Differential Interactions of Camptothecin Lactone and Carboxylate Forms with Human Blood Components†

Zihou Mi and Thomas G. Burke*

Division of Pharmaceutics, College of Pharmacy, and The Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210-1291

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ABSTRACT: The intrinsic fluorescent emissions from the lactone and carboxylate forms of camptothecin have been exploited in order to elucidate their markedly different interactions with the various components of human blood. In phosphate-buffered saline (PBS) at pH 7.4, human serum albumin (HSA) preferentially binds the carboxylate form with a 150-fold higher affinity than the lactone form; these interactions result in camptothecin opening more rapidly and completely in the presence of HSA than in the protein's absence [Burke, T. G., & Mi, Z. (1993) *Anal. Biochem. 212,* 285–287]. In human plasma, at pH 7.4 and 37 °C, we have observed camptothecin lactone to open rapidly and fully to the carboxylate form ($t_{1/2}$ = 11 min; % lactone at equilibrium, 0.2%). Substitution of a 10-hydroxy moiety into the camptothecin fluorophore makes the agent's emission spectrum highly sensitive to microenvironment polarity; we have observed pronounced blue shifting (from 530 to 430 nm) in the emission spectra of the hydroxy-substituted carboxylate both upon HSA association as well as upon drug dissolution in organic solvents of low dielectric strength. Hence, it appears that camptothecin carboxylate's fluorophore locates in a hydrophobic binding pocket in native HSA. Ionic interactions also appear to strongly affect binding between camptothecin carboxylate and the HSA binding pocket, since a 6-fold increase in solution salt concentration diminished camptothecin carboxylate binding by 10-fold. Our findings that HSA denaturation abolishes high-affinity binding indicate that interactions of the carboxylate drug form are specific for the native HSA conformation. Interestingly, high-affinity binding of the carboxylate appeared not to occur in the presence of other blood proteins, such as γ-globulin, α_1-acid glycoprotein, fibrinogen, and the oxy and deoxy forms of hemoglobin. In whole blood versus plasma, camptothecin was found to display enhanced stability ($t_{1/2}$ value of 22 min and a lactone concentration at equilibrium value of 5.3%). The enhanced stability of camptothecin in human blood was found to be due to drug associations with the lipid bilayers of red blood cells. Camptothecin lactone partitions into the lipid bilayers of erythrocytes, with the drug locating in a hydrophobic environment protected from hydrolysis.

The anticancer agent camptothecin is a water-insoluble natural product first isolated from the Chinese tree *Camptotheca acuminata* that has recently gained the U.S. Food and Drug Administration's approval for advancement to Phase II clinical trials in the treatment of human cancer [see Slichenmyer et al. (1993) and Potmesil (1994) for recent reviews]. Mechanistically, camptothecin and its more water-soluble synthetic analogues CPT-11[1] and topotecan are thought to control the proliferation of cancer cells by a mechanism of action that differs from those of other clinically approved anticancer medications. Camptothecin is thought to elicit its antitumor activity by interfering with the nicking/sealing activity of topoisomerase I (Hsiang et al., 1985, 1989; Hsiang & Liu, 1988; Jaxel et al., 1989). The drug stabilizes and forms a reversible enzyme–drug–DNA ternary complex, which is termed the cleavable complex. The formation of the cleavable complex specifically prevents the ligation step of the nicking/sealing cycle performed by the topoisomerase enzyme.

The structure of camptothecin (shown in Figure 1) and its potential as an antitumor agent were first determined in 1966 by Wall and co-workers. Camptothecin contains a α-hydroxy-δ-lactone ring moiety, a functionality that hydrolyzes under basic conditions, *i.e.*, at pH 7 or above, with the lactone readily opening up to yield the carboxylate form of the drug. Following several years of preclinical testing, interest in camptothecin as a potential anticancer agent heightened in the early 1970s to a point where administrators at the National Insitututes of Health chose to evaluate the compound in human trials. Due to poor water solubility, it was decided to evaluate the water-

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* Address correspondence to this author at the Division of Pharmaceutics, The Ohio State University College of Pharmacy, 500 W. 12th Ave., Columbus, OH 43210-1291. Telephone: (614) 292-2915. FAX: (614) 292-7766.

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[1] Abbreviations: PBS, phosphate-buffered saline containing 8 mM Na_2HPO_4, 1 mM KH_2PO_4, and unless specified otherwise, 137 mM NaCl and 3 mM KCl (pH 7.4); HSA, human serum albumin; CPT-11, 7-ethyl-10-[[[4-(1-piperidino)-1-piperidino]carbonyl]oxy]camptothecin; topotecan, 9-[(dimethylamino)methyl]-10-hydroxycamptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; AGP, α_1-acid glycoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PC or DMPC, L-α-dimyristoylphosphatidylcholine; DMPG, L-α-dimyristoylphosphatidylglycerol; PE, egg phosphatidylethanolamine; PS, L-α-phosphatidylserine from bovine brain; DMPA, L-α-dimyristoylphosphatidic acid; RBC, human red blood cells; 2,3-DPG, 2,3-diphospho-D-glyceric acid; SUV, small unilamellar vesicle; T_m, gel-to-liquid crystalline phase transition temperature; TES, buffer containing 0.05 M TES (2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid), pH 7.4; P_{50}, oxygen partial pressure at which the hemoglobin sample is 50% oxygenated; a, steady-state anisotropy; a_o, anisotropy in the absence of depolarizing rotations; τ, excited-state lifetime; K, overall association constant; K_{SV}, Stern–Volmer quench constant; V, static quench constant; k_q, bimolecular collisional quench constant.

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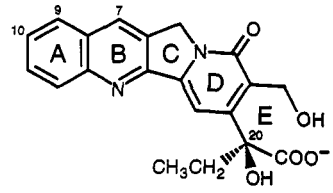
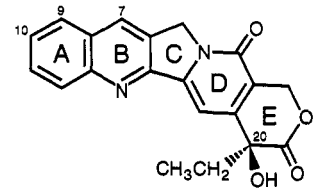


CARBOXYLATE FORM



LACTONE FORM

FIGURE 1: Structures of the lactone and carboxylate forms of camptothecin.

soluble carboxylate form of camptothecin in the initial clinical trials (Gottlieb et al., 1970; Muggia et al., 1972). Despite displays of antitumor activity in several patients, testing of camptothecin was suspended due to its formidable and unpredictable toxicity.

Today, over 20 years after the initial clinical trials, a more detailed mechanistic understanding of camptothecin's antitumor activity exists. It is now known that the biological activity of camptothecin both *in vitro* and *in vivo* is significantly greater for the lactone than for the carboxylate form (Wani et al., 1987a,b; Jaxel et al., 1989; Slichenmyer et al., 1993; Potmesil, 1994). Data suggest that a closed α-hydroxy-δ-lactone ring is an important structural requirement both for passive diffusion of drug into cancer cells as well as for successful interaction with the topoisomerase I target (Hsiang et al., 1985). The relatively recent advances in the understanding of the mechanism of action of camptothecin have resulted in a renewed clinical interest in the agent (Potmesil, 1994).

Factors influencing the lactone–carboxylate equilibrium of camptothecin thus are regarded as clearly important determinants of the agent's function. Recent studies from this laboratory indicate that the lactone and carboxylate forms of camptothecin exhibit markedly different interactions with human serum albumin (HSA) (Burke & Mi, 1993a). Time-resolved fluorescence spectroscopic measurements taken on the intensely fluorescent lactone and carboxylate forms of camptothecin have provided direct information on the differential nature of their interactions with HSA: while camptothecin lactone binds HSA with moderate affinity, the ring open carboxylate form displays a dramatic 150-fold enhancement in its apparent binding constant for HSA (Burke & Mi, 1993a). When the lactone form of camptothecin is put in solution in the presence of HSA, the protein's preferential binding of camptothecin carboxylate shifts the equilibrium and results in the lactone ring opening more rapidly and completely (Burke & Mi, 1993a). Such rapid and complete ring opening is not observed for camptothecin dissolved in PBS only. The end result is that, in the presence of physiologically relevant levels of human albumin, the biologically active form of camptothecin has a very short half-life (approximately 12 min). Similarly, 2 h after drug addition to human plasma, >99% of the drug has converted to camptothecin carboxylate (Burke & Mi, 1993a,b), the inactive and potentially toxic (Slichenmyer et al., 1993) form of the drug.

In the present report, we employ HPLC methodology, steady-state and time-resolved fluorescence spectroscopy, and flow cytometric techniques to more fully elucidate the markedly different interactions of the lactone and carboxylate forms of camptothecin with HSA and the various other human blood components. Our results indicate that camptothecin carboxylate binds to a hydrophobic binding pocket on HSA. Besides hydrophobic interactions, our studies reveal that ionic interactions between camptothecin carboxylate and protein also promote binding. Camptothecin carboxylate–HSA associations exhibit a high degree of specificity strongly modulated by changes in both the drug's chiral configuration and the protein's native conformation. In contrast to the manner in which ring opening promotes HSA associations, the lactone form of camptothecin binds the lipid bilayers of red blood cells 5 times more strongly than the carboxylate form. Here we demonstrate that RBCs stabilize the biologically active form of camptothecin by allowing the lactone ring to partition into the lipid bilayers of the erythrocyte membranes, thereby protecting the moiety from hydrolysis.

MATERIALS AND METHODS

Chemicals. Samples of 20(*S*)-camptothecin and 20(*R*)-camptothecin were obtained from the laboratories of Drs. Monroe Wall and Mansukh Wani (Research Triangle Institute, Research Triangle Park, NC). Samples of 20(*S*)-camptothecin carboxylate sodium salt and 10-hydroxycamptothecin (*S* isomer) were obtained from the National Cancer Institute, Division of Cancer Treatment (Bethesda, MD). Each of the agents was of high purity (>98%) as determined by HPLC chromatographic assays using fluorescence detection. Stock solutions of the drugs were prepared in dimethyl sulfoxide (DMSO) (ACS spectrophotometric grade, Aldrich, Milwaukee, WI) at a concentration of 2×10^{-3} M and stored in the dark at −20 °C. A 1 mM working solution of 10-hydroxycamptothecin carboxylate was prepared by adding 1 part DMSO drug stock solution to 1 part PBS buffer (pH 10). Crystallized HSA obtained from Sigma Chemical (St. Louis, MO) was used in our studies as before (Burke & Mi, 1993a,b, 1994). Samples of γ-globulin, α_1-acid glycoprotein (AGP), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and fibrinogen from human sources were obtained from Sigma. 2,3-Diphospho-D-glyceric acid pentasodium salt was also obtained from Sigma. L-α-Dimyristoylphosphatidylcholine (PC), L-α-dimyristoylphosphatidic acid (PA), L-α-phosphatidylserine (PS) sodium salt, bovine brain sphingomyelin, and transphosphatidylated egg L-α-phosphatidylethanolamine (PE) were procured from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. All other chemicals 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.

Preparation of Whole Blood and Fractionated Blood Samples. Whole human blood was obtained from a healthy male donor by drawing blood into sterile vacutainers containing either ethylenediaminetetraacetic acid (EDTA) or heparin to prevent clot formation. Human plasma samples as well as outdated packed red blood cells were obtained from the Red Cross of Ohio and were used without further processing. Human plasma samples for drug analysis studies were continuously aerated with "blood gas" (MEDIBLEND, Linde Medical Gases, CT) in order to maintain constant pH (7.5 ± 0.1). Suspensions containing plasma-free red blood cells were prepared in PBS buffer as follows: outdated RBCs were centrifuged at 4 °C at 1000*g* for 15–20 min, and the supernatant was discarded. The RBCs were then gently

resuspended in an equal volume of ice-cold PBS and again centrifuged at 1000*g*. This step was repeated four times. Finally, the washed RBCs were suspended in PBS buffer for drug stability studies, and their concentration was determined using a hemocytometer. RBC preparations were used the day of isolation and were kept at 4 °C until immediately prior to use.

Human hemoglobin was purified from outdated RBCs using the methodology of Rabiner (1967) with modification: packed, plasma-free RBCs in glass centrifuge tubes were lysed by the addition of an equal volume of cold water, followed by the addition of chloroform (5% v/v). The mixture was stirred for 30 min at 4 °C and then centrifuged at this temperature at 3000*g* for 10 min to remove the major portion of cell debris and the chloroform-containing viscous phase. The supernatant containing hemoglobin was further centrifuged at 4 °C and 9000*g* for 30 min followed by isolation of the hemoglobin-containing supernatant from the pelleted cell debris. The hemoglobin solution was stripped of organic phosphates by extensive dialysis at 4 °C against 0.5 mM TES (2-[[tris-(hydroxymethyl)methyl]amino]ethanesulfonic acid) buffer containing 0.1 M NaCl (pH 7.4). The dialysis buffer was initially changed at 1 h intervals for the first 6 h, followed by changes every 6 h for the next 24 h. An additional step for removing ionic impurities involved passing the hemoglobin solution through a column of Sephadex G-25 ion exchange resin that was prewashed and preequilibrated with pure water at 4 °C. The purified hemoglobin solution was concentrated 2-fold using Amicon (Beverly, MA) Centriprep concentrators by centrifuging at 3000*g* and 4 °C, followed by storage at −20 °C until use. The total hemoglobin content was determined by conversion to cyanomethemoglobin using Drabkin's reagent, followed by absorbance determination at 540 nm using an extinction coefficient of 44 000 M^{-1} cm^{-1} (Eilers, 1967). The hemoglobin solution was diluted to physiological levels in TES buffer, and drug stability parameters were determined. Recordings depicting the equilibrium binding of oxygen to hemoglobin were obtained using a Hemox Analyzer (TCS Medical Products, Huntingdon Valley, PA). P_{50} values were calculated from oxygenation curves. A P_{50} value is defined as the partial oxygen pressure (pO_2) value at which 50% oxygen saturation of the sample occurs.

Erythrocyte ghosts were prepared by the method of Dodge et al. (1963). Briefly, plasma-free RBCs in PBS were hemolyzed at 4 °C by 15-fold dilution with hypotonic phosphate buffer (0.01 M sodium phosphate, pH 7.4). After gentle swirling for 15 min, the hemolyzed cell suspension was centrifuged for 20 min at 20000*g* and 4 °C. The cell membrane pellet was washed three times with 30–40 vol of hypotonic phosphate buffer; each time the cell membrane pellet was recovered by centrifugation for 40 min at 20000*g* and 4 °C. After final washing, the pellet color appeared almost white; it was then resuspended in 3–4 vol of 0.1 M KOH and incubated for 45 min at 37 °C to obtain resealed erythrocyte ghosts (Dodge et al., 1963). The ghost preparations were washed three times with PBS prior to an experiment. Everted erythrocyte ghosts were prepared by a previously described method (Garcia-Sancho & Alvarez, 1989), with modification. Briefly, plasma-free RBCs were hemolyzed by mixing packed cells with 40 vol of ice-cold lysing buffer solution containing 0.1 mM EDTA and 2.5 mM HEPES (pH 7.6), followed by vigorous stirring for 30 min at 4 °C. The lyzed cells were centrifuged for 40 min at 20000*g* and 4 °C, followed by washing of the cell membrane pellet four times with lysing solution. The cell membranes were resuspended in about 10 vol of the lysing solution and resealed *inside-out* by placement in an incubation bath at 37 °C for 45 min. The resealed membranes were washed and centrifuged in PBS buffer (20000*g* for 40 min) four times at 4 °C in order to obtain a pellet of everted erythrocyte ghosts. Erythrocyte ghost and everted ghost preparations were assayed for phospholipids by the Stewart assay (New, 1990). Vesicle preparations were adjusted to approximate physiological lipid levels (4 mg/mL) prior to being utilized in stability studies.

Lipid Vesicle Preparation. Small unilamellar vesicle (SUV) suspensions were prepared the day of an experiment as described previously (Burke & Tritton, 1985a; Burke et al., 1988). Briefly, lipid mixtures approximating the composition of the lipid bilayers of RBCs (PC:PE:sphingomyelin:PS:PA at a mole ratio of 1:1:1:0.5:0.07, respectively) were dissolved in $CHCl_3$. A stream of nitrogen was then passed through the solution to dry the film. Following the removal of trace levels of solvent by storage *in vacuo*, stock lipid suspensions containing 200 mg/mL lipid in PBS were prepared by vortex mixing for 5–10 min at 50–55 °C. Nitrogen gas was slowly passed through the liposome solution to reduce O_2 levels prior to sonication. The glass tube's headspace was purged with N_2 and the tube tightly sealed. The lipid dispersions were then sonicated using a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY) for 3–4 h until they became optically clear. The pH of these SUV suspensions was adjusted to 7.4 using small quantities of 2.5 M NaOH in PBS, followed by additional sonication. Vesicle suspensions were annealed for 30 min at 37 °C prior to use in an experiment.

Kinetics of Lactone Ring Opening Determined by HPLC Methods. The rates of lactone ring opening due to hydrolysis for 20(*S*)-camptothecin and 20(*R*)-camptothecin were determined by the quantitative reversed-phase high-performance liquid chromatography (HPLC) assays of Supko and Malspeis (1991, 1992) and Underberg et al. (1990), with modification (Burke & Mi, 1993a,b, 1994). Separation of the parent from the carboxylate drug form was accomplished on an HPLC setup consisting of the following: a Waters Model 501 pump, a Waters U6-K injector, a Waters NovaPak-C_{18} 5 μm particle size reversed-phase column, a Waters Model 470 scanning fluorescence detector, and a Hewlett-Packard Model HP3396 Series II integrator for data processing. An isocratic mobile phase was employed consisting of 32% acetonitrile, 67% 0.1 M acetate buffer (pH 5.5), and 1% 0.1 M sodium dodecyl sulfate. For analyte detection, an excitation wavelength of 370 nm and an emission wavelength of 438 nm were used for the two camptothecin isomers; for 10-hydroxycamptothecin an excitation wavelength of 392 nm and an emission wavelength of 561 nm were used. Flow rates of 1 mL/min were employed, and the retention times for the 20(*R*)- and 20(*S*)-camptothecin lactone forms were approximately 7 min, while the carboxylate forms rapidly eluted from the column with short retention times of approximately 2.0 min. Solutions of drugs at concentrations of 1 μM in PBS (pH 7.4), with and without purified blood components or model membranes, were prepared and incubated at 37 °C. Immediately after the drug stock solution was mixed with samples and at intervals of 5 min thereafter, 20 μL aliquots were taken out of each solution and mixed with 180 μL of dry ice-chilled methanol (approximately −50 °C) followed by vigorous vortex mixing for 30 s. Following centrifugation at 8000*g* for 1 min, each mixture was diluted 10-fold in the mobile phase. Samples were then either directly injected using 90 μL injection volumes or, alternatively, kept frozen on dry ice for subsequent analysis during the day of the experiment. The fraction of intact lactone (f) versus time (t) data for camptothecin, its 20(*R*) isomer, and the 20(*S*) isomer of 10-hydroxycamptothecin were fit to

eq 1 by the method of nonlinear least-squares analysis using SigmaPlot 5.0 (Jandel Scientific, Corte Madera, CA):

$$f = a + b \exp(-k_1 t) \quad (1)$$

where the adjustable parameter k_1 is the pseudo-first-order rate constant for hydrolysis of the lactone ring, the adjustable parameter a corresponds to the concentration of the intact lactone form at equilibrium, and $(a + b)$ equals the total intact lactone form of the drug at $t = 0$ (*i.e.*, the time at which the drug was first placed in aqueous solution).

Fluorescence Spectroscopy. Fluorescence measurements were obtained on an SLM Model 4800C spectrofluorometer. The spectrometer was equipped with a thermostated cuvette compartment and interfaced with an IBM PS/2 Model 55 SX computer. Excitation and emission spectra were recorded with an excitation resolution of 8 nm and an emission resolution of 4 nm without the use of filters. In all cases, spectra were corrected for background fluorescence and scatter by the subtraction of the spectrum of a blank sample. Steady-state fluorescence intensity measurements were taken in the absence of polarizers. Fluorescence lifetime measurements were also taken in the absence of polarizers and were determined by the method of phase shift (Spencer & Weber, 1969) using exciting light modulated at 30 MHz. Free and protein-bound drug fractions in HSA solutions were determined using a fluorescence lifetime titration technique described previously (Burke & Mi, 1993a,b, 1994). Binding isotherms were generated by titrating a total drug concentration of 20 μM with varying amounts of HSA. Camptothecin lactone and carboxylate fluorescent emissions were induced using excitation light of 378 nm; the emissions were isolated from scattered light and miscellaneous fluorescent impurities by using a 420 nm long pass filter on each channel (Oriel Corp.). For 10-hydroxycamptothecin lactone and carboxylate, exciting light of 375 nm and emission filters of 399 nm were used.

Steady-state anisotropy (a) measurements were determined with the instrument in the "T-format", allowing for the simultaneous measurement of two polarized intensities. Anisotropy values were calculated using SLM 4800C software (Version 1.61). The alignment of polarizers was checked routinely using a dilute aqueous suspension of 0.25 μm polystyrene microspheres (Polysciences, Inc., Warrington, PA), and anisotropy values of >0.99 were obtained. Anisotropy measurements for the two camptothecin forms were conducted using the long pass filters described above on each emission channel. The combination of excitation light and emission filters allowed us to adequately separate fluorescence from the background signal. The contribution of background fluorescence, together with scattered light, was less than 10% for the mixed-lipid vesicles. Since the lactone rings of the drugs of interest undergo hydrolysis in aqueous medium (pH 7.4), with half-lives of approximately 20 min, all measurements were completed within the shortest possible time (*ca.* 0.5–1 min) after the drug stock solution was mixed with thermally preequilibrated solutions. As described previously (Burke & Tritton, 1985a; Burke et al., 1993), the method of fluorescence anisotropy titration was employed in order to determine the concentrations of the free and bound species of drug in liposome suspensions containing a fixed drug concentration and varying lipid concentrations. Experiments were conducted in glass tubes deactivated by the process of siliconization. The overall association constants are defined as $K = [A_B]/([A_F][L])$, where $[A_B]$ represents the concentration of bound drug, $[A_F]$ represents the concentration of free drug, and [L] represents the total lipid concentration of the sample. Double-reciprocal plots of the binding isotherms [1/(bound fraction of drug) versus 1/[lipid]] were linear. K values were determined from their slopes (Burke & Tritton, 1985a) by the method of linear least-squares analysis. A computer program based on the $K = [A_B]/([A_F][L])$ relationship was used to predict bound drug levels for situations where K values and total lipid and total drug concentrations were known parameters.

Using methodologies employed previously (Burke & Tritton, 1985b; Burke et al., 1993), iodide quenching experiments conducted at constant ionic strength were used to compare the accessibility of free and bound drug to membrane-impermeable (Cranney et al., 1983; Jendrasiak, 1972) iodide quencher. Drug stock solutions were added to aliquots of liposome preparations such that the final drug, lipid, and halide concentrations were 2 μM, 0.29 M, and 0.5 M, respectively. Solutions also contained 2 mM sodium thiosulfate to prevent the oxidation of iodide. The iodide concentration was varied from 0 to 0.5 M. Lipid concentrations of approximately 0.29 M utilized in these experiments assured a bound drug fraction in excess of 97%. The modified form of the Stern–Volmer relationship was employed to analyze the static and dynamic quenching processes (Eftink & Ghiron, 1976):

$$F_o/Fe^{V[Q]} = 1 + K_{SV}[Q] \quad (2)$$

The terms F_o and F are the fluorescence intensities in the absence and presence of quencher concentration [Q], respectively, K_{SV} is the Stern–Volmer quenching constant where $K_{SV} = \tau k_q$ (k_q equals the collisional rate constant and τ equals the fluorescence lifetime of the fluorophore in the quencher's absence), and V is the static quenching constant. Data were fit to this equation by determining the value of V that yielded the line with the best correlation coefficient for the plot of $F_o/Fe^{V[Q]}$ versus [Q].

Flow Cytometry. Flow cytometric analyses were conducted on a Coulter Epics Elite machine equipped with a 5 W water-cooled multiline argon ion laser. Light of 364 nm was used. Band-pass filters of 305–390 and 445–485 nm were used to measure scattered light and fluorescence emission, respectively. Cell densities of 2×10^6/mL and cell flow rates of 400–600 s^{-1} were employed in the study. Prior to the initiation of experiments, a reference channel was set using fluorescence microsphere standards (Standard-Brite, Coulter Co., Hialeah, FL), so that data generated on different days could be compared. RBC suspensions were first analyzed for about 1 min to obtain baseline values. Following drug addition, the suspensions were allowed to equilibrate for approximately 1 min in order for the fluorescence levels to reach their plateau. Maximum fluorescence intensity levels, corrected for baseline signals, were then used to depict drug accumulation in the cell suspensions. The accuracy of using the flow cytometric method for the determination of drug uptake was corroborated using the HPLC assays described above. Briefly, individual samples containing RBCs ($5 \times 10^6/\mu$L) suspended in PBS or plasma were incubated with carboxylate or lactone forms of the drug for 5 min at 37 °C. The cells were then washed with ice-cold PBS. Pelleted cells were extracted with 6 vol of methanol. Following the conversion of all drug to the lactone form by adding pH 3.0 buffer, camptothecin levels were quantitated. For RBCs exposed to drug in PBS, HPLC analysis showed net cellular drug levels for lactone-exposed cells (4.9 μM) to be 15 times greater than those for carboxylate-exposed cells (0.32 μM). For RBCs exposed to drug in plasma, HPLC analysis showed net cellular drug levels for lactone-exposed RBCs (2.0 μM) to be 20 times greater than those for carboxylate-exposed cells (0.1 μM). Thus, the HPLC results were in close agreement with the flow cytometric results of

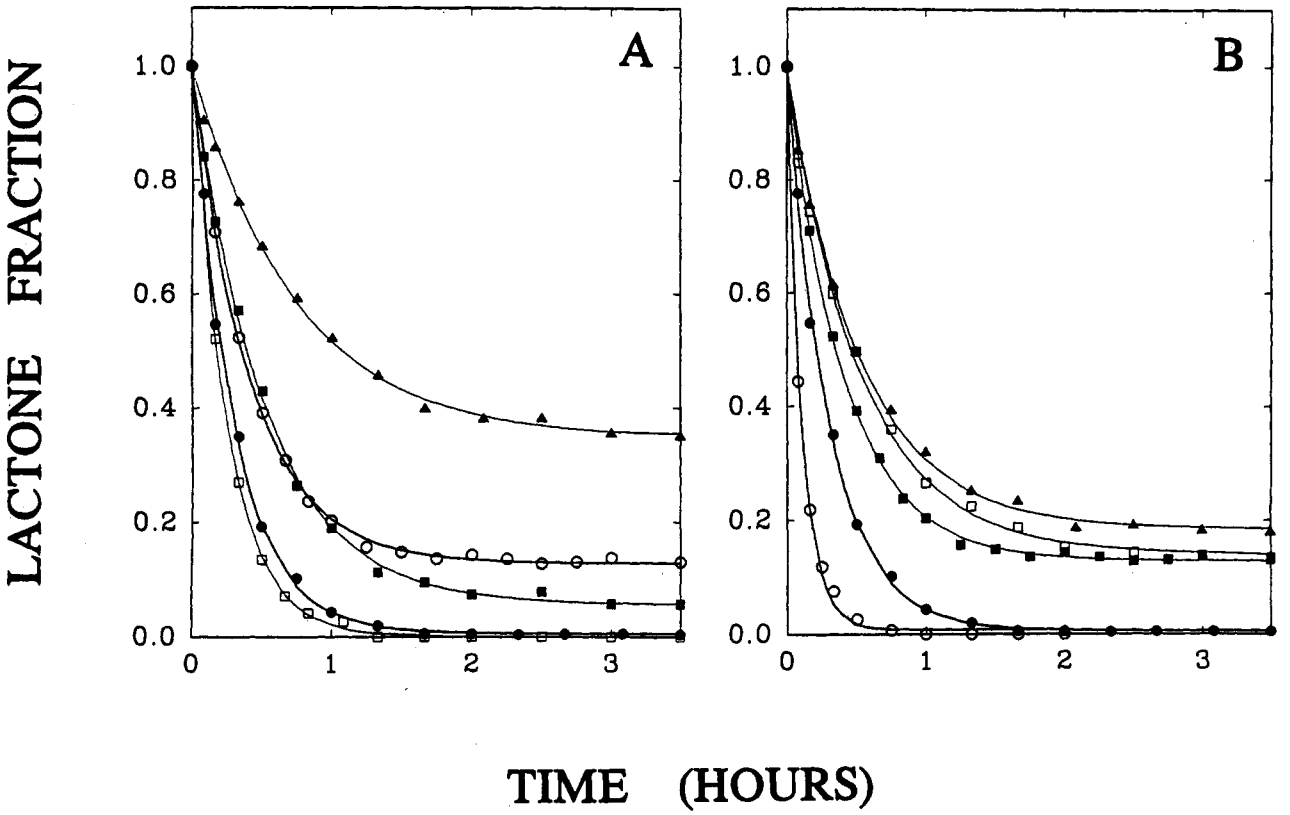


FIGURE 2: Kinetic evaluation of the rate of lactone ring opening for camptothecin. Panel A depicts stability data for the agent in the presence of human blood and purified human blood components: whole blood (■), human plasma (□), plasma-free red blood cells (▲), HSA (●), and PBS buffer (○). The hydrolysis data found in panel B demonstrate that camptothecin interactions with native HSA change dramatically with alterations in both drug and protein structure. Depicted are hydrolysis profiles of camptothecin in the presence of γ-globulin (▲), HSA (●), denatured HSA (□), and PBS buffer (■). Panel B also compares the markedly different stability parameters in the presence of native HSA observed between the 20(*S*) and 20(*R*) camptothecin forms [represented by the ● and ○ symbols, respectively]. Stability parameters are summarized in Table 1 and in the Results section. All experiments were conducted in PBS (pH 7.40 ± 0.05) at 37 °C unless specified otherwise (Table 1). Drug and RBC concentrations of 1 μM and 5×10^6 cells/μL, 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 is typically 5% or less.

Figure 6. Flow cytometric experiments were carried out in triplicate, and the data were plotted as the mean ± SD.

RESULTS

Opposite Effects of Human Serum Albumin versus Red Blood Cell Membranes on the Stability of Camptothecin's Lactone Ring. Figure 2 depicts changes in lactone concentration as a function of time for 1 μM samples of camptothecin in human blood, PBS buffer, and PBS buffer containing physiologically relevant levels of individual blood components. The kinetic and equilibrium parameters for the hydrolysis of camptothecin in the samples of interest are summarized in Table 1. Analysis of the data shows that the hydrolysis of camptothecin free in PBS solution proceeded with a half-life ($t_{1/2}$ value) of approximately 24 min and achieved a final carboxylate-to-lactone equilibrium ratio of 83:17 (Burke & Mi, 1994).

Interestingly, Table 1 demonstrates that a more rapid and virtually complete hydrolysis of camptothecin was observed both in the presence of human plasma and in PBS buffer containing HSA concentrations ranging from 5 mg/mL to physiologically relevant levels (Burke, 1980) of 30–50 mg/mL. In each case, camptothecin $t_{1/2}$ values of less than 13 min and final carboxylate:lactone ratios of greater than 99.0:1.0 were observed.

In marked contrast to the greater instability of camptothecin's lactone ring observed in the presence of HSA, physiologically relevant levels of other major proteins found in human plasma, such as γ-globulin and fibrinogen, exhibited little to no effect on drug hydrolysis. For example, camptothecin in PBS in the presence of γ-globulin (20 mg/mL) displayed a $t_{1/2}$ value of 21.9 ± 2.3 min and a % lactone at equilibrium value of 18.3%. Also in contrast to the effect of HSA on the rate and extent of camptothecin lactone ring opening, fibrinogen in PBS solution at a concentration of 3 mg/mL resulted in $t_{1/2}$ and % lactone at equilibrium values of 21.1 ± 1.0 min and 15.3%, respectively. The stability parameters for camptothecin in the presence of γ-globulin and fibrinogen described above are close to the stability parameters observed for camptothecin in the presence of PBS only ($t_{1/2}$ and % lactone at equilibrium values of 23.8 ± 1.3 min and 17.0%, respectively).

In contrast with the plasma components mentioned above (*e.g.*, HSA, which significantly promoted camptothecins's ring opening, and γ-globulin and fibrinogen, plasma proteins that displayed little influence on camptothecin's stability), several other components present in human plasma exhibited a stabilizing influence on camptothecin's lactone ring structure. One such component is the α_1-acid glycoprotein (AGP), a major acute phase reactant protein. In human plasma the concentration of AGP can vary from levels of 0.55–1.2 mg/mL (Schmid et al., 1973) in healthy individuals to levels 2–4-fold higher (Schmid et al., 1975) in individuals with disease states such as infection (Sann, 1984) or cancer (Fish et al., 1982). As summarized in Table 1, AGP at physiologically normal levels of 1 mg/mL exhibited relatively little influence, when compared to drug in PBS solution only, on the rate and extent of camptothecin's lactone ring opening ($t_{1/2}$ = 22.6 ± 2.7 min and a % lactone at equilibrium value of 19.6%). However, elevated levels of AGP were found to be more

Table 1: Summary of the Stability Parameters of Camptothecin in Human Blood, PBS Buffer, and PBS Buffer Containing Individual Blood Components[a]

components	concentration (mg/mL)	$t_{1/2}$ (min)	% lactone at equilibrium
phosphate-buffered saline[b]	na	23.8 ± 1.3	17.0 ± 2.0
whole blood	na	21.6 ± 2.6	5.3 ± 0.6
plasma fraction components			
plasma[c]	na	10.6 ± 0.7	<0.2
albumin	5.0	13.4 ± 1.1	<1.0
albumin	20.0	12.6 ± 0.5	<0.5
albumin	30.0	11.9 ± 0.3	<0.5
albumin	50.0	11.9 ± 0.8	<1.0
albumin (denatured)	5.0	22.1 ± 1.0	14.0 ± 1.0
γ-globulin	20.0	21.9 ± 2.3	18.3 ± 1.6
α_1-acid glycoprotein	1.0	22.6 ± 2.7	19.6 ± 0.6
α_1-acid glycoprotein	2.5	29.2 ± 1.4	22.7 ± 1.4
α_1-acid glycoprotein	5.0	33.7 ± 4.0	25.3 ± 2.3
low-density lipoprotein	6.5	64.0 ± 6.3	44.9 ± 9.4
high-density lipoprotein	3.0	28.6 ± 1.3	0.0 ± 1.0
fibrinogen	3.0	21.1 ± 1.0	15.3 ± 1.3
formed elements fraction components			
RBCs, HSA-free	*d*	30.8 ± 2.8	34.6 ± 1.0
RBC ghosts	*d*	28.1 ± 2.8	25.6 ± 1.3
everted RBC ghosts	*d*	24.9 ± 2.1	21.9 ± 0.7
platelets	*d*	20.1 ± 1.2	22.3 ± 1.9
hemoglobin[e] (P_{50} = 6 mmHg)	150	20.0 ± 2.1	25.6 ± 0.5
hemoglobin[e] (P_{50} = 26 mmHg)	150	17.5 ± 1.5	20.6 ± 1.7

[a] Stability determinations were made using HPLC methodology, as described in the Materials and Methods section. All experiments were conducted at pH 7.4 and 37 °C using a 1 μM drug concentration unless specified otherwise. [b] Lactone ring opening is a reversible process. For camptothecin carboxylate in PBS at 37 °C, the rate of ring closure occurred with a $t_{1/2}$ value of approximately 17 min with a % lactone at equilibrium measured to be 16%. [c] Plasma samples were aerated with "blood gas" (MEDIBLEND, Linde Medical Gases, CT) to maintain constant pH (pH 7.5 ± 0.1).[d] The densities of HSA-free RBCs (5 ± 1) × 10^6 cells/μL and platelets ((2.5 ± 0.5) × 10^6 cells/μL) utilized in these experiments are similar to those levels found in blood. [e] Measurements made in TES buffer, pH 7.4.

effective at enhancing camptothecin's solution half-life. For camptothecin in PBS coincubated with 5 mg/mL AGP, the following stability parameters were observed: a $t_{1/2}$ value of 33.7 ± 4.0 min and a % lactone at equilibrium value of 25.3 ± 2.3.

Also, the low-density lipoprotein (LDL) fraction from human plasma at a physiologically relevant concentration of 6.5 mg/mL (Burke, 1980) was found to be quite effective at stabilizing camptothecin's lactone ring moiety; a $t_{1/2}$ value of 64 min and a % lactone at equilibrium value of 45% were observed. Such significant stabilization was not observed in the presence of HDL at physiologically relevant levels of 3 mg/mL (Gotto, 1987), where $t_{1/2}$ and % lactone at equilibrium values of 28.6 ± 1.3 and 0.0 ± 1.0%, respectively, were observed.

Taken together, our data indicate that HSA exhibits a pronounced destabilizing influence on camptothecin's lactone ring, while the other major components of human plasma (*i.e.*, γ-globulin, AGP, LDL, fibrinogen) either exhibited little or no effect on the kinetics of lactone ring opening or, unlike HSA, prevented rather than promoted the loss of the intact lactone ring form of the drug. Despite the presence of components other than HSA that are found in human plasma, the stability of camptothecin in plasma parallels that of drug stability in PBS solution containing HSA only. Presumably both HSA's high abundance in human plasma (*i.e.*, HSA levels exceed those of any other component of plasma) and the protein's high affinity for camptothecin carboxylate are two factors that collectively explain why results obtained in human plasma are paralleled so closely by findings in PBS solution containing HSA only (*i.e.*, PBS solution containing HSA in the absence of other protein and lipoprotein components contained in human plasma).

Table 1 also contains information concerning camptothecin interactions with several formed element fractions of human blood. Interestingly, physiologically relevant concentrations of HSA-free RBCs of 5 × 10^6 cells/μL (Burke, 1980) exhibited a significant stabilizing effect on camptothecin's lactone ring, with analysis of the stability profiles revealing an enhanced $t_{1/2}$ value of 30.8 ± 2.8 min and a % lactone at equilibrium value of 34.6%. In our work we also examined RBC ghosts (both right-side-out and everted membrane vesicle preparations), and we found that the presence of both types of membrane preparations enhanced camptothecin's $t_{1/2}$ value slightly (*i.e.*, relative to $t_{1/2}$ values for drug free in solution). The presence of membrane preparations also increased the observed % lactone at equilibrium values (as summarized in Table 1).

The hemoglobin component of RBCs was found to be significantly less effective, relative to the membrane-containing whole RBCs, at stabilizing camptothecin's lactone ring. Physiologically relevant concentrations of an oxyhemoglobin preparation (P_{50} = 6 mmHg) displayed a $t_{1/2}$ value of 20.0 ± 2.1 min and a % lactone at equilibrium value of 25.6 ± 0.5%. Hemoglobin preparations with reduced oxygen binding capacities due to the presence of the natural allosteric modifier 2,3-DPG were also studied. The DPG-containing hemoglobin sample displaying a P_{50} value of 26 mmHg (which is close to levels exhibited by RBCs in circulation) was prepared, and camptothecin stability in the sample (observed $t_{1/2}$ value of 17.5 ± 1.5 min and % lactone at equilibrium value of 20.6 ± 1.7%) was, as in the case of the oxyhemoglobin sample, found to be less effective relative to intact RBCs and RBC ghosts at stabilizing the drug's lactone ring. Finally, a physiologically relevant concentration of platelets of 2.5 × 10^5 cells/μL (Burke, 1980) was found to be poorly effective at stabilizing the lactone ring structure, with a $t_{1/2}$ value of 20.1 ± 1.2 min and a % lactone at equilibrium value of 22.3 ± 1.9% being observed.

In summary, our data indicate that RBCs exert a stabilizing influence on camptothecin's lactone ring. Analysis of the stability data contained in Table 1 clearly indicates that the RBC membrane component appears to be the fraction primarily responsible for camptothecin in whole blood displaying a greater $t_{1/2}$ value (21.6 ± 2.6 min) and a greater % lactone at equilibrium value (5.3 ± 0.6%) than the corresponding values observed for the drug in human plasma

only ($t_{1/2}$ value of 10.6 ± 0.7 min and % lactone at equilibrium value of <0.2%).

Camptothecin Carboxylate's Interactions with HSA Are Highly Dependent upon Both the Drug's Chiral Configuration and the Protein's Native Structure: Binding Interactions Exhibit Both Hydrophobic and Ionic Components. Data concerning the structural specificity of camptothecin–HSA interactions are provided in panel B of Figure 2. Our data indicate that the destabilizing effect of HSA on camptothecin's lactone ring is strongly affected by alterations in both native protein structure as well as drug configuration. As just discussed in the previous part of the Results section, the presence of native HSA results in rapid and complete ring opening (a $t_{1/2}$ value of 11.9 min and a % lactone at equilibrium value of less than 0.5%). But panel B of Figure 2 (as well as the data summarized in Table 1) indicates that neither γ-globulin ($t_{1/2}$ value of 21.9 min and a % lactone at equilibrium of 18.3%) nor heat-denatured HSA ($t_{1/2}$ value of 22.1 min and a % lactone at equilibrium of 14.0%) exerts such a pronounced destabilizing influence on camptothecin's lactone ring as native HSA.

Panel B of Figure 2 demonstrates that the configuration of drug structure also impacts prominently on lactone ring stability in the presence of native HSA. Both the *R* and *S* isomers of camptothecin were studied in PBS, with and without the presence of physiologically relevant HSA concentrations. While both the *S* and *R* isomers free in PBS solution displayed similar $t_{1/2}$ values of 23.8 and 23.3 min, respectively, and % lactone at equilibrium ratios of 83:17 (data not shown), the $t_{1/2}$ values for the two isomers in PBS with 50 mg/mL levels of HSA present were noticeably different (*S* form: $t_{1/2}$ = 11.9 min, % lactone at equilibrium <0.5%; *R* form: $t_{1/2}$ = 4.5 min, % lactone at equilibrium <0.5%). Thus, a change in configuration at the carbon-20 position from *R* to *S* slowed the hydrolysis reaction of camptothecin in the presence of HSA by almost 3-fold, as evidenced by the observed difference in $t_{1/2}$ values. As we have done in the past (Burke & Mi, 1993a,b, 1994), the method of fluorescence lifetime titration was used to determine that the carboxylate form of 20(*R*)-camptothecin associated with native HSA with an affinity of 1.1×10^4 [M amino acid residues (aa)]$^{-1}$ (data not shown), while the lactone form of 20(*R*)-camptothecin displayed a *K* value of 40 (M aa)$^{-1}$. Comparison of these values with same parameters previously determined (Burke & Mi, 1993a) for the 20(*S*) configuration of camptothecin [carboxylate form: $K = 4700$ (M aa)$^{-1}$; lactone form: $K = 270$ (M aa)$^{-1}$] indicates that the carboxylate form of the 20(*R*) isomer binds HSA some 2.5 times more avidly than the 20(*S*) form, while the lactone form of 20(*R*) binds almost 7 times less strongly than the lactone form of the 20(*S*) form. Together, the enhanced affinity of HSA for 20(*R*)-camptothecin carboxylate and the reduced affinity of HSA for the 20(*R*)-camptothecin lactone (relative to the values of the corresponding forms of the 20(*S*) configuration) correlate with more rapid ring opening. The above factors provide a physical chemical explanation for the more rapid opening of the lactone ring observed in the case of the 20(*R*) isomer.

Information regarding the physical nature of camptothecin carboxylate's binding site on the native HSA macromolecule is revealed in Figures 3 and 4. Substitution of a 10-hydroxy moiety into the camptothecin fluorophore results in an agent whose emission spectrum is highly sensitive to the polarity of the solvent or binding microenvironment. Earlier studies from this laboratory (Burke & Mi, 1993b, 1994) have shown that the behavior of 10-hydroxycamptothecin in the presence of native HSA parallels the behavior of camptothecin. Like

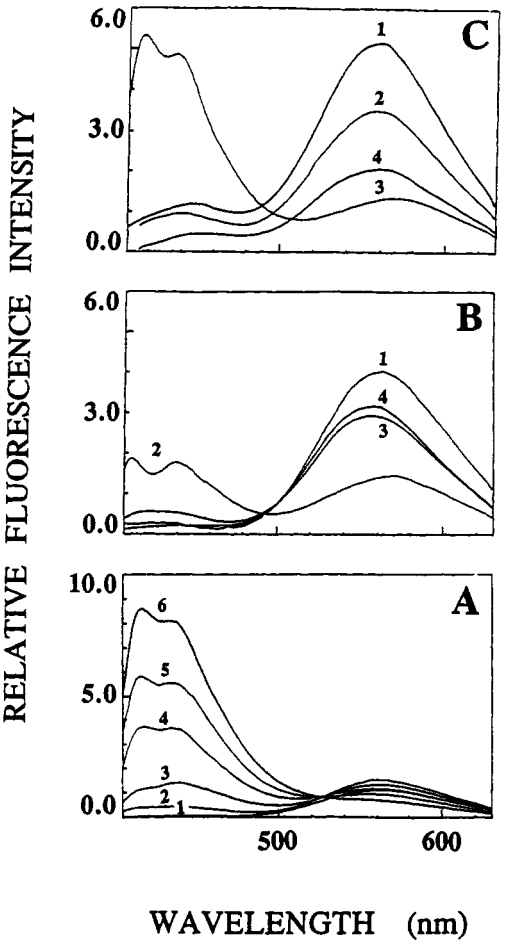


FIGURE 3: Dependence of the fluorescence emission spectra of 10-hydroxycamptothecin lactone and carboxylate forms on solvent polarity (panel A) and macromolecular binding microenvironment (panels B and C). Panel A depicts a strong blue shifting of the lactone's emission spectra in H_2O/methanol solutions upon decreasing dielectric constant: (1) 100% H_2O; (2) 90% methanol; (3) 95% methanol; (4) 98% methanol; (5) 99% methanol; and (6) 100% methanol. Panels B and C show the emission spectra of the lactone and carboxylate forms of camptothecin, respectively, in PBS buffer containing the following individual blood components: (1) PBS buffer only; (2) PBS containing erythrocyte ghosts (4 mg/mL) in suspension; (3) PBS buffer containing 20 mg/mL native HSA; and (4) heat-denatured HSA (10 mg/mL). Note that the lactone form of 10-hydroxycamptothecin carboxylate exhibits the greatest blue shifting of the spectrum in the presence of erythrocyte membrane (panel B, tracing 2). In contrast, the carboxylate form of the drug displays only slight blue shifting in the presence of erythrocyte membrane (panel C, tracing 2), while displaying marked blue shifting in the presence of native HSA (panel C, tracing 3). Also note how HSA denaturation abolishes the blue shifting exhibited by the carboxylate form (panel C, tracing 4).

camptothecin, the 10-hydroxy analogue is preferentially bound by HSA in the carboxylate form. Its similar stability behavior, combined with its exquisite spectral sensitivity, makes 10-hydroxycamptothecin an excellent probe to employ in characterizing the macromolecular associations of camptothecin.

Panel A of Figure 3 depicts a strong blue shifting displayed by 10-hydroxycamptothecin lactone's emission spectra upon decreasing the dielectric constant of the H_2O/methanol solvent system in which the compound is dissolved. In 100% water, the agent displays an emission maximum (λ_{MAX}) value of 562 nm. An increase in the methanol content from 0% to 90% results in a decrease in the fluorescence intensity at 562 nm and an increase in emission in the 400–440 nm region. Upon further increasing the methanol content (from 90% to 95% and then to levels of 98%, 99%, and 100%), the peak intensity at 560 nm continues to decrease, but more significantly, the

signal in the 400–440 nm region increases to very high levels (from 0 for 100% H_2O to in excess of 8 for 100% methanol). Almost identical spectral shifting was observed for the carboxylate form of 10-hydroxycamptothecin upon changing the solvent from H_2O to methanol (data not shown). Similar blue shifting of 10-hydroxycamptothecin's emission spectrum was observed upon drug dissolution in nonpolar solvents such as *n*-butanol and chloroform (data not shown). Since the emission properties of 10-hydroxycamptothecin carboxylate do not change upon reduction of the solution pH to a value of 3.0 (data not shown), the observed blue shifting does not appear to be due to protonation of the aromatic hydroxyl group. Thus, it appears that microenvironment polarity is the main determinant of the emission spectral properties.

The fluorescent emission spectral properties of the lactone and carboxylate forms of 10-hydroxycamptothecin in the presence of various individual components of human blood are presented in panels B and C of Figure 3. Panel B contains the spectra of the lactone form, while panel C depicts spectra from the carboxylate form. In these experiments, aliquots of drug stock solutions were rapidly mixed with thermally preequilibrated samples, and the emission spectra were rapidly initiated and completed (*e.g.*, in less than 4 min). This procedure allowed us to record the emission spectra of relatively homogeneous lactone or carboxylate samples prior to the occurrence of any significant change in the lactone–carboxylate equilibrium, as assessed using HPLC methodology.

Panel B of Figure 3 indicates that the lactone form of 10-hydroxycamptothecin in PBS solution displays an emission maximum of 560 nm (similar to the spectrum of the drug dissolved in pure water (panel A)). Also shown in panel B are emission spectra for 10-hydroxycamptothecin lactone taken in the presence of the following: PBS buffer containing erythrocyte ghosts (4 mg/mL) in suspension (tracing 2); PBS buffer containing native HSA at a concentration of 20 mg/mL (tracing 3); and heat-denatured HSA at a concentration of 10 mg/mL (tracing 4). Comparison of the spectra in Figure 3, panel B, indicates that by far the most prominent change in the fluorescence emission spectra of 10-hydroxycamptothecin lactone occurs in the presence of RBC membranes. The strong blue shifting in the case of the RBC membrane sample is suggestive of partitioning of the agent into the lipid bilayer regions of low dielectric constant (with the spectral changes paralleling those induced by changes in solvent polarity as depicted in Figure 3, panel A). There is also evidence of a subtle blue shifting of 10-hydroxycamptothecin lactone's spectra upon associations with native HSA (albeit not to the high levels observed in the presence of the RBC membrane preparation), with the observed spectral blue shifting diminished considerably upon denaturation of the HSA (tracing 4).

Comparison of the spectra of the carboxylate form of 10-hydroxycamptothecin (which appears in Figure 3, panel C) with spectral tracings of the lactone form (panel B) clearly indicates that significant differences exist in the nature of the interactions of the two camptothecin forms with human blood components. For example, the greatest degree of blue shifting observed in the case of the lactone form occurred when RBC membranes were present. The less lipophilic carboxylate form of 10-hydroxycamptothecin, as expected, exhibited significantly less blue shifting than the lactone form in the presence of equivalent concentrations of the RBC membranes. However, in the presence of HSA, the carboxylate form of the drug displays an intense blue shifting of its emission spectra to a degree much greater than the blue shifting exhibited by 10-hydroxycamptothecin lactone in the presence of equivalent concentrations of HSA. The marked blue shifting of the

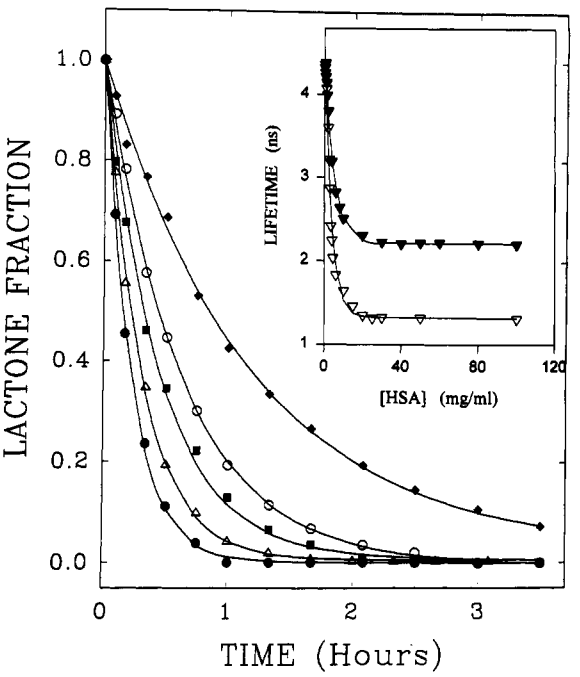


FIGURE 4: Ionic strength dependence of camptothecin stability in HSA solutions containing the following NaCl concentrations: 0 M (●), 0.15 M (△), 0.4 M (■), 0.7 M (○), and 1.0 M (◆). Experiments were conducted in PBS buffer at 37 °C using drug and protein concentrations of 1 μM and 20 mg/mL, respectively. The inset shows binding isotherms for camptothecin carboxylate (20 μM) interacting with native HSA in PBS buffer at 37 °C. The isotherms were generated using the method of fluorescence lifetime titration. The inset compares carboxylate binding to HSA at NaCl concentrations of 0.15 M (▽) and 1.0 M (▼). Note that at the higher salt concentration binding is diminished (see Results section for additional details).

emission spectra of 10-hydroxycamptothecin carboxylate in the presence of native HSA was not observed when heat-denatured HSA was employed in the study (see tracing 4 of Figure 3, panel C).

The data appearing in Figure 3A examining the solvent dependency of 10-hydroxycamptothecin's fluorescent emission properties indicate that spectral blue shifting is induced by fluorochrome relocation from water to a more hydrophobic solvent environment. From the data presented in panel B of Figure 3, it appears that 10-hydroxcamptothecin lactone interacts with the hydrophobic milieu provided by an RBC membrane. In marked contrast, the carboxylate form of 10-hydroxycamptothecin preferentially associates with a hydrophobic binding pocket contained in the native HSA structure over the RBCs. Clearly, from the spectral data that is presented in panel C of Figure 3, it appears that hydrophobic interactions are involved in promoting the association of the carboxylate form of 10-hydroxycamptothecin with native HSA.

However, consideration of the data presented in Figure 4 suggests that ionic interactions between the native HSA drug binding pocket and camptothecin carboxylate are also involved in promoting tight binding. Figure 4 examines the stability of 1 μM camptothecin in the presence of 20 mg/mL native HSA in PBS solutions of varying ionic strengths. The measured $t_{1/2}$ values for the various NaCl concentrations were as follows: 0 M, 9.3 min; 0.15 M, 12.6 min; 0.40 M, 18.8 min; 0.70 M, 26.1 min; and 1.00 M, 48.8 min. Each of the samples of varying salt concentrations displayed a % lactone at equilibrium value of <0.1. Our HPLC stability data clearly demonstrate that the rate of lactone ring opening for camptothecin in the presence of native HSA is reduced by increasing the electrolyte concentration of the surrounding solution.

Direct assessment of the impact of increasing salt concentration on camptothecin carboxylate interactions with native HSA is presented in the inset of Figure 4. The method of fluorescence lifetime titration was employed to determine how varying NaCl concentrations of 0.15 versus 1.0 M affected carboxylate interactions with HSA. Note that at 1.0 M NaCl the fluorophore's lifetime value changes less rapidly relative to the change observed at the lower ionic strength. Whereas the free drug's lifetime (τ_F) value for camptothecin carboxylate was measured to be 4.3 ns at both ionic strengths studied, the HSA-bound (τ_B) values for 0.15 and 1.0 M NaCl were 1.3 and 2.3 ns, respectively. Analysis of the lifetime titration data presented in the inset of Figure 4 using methods described previously (Burke & Mi, 1993a,b, 1994) yielded the following association constants: $K = 4700$ (M aa)$^{-1}$ at 0.15 M NaCl; $K = 460$ (M aa)$^{-1}$ at 1.0 M NaCl. Whereas the K value for the carboxylate form of camptothecin binding to HSA decreased by approximately 10-fold upon increasing ionic strength, little change was detected in the K value for the lactone form of camptothecin [the K term remained relatively invariant with increasing salt concentrations at a value of 30–40 (M aa)$^{-1}$ (data not shown)]. Our data indicate that the slower rate of camptothecin hydrolysis observed at the higher salt concentrations correlates with a 10-fold-reduced affinity of HSA for camptothecin carboxylate. The finding that at each of the different salt concentrations the % lactone at equilibrium value falls to <0.1 is also consistent with the finding that the K value of the carboxylate form exceeds that of the lactone form at each of the salt concentrations studied.

Camptothecin Lactone Preferentially Partitions into Red Blood Cell Membranes over the Carboxylate Form: RBC Interactions Act To Conserve the Biologically Active Drug Form. In previous studies, we employed the fluorescence emission properties of camptothecin to characterize its interactions with small unilamellar vesicles composed of DMPC and DMPG (Burke et al., 1992, 1993). We have demonstrated, using the method of fluorescence anisotropy titration (Burke & Tritton, 1985a; Burke et al., 1992, 1993), that camptothecin binds homogeneous lipid vesicles composed of either DMPC or DMPG with an affinity of 100 M^{-1} (Burke et al., 1993). In our work we showed that the drug intercalates into the acyl chain region of the bilayer, with these lipid bilayer interactions stabilizing and protecting the lactone ring from hydrolysis.

Here we employ the method of fluorescence anisotropy titration to compare the associations of camptothecin lactone and camptothecin carboxylate with SUVs composed of PC:PE:sphingomyelin:PS:PA (mole ratio of 1:1:1:0.5:0.07, respectively). This lipid mixture closely approximates the lipid composition of human RBCs (Chapman, 1968). To avoid the interconversion of the lactone and carboxylate forms of camptothecin, which occurs in aqueous solution at pH 7.4, measurements taken on the two different forms were completed within the shortest possible time (*ca.* 0.5–1 min) after the drug stock solution was mixed with thermally preequilibrated solutions (see Materials and Methods for addition details).

Figure 5 depicts the binding isotherms of the lactone and carboxylate forms of camptothecin interacting with the SUVs. The figure demonstrates that the steady-state fluorescence anisotropy values of the lactone and carboxylate forms of camptothecin are highly sensitive to associations with the SUVs. An approximate 15-fold enhancement in the anisotropy value of camptothecin lactone is observed upon the binding of drug to SUVs. Whereas the anisotropy value of the lactone form of camptothecin increases rapidly with increasing lipid

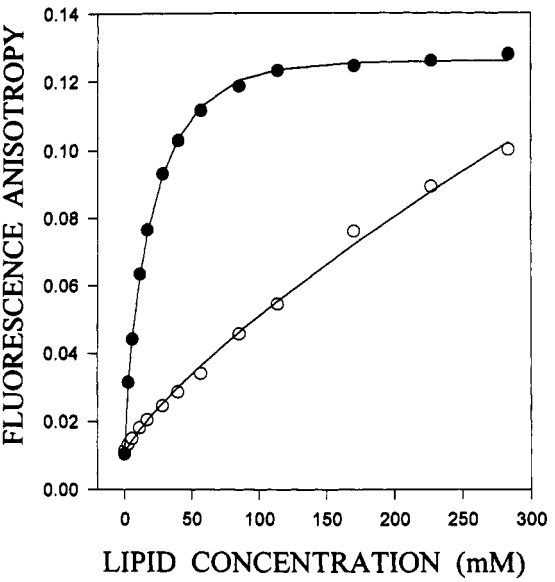


FIGURE 5: Binding of camptothecin (●) and its carboxylate form (○) to SUVs composed of PC:PE:sphingomyelin:PS:PA (mole ratio of 1:1:1:0.5:0.07, respectively). Note that the lactone form of camptothecin exhibits a higher affinity for the lipid vesicles compared with the affinity exhibited by its carboxylate form. Experiments were conducted rapidly after the addition of drug (~30 s) at total drug concentrations of 40 μM in PBS buffer at 37 °C. Binding constants were determined using double-reciprocal plots as described in the Materials and Methods section.

concentration, the rate of change of the anisotropy value of the carboxylate is slower relative to the lactone form. Analysis of the binding isotherms using double-reciprocal plots (Burke & Tritton, 1985a; Burke et al., 1992, 1993) yielded an association constant of 130 M^{-1} for camptothecin lactone. This value is similar to K values of 100 M^{-1} determined previously for homogeneous SUVs composed of DMPC or DMPG (Burke et al., 1993). The carboxylate form was found to display a K value of 20 M^{-1} for SUVs composed of RBC lipids. Using HPLC methodology, we confirmed that the presence of SUVs composed of the RBC lipid mixtures at a lipid concentration of 200 mg/mL in PBS buffer (pH 7.4) at 37 °C was capable of enhancing the $t_{1/2}$ value of camptothecin to 110 min, with a % lactone at equilibrium value of 70% (data not shown). Thus, it appears that camptothecin is capable of partitioning into the lipid bilayers of RBCs, a phenomenon that acts to maintain the drug's lactone ring in its closed form.

Also, using the membrane-impermeable iodide as quencher and previously described methodology (Burke & Tritton, 1985b; Burke et al., 1993), we demonstrated that camptothecin associated with SUVs composed of RBC lipid mixtures was more difficult to quench [static quench constant (V) = 0 M^{-1} and K_{SV} = 5.6 M^{-1} compared with V = 1.8 M^{-1} and K_{SV} = 51.8 M^{-1} for camptothecin free in solution (data not shown)].

Direct assessment of the relative partitioning of camptothecin lactone and camptothecin carboxylate into human RBCs was evaluated using the technique of flow cytometry. Figure 6 shows the concentration-dependent uptake of camptothecin's lactone and carboxylate forms by HSA-free red blood cells in PBS buffer, as well as by RBCs present in human plasma. Our results demonstrate that the lactone form of camptothecin readily partitions into HSA-free RBCs suspended in PBS solution; the fluorescence intensity of the lactone-treated cells is significantly greater than the fluorescence signal from cells incubated with an identical concentration of the carboxylate form of the drug.

For RBCs suspended in human plasma, we observed that lactone uptake was decreased significantly relative to RBCs

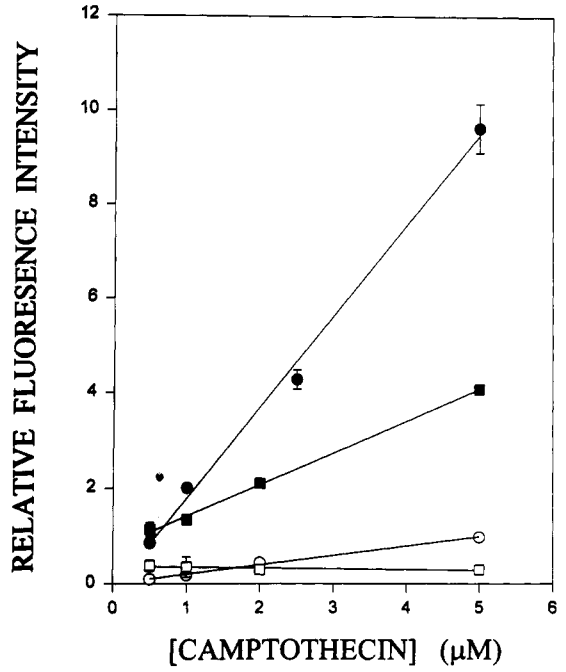


FIGURE 6: Flow cytometric analysis concerning the cellular accumulation of the lactone and carboxylate forms of camptothecin within human red blood cells. Shown here are the dose-dependent uptake of camptothecin's lactone (●) and carboxylate forms (○) by red blood cells in PBS buffer, as well as in human plasma (■ for the lactone form and □ for the carboxylate form). Experiments were conducted using a cell density of 2×10^6/mL and a flow rate of approximately 550 s^{-1} at ambient temperature. Data were plotted as mean ± SD (n = 3). See the Materials and Methods section for additional experimental details.

suspended in HSA-free PBS (Figure 6). The observed decrease in the cellular accumulation of camptothecin into RBCs in the presence of HSA is suggestive of drug association with the extracellular protein, a process that reduces the accumulation of the drug in the RBCs. The cellular uptake of camptothecin carboxylate in the presence of HSA-containing plasma was diminished to even lower levels (*i.e.*, relative to the lactone form). This finding is most likely due to the following two factors: (1) the high-affinity binding of the carboxylate to the HSA present in plasma and (2) the lower membrane partitioning of the charged carboxylate form of the drug.

To more accurately quantitate total camptothecin lactone and carboxylate levels inside RBCs following drug exposure, an HPLC assay was employed as described in the Materials and Methods section. For RBCs ($5 \times 10^6/\mu$L) suspended in PBS, analysis showed total intracellular drug levels for lactone-exposed cells to be 4.9 μM, which is some 15 times greater than the corresponding levels for carboxylate-exposed cells (0.32 μM). For RBC suspensions in plasma, HPLC analysis showed total net cellular drug levels (*i.e.*, lactone and carboxylate species combined) for lactone-exposed RBCs (2.0 μM) to be 20 times greater than those for carboxylate-exposed cells (0.1 μM). These results are in close agreement for the relative differences in drug accumulation determined by the flow cytometric technique.

DISCUSSION

Markedly Different Interactions of the Carboxylate and Lactone Forms of Camptothecin with Human Blood Components: Impact on the Bloodstream Stability of Camptothecin's Lactone Ring. In this report, we have employed fluorescence spectroscopic and flow cytometric methodologies to compare the very different interactions of the camptothecin lactone and carboxylate forms with purified human blood components. Biophysical and cytometric data have been generated that allowed us to interpret how the differential interactions of the lactone and carboxylate forms of camptothecin with human blood components impact on the stability of the drug's lactone ring. We have found that the two blood components that exert the most prominent influence on the rate of camptothecin's lactone ring opening are the RBC and HSA fractions, with the presence of these two different macromolecular assemblies having opposite effects on the lactone–carboxylate equilibrium: RBCs and HSA shift the system to the left and right, respectively.

Fluorescence spectroscopic methodologies were employed to quantitatively assess the binding affinities of the carboxylate and lactone forms of camptothecin for RBC lipid membranes and HSA, and the measured association constants have proven to be useful in explaining the differential rate of lactone ring opening for camptothecin in human plasma versus RBC-containing whole blood. Camptothecin lactone was found to exhibit a K value of 130 M^{-1} for RBC lipid vesicles, compared to a value of 20 M^{-1} for the ring-opened form. On the basis of the measured association constants listed above, it can be expected that the lactone form of camptothecin, relative to the carboxylate, will partition more readily into the lipid bilayer domains of RBCs.

As shown in our flow cytometric results presented in Figure 6, this indeed appears to be the case, with fluorescence levels from RBCs exposed to the lactone form of the drug more than 10 times greater than the fluorescence levels from RBCs incubated with identical levels of the carboxylate form of the drug. Both camptothecin carboxylate and camptothecin lactone contain an identical fluorophore. The fluorescent nucleus of camptothecin is at the opposite end of the molecule, quite distant from the γ-hydroxy-δ-lactone ring that hydrolyzes, and from model membrane studies it is known that the fluorescence emission spectra of bilayer-bound lactone versus bilayer-bound carboxylate are nearly the same. Thus, the high fluorescence levels that are detected from the lactone-treated RBCs versus carboxylate-treated RBCs (Figure 6) are thought to be due to the higher levels of partitioning of the lactone form of camptothecin (*i.e.*, not due to intrinsic differences in fluorescence intensities of membrane-bound species), and this finding of enhanced cellular accumulation of the lactone form was corroborated independently using HPLC methodology (see Results section). Our finding of an apparent enhancement in cellular accumulation for the lactone form versus carboxylate form also correlates well with the approximately 6–7-fold higher level of lipophilicity ($K_{lactone}$ = 130 M^{-1}, $K_{carboxylate}$ = 20 M^{-1}) observed for the lactone form of the drug, as discussed earlier.

The preferential binding of the lactone form of camptothecin to the lipid bilayers of RBCs results in altered kinetics for camptothecin lactone ring hydrolysis. In the presence of physiologically relevant levels of RBCs, the rate of lactone ring opening in PBS at 37 °C occurs with a $t_{1/2}$ value of 31 min, and lactone ring stability improves to an equilibrium value of 35%. These values compare favorably to a $t_{1/2}$ value of 24 min and a 17% lactone at equilibrium value for drug free in PBS solution.

However, the results presented in Table 1 demonstrate that the stabilizing influence of RBCs on camptothecin lactone ring stability is markedly attenuated by the presence of physiologically relevant concentrations of HSA. We have elaborated here and elsewhere (Burke & Mi, 1993a) on the capacity of HSA to preferentially bind the carboxylate form of camptothecin with high affinity, and this avid association

between the carboxylate form of camptothecin and HSA is responsible for the lactone ring of camptothecin opening more rapidly and completely in the presence of the protein. In whole blood, the combined presence of HSA and RBCs resulted in an intermediate $t_{1/2}$ value for camptothecin of 21.6 min, with a % lactone at equilibrium value of 5.3%.

HSA is the principal carrier of water-insoluble fatty acids in blood. This blood protein has long been noted for its ability to bind with high-affinity small hydrophobic molecules carrying a net negative charge (He & Carter, 1992; Carter & Ho, 1994). Such ligand interactions with HSA occur at two distinct hydrophobic sites known as the IIA and IIIA binding pockets (He & Carter, 1992; Carter & Ho, 1994). The following three factors discussed in this report are consistent with camptothecin carboxylate interacting with a hydrophobic site such as the IIA or IIIA binding pockets of HSA: (1) like many fatty acids and other ligands known to interact with the IIA and IIIA binding pockets, camptothecin carboxylate is a relatively small, hydrophobic molecule containing a negatively charged carboxylate at physiological pH; (2) heat-denatured HSA was found to markedly attenuate the binding between camptothecin carboxylate and HSA, suggestive of binding interactions with a structurally specific cavity in HSA; and (3) the spectral shifting of 10-hydroxycaptothecin carboxylate upon association with native HSA is consistent with the involvement of a hydrophobic binding pocket. Crystallographic analyses of camptothecin carboxylate–HSA complexes are in progress, and the specific location of the camptothecin carboxylate binding site should be forthcoming.

Clinical Significance of the Differential Interactions of Camptothecin Lactone and Carboxylate Forms with Human Blood Components. It is widely recognized that drug effect is often closely correlated with the free or unbound drug concentration in blood or plasma [see MacKichan (1992) and Rowland & Tozar (1989) and references therein]. In the case of camptothecin, it is the lactone form of the agent that is regarded to be the biologically active form. Thus, from both the pharmacokinetic and pharmacodynamic viewpoints, it is the free or unbound concentrations of camptothecin lactone in blood or plasma water that can be utilized as the parameters that are most likely to be predictive indicators of anticancer activity (Potmesil, 1994).

Our studies indicate that upon the introduction of camptothecin lactone into the bloodstream, the closed ring form of the drug will persist for several minutes ($t_{1/2}$ = 21.6 min). During this time frame, some of the camptothecin lactone will reversibly associate with RBC, HSA, LDL, and the other components of human blood. Because of the great abundance of RBCs and HSA in blood, it seems reasonable to assume that the largest percent of the bound drug fractions will be associated with these two blood components. As mentioned in the Results section, RBC associations act to impact favorably on camptothecin's lactone ring stability by extending the half-life value of the biologically active lactone form of the drug in the bloodstream.

However, almost immediately following the introduction of camptothecin into human blood, a certain fraction of the administered lactone drug form will begin to hydrolyze. The high-affinity binding of camptothecin carboxylate by HSA acts to reduce the free or unbound levels of camptothecin carboxylate in plasma water, thereby shifting the overall lactone–carboxylate equilibrium further to the right. With a half-life of 21.6 min, the total level of camptothecin lactone in blood (*i.e.*, unbound camptothecin lactone in plasma water, as well as camptothecin lactone reversibly associated with RBC, HSA, LDL, *etc.*) will fall until a steady-state equilibrium value of 5.3% is reached.

In the case of direct administration of the carboxylate form of camptothecin into the bloodstream, as was done in the initial clinical trials of the agent (Gottlieb et al., 1970; Muggia et al., 1972; Moertel et al., 1972; Gottlieb & Luce, 1972), our results suggest that the open ring form of the drug will rapidly and extensively associate with HSA with high affinity. Although the associations of camptothecin carboxylate are reversible, they are sufficiently strong to act to keep the drug in close contact with the protein. In support of this point of tight binding between HSA and camptothecin carboxylate, recent experiments from our laboratory have demonstrated that the rotational motions of camptothecin carboxylate in the presence of HSA slow to a point where the drug exhibits little local motion and rotates globally (T. G. Burke et al., unpublished) at a rate similar to the rate of rotation of an albumin molecule (Wang & Bright, 1993).

On the basis of the findings presented here, it seems logical to anticipate that camptothecin carboxylate in blood, for the most part, will remain closely associated with HSA. The tissue distribution of camptothecin carboxylate from the point of administration onward is strongly influenced by its tight associations with HSA. In contrast to the carboxylate form, the lactone form of the drug exhibits reduced binding to HSA. Our results predict that, immediately following the introduction of camptothecin lactone into the bloodstream, a fraction of the lactone drug form weakly associates with RBCs (relative to the strength of camptothecin carboxylate–HSA interactions). Thus, the free or unbound concentration of camptothecin lactone, the biologically active species, can be expected, during the first hour, to be greater for drug administered in the lactone form rather than drug administered in the carboxylate form. From a therapeutic viewpoint, higher levels of unbound lactone in the bloodstream will facilitate greater penetration of this biologically active, more membrane-permeable form into tissue. Both in extensive testing in animal models and in the more limited testing in human trials, camptothecin administered in its lactone form has been found to be significantly more effective than drug administered in the carboxylate form [Giovanella et al., 1989; Potmesil (1994) and references therein].

Potential Approaches for Enhancing the Stability of Camptothecin Lactone and Related Analogues in the Bloodstream. In this report, we have demonstrated that the dominant influence that the presence of native HSA exerts on the rate of camptothecin ring opening can be modulated by a significant 2.5-fold by a subtle change in chiral configuration. The 20(*R*) isomer of camptothecin, which opens more rapidly in the presence of HSA due to the enhanced affinity of the carboxylate form for HSA, is thought to be intrinsically less effective against the topoisomerase I target (Jaxel et al., 1989).

Also concerning the structural basis of the camptothecin interaction with HSA and the impact of these associations on drug stability, we have recently demonstrated that ethyl substitution at the 7-position markedly improves drug stability in the presence of HSA (Burke & Mi, 1993b, 1994). Using the method of fluorescence lifetime titration, we have shown that substitution of a 7-ethyl group onto 10-hydroxycamptothecin (resulting in the SN-38 agent) significantly enhances drug stability by promoting preferential associations of the lactone form of the drug with the blood protein (Burke & Mi, 1993b, 1994). Other specific modifications at the 7- and 9-positions of the quinoline nucleus, such as those contained in CPT-11 and topotecan, enhance drug stability in the

presence of HSA (Burke & Mi, 1994). From the work cited above, it appears that significant gains in drug stability in the presence of HSA can be obtained through analogue development. Of course, in analogue development, it is also important to consider intrinsic drug potency at the topoisomerase I target. In the case of SN-38, the agent turns out to display both enhanced stability in the bloodstream (Burke & Mi, 1993b, 1994) as well as potent activity against the topoisomerase I target (Kaneda et al., 1990).

An alternate approach to drug stabilization in the bloodstream involves liposomal formulation. Preliminary work from this laboratory indicates that the lactone rings of camptothecin (Burke et al., 1994) and topotecan (Burke & Gao, 1994) can be partially stabilized at physiological pH values by packaging the agents within lipid vesicles with a reduced internal pH of 5 (at pH 5, camptothecins remain in their lactone form and gel phase lipid bilayers provide a barrier, hindering drug interactions with serum albumin). Thus, our early experiments with liposomes suggest that these particles may be of potential utility for improving the half-lives of camptothecin drugs in the presence of HSA. At present, we are investigating passive as well as active targeting mechanisms for directing these liposomal drug carriers to tumors.

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REFERENCES

Bowman, B. H. (1993) in *Hepatic Plasma Proteins*, Academic Press, San Diego, CA.
Burke, S. R. (1980) in *The Composition and Function of Body Fluids*, C. V. Mosby Co., St. Louis, MO.
Burke, T. G., & Tritton, T. R. (1985a) *Biochemistry 24*, 1768–1776.
Burke, T. G., & Tritton, T. R. (1985b) *Biochemistry 24*, 5972–5980.
Burke, T. G., & Mi, Z. (1993a) *Anal. Biochem. 212*, 285–287.
Burke, T. G., & Mi, Z. (1993b) *J. Med. Chem. 36*, 2580–2582.
Burke, T. G., & Gao, X. S. (1994) *J. Pharm. Sci. 83*, 967–969.
Burke, T. G., & Mi, Z. (1994) *J. Med. Chem. 37*, 40–46.
Burke, T. G., Sartorelli, A. C., & Tritton, T. R. (1988) *Cancer Chemother. Pharmacol. 21*, 374–380.
Burke, T. G., Staubus, A. E., Mishra, A. K., & Malak, H. (1992) *J. Am. Chem. Soc. 114*, 8318–8319.
Burke, T. G., Mishra, A. K., Wani, M. E., & Wall, M. E. (1993) *Biochemistry 32*, 5352–5364.
Burke, T. G., Mi, Z., Gao, X., & Wheeler, M. T. (1994) *Proc. Am. Assoc. Cancer Res. 35*, 416.
Carter, D. C., & Ho, J. X. (1994) *Adv. Protein Chem. 45*, 153–203.
Chapman, D. (1968) in *Biological Membranes, Physical Fact and Function*, Academic Press, Inc., New York.
Cranney, M., Cundall, R. B., Jones, G. R., Richards, J. T., & Thomas, E. W. (1983) *Biochim. Biophys. Acta 735*, 418–425.
Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119.
Eftink, M., & Ghiron, C. A. (1976) *J. Phys. Chem. 80*, 486–493.
Eilers, R. J. (1967) *Am. J. Clin. Pathol. 47*, 212.
Fish, R. G., Yap, A. K. L., & James, K. (1982) *Anal. Biochem. 15*, 4.
Garcia-Sancho, J., & Alvarez, J. (1989) *Methods Enzymol. 173*, 368.
Giovanella, B. C., Stehlin, J. S., Wall, M. C., Nicholas, A. W., Liu, L. F., Silber, R., & Potmesil, M. (1989) *Science (Washington, DC) 246*, 1046–1048.
Gottlieb, J. A., & Luce, J. K. (1972) *Cancer Treatment Rep. 56*, 103–105.
Gottlieb, J. A., Guarino, A. M., Call, J. B., Oliverio, V. T., & Block, J. B. (1970) *Cancer Chemother. Rep. 54*, 461.
Gotto, A. M., Jr. (1987) in *Plasma Lipoproteins*, Elsevier Science Publishing, Inc., New York.
He, X. H., & Carter, D. C. (1992) *Nature 358*, 209–215.
Hsiang, Y-H., & Liu, L. F. (1988) *Cancer Res. 48*, 1722–1726.
Hsiang, Y.-H., Hertzberg, R., Hecht, S., & Liu, L. F. (1985) *J. Biol. Chem. 260*, 14873–14878.
Hsiang, Y.-H., Lihou, M. G., & Liu, L. F. (1989) *Cancer Res. 49*, 5077–5082.
Jaxel, C., Kohn, K. W., Wani, M. C., Wall, M. E., & Pommier, Y. (1989) *Cancer Res. 49*, 5077–5082.
Jendrasiak, G. L. (1972) *Chem. Phys. Lipids 9*, 133–146.
Kaneda, N., Nagata, H, Furata, T., & Yokokura, T. (1990) *Cancer Res. 50*, 1715–1720.
MacKichan, J. J. (1992) in *Applied Pharmacokinetics: Principles and Therapeutic Drug Monitoring* (Evans, W. E., Schentag, J. J., & Jusko, W. J., Eds.), Applied Therapeutics, Inc.
Moertel, C. G., Schutt, A. J., Reitemeier, R. J., & Hahn, R. G. (1972) *Cancer Chemother. Rep. 56*, 95.
Muggia, F. M., Creaven, P. J., Hansen, H. H., Cohen, M. H., & Sealwry, O. S. (1972) *Cancer Treatment Rep. 56*, 515.
New, R. R. C. (1990) in *Liposomes, A Practical Approach*, Oxford University Press, New York.
Potmesil, M. (1994) *Cancer Res. 54*, 1431–1439.
Rabiner, S. F., Herbert, J. R., Lopes, H., & Friedman, L. H. (1967) *J. Exp. Med. 126*, 1127.
Rowland, M., & Tozar, T. N. (1989) in *Clinical Pharmacokinetics: Concepts and Applications*, Lea and Febiger, Philadelphia, PA.
Sann, L., Bienvenu, F., Bourgeois, J., & Bethenod, M. J. (1984) *J. Pediatr. 105*, 977.
Schmid, K. (1975) in *The Plasma Proteins: Structure, Function, and Genetic Control* (Putnam, F. W., Ed.), pp 184–228, Academic Press, New York.
Schmid, K., Kaufmann, H., Iseura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M., & Nanno, S. (1973) *Biochemistry 12*, 2711–2724.
Slichenmyer, W. J., Rowinsky, E. K., Donehower, R. C., & Kaufmann, S. H. (1993) *J. Natl. Cancer Inst. 85*, 271–291.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361–376.
Supko, J. G., & Malspeis, L. (1991) *J. Liq. Chromatogr. 14*, 1779–1789.
Supko, J. G., & Malspeis, L. (1992) *J. Liq. Chromatogr. 15*, 3261–3283.
Underberg, W. J. M., Goossen, R. M. J., Smith, B. R., & Beijnen, J. H. (1990) *J. Pharm. Biomed. Anal. 8*, 681–683.
Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. T., & Sim, G. A. (1966) *J. Am. Chem. Soc. 88*, 3888–3890.
Wang, R., & Bright, F. V. (1993) *Appl. Spectrosc. 47*, 792–799.
Wani, M. C., Nicholas, A. W., Manikumar, G., & Wall, M. E. (1987a) *J. Med. Chem. 30*, 1774–1779.
Wani, M. C., Nicholas, A. W., & Wall, M. E. (1987b) *J. Med. Chem. 30*, 2317–2319.