The Structural Basis of Camptothecin Interactions with Human Serum Albumin: Impact on Drug Stability†,‡

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Received September 7, 1993•

The intense intrinsic fluorescence emissions from several clinically relevant camptothecin drugs have been exploited in order to study the structural basis of drug binding to human serum albumin. Both HPLC and time-resolved fluorescence spectroscopic methodologies were employed to characterize the associations of camptothecins with HSA in phosphate-buffered saline (pH 7.4) at 37 °C. The α-hydroxy δ-lactone ring moiety of camptothecin (C), 10-hydroxycamptothecin (HC), 10,11-(methylenedioxy)camptothecin (MC) and 9-chloro-10,11-(methylenedioxy)camptothecin (CMC) was in each case observed to hydrolyze more rapidly and completely in the presence of HSA than in the protein's absence. Binding isotherms constructed by the method of fluorescence lifetime titration showed that HSA bound preferentially the carboxylate forms of C, HC, MC, and CMC over their lactone forms, thereby providing an explanation for the shift to the right in the lactone–carboxylate equilibrium observed for each compound upon HSA addition. In marked contrast, three analogues (SN-38, CPT-11, and topotecan) all displayed enhanced stabilities in the presence of HSA. While the lifetimes of CPT-11, topotecan, and the carboxylate forms of both drugs were insensitive to the addition of HSA, the lifetimes of both SN-38 and its carboxylate form did titrate upon HSA addition. Analysis of binding isotherms constructed for the albumin interactions of SN-38 and its carboxylate form demonstrated a higher overall association constant for the lactone form [640 (M amino acid (aa) residues)$^{-1}$] relative to the carboxylate form [150 (M aa)$^{-1}$]. Our studies indicate that specific modifications at the 7- and 9-positions of the quinoline nucleus, such as those contained in CPT-11, topotecan, and SN-38, enhance drug stability in the presence of HSA. In the case of SN-38, the enhanced stability was shown to be due to preferential associations between the drug's lactone form and the blood protein.

Introduction

The α-hydroxy δ-lactone ring moiety of camptothecin anticancer drug hydrolyzes under physiological conditions, *i.e.*, at pH 7 or above,[1] with the lactone ring of each drug readily opening up to yield an inactive carboxylate form.[2-4] Anticancer activities of camptothecins both *in vitro* and *in vivo* are significantly greater for the lactone versus the carboxylate forms,[2-5] with a closed α-hydroxy δ-lactone ring being an important structural requirement for both passive diffusion of drug into cancer cells, as well as for successful drug interaction with the pharmacological target, topoisomerase I.[6]

Thus factors influencing the lactone–carboxylate equilibria of camptothecins are important determinants of their biological activities. Recent studies from this laboratory indicate that the lactone and carboxylate forms of camptothecin exhibit markedly different interactions with human serum albumin.[7] 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 binds human serum albumin (HSA) with moderate affinity, the ring-open carboxylate form displays a dramatic 150-fold enhancement in its apparent binding constant for HSA.[7] 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 chemical equilibrium and results in the lactone ring opening more rapidly and completely relative to drug in aqueous solution.[7] 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), and 2 hours after drug addition to human plasma >99% of the drug has converted to camptothecin carboxylate,[8] the biologically inactive and potentially toxic[5] form of the drug.

In the present report we employ both HPLC methodologies and time-resolved fluorescence spectroscopy to study how variations of camptothecin's structure affect drug interactions with HSA and how these interactions impact the stability of the α-hydroxy δ-lactone pharmacophore of each agent. Our results demonstrate that drug stability in the presence of HSA can be strongly modulated through structural modification. Moreover, the data presented herein indicate that specific structural modifications such as 7-ethyl substitution promote reversible binding between the *lactone* form of the drug and the blood protein, thereby enhancing drug stability.

Experimental Section

Chemicals. Samples of camptothecincarboxylate sodium salt, 10-hydroxycamptothecin, 10,11-(methylenedioxy)camptothecin, 9-aminocamptothecin, and topotecan were obtained from the

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† Abbreviations: HSA, human serum albumin; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline containing 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, and 3 mM KCl (pH 7.4); τ, excited-state lifetime; ns, nanosecond.

‡ Part of this work has appeared earlier in preliminary form as a Communication to the Editor (Burke, T. G.; Mi, Z. *J. Med. Chem.* **1993**, 2580-2582).

• Abstract published in *Advance ACS Abstracts*, December 1, 1993.

0022-2623/94/1837-0040$04.50/0

National Cancer Institute, Division of Cancer Treatment. Samples of camptothecin and 9-chloro-10,11-(methylenedioxy)-camptothecin (CMC) were generously provided by the laboratories of Drs. Monroe Wall and Mansukh Wani. We gratefully acknowledge Yakult Honsha Pharmaceutical, Tokyo, Japan, for generously providing samples of SN-38 and CPT-11. All of the camptothecins were in the 20*S* configuration. Stock solutions of the drugs were prepared in dimethyl sulfoxide (A.C.S. spectrophotometric grade, Aldrich, Milwaukee, WI) at a concentration of 2×10^{-3} M and stored in dark at 4 °C. By adding 1 part DMSO drug stock solution to 1 part PBS buffer (pH 10), 1 mM working stock solutions of the corresponding carboxylate forms of each camptothecin drug were prepared. Crystallized HSA obtained from Sigma Chemical, St. Louis, MO, was used in our studies as before.[7,8] All other chemicals were reagent grade and were used without further purification.

Kinetics of Lactone Ring Opening. The rates of lactone ring opening due to hydrolysis for the camptothecins were determined by the quantitative reversed-phase high-performance liquid chromatography (HPLC) assays,[9-12] with modifications.[13,14] Separation of parent drug from carboxylate form was achieved using a HPLC setup consisting of the following: a Waters Model 501 pump, a Waters U6-K injector, a Beckman Ultrasphere Octadecylsilane (ODS) 5-μm particle size C_{18} reversed-phase column, a Waters Model 470 scanning fluorescence detector, and a Hewlett-Packard Model HP3396 Series II integrator for data processing. For camptothecin, topotecan, and 9-chloro-10,11-(methylenedioxy)camptothecin the chromatographic assays conditions have been summarized elsewhere.[14] For 10,11-(methylenedioxy)camptothecin the mobile phase consisted of 25% acetonitrile, 74% 0.1 M acetate buffer, and 1% 0.1 M SDS. For CPT-11, the mobile phase consisted of 13% acetonitrile, 20% ethanol, and 67% 0.8% $(NH_4)_2CO_3$, final pH 7.0. For 10-hydroxycamptothecin, the mobile phase consisted of 25% acetonitrile, 74% 0.1 M acetate buffer, and 1% 0.1 M SDS. For SN-38, the mobile phase consisted of 72% 0.1 M acetate buffer, 27% acetonitrile, and 1% 0.1 M SDS. For 9-aminocamptothecin, the mobile phase consisted of 72% acetate buffer and 28% acetonitrile, and the postcolumn acidification was used to decrease the pH of the eluant for improved sample detectability (post-column pump flow rate, 0.1 mL/min; acid reservoir, 1 M H_3PO_4). The fluorescence detector excitation (λ_{ex}) and emission (λ_{em}) settings for the various camptothecin congeners were as follows: λ_{ex} of 370 nm and λ_{em} of 440 nm for camptothecin, 9-chloro-10,11-(methylenedioxy)camptothecin, and 10,11-(methylenedioxy)camptothecin; λ_{ex} of 392 nm and λ_{em} of 561 nm for 10-hydroxycamptothecin; λ_{ex} of 380 nm and λ_{em} of 470 nm for 9-aminocamptothecin, λ_{ex} of 363 nm and λ_{em} of 537 nm for topotecan; λ_{ex} of 373 nm and λ_{em} of 428 nm for CPT-11; and λ_{ex} of 383 nm and λ_{em} of 560 nm for SN-38. Solutions of drug at a concentration of 1 μM in PBS (pH 7.40 ± 0.05) with or without HSA were prepared and incubated at 37 °C. Immediately after mixing the drug stock solution with PBS or PBS/HSA solution and at intervals of 5 min thereafter, 20-μL aliquots were withdrawn and mixed with 1 mL of distilled H_2O. The mixtures were either injected into HPLC directly (90-μL injection volumes were used) or frozen rapidly under dry ice for analysis at a later time. No significant differences in drug stabilities were observed between HSA concentrations of 20 mg/mL and physiological levels of 50 mg/mL (*e.g.* for camptothecin in the presence of 20 mg/mL HSA a $t_{1/2}$ value of 12.5 ± 0.8 min was observed with a final camptothecin lactone concentration of <0.5%; for camptothecin in 50 mg/mL HSA a $t_{1/2}$ = 11.9 ± 0.5 min was observed with a final lactone concentration of 0.5%). Thus no significant differences were detected and the 20 mg/mL HSA levels were used in our studies because of lower viscosity which aided in pipetting precision.

The fractions of intact lactone (f) vs time (t) data for the camptothecins were fit to eq 1 found below by the method of nonlinear least squares:

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

where the adjustable parameter k_1 is the first-order rate constant of hydrolysis of the lactone ring, from which the half-life of hydrolysis ($t_{1/2} = \ln 2/k_1$) can be calculated. 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 $t = 0$ when the agent of interest was first put in solution. Fitting of experimental data to the equation above was accomplished using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA).

Fluorescence Instrumentation. Steady-state and time-resolved fluorescence measurements were obtained on a SLM Model 4800C spectrofluorometer with a thermostated cuvette compartment. This instrument was interfaced with an IBM PS/2 Model 55 SX computer. All fluorescence measurements were made in the absence of polarizers. Fluorescence excited-state lifetimes were determined by the method of phase shift[15] using excitation light modulated at 30 MHz. To determine the fraction of free and bound species in HSA solutions, binding isotherms were generated by titrating a total drug concentration of 20 μM with varying amounts of HSA. Fluorescence from the samples were isolated from scattered light using an appropriate long pass filter as specified below. Intensity levels were typically in excess of 98% and did not fall below 90% even in the most concentrated HSA solutions.

Lifetime measurements for camptothecin, camptothecincarboxylate, and 10,11-(methylenedioxy)camptothecin were conducted using exciting light of 370- and 420-nm long pass filters on the emission channel in order to isolate the drug's fluorescence signal from background scatter and/or residual fluorescence. For other drugs, the following excitation wavelengths and emission filters, respectively, were used: topotecan (400-nm, 500-nm long pass filter), 9-aminocamptothecin (375-nm, 420-nm long pass filter), CMC (370-nm, 399-nm long pass filter), 10-hydroxycamptothecin and SN-38 (375-nm, 500-nm long pass filter), and CPT-11 (375-nm, 399-nm long pass filter). All emission filters were obtained from Oriel Corp (Stamford, CT). The combination of exciting light and emission filters allowed us to adequately separate fluorescence from background signal. The contribution of background fluorescence, together with scattered light, was typically less than 2% of the total intensity. All experiments were conducted in a tightly controlled pH range of 7.40 ± 0.05.

Determination of Association Constants. The method of fluorescence lifetime titration was used to characterize the association constants of the lactone and carboxylate forms of camptothecins with HSA. PBS and PBS/HSA solutions of pH 7.4 were pre-equilibrated at 37 °C in a quartz cuvette present in the thermostated sample compartment. Lifetime measurements were completed within 1 min following the addition of drug stock solution. This type of rapid measurement of excited-state lifetime allowed us to gather binding data for essentially pure lactone or carboxylate species, before any significant change in the equilibrium occurred. The fractions of free and bound species were determined according to

$$\tau = f_{F/F} + f_{B/B} + f_{H/H} \quad (2)$$

where τ is the measured lifetime and τ_F, τ_B, and τ_H are the lifetimes of free drug, bound drug, and HSA, respectively. The terms f_F, f_B, and f_H refer to the fraction of the total detected signal that originates from free drug, bound drug, and HSA, respectively, where $f_F + f_B + f_H = 1$. The τ_H value was determined to be 3.2 ns; f_H was determined by taking the ratio of fluorescence intensity of the HSA solution in the absence of drug divided by the fluorescence intensity of the same HSA sample in the presence of drug. Typically the f_H value was less than 0.02, and even in the most concentrated HSA samples f_H did not exceed 0.10. The bound lifetime value (τ_B) was determined by extrapolation to infinite HSA concentration using the method of nonlinear least-squares while fitting binding data to the following equation:

$$\tau_C = \tau_B + b \exp(-c[\text{HSA}]) \quad (3)$$

where τ_C is the corrected lifetime which equals to the measured lifetime (τ) minus $f_H\tau_H$. The terms b and c are curve-fitting parameters.

The apparent association constants and binding-site stoichiometries were determined as described previously.[7,8] The procedure involved plotting the free drug fraction, $(\tau - \tau_B)/(\tau_F - \tau_B)$, vs the logarithm of the molar concentration of amino acid residues. The data were then fit to the following equation by the

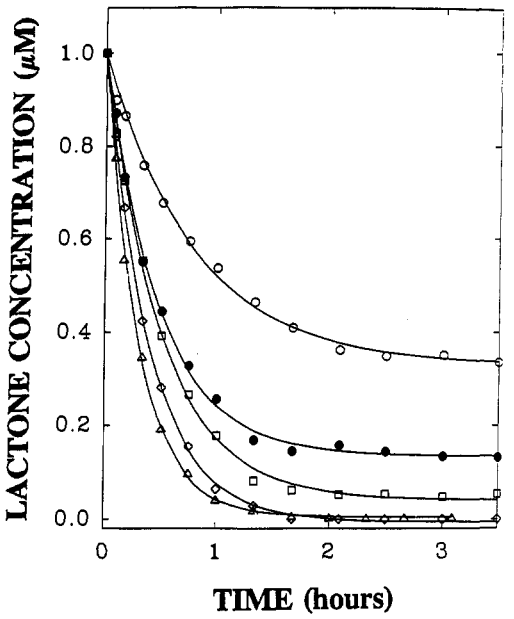


Figure 1. Kinetic evaluation of the rate of lactone ring opening for SN-38 (○), 10-hydroxycamptothecin (□), 9-chloro-10,11-(methylenedioxy)camptothecin (◇), and camptothecin (△) in the presence of human serum albumin. The symbol ● represents the hydrolysis of SN-38 in the absence of HSA. Total drug and HSA concentrations of 1 and 290 μM, respectively, were employed in the study. All experiments were conducted in PBS (pH 7.40 ± 0.05) at 37 °C. Shown are the averages of at least three independent kinetic runs with the same sampling schedules. The standard deviation of each point is typically 5% or less.

method of nonlinear least squares:

$$K = [D_b]/([D_f][aa]^n) \quad (4)$$

where K represents the apparent association constant, $[D_b]$ represents the concentration of bound drug, $[D_f]$ represents the concentration of free drug, [aa] represents the concentration of amino acid residues, and n represents the stoichiometric factor (*i.e.* the number of amino acid residues per binding site). All nonlinear least-squares analyses were conducted on a Dell System 386 personal computer using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA).

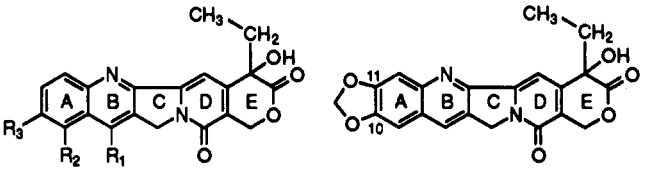
Results

Figure 1 depicts changes in lactone concentration as a function of time for camptothecin drugs in phosphate-buffered saline (PBS) solution at pH 7.4 and 37 °C. Stability data is shown for SN-38 in PBS at 37 °C, both in the presence and absence of 20 mg/mL (2.9×10^{-4} M) HSA. Also shown in Figure 1 are data for several other camptothecin congeners in PBS solution with HSA present. The kinetic and equilibrium parameters for the hydrolysis of all of the camptothecins examined in this study are summarized in Table 2.

Analysis of the data from Figure 1 showed that the hydrolysis of SN-38 free in solution proceeded with a half-life ($t_{1/2}$ value) of 20 min (final carboxylate to lactone ratio of 87:13). Similar equilibrium parameters were observed for camptothecin, 10-hydroxycamptothecin, 9-aminocamptothecin, topotecan, and CPT-11 upon standing in PBS solution in the absence of HSA, with the measured equilibrium carboxylate:lactone ratios consistently falling within a similar range (*i.e.* 13–21% lactone) as summarized in Table 2.

In the case of SN-38, it is of interest to note that the presence of HSA was found to dramatically slow lactone ring opening (*i.e.* the $t_{1/2}$ value of SN-38 increased from 19.9 min in the absence of HSA to 35.0 min in the presence of HSA). A strong shift in the equilibrium lactone–carboxylate ratio to the left in favor of the lactone form (from 13% in the absence of HSA to 38% in the protein's presence) was also observed to occur due to the presence of HSA. In a similar manner, topotecan was observed to display enhanced stabilization in the presence of HSA, with a 6-min increase in the half-life value and a 38% increase in the lactone concentration at equilibrium detected due to the presence of HSA (Table 2). CPT-11 also displayed an enhancement in stability upon HSA addition, although not to the levels achieved as in the case of SN-38 (Table 2).

Table 1. Structures of the Camptothecins (Shown in the Top Right Is 10,11-(Methylenedioxy)camptothecin)



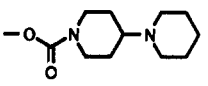
compound	R_1	R_2	R_3
camptothecin	H	H	H
10-hydroxycamptothecin	H	H	OH
topotecan	H	$CH_2NH(CH_3)_2$	OH
9-aminocamptothecin	H	NH_2	H
CPT-11	C_2H_5	H	
SN-38	C_2H_5	H	OH

Table 2. Summary of the Kinetic and Equilibrium Parameters for the Hydrolysis of Camptothecin Drugs in PBS Buffer at 37 °C in the Presence and Absence of Human Serum Albumin[a]

compound	solution	$t^{1/2}$ (min)	% lactone form at equilibrium
camptothecin[b]	PBS	23.8 ± 1.3	17 ± 2
camptothecin	HSA	12.5 ± 0.5	<0.5
10-hydroxycamptothecin[c]	PBS	22.1 ± 2.0	16 ± 1
10-hydroxycamptothecin[c]	HSA	21.1 ± 2.0	4 ± 1
9-aminocamptothecin	PBS	17.9 ± 2.0	19 ± 1
9-aminocamptothecin	HSA	23.4 ± 2.0	<0.5
topotecan	PBS	22.8 ± 1.0	13 ± 1
topotecan	HSA	28.9 ± 2.0	18 ± 1
CPT-11	PBS	25.6 ± 2.0	13 ± 2
CPT-11	HSA	29.0 ± 2.0	21 ± 1
SN-38[c]	PBS	19.9 ± 1.0	13 ± 1
SN-38[c]	HSA	35.0 ± 0.2	38 ± 1
10,11-(methylenedioxy)camptothecin	PBS	31.7 ± 2.0[d]	24 ± 2[d]
10,11-(methylenedioxy)camptothecin	HSA	20.7 ± 0.8	<0.5
9-chloro-10,11-(methylenedioxy)-camptothecin	PBS	34.6 ± 1.0[d]	21 ± 2[d]
9-chloro-10,11-(methylenedioxy)-camptothecin	HSA	16.9 ± 2.5	<0.5

[a] Hydrolysis of drugs was monitored using HPLC assays as described in the Experimental Section. The $t_{1/2}$ and percent lactone form at equilibrium values were determined from decay profiles (*e.g.* Figure 1) by using the method of nonlinear least-squares analysis as determined in the Experimental Section. Drug and albumin concentrations of 1 and 290 μM, respectively, were employed in these studies. All experiments were conducted in PBS at 37 °C. [b] The measured $t_{1/2}$ and percent lactone form at equilibrium values for a high-purity camptothecin sample (>99% by HPLC) obtained by recrystallization processes are higher than reported previously for a less pure sample.[7] [c] Previously reported values.[8] [d] Compounds containing the addition of a 10,11-methylenedioxy ring system exhibit a greater tendency to self-associate in PBS (T. Burke, unpublished results), thereby providing a possible explanation for the reduced rates and reduced levels of hydrolysis exhibited by these agents when free in solution.

Table 3. Summary of the Excited-State Lifetime Values for Camptothecin Drugs Free in Solution and Bound to Human Serum Albumin[a]

compound	lactone form		carboxylate form	
	τ_{free} (ns)	τ_{bound} (ns)	τ_{free} (ns)	τ_{bound} (ns)
camptothecin	4.2	2.7	4.3	1.3
10-hydroxycamptothecin	4.0	5.4	4.0	5.5
topotecan	6.0	6.0	6.1	6.1
CPT-11	3.7	3.7	4.0	4.0
SN-38	4.0	5.7	4.1	5.5
10,11-(methylenedioxy)camptothecin	2.0	1.3	2.4	1.3
9-chloro-10,11-(methylenedioxy)-camptothecin	1.9	1.9	2.5	1.1

[a] Lifetime measurements were conducted as described in the Experimental Section. The lifetimes of bound fluorophores were determined by extrapolating the lifetime titration profiles (Figure 2) to infinite HSA concentrations using the method of nonlinear least-squares analysis (see Experimental Section). Lifetime data of 9-aminocamptothecin were not determined due to the poor fluorescence properties of this agent at pH of 7.4.

Quite unlike SN-38, topotecan, and CPT-11, the α-hydroxy δ-lactone ring moiety of the following congeners opened more rapidly and completely due to the presence of HSA: 10-hydroxycamptothecin, camptothecin, 9-aminocamptothecin, 10,11-(methylenedioxy)camptothecin, and 9-chloro-10,11-(methylenedioxy)camptothecin. In the case of the latter four drugs, essentially complete (99% or greater) conversion to the carboxylate form of each drug was observed.

Our interest in elucidating the underlying factors for the differential stabilities of the camptothecins in the presence of HSA led us to study spectroscopically their intense intrinsic fluorescence emissions. The fluorescence from camptothecin is associated with the extended conjugation of the quinoline ring system; alterations in the A ring, as in the case of all of the analogues of camptothecin listed in Table 1, affect the fluorescence properties of the compound. Thus it is not surprising that the excited-state lifetimes of the fluorophores free in solution differ from one another (Table 3). For example, camptothecin displayed a τ value in PBS or 4.2 ns, while under identical conditions topotecan and 10,11-(methylenedioxy)camptothecin displayed higher and lower τ values of 6.0 and 2.0 ns, respectively.

Because fluorescence lifetime measurements provide a direct means of assessing a change in the binding environment of a fluorophore,[16] we employed lifetime measurements to study drug interactions with HSA. Figure 2 shows how the τ values for the lactone and carboxylate forms of camptothecin, topotecan, and 10-hydroxycamptothecin titrate in PBS (pH 7.4) due to the addition of HSA. While both forms of camptothecin and both forms of 10-hydroxycamptothecin exhibited changes (a decrease in τ values for camptothecin and camptothecincarboxylate, an increase in τ values for 10-hydroxycamptothecin and 10-hydroxycamptothecincarboxylate) upon the addition of HSA, the τ values for the lactone and carboxylate forms of topotecan were found to be insensitive to the presence of HSA (*i.e.* these fluorophores exhibited no significant change in τ value upon HSA addition).

The τ value of camptothecincarboxylate changes markedly upon the addition of the HSA titrant; its lactone form also changes, but to a lesser extent than the carboxylate. By using the method of nonlinear least-squares analysis to fit plots of τ versus albumin concentration (as described in the Experimental Section), the excited-state lifetimes of albumin-bound fluorophores (τ_B) were determined. These τ_B values for camptothecin, as well the τ_B values for the other congeners of interest, are summarized in Table 3.

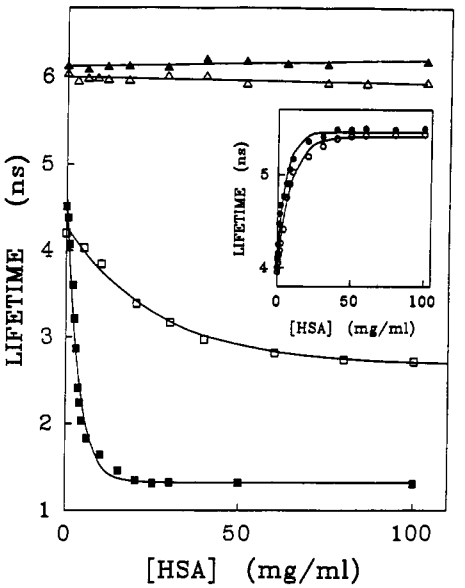


Figure 2. Binding of camptothecin analogues to HSA as monitored by the method of fluorescence lifetime titration (see Experimental Section). The differential effect of HSA on the excited-state lifetimes of lactone forms and carboxylate forms of camptothecin (□, ■), topotecan (△, ▲), and 10-hydroxycamptothecin (○, ●) are shown. The hollow symbols represent the lactone forms and the filled symbols represent the carboxylate forms. All experiments were conducted in PBS (pH 7.4) at 37 °C using fluorophore concentrations of 20 μM. The average of triplicate runs are shown, and each point has a variance of 7% or less.

Camptothecin exhibits a HSA-bound τ_B value of 2.7 ns, while the corresponding τ_B value for its carboxylate form was determined to be 1.3 ns. The significantly reduced τ_B value observed for the carboxylate species is direct evidence suggesting that the carboxylate fluorophore's binding microenvironment on HSA is appreciably different than that experienced by camptothecin, although the exact factors responsible for this finding remain to be elucidated.

Analysis of plots of free drug fraction vs the logarithm of the molar concentration of amino acid residues, as shown in Figure 3 and described in the Experimental Section, allowed for quantitation of binding constants. Figure 3 shows the fraction of the total fluorophore free in solution (as determined by the relationship $(\tau - \tau_B)/(\tau_F - \tau_B)$) versus albumin concentration for the lactone and carboxylate forms of 10,11-(methylenedioxy)camptothecin, 10-hydroxycamptothecin, and SN-38. The titration profiles for the lactone and carboxylate forms of each fluorophore differ, a direct indication that their binding affinities for HSA differ. For example, nonlinear least-squares analyses of the titration curves depicted in Figure 3 yield binding constants of 2200 (M amino acid residues (aa))$^{-1}$ for 10,-11-(methylenedioxy)camptothecincarboxylate and 10 (M aa)$^{-1}$ for 10,11-(methylenedioxy)camptothecin lactone. The n values for the carboxylate and lactone forms of 10,11-(methylenedioxy)camptothecin were determined to be 1.7 and 1.3, respectively. Thus our modeling of the binding data indicate that the carboxylate and lactone forms of 10,11-(methylenedioxy)camptothecin display a similar number (approximately 1.5) of amino acids per receptor

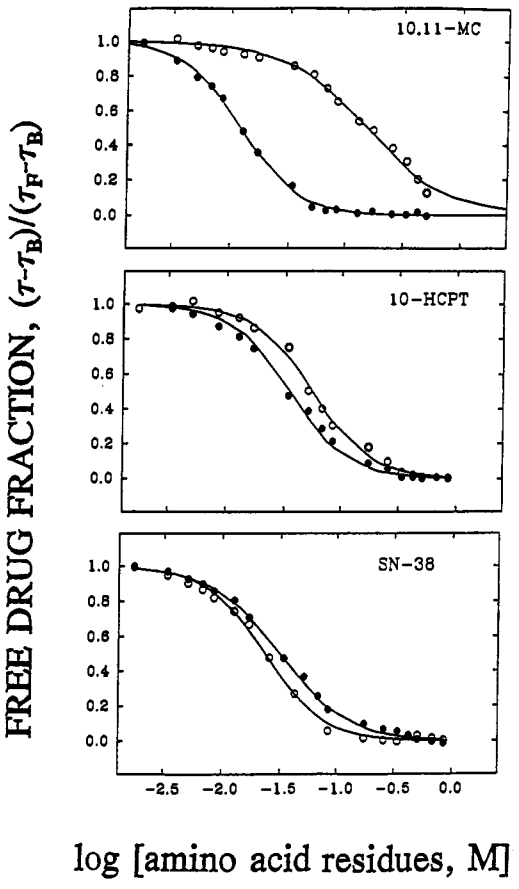


Figure 3. Replots of lifetime data of 10,11-(methylenedioxy)-camptothecin (top panel), 10-hydroxycamptothecin (middle panel), and SN-38 (bottom panel) in the form of free drug fraction, $(\tau-\tau_B)/(\tau_F-\tau_B)$, versus HSA concentrations expressed in terms of the log[amino acid (aa) residues]. τ_F and τ equal the lifetime of the fluorophore in the absence and presence of a given HSA concentration, and τ_B equals the lifetime of the bound fluorophore. The hollow symbols represent the lactone forms and the filled symbols represent the carboxylate forms. The solid lines represent the best nonlinear least-squares fits of the data sets.

site but that the carboxylate form of the drug displays a significant, 220-fold higher affinity for HSA relative to the drug's lactone form. Large differences (150-fold or greater) in K values between lactone and carboxylate forms were also observed in the cases of camptothecin [4700 (M amino acid residues (aa))$^{-1}$ for camptothecin carboxylate versus 30 (M aa)$^{-1}$ for camptothecin] and 9-chloro-10,-11-(methylenedioxy)camptothecin [1200 (M amino acid residues (aa))$^{-1}$ for 9-chloro-10,11-(methylenedioxy)camptothecincarboxylate versus ca. 0 (M aa)$^{-1}$ for the corresponding lactone form].

Also shown in Figure 3 is binding data for the lactone and carboxylate forms of 10-hydroxycamptothecin. Our modeling of the data indicates that 10-hydroxycamptothecincarboxylate and 10-hydroxycamptothecin display a similar number (approximately 2) of amino acids per binding site, but the carboxylate form displays a statistically significant 2-fold higher affinity relative to the lactone form. The findings that the carboxylate forms of 10-hydroxycamptothecin, 10,11-(methylenedioxy)camptothecin, 9-chloro-10,11-(methylenedioxy)camptothecin, and camptothecin preferentially bind HSA relative to their corresponding lactone forms provide a mechanistic explanation for the shift in the lactone–carboxylate equilibria to the right observed for these four agents upon the addition of HSA (see Table 2).

Table 4. Summary of the Association Constants and n Values for the Binding of the Lactone and Carboxylate Forms of Camptothecin Drugs to Human Serum Albumin[a]

	K value (M aa)$^{-1}$		n value	
compound	lactone	carboxylate	lactone	carboxylate
camptothecin	30	4700	1.8	2.3
10,11-(methylenedioxy)-camptothecin	10	2200	1.3	1.7
9-chloro-10,11-(methylenedioxy)-camptothecin	nd	1200	nd	1.6
10-hydroxycamptothecin	130	260	1.7	1.7
SN-38	640	150	1.7	1.4

[a] Binding isotherms were constructed by lifetime titration in PBS, pH 7.4 at 37 °C. Binding parameters were determined by curve fitting the binding data in Figure 3 to eq 4 by the method of nonlinear least-squares (see the Experimental Section for details). Binding parameters for CPT-11 and topotecan were not determined because the lifetimes of these two agents did not change upon the addition of HSA. 9-Aminocamptothecin is only weakly fluorescent at pH 7.4, and therefore it was not possible to determine drug binding parameters by the lifetime titration method.

The 9-aminocamptothecin analogue has weak fluorescence at physiological pH, and thus we were unable to employ lifetime measurements in quantitating the binding of its lactone and carboxylate forms to HSA. However, kinetic data determined from HPLC analysis (Table 2) shows that 9-aminocamptothecin opens more completely (>99%) in the presence of HSA than the protein's absence. Thus it appears that 9-aminocamptothecin, like 10-hydroxycamptothecin, 10,11-(methylenedioxy)camptothecin, 9-chloro-10,11-(methylenedioxy)camptothecin, and camptothecin, preferentially binds HSA in its carboxylate rather than lactone form.

Of the five camptothecin compounds listed in Table 4, the behavior of SN-38 is unique. Unlike the other four agents which open more rapidly and completely with HSA present, SN-38 displays a significantly longer half-life in the presence of HSA (35 min) versus in the absence of HSA (20 min). The data contained in Table 4 underscores other differences between the behavior of SN-38 with camptothecin, 10,11-(methylenedioxy)camptothecin, 10-hydroxycamptothecin, and 9-chloro-10,11-(methylenedioxy)camptothecin. Whereas the latter four drugs preferentially bind HSA in their carboxylate form, SN-38 preferentially binds HSA in its *lactone* form.

Discussion

Arguably the most important determinant of the activities for each camptothecin anticancer drug is the equilibrium between the lactone and carboxylate forms. In this report we have shown that alteration of the A and B rings of camptothecin strongly modulates drug stability in the presence of HSA. While some analogues such as SN-38, topotecan, and CPT-11 were more stable in the presence of HSA, others were less stable.

The drugs which were less stable in the presence of HSA include 9-aminocamptothecin, camptothecin, 10-hydroxycamptothecin, 10,11-(methylenedioxy)camptothecin, and 9-chloro-10,11-(methylenedioxy)camptothecin. These agents were found to hydrolyze rapidly in the presence of HSA; within 2 h the lactone–carboxylate equilibrium for each agent went almost completely (96–99%) to the right. For the above compounds which are amenable to fluorescence spectroscopic investigation (*i.e.* the latter four agents), the enhanced rates of hydrolysis correlated with

preferential binding of the carboxylate forms of the drugs by HSA. Our results are consistent with results from other laboratories concerning the preferential binding of the carboxylate form of camptothecin by HSA.[17,18]

Human serum albumin is the principal carrier of water-insoluble fatty acids in the bloodstream. The albumin protein has long been noted for its preferential binding of small, hydrophobic molecules which carry a negative charge.[19] The principal regions of ligand association with HSA occur at two distinct, hydrophobic regions commonly referred to as the IIA and IIIA binding pockets.[19] Similar to a fatty acid, camptothecincarboxylate is also a small, hydrophobic molecule carrying a negative charge at physiological pH. In this report we have shown that HSA preferentially binds the carboxylate form of camptothecin with high specificity (*i.e.* there exists a 150-fold difference in binding affinity between the carboxylate and lactone forms of camptothecin).

Thus it appears likely that camptothecincarboxylate associates with HSA in either the IIA or IIIA hydrophobic binding pockets. The proposal that camptothecincarboxylate interacts with a specific binding cavity on HSA is supported by recent findings using heat-denatured HSA. Alteration of the secondary and tertiary protein structure of HSA achieved by heating to 100 °C was found to markedly attenuate the binding between HSA and camptothecincarboxylate at 37 °C (Burke and Mi, unpublished results).

Data contained in Table 4 further indicates that HSA binding of camptothecincarboxylate exhibits a high level of structural specificity. Comparison of the association constants of the various carboxylate forms indicates binding can be strongly modulated by simple structural modification of the camptothecincarboxylate molecule. Inclusion of the 10,11-methylenedioxy ring resulted in an approximate 50% reduction in carboxylate binding, while the 9-chloro-10,11-methylenedioxy substituent decreasing binding 74%. More impressively, however, was the manner in which 10-hydroxy substitution dropped the binding of the carboxylate by 94%. 10-Hydroxy substitution, in combination with 7-ethyl substitution, dropped binding of camptothecincarboxylate by 97%.

Reduction of the binding of the carboxylate form, together with the promotion of the binding of the lactone form, results in analogues which preferentially associate with HSA in the lactone form. Fluorescence lifetime measurements concerning the HSA interactions of SN-38 indicate that HSA preferentially binds the *lactone* form of this drug, thereby providing a biophysical explanation for the favorable stability properties displayed by this analogue in the presence of human serum albumin. Topotecan and CPT-11 also display enhanced stabilities in the presence of HSA. The favorable stabilities observed in the cases of topotecan and CPT-11 suggest that these drugs preferentially bind HSA in their lactone forms, albeit direct confirmation of this point using lifetime measurements was not possible due to the inherent insensitivities of the respective fluorochrome of each drug. However, we have recently employed time-resolved fluorescence anisotropy measurements to determine that both the lactone and carboxylate forms of topotecan and CPT-11 bind HSA weakly relative to camptothecincarboxylate (Burke and Malak, unpublished).

Our findings concerning the stabilities of several camptothecins (camptothecin, 10-hydroxycamptothecin, SN-38) in HSA solutions are paralleled closely by data generated using human plasma samples.[8] For example, camptothecin displayed a $t_{1/2}$ value of 10.6 min in human plasma,[8] which is quite similar with a value of 12.6 min for HSA solution (Table 2). The percent lactone form at equilibrium values for camptothecin in human plasma and purified HSA solution were determined to be <0.2 and <0.5, respectively. For SN-38, a $t_{1/2}$ value of 34.3 min in human plasma was observed,[8] which compares closely with a value of 35.0 min for HSA solution (Table 2). The percent lactone form at equilibrium values for SN-38 in human plasma and purified HSA solution were determined to be 24 and 38, respectively.

In summary, we have demonstrated that structural modification of camptothecin markedly enhances drug stability in the presence of human albumin. Enhancements in the stabilities of the camptothecins can be accomplished by inclusion of structural features such as a 7-ethyl substituent which reduce binding of the carboxylate form while promoting the reversible binding of the lactone form. As is demonstrated in the case of SN-38, significant gains in stability in the presence of HSA can be achieved without loss of intrinsic potency.[12] From our work it thus appears that the rational development of more active camptothecins would benefit from the further elucidation of the structural basis of drug interactions with HSA.

Acknowledgment. T.G.B. gratefully acknowledges the support of the Wendy Will Case Cancer Fund and The Ohio State University Comprehensive Cancer Center Core Grant (NIH CA 16058-19). Ying Jiang is gratefully acknowledged for her excellent technical assistance.

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