Ethyl Substitution at the 7 Position Extends the Half-Life of 10-Hydroxycamptothecin in the Presence of Human Serum Albumin

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Camptothecin and related analogues possess the ability to halt the growth of a wide range of animal and human tumors.[1-4] In exerting their antitumor action the camptothecins display a unique mechanism of action: stabilization of the binding of topoisomerase I to DNA, leading to DNA fragmentation.[4] An important structural requirement for successful interaction with the topoisomerase I target[5] and antitumor potency *in vivo*[6] is a closed α-hydroxy lactone ring moiety. Unfortunately, this functionality hydrolyzes under physiological conditions, *i.e.*, at pH 7 or above, with the lactone ring readily opening up to yield the inactive carboxylate form of the drug.[4,5] In this report we employ both HPLC methodologies[7-10] and time-resolved fluorescence spectroscopy to demonstrate that substitution of a 7-ethyl group into the 10-hydroxycamptothecin molecule significantly enhances drug stability in the presence of HSA by promoting preferential associations of the lactone form of the drug with the blood protein.

The structure of 7-ethyl-10-hydroxycamptothecin (SN-38), a highly active compound generated *in vivo* by deesterification of its inactive prodrug analogue 7-ethyl-10-[[[4-(1-piperidino)-1-piperidino]carbonyl]oxy]camptothecin (CPT-11),[10] is shown in Figure 1. Also depicted in Figure 1 are changes in lactone concentration as a function of time for 1 μM solutions of SN-38 as well as its de-ethylated congener 10-hydroxycamptothecin. Studies were conducted in phosphate-buffered saline (PBS)[11] solution at pH 7.4 and 37 °C. Data is shown for drug in the presence and absence of 20 mg/mL (290 μM) HSA, and the stability data is summarized in Table I.

Hydrolysis of both 10-hydroxycamptothecin and SN-38 free in solution proceeded with similar half-lives ($t_{1/2}$ values) of 20 and 22 min, respectively (with final carboxylate to lactone ratio of 84:16 and 87:13, respectively). However, the stability profiles of the two agents differ markedly in the presence of a relatively dilute 20 mg/mL concentration of HSA [albumin levels in humans typically range from 35 to 55 mg/mL[12]]. The effect of HSA on 10-hydroxycamptothecin's stability was to shift the lactone–carboxylate equilibrium far to the right in favor of the carboxylate (96%). Similar to 10-hydroxycamptothecin, camptothecin also opens more rapidly and completely (>99%) due to the presence of HSA[13] (Table I).

In stark contrast to 10-hydroxycamptothecin and camptothecin, SN-38 exhibits enhanced stability in the presence of HSA. The half-life of SN-38 in the presence of HSA was 35 min, as compared with 20 min in the absence of HSA (Table I). Moreover, the percent lactone form at equilibrium is very much higher (38%) in the presence of HSA versus in the absence of HSA (13%).

Our interest in understanding mechanistically the differential interactions of camptothecins with HSA led us to study spectroscopically each drug's intense intrinsic fluorescence emission. As that fluorescence lifetime measurements provide a direct means of assessing a change in the binding environment of a fluorophore,[14] we employed this technique in our work. Figure 2 shows a plot of the fraction of the total fluorophore free in solution (as determined by the relationship $(\tau - \tau_B)/(\tau_F - \tau_B)$ versus albumin concentration[13]). The titration profiles vary for each fluorophore, a direct indication that their binding

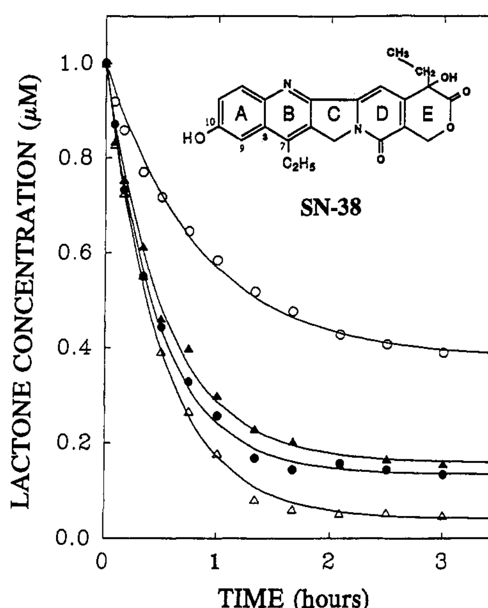

Figure 1. Kinetic evalution of the rate of lactone ring opening for 10-hydroxycamptothecin and SN-38. Stability profiles are shown for both agents free in PBS solution (● for SN-38), ▲ for 10-hydroxycamptothecin) and in the presence of HSA (○ for SN-38, △ for 10-hydroxycamptothecin). Total drug and HSA concentrations of 1 and 290 μM, respectively, were employed. Experiments were conducted in PBS at 37 °C. Shown is the average of at least three independent kinetic runs with the same sampling schedule. The standard deviation of each point is typically 5% or less.

Table I. 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	$t_{1/2}$ value (min)	% lactone form at equilibrium
camptothecin[b]	PBS	16.8 ± 0.5	13 ± 1
camptothecin[b]	HSA	12.6 ± 0.5	<0.5
camptothecin	plasma	10.6 ± 0.7	<0.2
10-hydroxycamptothecin	PBS	22.1 ± 2.0	16 ± 1
10-hydroxycamptothecin	HSA	21.1 ± 2.0	4 ± 1
10-hydroxycamptothecin	plasma	18.6 ± 1.0	6 ± 1
SN-38	PBS	19.9 ± 1.0	13 ± 1
SN-38	HSA	35.0 ± 0.2	38 ± 1
SN-38	plasma	34.3 ± 0.5	24 ± 2

[a] Hydrolysis of drugs was monitored using HPLC assays as described.[7-10] The $t_{1/2}$ and % lactone form at equilibrium values were determined from decay profiles (Figure 1) by the method of nonlinear least squares. Drug and albumin concentration of 1 and 290 μM were employed in these studies. Plasma samples were continuously aerated with "blood gas" (MEDIBLEND, Linde Medical Gases, CT) in order to maintain constant pH (7.6 ± 0.1). All experiments were conducted at 37 °C. [b] These values are reported elsewhere.[13]

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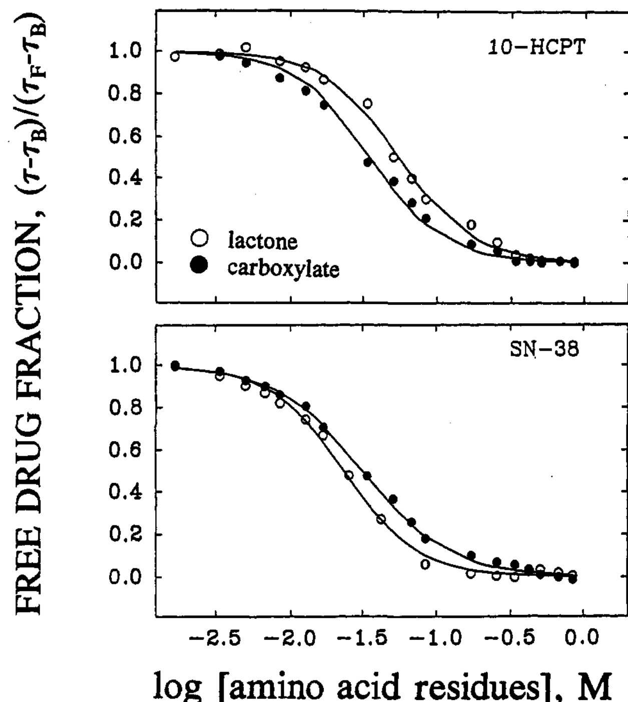

Figure 2. The binding of the lactone and carboxylate forms of 10-hydroxycamptothecin (top panel) and SN-38 (bottom panel) as monitored by the method of fluorescence lifetime titration.[15] For each drug the differential effect of HSA on the excited-state lifetimes of lactone (○) and carboxylate (●) forms are shown. Shown is a plot of the lifetime data in the form of $(\tau - \tau_B)/(\tau_F - \tau_B)$ versus HSA concentation (expressed in terms of log-[amino acid (aa) residues]).[13] τ_F and τ equal the lifetime of the fluorophore in the absence and presence of a given HSA concentration, and τ_B is equal to the lifetime of the bound fluorophore. The solid lines represent the best nonlinear least-squares fits of the dat sets.

Table II. 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[b]	30	4700	1.8	2.3
10-hydroxy-camptothecin	135 ± 16	264 ± 34	1.7 ± 0.1	1.7 ± 0.1
SN-38	636 ± 29	148 ± 34	1.7 ± 0.1	1.4 ± 0.2

[a] Binding isotherms were constructed by lifetime titration.[15] Binding parameters were determined by curve fitting the data to equation $K = [D_b]/[D_f][aa]^n$ by the method of nonlinear least squares, where $[D_b]$ is the concentration of bound drug, $[D_f]$ is the concentration of free drug, [aa] is the concentration of amino acid residues, and *n* is the number of amino acid per binding site. All experiments were conducted in PBS at 37 °C. [b] These values are reported elsewhere as the average of the duplicate determinations.[13]

affinities for HSA differ. Nonlinear least-squares analyses of the titration curves in Figure 2 yield apparent binding constants (*K* values) and *n* values (the number of amino acid residues per binding site), and these parameters are summarized in Table II.

Our modeling of the binding 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. An even more pronounced difference (150-fold) in *K* values between the lactone and carboxylate forms was observed in the case of camptothecin: 4700 (M amino acid residues (aa))$^{-1}$ for camptothecin carboxylate and 30 (M aa)$^{-1}$ for camptothecin lactone.[13] The findings that the carboxylate forms of 10-hydroxycamptothecin and camptothecin preferentially bind HSA relative to their corresponding lactone forms provide a mechanistic explanation for the shift in the lactone–carboxylate equilibrium to the right observed for these two agents upon the addition of HSA.

Quite different though is the behavior of SN-38 in the presence of HSA. Unlike the other two drugs, 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 time-resolved spectroscopic data contained in Table II also underscore differences between the behavior of SN-38 with both 10-hydroxycamptothecin and camptothecin. Whereas the latter two drugs preferentially bind HSA in their carboxylate form, SN-38 preferentially binds HSA in its *lactone* form. Our findings in HSA solutions are paralleled closely by data generated using human plasma samples (Table I), with SN-38 once again exhibiting significantly enhanced stability.

In summary, our results indicate that incorporation of an ethyl substituent into 10-hydroxycamptothecin at the 7 position markedly enhances drug stability in the presence of human albumin. This is due to the occurrence of a favorable, reversible binding between the lactone form of the drug and HSA. Owing to the lactone–HSA interactions, a high percentage of SN-38, relative to other camptothecins, will be transported in its intact and biologically-active form. The 7-ethyl substituent contained in SN-38 is also responsible for a three-fold or greater enhancement in the drug's affinity for lipid bilayers relative to the affinity of camptothecin and 10-hydroxycamptothecin (Burke et al., unpublished results). The increased lipophilicity of SN-38, together with its preferential binding to HSA in the lactone form, should contribute to promoting distribution of the active lactone form in the body. The notion of stabilizing camptothecins in the bloodstream by promoting reversible associations between HSA and their lactone forms (*i.e.* to use HSA as a carrier to optimize distribution of the active form throughout the body) is highly attractive and our present results indicate that analogues alkylated at the 7-position are logical candidates for development and evaluation.

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(15) Fluorescence lifetimes were taken on a SLM Model 4800C spectrofluorometer and were determined by the method of phase shift[14] using excitation light modulated at 30 MHz. 10-Hydroxycamptothecin was provided by the National Cancer Institute. SN-38 was obtained through the generosity of Yakult Honsha (Tokyo). High-purity HSA was obtained from Sigma Chemical. 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 by a 500-nm long-pass filter. Intensity levels were typically in excess of 98% and did not fall below 95% even in the most concentrated HSA solutions. The following lifetime values for free and HSA-bound drugs were determined: 10-hydroxycamptothecin lactone, τ_F = 3.9 ns, τ_B = 5.4 ns; 10-hydroxycamptothecin carboxylate, τ_F = 4.0 ns, τ_B = 5.5 ns; SN-38 lactone, τ_F = 4.1 ns, τ_B = 5.7 ns; SN-38 carboxylate, τ_F = 4.1 ns, τ_B = 5.5 ns. In Figure 2 the average of triplicate runs are shown, and each point has a variance of 7% or less.