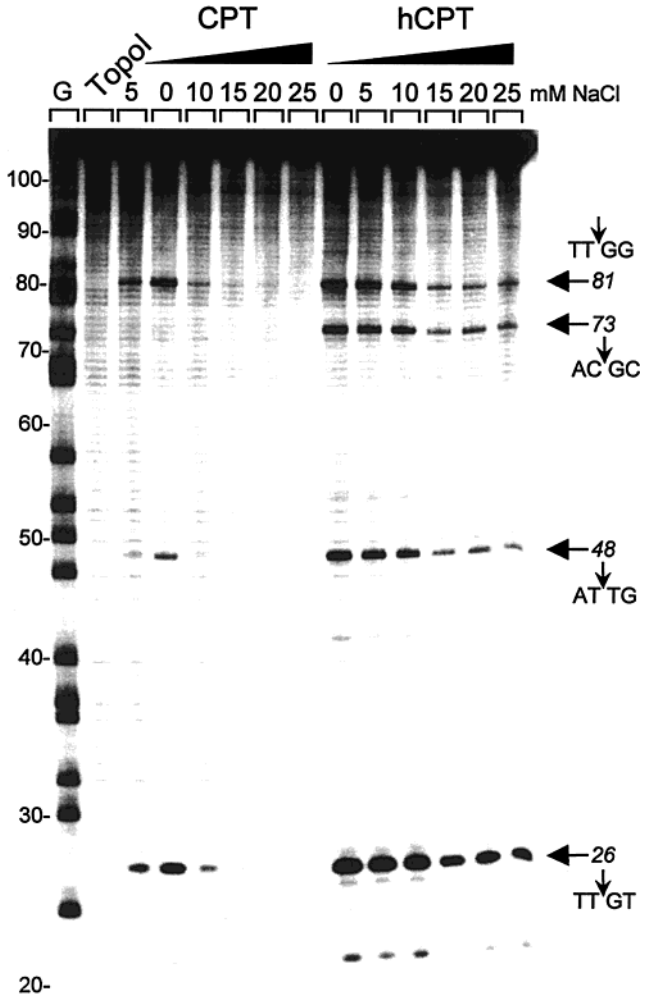

FIGURE 4: Salt-induced reversibility of CPT and hCPT-induced DNA−topoisomerase I covalent complexes. The 3′-end-labeled 117-mer pBS fragment was incubated in a 50 mM Tris buffer containing 50 mM KCl and 10 mM $MgCl_2$ with 20 μM drug and 20 units of topoisomerase I for 15 min in the presence of drug prior to adding NaCl. Samples were incubated for a further 15 min at 37 °C and then treated with SDS−proteinase K and precipitated.

complexes can be detected in P388 murine leukemia cells treated with hCPT than those treated with CPT. The level of protein−DNA complexes is significantly higher with hCPT than with CPT. Similar results were obtained when using human colon cancer HT29 cells (data not shown). In addition, to ascertain whether the protein−DNA complexes obtained in the presence of the drugs contain topoisomerase I, we set up an immunoblot assay to identify the drug-stabilized topoI−DNA complexes directly in the cells. This in vivo link assay has previously been used to detect CPT-stabilized covalent complexes in clinical samples (*22*) as well as to investigate the topoI poisoning activity of drugs like actinomycin D (*23*). The results of the immunoblot analysis are presented in Figure 7. In the control samples with no drug, topoI was found exclusively at the top of the CsCl gradient as free protein. In sharp contrast, with cells treated for 1 h with 50 μM CPT or hCPT, topoI was found in the top fractions as well as in fractions 8−10 near the bottom of the gradient where the nucleic acids were localized (as judged from the absorbance measurements at 260 nm). The same results were obtained using 10 and 20 μM drug and 3 h of drug treatment (data not shown). Although the immunoassay is essentially qualitative, it is interesting to note that

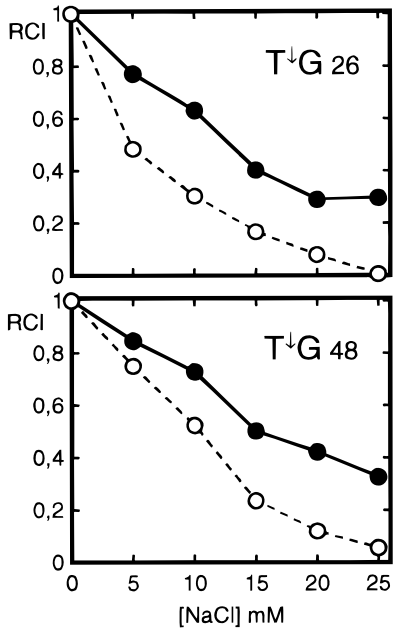

FIGURE 5: Differential reversibility of (●) hCPT- and (○) CPT-induced cleavable complexes in the presence of increasing concentrations of NaCl. The plots show the variations of the relative cleavage intensity (RCI) at positions 26 (GAAT$^{\downarrow}$TGTA) and 48 (CGTT$^{\downarrow}$GTAA) of the 117 bp DNA. Data were compiled from quantitative analysis of two sequencing gels such as the one shown in Figure 4 and must be considered as a set of averaged values.

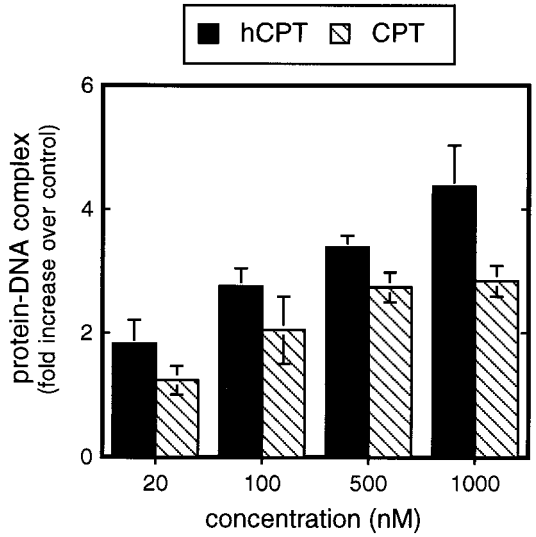

FIGURE 6: Formation of DNA−protein complexes in P388 cells. The cells were incubated with the drug at the indicated concentration (μM) for 1 h prior to precipitation.

CPT-trapped covalent complexes are restricted to fractions 9 and 10 only, whereas they are also found in fraction 8 with hCPT, suggesting that the homologous drug produces more covalent complexes than the parent compound, as expected from the in vitro data and the protein−DNA complexes quantification in Figure 6. These immunoblotting experiments demonstrate that endogenous topoI can be trapped onto DNA by hCPT in P388 leukemia cells.

The cytotoxicity of the drugs was evaluated using leukemia cells sensitive (P388) and resistant (P388CPT5) to CPT. The IC_{50} values collated in Table 1 indicate that hCPT is more cytotoxic than CPT. The higher cytotoxicity of hCPT may arise from its superior effects in terms of topoI inhibition

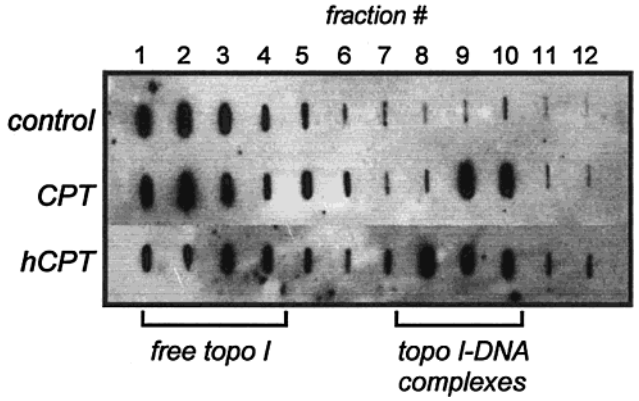

FIGURE 7: Immunoblot analysis of drug-stabilized topoisomerase I−DNA covalent complexes in P388 murine leukemia cells. 10^7 exponentially growing P388 cells were incubated with CPT or hCPT at 50 μM for 1 h at 37 °C. The lysates were applied onto a CsCl gradient and centrifuged overnight. Twelve fractions were collected and analyzed by the slot blot method described under Materials and Methods. Fractions 1−4 contain free topoisomerase I. DNA−topoisomerase I covalent complexes can be detected in fractions 8−10 of the drug-treated samples.

Table 1: Cytotoxicity (IC_{50}, nM)

	P388[a]	P388CPT5
CPT	17.00 ± 1.66[b]	5938 ± 876
hCPT	3.00 ± 0.39	3368 ± 361

[a] P388 and P388CPT5 murine leukemia cells are sensitive and resistant to camptothecin, respectively. [b] IC_{50} values (nM) refer to the concentrations of drugs giving 50% of growth inhibition after 72 h incubation.

compared to CPT. The use of the P388CPT5 cell line resistant to CPT provides another line of evidence that topoI is involved in the cytotoxicity of hCPT. The resistance of the P388CPT5 cells has been attributed to the expression of a deficient form of topoI as a result of a mutation in the *top1* gene of these cells (*17*). Under the experimental conditions used (3 days continuous exposure), hCPT proved to be considerably more toxic to P388 cells than to P388CPT5 cells (Table 1). There is no doubt that the P388CPT5 cells are cross-resistant to hCPT. We can therefore conclude that topoI most likely represents a primary cellular target for the homoCPT derivative.

DISCUSSION

As mentioned in the introduction, clinical trials with the water-soluble sodium salt of CPT were discontinued because of poor efficacy and excessive toxicity which were clearly attributed to the carboxylate form. Although CPT analogues, such as TPT and CPT-11, are now administered in their lactone forms, the lactone−carboxylate interconversion remains fairly rapid and reversible. In contrast, the hydrolysis of the lactone ring of hCPTs is slow and irreversible. This different hydrolytic profile of the seven-membered lactone may account for the improved in vivo antitumor efficacy of hCPT (*13*). However, the observed differences may also reflect different transport activity of the cell system. The present study shows that, at the molecular level, the β-hydroxylactone ring of hCPT may also play an important and positive role in the poisoning of topoI.

DNA cleavage experiments reveal significant differences between hCPT and CPT in terms of sequence specificity of

topoI cleavage sites. Not only is hCPT more efficient than CPT at inducing cleavage at T↓G sites, but, remarkably, the enzyme cleaves at additional sites, unseen with CPT and containing an AAC↓G motif. These new, hCPT-specific, cleavage sites may allow topoI to induce DNA damages at genes that are normally not affected by CPT and could, therefore, broaden the cytotoxicity profile of the drug. However, since the shapes of CPT and hCPT only differ by the size of their lactone ring, it is quite paradoxal to identify new cleavage sites at C↓G sequences which are specifically associated with a larger lactone ring.

Cleavage at TG sites in the presence of CPT is believed to result from the interaction of topoI with the T residue combined with the stacking of the CPT molecule with the adjacent G residue (*20*, *25*). Two distinct models for the CPT−topoI−DNA ternary complex architecture have been recently proposed by Redinbo et al. (*26*), and Fan et al. (*27*). Both models postulate the stacking of CPT with the G+1 residue. Since the hCPT-specific sites also favor a G+1 residue, the stacking interaction with the +1 base is apparently not affected by the size of the lactone ring. The model of Redinbo et al. (*26*) postulates the 21-CO and 20-OH groups of the CPT lactone to interact with, respectively, the Arg364 and Asp533 residues of human topoI. Interestingly, to remove steric clashes with the side chain of Asp533 and the −1 base, the terminal carbon (C-18) of the ethyl group had to be slightly rotated relative to its position in the CPT crystal structure (*28*), in order to build the model. It is plausible that, as a consequence of the expansion of the lactone ring, the ethyl group in hCPT is now better positioned and allows more space for the −1 base, and, consequently, a cytosine may now also enter this cavity facing the ethyl group, as does a thymine residue.

Pommier and co-workers (*12*, *27*) have proposed that a lactone ring opening reaction could represent a second step for topoI poisoning by CPT, resulting in a covalent intermediate involving the E-ring carboxyl group and a nucleophile from either topoI or DNA. One would expect the lower reactivity of the hCPT lactone to decrease its propensity to form such a covalent intermediate, but the results reported here tend to show that, on the contrary, the reduced reactivity is accompanied by an increased affinity toward topoI−DNA complexes. That this hypothetical formation of a covalent bond with DNA or topoI is not a limiting step could imply a certain level of general acid−base catalysis exerted by the cleavable complex toward E-ring opening. Would that be the case, the lower reactivity of the hCPT lactone may be overcome to trigger the covalent bond formation, and the higher stability of the resulting ternary adduct, involving a β-hydroxyacyl linkage instead of an α-hydroxyacyl one, could match our observations. Further structure−activity studies will be needed to investigate these options.

An interesting feature of the hCPT-induced topoI−DNA covalent complexes is the observed enhanced salt stability in comparison to CPT. TopoI poisoning-induced cell death requires cleavable complexes (single-strand breaks) to be converted into DNA damage (double-strand breaks) (*24*). A strict correlation between DNA cleavage and cytotoxicity is seldom observed in the CPT series (*4*), and an enhanced potency of enzyme inhibition may fail to increase cytotoxicity, due to differences in cellular penetration and distribution in the various cellular compartments. But CPT and hCPT, which are structurally very close, can reasonably be expected to behave similarly in that respect, and the higher stability of hCPT-induced covalent complexes compared to those induced by CPT may increase the probability of conversion to double-strand breaks. The cellular studies further argue in that sense, as an increased cytotoxicity of hCPT is observed on the P388 mouse leukemia. The immunoblot and protein−DNA complex precipitation assays suggest that topoI represents a molecular target for hCPT, and cross-resistance P388CPT5 cells to CPT, and hCPT, further support this view.

Together with other recent studies (*13*, *15*), this work confirms hCPT as a promising template for the elaboration of new anticancer agents. The introduction of a methylene spacer between the alcohol moiety and the carbonyl function of CPT lactone brings relatively modest, but significant, quantitative improvement in terms of cleavable complex stabilization and cytotoxicity. More importantely, it brings some qualitative changes in terms of pharmacokinetics, and, as reported here, DNA sequence specificity, which may offer new therapeutic opportunity. Clinical trials with BN 80915, a difluorinated hCPT, have been recently initiated.

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