Reduced Albumin Binding Promotes the Stability and Activity of Topotecan in Human Blood†

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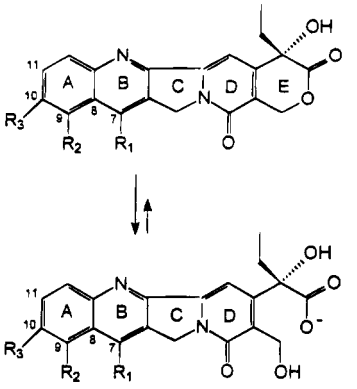
ABSTRACT: Topotecan, a semisynthetic water-soluble analogue of camptothecin, is the first topoisomerase I targeting anticancer agent to enter comparative phase III clinical trials. Here we elucidate the biophysical factors underlying the markedly improved bloodstream stability and cytotoxic activity of topotecan relative to camptothecin. Each agent contains an α-hydroxy-δ-lactone ring that hydrolyzes under physiological pH to yield a biologically-inactive carboxylate form. In human plasma, camptothecin lactone converts rapidly and completely to its carboxylate form due to a 200-fold binding preference by serum albumin (HSA) for the latter [Mi, Z., & Burke, T. G. (1994) *Biochemistry 33*, 10540−12545]. Time-resolved fluorescence anisotropy measurements reveal that neither topotecan lactone nor carboxylate associates with HSA, thereby resulting in a significantly higher level of lactone stability in plasma for topotecan ($t_{1/2}$ = 23.1 min, percent lactone at equilibrium of 17.6) relative to camptothecin ($t_{1/2}$ = 10.6 min, percent lactone at equilibrium of <0.2). Moreover, studies with HL-60 human promyelocytic leukemia cells reveal that a physiologically-relevant level (40 mg/mL) of HSA dramatically attenuates the cytotoxic activity of camptothecin in excess of 2600-fold (for a 72 h exposure, the IC_{50} value of 1.5 nM in the absence of HSA increased to 4 μM in the presence of HSA). The activities of other clinically relevant anticancer analogues, 9-aminocamptothecin and SN-38, were also strongly modulated by the presence of 40 mg/mL HSA. In marked contrast, the presence of HSA effected no change on the cytotoxic activity of topotecan (IC_{50} = 12 nM both in the absence and in presence of HSA). Our data characterize important differences in the chemical dynamics between camptothecin and topotecan in human blood which correlate with the favorable anticancer activity of the latter agent observed in the presence of HSA.

Topotecan, a 9-dimethylaminomethyl-10-hydroxy analogue of camptothecin (Table 1), is the first topoisomerase I-targeted cytotoxic agent to enter comparative phase III clinical trials. Camptothecin had entered clinical trials for the treatment of cancer in the early 1970s (Gottlieb et al., 1970; Muggia et al., 1972) but was abandoned shortly thereafter due to its unacceptable toxicities (Slichenmyer et al., 1993; Potmesil, 1994). Displaying enhanced aqueous solubility relative to camptothecin (Slichenmyer et al., 1993; Potmesil, 1994; Fassberg & Stella, 1992), topotecan has been shown to display significant inhibitory activity over a broad spectrum of animal and human tumors (Christian & Trimble, 1994; Rowinsky et al., 1994; Houghton et al., 1992) and is presently undergoing worldwide clinical evaluation.

Both camptothecin and topotecan inhibit topoisomerase I by stabilizing the DNA−topoisomerase I cleavable complex, preventing religation of DNA molecules (Slichenmyer et al., 1993; Potmesil, 1994). In several cell lines, the levels of topoisomerase I (Niwa et al., 1994) and the amounts of cleavable complex (Ellis et al., 1994) were found to correlate with drug-induced cytotoxicities. In humans, the levels and activities of topoisomerase I have been found to be elevated in colon and prostate cancers (Husain et al., 1994) as well as in acute lymphocytic leukemia (Rowinsky et al., 1994) compared to normal tissues. These increases in topoisomerase I content of cancer cells have potential therapeutic implications as reviewed previously (Slichenmyer et al., 1993; Potmesil, 1994).

Table 1: Structures of Lactone (Upper) and Carboxylate (Lower) Forms of Camptothecin and Topotecan



	R_1	R_2	R_3
camptothecin	H	H	H
topotecan	H	$CH_2NH(CH_3)_2$	OH

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Camptothecin, topotecan, and related analogues each contain an α-hydroxy-δ-lactone ring functionality which hydrolyzes under physiological conditions, *i.e.*, at pH 7 or above, with the lactone moiety readily opening up to yield the carboxylate form. It is now known that the biological activities of camptothecins both *in vitro* and *in vivo* are significantly greater for the lactone over the carboxylate species (Slichenmyer et al., 1993; Potmesil, 1994; Wani et al., 1987a,b; Jaxel et al., 1989); available data indicate that a closed lactone ring is also an important structural requisite both for passive diffusion of drug into cancer cells as well as for successful interaction with the topoisomerase I target (Hsiang et al., 1985; Hertzberg et al., 1989). Thus, factors influencing the lactone−carboxylate equilibria of camptothecins are regarded as critical determinants of drug function.

Recently this laboratory has exploited the intrinsic fluorescent emissions from the lactone and carboxylate forms of camptothecin in order to elucidate drug interactions with human blood components (Burke & Mi, 1993a; Mi & Burke, 1994a,b). In phosphate-buffered saline (PBS) at pH 7.4, frequency-domain lifetime fluorometry revealed that human serum albumin (HSA) preferentially binds camptothecin carboxylate with a 200-fold higher affinity than camptothecin lactone (Mi & Burke, 1994b); this differential binding for carboxylate over lactone results in camptothecin opening more rapidly and completely in the presence of HSA than in the absence of the protein (Burke & Mi, 1993a,b, 1994; Mi & Burke, 1994a,b). In human plasma, pH 7.4 and 37 °C, camptothecin lactone opens rapidly and completely to the carboxylate form with a $t_{1/2}$ value of 11 min and an almost negligible 0.2% lactone at equilibrium.

In the present report, we document marked differences between camptothecin and topotecan in their interactions with human blood components. Time-resolved fluorescence anisotropy measurements reveal that neither the lactone nor the carboxylate form of topotecan associates with HSA, thereby resulting in significantly higher lactone levels in both human plasma and blood for topotecan relative to camptothecin. Moreover, the presence of physiologically-relevant levels of HSA markedly attenuates by greater than 3 orders of magnitude the cytotoxic activity of camptothecin against leukemia cells while HSA has no effect on the potency of topotecan.

MATERIALS AND METHODS

Chemicals. Samples of camptothecin and topotecan were obtained from the Stehlin Foundation, Houston, TX, and the National Cancer Institute, Division of Cancer Treatment, respectively. Both drugs were in the 20*S* configuration and were of high purity (>98%) as determined by HPLC assays with fluorescence detection (Burke & Mi, 1994; Mi & Burke, 1994a,b). Drug stock solutions (2×10^{-3} M) were prepared in dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. Working solutions of camptothecin and topotecan carboxylate were prepared by a 1:1 dilution of DMSO stock solution in PBS buffer (pH 10) to a final concentration of 1×10^{-3} M. Crystallized HSA of high purity (>97%) from Sigma Chemical Co. (St. Louis, MO) was used as before (Burke & Mi, 1994; Mi & Burke, 1994a,b). HSA stock solutions were prepared in PBS buffer with a final pH of 7.40 ± 0.05. HSA concentrations were determined by the UV absorbance at 278 nm using an extinction coefficient of 39 800 M^{-1} cm^{-1} (Porter, 1992). RNase A and RNase T1 were obtained from Sigma and GIBCO (Grand Island, NY), respectively, and were free of DNase and protease. Propidium iodide and proteinase K free of DNase and RNase were obtained from Sigma. Agarose, Hank's balanced salt solution (HBSS), and 100 bp DNA ladder were obtained from GIBCO. All other agents were reagent grade and were used without further purification. High-purity water provided by a Milli-Q UV PLUS purification system (Bedford, MA) was utilized in all experiments.

Fluorescence Measurements. Time-resolved fluorescence intensity and anisotropy decay curves were generated by the technique of time-correlated single photon counting (Birch & Imhof, 1991). Samples were excited at 370 nm using the frequency-doubled output of a pyridine dye laser pumped synchronously by a mode-locked Coherent Antares Nd:YAG laser. Fluorescence emissions were detected by a microchannel plate photomultiplier tube through a 400 nm longpass filter. The instrumental response functions were determined using Ludox (Dupont, Wilmington, DE), a colloidal silica scattering solution. Fluorescence emissions were sampled for 100 s for both parallel and perpendicular polarizer orientations, and peak counts of 10−20K were obtained for parallel components by adjusting the neutral density filters in the paths of both excitation and emission light beams. Background fluorescence was sampled in the absence of drug for each measurement, which was always less than 2% of the fluorescence signal. Drug concentrations of 1×10^{-6} M and HSA concentrations of 7.25×10^{-6} M in PBS were used in all measurements. Data analyses of total fluorescence decays and anisotropy decays were performed using an IBH decay analysis software (Version 4) from IBH Consultants Limited (Scotland, U.K.) (Imhof, 1989).

Kinetics of Lactone Ring Opening. The hydrolysis kinetics of topotecan in the presence of different blood components were determined by a quantitative C18 reversed-phase high-performance liquid chromatography (HPLC) assay as described before (Burke & Mi, 1994; Mi & Burke, 1994a,b). The preparation of whole blood and fractionated blood samples was carried out as described previously (Mi & Burke, 1994a).

Cells. The HL-60 human promyelocytic leukemia cell line was kindly provided by Dr. Zbigniew Darzynkiewicz from New York College of Medicine, Valhalla, NY. The cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin (PSN antibiotics mixture, GIBCO), and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was assessed by the trypan blue exclusion assay. Only exponentially growing, viable cells were used in the studies.

Drug Uptake. Intracellular accumulation of camptothecin and topotecan was studied using a flow cytometric approach as described before (Mi & Burke, 1994a). Briefly, HL-60 cells in exponential growth phase were washed twice with PBS and resuspended in either PBS only or PBS containing 40 mg/mL HSA at a density of 1×10^6 cells /mL. Each suspension was subject to flow cytometry for 1 min to obtain a base line value prior to the addition of drug stock solution. Both camptothecin and topotecan display strong intrinsic

fluorescence emissions, and cellular fluorescence intensities due to drug exposure were monitored continuously by the flow cytometer until plateau values were reached (typically from 5 to 10 min following drug addition to the cell suspension). Thus, the maximum fluorescence intensity levels, corrected for base line signals, were used to represent total drug accumulation in the cell suspensions. A Coulter Epics Elite flow cytometer equipped with a 5 W water-cooled argon ion laser operating at 364 nm was used in the experiments. Two band-pass filters of 445−485 and 490−625 nm were used to separate the fluorescence emission from the scattered light for camptothecin and topotecan, respectively. Another band-pass filter of 305−390 nm was used to measure the right-angle scattered light. Prior to the initiation of experiments, a reference channel was set so that data generated from different days were comparable. All experiments were carried out in triplicate, and the results were plotted as mean ± SD.

Drug-Induced Apoptosis. Agarose gel electrophoresis (Sambrook et al., 1989; Ray et al., 1994) was performed to monitor the 180−200 bp DNA ladder associated with drug-induced apoptosis; 2×10^6 HL-60 cells were incubated with 0.2 and 0.5 μM camptothecin or topotecan for 3 h in the presence and absence of 40 mg/mL HSA. The cells were washed twice with PBS and incubated at 37 °C for 1 h in 100 μL of lysis buffer composed of 200 mM NaCl, 10 mM Tris-HCl (pH 8.0), 40 mM EDTA, 0.5% SDS, 200 ng/μL RNase A, and 10 units/μL RNase T_1. Digestion buffer (400 μL) composed of 200 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% SDS, and 0.5 mg/mL proteinase K was then added to the solutions which were subsequently incubated at 60 °C overnight. The mixtures were extracted with an equal volume of phenol/chloroform (1:1, pH 8.0). The aqueous phases were further extracted with an equal volume of chloroform, followed by the addition of $MgCl_2$ to 10 mM to enhance the precipitation of small DNA fragments. Total DNA was precipitated by the addition of 2 volumes of absolute ethanol. The DNA pellets were air-dried and dissolved in 50 μL of 10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA buffer. The DNA concentrations were checked by the UV absorbance at 260 nm and adjusted to assure equivalent loading. DNA samples were then mixed with 0.1 volume of loading dye (0.5% bromophenol, 0.5% xylene cyanol, 25% glycerol) and loaded onto the 1.8% agarose gel in TAE (40 mM Tris-HCl, 40 mM sodium acetate, 1.0 mM EDTA, pH 8.3) buffer. Gel electrophoresis was carried out at 2 V/cm for 12 h at 4 °C. The gels were subsequently stained with 0.5 μg/mL ethidium bromide for 30 min, destained with distilled water for 30 min, and photographed by a Biophotonics Gel Print 2000i Camera System (Ann Arbor, MI) using a 320 nm UV transilluminator.

Drug-induced apoptosis was also studied using a flow cytometric assay (Hotz et al., 1994; Darzynkiewicz et al., 1994). Briefly, drug-treated HL-60 cells were washed with Hank's balanced salt solution (HBSS) and fixed in 70% cold ethanol (−20 °C) overnight. Fixed cells were resuspended in the DNA extraction buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8), diluted 10-fold in HBSS, and incubated for 5 min at room temperature. After extraction, cells were incubated in the dark in HBSS solution containing 20 μg/mL propidium iodide and 0.1 mg/mL proteinase K for 30 min at 37 °C. DNA histograms were generated on a Coulter Elite flow cytometer with an air-cooled argon ion laser operating at 488 nm. A long-pass filter of 610 nm was used to separate the fluorescence emission from scattered light. Relative cell populations in G_1/G_0, S, G_2/M, and undergoing apoptosis (Ap) were determined using the Multicycle cell cycle analysis software obtained from Phoenix Flow System (San Diego, CA).

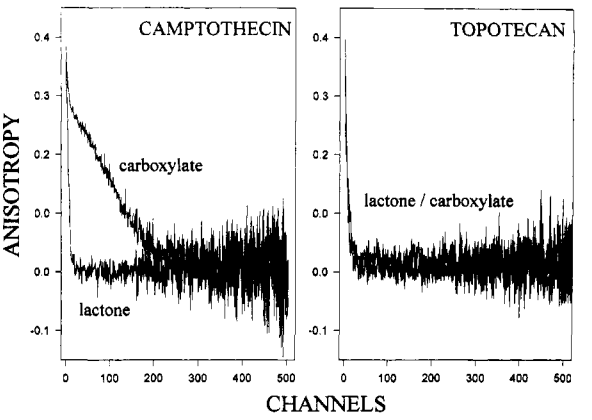

FIGURE 1: Time-resolved fluorescence anisotropy decays of the lactone and carboxylate forms of camptothecin (left panel) and topotecan (right panel) in the presence of 7.25×10^{-6} M HSA. Decay data were generated by the time-correlated single photon counting techniques and analyzed using IBH decay analysis software as described under Materials and Methods. For camptothecin, a rotational correlation time of 90 ps was determined for the lactone form, and double correlation times of 70 ps and 16 ns were determined for the carboxylate form. In the case of topotecan, however, both lactone and carboxylate forms displayed rapid rotational motion with the same rotational correlation time of 180 ps. The time calibration in the figure is 0.033 ns/channel.

Cytotoxicity Assays. Cytotoxicities of camptothecins were determined using the growth inhibition method. HL-60 cells (5×10^5 cells/mL) were exposed continuously for 24 and 72 h periods at 37 °C with 6−7 sequentially diluted drug concentrations. After treatment, aliquots of cells were mixed with an equal volume of 1.0 mg/mL solution of erythrocin B (Hay, 1992), and unstained, viable cells were counted with a hemacytometer. The percentage of viable cells was estimated by scoring 150−300 cells per sample.

RESULTS AND DISCUSSION

Time-Resolved Fluorescence Anisotropy Measurements Provide No Evidence for Topotecan Lactone and Carboxylate Forms Interacting with HSA. Figure 1 depicts the time-resolved anisotropy decays of camptothecin (left panel) and topotecan (right panel) in PBS buffer (37 °C) containing 7.25×10^{-6} M HSA. In the case of camptothecin, the lactone form displayed a rapid anisotropy decay with a rotational correlation time (θ value) of 90 ps. This correlation time of 90 ps for camptothecin lactone in the presence of HSA is identical to its rotational correlation time observed in PBS solution only (data not shown), confirming that camptothecin lactone does not readily interact with HSA at dilute concentrations of 7.25×10^{-6} M (Mi & Burke, 1994b). However, the carboxylate form of camptothecin displayed a dramatically slower decay (Figure 1, left panel) consistent with strong binding between camptothecin carboxylate and HSA (Mi & Burke, 1994b).

Data analysis of the camptothecin carboxylate−HSA data recovered two rotational correlation times of 70 ps (fractional intensity of 0.12) and 16 ns (fractional intensity of 0.88). We attribute the fast motion to the free drug while the more

Table 2: Stability Parameters of Topotecan in PBS, Whole Blood and Plasma, and PBS Buffer Containing Individual Blood Components[a]

components	concn (mg/mL)	$t_{1/2}$ (min)	% lactone at equilibrium
phosphate-buffered saline	na	23.6 ± 1.7	15.4 ± 0.7
whole blood[b]	na	46.9 ± 3.3	22.6 ± 2.0
plasma fraction components			
plasma[c]	na	23.1 ± 1.2	17.6 ± 1.1
albumin	40.0	33.1 ± 2.4	16.2 ± 1.4
albumin (denatured)	5.0	25.2 ± 2.1	15.5 ± 0.5
γ-globulin	20.0	27.0 ± 0.8	15.1 ± 0.5
α_1-acid glycoprotein	2.5	28.1 ± 1.4	19.5 ± 0.5
low-density lipoprotein	6.5	32.4 ± 0.9	7.8 ± 0.8
high-density lipoprotein	3.0	32.6 ± 1.7	15.1 ± 1.6
fibrinogen	3.0	27.1 ± 0.5	13.8 ± 0.3
formed element fraction components			
HSA-free erythrocytes	*d*	34.7 ± 3.5	34.5 ± 2.4
erythrocyte ghosts	*d*	32.1 ± 1.0	15.5 ± 1.4
everted erythrocyte ghosts	*d*	25.1 ± 1.0	20.6 ± 1.1
platelets	*d*	26.9 ± 0.7	13.3 ± 2.2
hemoglobin	150	24.2 ± 1.0	13.1 ± 1.0

[a] Stability determinations were made using HPLC methodologies as described under Materials and Methods. All experiments were conducted at pH 7.4, 37 °C, using 1 μM drug concentration unless specified otherwise. Some preliminary stability parameters for topotecan (in PBS, HSA, plasma, whole blood) were reported earlier in a communication (Burke et al., 1995). [b] Summary of five independent determinations. [c] Plasma samples were aerated with "Blood Gas" (MEDIBLEND, Linde Medical Gases, CT) to maintain constant pH (pH 7.4−7.5). [d] The densities of HSA-free erythrocytes ($5 \times 10^6 \pm 1 \times 10^6$ cells/μL) and platelets ($2.5 \times 10^5 \pm 0.5 \times 10^5$ cells/μL) utilized in these experiments are similar to those levels found in blood.

prominent signal component of slower motion is attributed to protein-associated drug. The fluorescence decay time (τ) of HSA-bound camptothecin carboxylate is 1.1 ns; because the dynamic reach of anisotropy decay studies is considered to be $\theta/\tau < 10$, the θ value of 16 ns for HSA-bound camptothecin carboxylate should be regarded as an approximation. In the case of topotecan, both lactone and carboxylate forms display similar fast decays in the presence of HSA (Figure 1, right panel). Data analysis recovered a single rotational correlation time of 180 ps in both cases. Essentially the same rotational correlation time was recovered for topotecan only in PBS solution (data not shown). Thus, our data reveal dramatic differences between the HSA interactions of camptothecin carboxylate vs topotecan carboxylate; in the latter case, no evidence of association to HSA was observed.

Reduced Albumin Interactions Correlate with the Improved Stability of Topotecan in Human Plasma and Blood. Table 2 summarizes the kinetic and equilibrium parameters for the hydrolysis of topotecan in human blood, PBS buffer, and PBS buffer containing physiologically relevant levels of individual blood components. Experiments were conducted at 37 °C using 1 μM drug concentrations. Analysis of the HPLC stability data shows that the hydrolysis of topotecan in PBS occurred with a half-life ($t_{1/2}$ value) of 23.6 min and achieved a final carboxylate-to-lactone equilibrium of 85:15. The stability data for topotecan in PBS are similar to the stability data in PBS for camptothecin (Mi & Burke, 1994a) and other camptothecin analogues (Burke & Mi, 1994; Burke et al., 1995).

The addition of HSA (40 mg/mL) to PBS buffer containing topotecan was found to slightly enhance drug stability. In the presence of HSA, drug half-life increased to 33.1 min with a percent lactone at equilibrium value of 16.2%. The

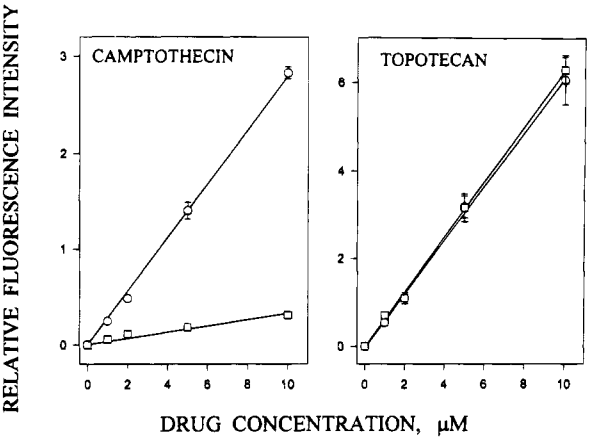

FIGURE 2: Uptake of camptothecin (left panel) and topotecan (right panel) into HL-60 cells using a flow cytometric analysis in the presence (□) and absence (○) of 40 mg/mL human serum albumin. Note that HSA attenuates the net cellular accumulation of camptothecin by approximately 15-fold while HSA effected no change on the net cellular accumulation of topotecan.

presence of other plasma proteins such as γ-globulin, α_1-acid glycoprotein, and fibrinogen also slowed the rate of topotecan hydrolysis in PBS. Red blood cells and platelets suspended in PBS solution also exhibited slight stabilizing effects on the lactone ring of topotecan. Stabilization of topotecan and other camptothecin congeners in the presence of membranes like RBCs and platelets is known to occur by the preferential partitioning of lactone drug forms into the acyl chain region of the membranes (Burke et al., 1992, 1993; Mi & Burke, 1994a). The presence of LDL and HDL was observed to slow drug hydrolysis ($t_{1/2}$ value of approximately 32 min), with LDL lowering the percent lactone at equilibrium value to 8%. Half-lives and equilibrium values for topotecan in plasma ($t_{1/2}$ = 23.1 min, percent lactone at equilibrium of 17.6%) and whole blood ($t_{1/2}$ = 46.9 min, percent lactone at equilibrium of 22.6%) were also determined.

The topotecan stability data contained in Table 2 contrast markedly with stability data for camptothecin (Mi & Burke, 1994a). Due to the 200-fold binding preference exhibited by HSA for camptothecin lactone over camptothecin carboxylate (Mi & Burke, 1994b), camptothecin displays rapid ($t_{1/2}$ = 11 min) and complete (<0.2% lactone at equilibrium) hydrolysis in plasma. Thus, the reduced HSA interactions of topotecan relative to camptothecin (Figure 1) provide an explanation for the 60-fold higher level of topotecan lactone observed in human plasma. In whole blood, the destabilizing effect of HSA on camptothecin stability is mitigated somewhat by the ability of the lipophilic (Burke et al., 1992, 1993) lactone form to partition into red blood cell membranes (Mi & Burke, 1994a). While camptothecin in whole blood displayed a percent lactone at equilibrium level of 5.3% (Mi & Burke, 1994a), the stability of topotecan in whole blood was found to be significantly higher with a percent lactone at equilibrium value of 22.6% (Table 2).

The Presence of HSA Markedly Reduces the Cellular Uptake, Drug-Induced Apoptosis, and Anticancer Activities of Camptothecin, but Not Topotecan. Figure 2 depicts the net cellular accumulation of camptothecin and topotecan in the presence and absence of physiologically relevant levels of HSA as determined using flow cytometric analysis (Mi & Burke, 1994a). These experiments involved the addition

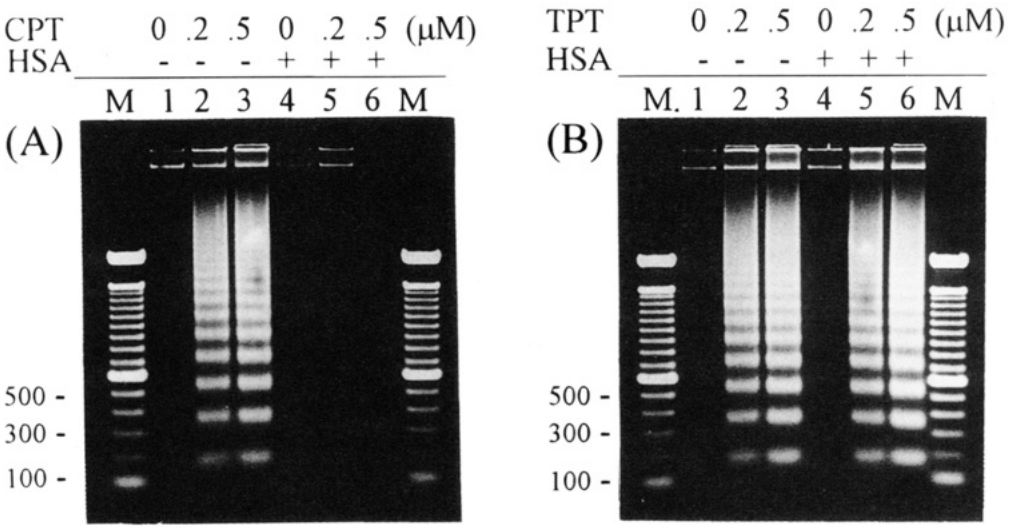

FIGURE 3: Agarose gel electrophoresis of DNA fragmentation in HL-60 cells induced by camptothecin and topotecan. 1×10^6 cells were treated with camptothecin (A) and topotecan (B) for 3 h in the presence (+, lanes 4−6) and absence (−, lanes 1−3) of 40 mg/mL human serum albumin. Lane M: 100 bp DNA ladder.

of drug to HL-60 cell suspensions followed by monitoring drug fluorescence intensity levels until plateau levels were reached (see Materials and Methods for additional details). Due to extensive drug−protein associations, the uptake of camptothecin into HL-60 human leukemia cells was attenuated more than 15-fold by the presence of 40 mg/mL HSA (Figure 2, left panel). In dramatic contrast, however, the presence of equivalent amounts of HSA had no effect on the net accumulation of topotecan by HL-60 cells (Figure 2, right panel).

Topotecan and the other members of the camptothecin family are known to induce both single-stranded as well as internucleosomal types of DNA cleavage in leukemia cells (Yoshida, 1993) and other cancer cells [see Gupta et al. (1995) and references cited therein]. Upon cellular access, the various camptothecin analogues are thought to interact with both topoisomerase I and DNA, forming stable ternary complexes which ultimately lead to replication fork arrest and DNA damage (Gupta et al., 1995). The formation of distinct DNA fragments of 180−200 bp lengths prior to cell death is a biochemical hallmark of apoptotic agents such as the camptothecins (Bortner et al., 1995).

To compare how HSA modulates the induction of apoptosis in HL-60 cells treated with camptothecin vs topotecan, we employed an agarose gel electrophoresis assay (Sambrook et al., 1989; Ray et al., 1994). This assay provides an effective means of following DNA fragmentation. Figure 3 demonstrates that, in the absence of HSA, both camptothecin (panel A) and topotecan (panel B) are able to induce apoptosis in HL-60 cells after 3 h incubation at drug concentrations of 0.2 μM or higher (see lanes 2 and 3). For camptothecin-treated cells, the presence of 40 mg/mL HSA markedly altered the fragmentation data with essentially no DNA fragmentation being observed at drug concentrations of 0.2 μM and 0.5 μM (lanes 5 and 6 in panel A). However, the ability of topotecan to induce the 180−200 bp DNA ladder in HL-60 cells (lanes 5 and 6 of panel B) was unaffected by the presence of 40 mg/mL HSA.

Additional information concerning the differential effects of HSA on the apoptotic activities of camptothecin vs topotecan was obtained using flow cytometry. Figure 4 is a series of DNA histograms which depict the relative HL-60

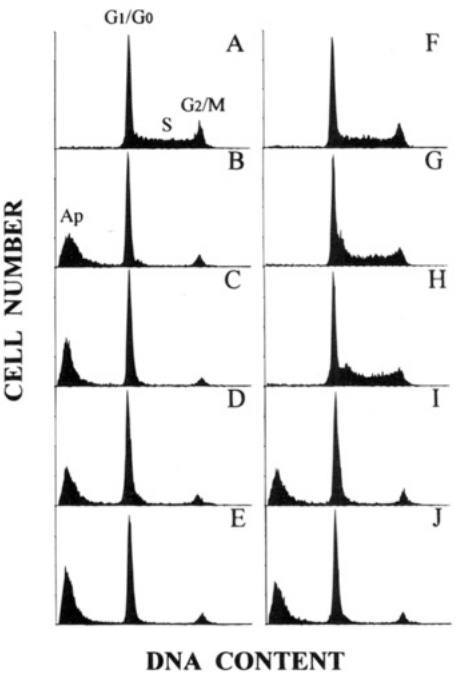

FIGURE 4: DNA content frequency distributions of HL-60 cells treated by camptothecin and topotecan in the absence (A−E) and presence (F−J) of 40 mg/mL HSA as determined by a flow cytometric method of analysis (described under Materials and Methods). A and F, controls; B and G, treated with 0.2 μM camptothecin; C and H, treated with 0.5 μM camptothecin; D and I, treated with 0.2 μM topotecan; E and J, treated with 0.5 μM topotecan. Ap: apoptotic cells.

cell populations after treatment with camptothecin and topotecan in the presence and absence of HSA. Before drug treatment and in the absence of HSA, approximately 45% of the HL-60 cells were in G_1 phase, 40% in S phase, and 15% in G_2/M phase (Figure 4, panel A).

The appearance of cells with low DNA stainability [lower than that of the G_1 cells (sub-G_1 peak)] in cultures treated with cytotoxins is considered a characteristic marker of cell death by apoptosis (Darzynkiewicz et al., 1994). Following 3 h of camptothecin treatment at drug concentrations of 0.2 μM (Figure 4, panel B) and 0.5 μM (Figure 4, panel C) in the absence of HSA, about 37−40% of the cells were

Table 3: Cytotoxicities of Camptothecin, Topotecan, and Related Analogues in HL-60 Cells Using a Cell Growth Inhibition Assay in the Presence and Absence of Physiological Levels of Human Serum Albumin[a]

agents	incubation time (h)	IC_{50} (μM) without HSA	IC_{50} (μM) with 40 mg/mL HSA
camptothecin	24	0.037 ± 0.008	6 ± 1
	72	0.0015 ± 0.0002	4 ± 1
9-aminocamptothecin	24	0.17 ± 0.05	0.42 ± 0.05
	72	0.0014 ± 0.0003	0.31 ± 0.02
topotecan	24	0.084 ± 0.009	0.096 ± 0.008
	72	0.012 ± 0.001	0.012 ± 0.002
CPT-11	24	3.6 ± 0.6	14 ± 1
	72	0.8 ± 0.1	2.0 ± 0.3
SN-38	24	0.008 ± 0.001	0.24 ± 0.03
	72	0.0015 ± 0.0002	0.041 ± 0.002

[a] Cell growth inhibition assays were carried out as described under Materials and Methods. Two incubation times of 24 and 72 h were used in the study. Note the marked increase in IC_{50} for camptothecin, 9-aminocamptothecin, CPT-11, and SN-38 in the presence of human serum albumin, while no significant change occurs in the case of topotecan as determined by the Student's t-test ($p = 0.15$). IC_{50}: drug concentration which induces a 50% growth inhibition.

observed to be in an apoptotic state while only 0−3% of the cells remained in S-phase. The changes in the populations of G_2/M and G_1 cells following drug treatment were relatively slight in comparison to the changes observed in the S-phase, and this observation agrees with literature citations indicating that camptothecin drugs are S-phase-sensitive (Del Bino et al., 1991).

In the presence of 40 mg/mL HSA, however, camptothecin-treated HL-60 cells did not undergo apoptosis (Figure 4, panels G and H). More careful study of the DNA histograms of HL-60 cells treated with camptothecin in the presence of HSA revealed an increase of cells in early S-phase (Figure 4, panels G and H). Similar DNA histograms have been reported previously (Traganos et al., 1993) for HL-60 cells exposed to very low concentrations (20 nM) of camptothecin. In our studies, the early S-phase arrest observed in panels G and H of Figure 4 is most likely due to the ability of HSA to bind the majority of drug and thereby reduce the free camptothecin levels to very low levels. In marked contrast to camptothecin-treated cells, topotecan-treated cells displayed similar apoptotic populations in the absence (Figure 4, panels D and E) and presence of 40 mg/mL HSA (Figure 4, panels I and J). Topotecan, unbound with virtually all of the drug available to partition into the HL-60 cells, readily induces apoptosis in the presence of HSA.

Table 3 summarizes the cytotoxicities of camptothecin, topotecan, and other clinically-relevant analogues (9-aminocamptothecin, CPT-11, and SN-38) in HL-60 cells in the absence and presence of physiologically relevant 40 mg/mL concentrations of HSA. Cytotoxicities for drug exposure periods of 24 and 72 h are expressed as IC_{50} values (see Materials and Methods). In the absence of HSA, topotecan is the second least potent drug among the five listed. But in the presence of 40 mg/mL HSA, the IC_{50} values for camptothecin, 9-aminocamptothecin, SN-38, and CPT-11 all increase dramatically while HSA effects no change on the IC_{50} value of topotecan. For drug exposure periods of 72 h, the increase in IC_{50} value due to the presence of HSA was a dramatic 2600-fold for camptothecin, 220-fold for 9-aminocamptothecin, 2.5-fold for CPT-11, and 27-fold for SN-38. As a result of the differential modulation of IC_{50} values of camptothecins by HSA, topotecan emerges as the most active drug against HL-60 cells (both 24 and 72 h exposures) in the presence of HSA. SN-38, regarded as one of the most potent camptothecin analogues yet to be identified in many cell lines (in HSA-free assays), is approximately 3-fold less potent than topotecan in the presence of HSA (Table 3).

In summary, the camptothecin-class topoisomerase I inhibitors are exceptionally dynamic drugs in biological systems, with their anticancer activities influenced strongly by hydrolytic and metabolic processes (Slichenmyer et al., 1993; Potmesil, 1994) as well as by protein binding. In the present study, we have demonstrated that HSA interactions can differentially modulate in a very marked fashion both the bloodstream stabilities and anticancer activities of topotecan vs camptothecin. Physiologically-relevant levels of HSA were found to dramatically attenuate the cytotoxic activity of camptothecin in HL-60 cells in excess of 2600-fold while the presence of HSA had no effect on the cytotoxic activity of topotecan. Our findings concerning the prominent differences in HSA binding among clinically-relevant camptothecin drugs and the corresponding effects on drug stabilities and activities provide ample reason to anticipate strong alterations in pharmacokinetic and pharmacodynamic parameters among the various members of this class of agents. Differences in HSA binding may be contributing factors to the clinical observations of a short, terminal half-life of 2−3 h for topotecan in the bloodstream of patients in contrast with half-lives of 10 h or longer for HSA-interactive analogues such as camptothecin, 9-aminocamptothecin, and SN-38 (Slichenmyer et al., 1993). It may also be possible that the activities of HSA-interactive agents such as camptothecin, 9-aminocamptothecin, and SN-38 may be more prone to patient-to-patient variations in albumin levels (known to range widely from 20 mg/mL to 50 mg/mL in cancer patients) than for a noninteractive agent such as topotecan. Ultimately, optimization of the therapeutic utilities of the camptothecins will benefit greatly from an integrated view of the chemical, biophysical, and biological processes which influence the activities of the various members of this promising class of anticancer drugs.

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