Lipid Bilayer Partitioning and Stability of Camptothecin Drugs†

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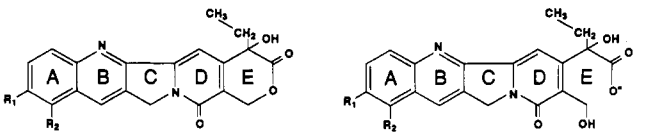
Received January 15, 1993; Revised Manuscript Received March 23, 1993

ABSTRACT: The intense intrinsic fluorescence emissions from several clinically relevant camptothecin drugs have been exploited in order to determine (1) the structural basis of drug binding to lipid bilayers, (2) the lipid bilayer stability of each drug's α-hydroxylactone moiety, a pharmacophore which is essential for antitumor activity, and (3) the site of drug binding in the bilayer. Equilibrium affinities of camptothecin and related congeners for small unilamellar vesicles composed of electroneutral dimyristoylphosphatidylcholine (DMPC) or negatively-charged dimyristoylphosphatidylglycerol (DMPG) were determined using the method of fluorescence anisotropy titration. Experiments were conducted in phosphate-buffered saline (PBS) at 37 °C and overall association constants (K values) were determined. Of the seven compounds studied, the new compound 9-chloro-10,11-methylenedioxy-(20*S*)-camptothecin (CMC) was found to display the highest membrane affinities (K_{DMPC} = 400 M^{-1}, K_{DMPG} = 320 M^{-1}), followed by 10,11-methylenedioxycamptothecin and camptothecin, which exhibited K_{DMPC} and K_{DMPG} values of 100 M^{-1} or greater. Topotecan displayed markedly reduced binding to lipid bilayers (K_{DMPC} = 10 M^{-1}, K_{DMPG} = 50 M^{-1}). HPLC assays were subsequently employed to assess the relative stabilities of the lactone ring of membrane-bound drugs. Our results clearly indicate that lipid bilayer interactions stabilize the lactone moiety of camptothecin drugs. In comparison to half-lives in PBS (37 °C) of 17 and 19 min for camptothecin and CMC, respectively, DMPC- or DMPG-bound drugs were found to be stable even for periods up to 72 h. Iodide quenching data indicate that membrane-bound camptothecin intercalates between the lipid acyl chains, in a protected environment well removed from the aqueous interface. In this manner lipid bilayer interactions stabilize the lactone ring structure of camptothecins and thereby conserve the biologically active form of each medication.

The promising anticancer drug camptothecin (Wall et al., 1966), as well as several related analogues such as topotecan, CPT-11, and 9-aminocamptothecin, have aroused considerable interest in recent years for their ability to halt the growth of a wide range of human tumors (Giovanella et al., 1989, 1991; Wall & Wani, 1991; Potmesil et al., 1991; Wani et al., 1986, 1987a,b; Sawada et al., 1991; Kingsbury et al., 1991; Johnson et al., 1989; Burris et al., 1992). The camptothecin family of drugs appears to have a unique mechanism of action: stabilization of the binding of topoisomerase I (an enzyme involved in the maintenance of DNA topology), leading to DNA fragmentation. DNA topoisomerase I is the only known molecular target of camptothecin in cancer cells (Hsiang et al., 1985; Hsiang and Liu, 1988), and the drug is thought to elicit its antitumor effect by interfering with the nicking/sealing activities 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, term the cleavable complex. The formation of the cleavable complex specifically prevents the ligation step of the nicking/sealing cycle of topoisomerase activity. Camptothecin for oral therapy entered phase I clinical trials at St. Joseph's Hospital, Houston, TX, in January of 1992, while the topotecan analogue has been under phase I and II clinical investigations for the past several years. The 9-aminocamptothecin analogue, first synthesized and studied at the Research Triangle Institute (Wani et al., 1986, 1987a,b) and further developed by the National Cancer Institute, is presently undergoing phase I clinical trials.

Table I: Structures of the Camptothecins[a]



LACTONE FORM CARBOXYLATE FORM

compound	R_1	R_2
camptothecin	H	H
9-aminocamptothecin	H	NH_2
9-nitrocamptothecin	H	NO_2
10-hydroxycamptothecin	OH	H
topotecan	OH	$CH_2NH(CH_3)_2$

[a] Topotecan is the hydrochloride of the structure shown.

Unfortunately, the antitumor activities of camptothecin, topotecan, and related congeners begin to deteriorate immediately following dissolution of the compounds in aqueous media. This is due to the presence of a hydrolyzable α-hydroxylactone ring moiety (ring E) in each drug's structure (shown in Table I). This functionality readily reacts in solution via acyl cleavage (Fassberg & Stella, 1992), yielding the biologically inactive carboxylate form of each medication. Previous investigators have shown that camptothecin carboxylate is not readily internalized by cancer cells (Wani et al., 1987a,b), and an intact α-hydroxylactone ring is known to be essential for activity against the topoisomerase I target (Jaxel et al., 1989; Hertzberg et al., 1989). Furthermore, it

† The very generous support provided of The Ohio State University College of Pharmacy and its Division of Pharmaceutics is gratefully acknowledged. T.G.B. gratefully acknowledges the support of the Wendy Will Case Cancer Fund. Part of this work has appeared earlier in preliminary form [Burke, T. G., Staubus, A. E., Mishra, A. K., & Malak, H. (1992) *J. Am. Chem. Soc. 114,* 8318–8319].

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0006-2960/93/0432-5352$04.00/0

has been well established that the α-hydroxylactone ring moiety is a critical structural feature for activity *in vivo* (Wall, 1969; Wall & Wani, 1991; Wani et al., 1987a,b; Nicholas et al., 1990).

In this report and elsewhere (Burke et al., 1992), we characterize a potentially important role for the lipid bilayer interactions of camptothecin drugs in determining their biological activities. Using the brilliant fluorescence emission from the substituted quinoline nucleus of these compounds, we have employed steady-state and frequency-domain fluorescence spectroscopic methodologies to characterize in detail the equilibrium associations of camptothecin drugs with small unilamellar vesicles composed of L-α-dimyristoylphosphatidylcholine (DMPC)[1] and L-α-dimyristoylphosphatidylglycerol (DMPG). The structural basis of drug associations with lipid bilayers has been examined, and we demonstrate that the association constants among the members of the camptothecin family vary over a 40-fold range. We also demonstrate that camptothecin binds membranes by intercalating between the acyl chains of the phospholipid membrane. Moreover, using HPLC methodologies we have shown that camptothecin drugs are stable when harbored within lipid bilayers. Because the pharmacologically active forms of camptothecins are shown here to be stable in lipid bilayers, drug interactions with lipid bilayers should be regarded as clearly favorable events for conserving the lactone ring structure of these clinically relevant anticancer medications.

MATERIALS AND METHODS

Chemicals. Samples of camptothecin, camptothecin carboxylate sodium salt, 10-hydroxycamptothecin, 10,11-methylenedioxycamptothecin, 9-aminocamptothecin, and topotecan were obtained from the Division of Cancer Treatment, National Cancer Institute. Samples of 9-chloro-10,11-methylenedioxycamptothecin (CMC), 9-amino-10,11-methylendioxycamptothecin (AMC), and 9-nitrocamptothecin were obtained from the laboratories of Drs. Monroe Wall and Mansukh Wani. All of the camptothecins were in the 20*S* configuration. Stock solutions of the drugs were prepared in dimethyl sulfoxide (ACS spectrophotometric grade, Aldrich, Milwaukee, WI) at a concentration of 2×10^{-3} M and stored in dark at 4 °C. L-α-Dimyristoylphosphatidylcholine (DMPC), L-α-dimyristoylphosphatidylglycerol (DMPG), and L-α-distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids, Alabaster, AL, and were used without further purification. All other chemicals were reagent grade and were used without further purification.

Vesicle Preparation. Small unilamellar vesicle (SUV) suspensions were prepared the day of an experiment by the method of Burke and Tritton (1985a). Briefly, stock lipid suspensions containing 200 mg/mL lipid in phosphate-buffered saline (PBS, pH 7.4) were prepared by Vortex mixing for 5–10 min above the T_M of the lipid. 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. A decrease in pH from 7.4 to 6.8 was observed for the SUV preparations of DMPG; therefore, 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. Each type of vesicle suspension was annealed for 30 min at 37 °C and then used in an experiment.

Fluorescence Instrumentation. Steady-state 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. Excitation and emission spectra were recorded with an excitation resolution of 8 nm and an emission resolution of 4 nm. In all cases spectra were corrected for background fluorescence and scatter from unlabeled lipids or from solvents by subtraction of the spectrum of a blank. Steady-state fluorescence intensity measurements were made in the absence of polarizers. Steady-state anisotropy (a) measurements were determined with the instrument in the T-format for 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 suspension of 0.25 μm polystyrene microspheres (Polysciences, Inc., Warrington, PA) in water, and anisotropy values of >0.99 were obtained.

Anisotropy measurements for camptothecin, camptothecin carboxylate, and 10,11-methylenedioxycamptothecin were conducted using exciting light of 370 nm and 420-nm long-pass filters on each 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), AMC and CMC (370 nm, 400-nm long-pass filter), and 10-hydroxycamptothecin (375 nm, 500-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 1% of the total intensity. Since the lactone rings of camptothecin and related congeners 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 mixing the drug stock solution with thermally preequilibrated solutions.

Determination of Equilibrium Binding Constants. As described previously (Burke & Tritton, 1985a), the method of fluorescence anisotropy titration was employed in order to determine the concentrations of free and bound species of drug in liposome suspensions containing a total drug concentration of 1×10^{-6} M and varying lipid concentrations. All 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) vs 1/[lipid]} were linear and 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 written to predict bound drug levels for situations where K values and total lipid and total drug concentrations were known parameters.

[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DMPG, L-α-dimyristoylphosphatidylglycerol; DSPC, L-α-distearoylphosphatidylcholine; SUV, small unilamellar vesicles; HPLC, high-performance liquid chromatography; 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); T_m, gel-to-liquid-crystalline phase transition temperature; a, steady-state anisotropy; a_0, anisotropy in the absence of depolarizing rotations; τ, excited-state lifetime; K, overall association constant; MDR, multidrug resistance.

Kinetics of Lactone Ring Opening. The rate of lactone ring opening due to hydrolysis for the camptothecins 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. 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 reversed-phase column, a Waters Model 470 scanning fluorescence detector, and a Hewlett-Packard Model HP3396 Series II integrator for data processing. For camptothecin, an isocratic mobile phase was employed which consisted of 32% acetonitrile, 67% 0.1 M acetate buffer (pH 5.5), and 1% 0.1 M sodium dodecyl sulfate. For topotecan the mobile phase consisted of 27% acetonitrile, 73% 0.03 M KH_2PO_2 buffer, 0.2% sodium dodecyl sulfate, and 0.2% triethylamine (pH 6.0). For CMC the mobile phase consisted of 24% acetonitrile, 67% 0.1 M acetate buffer, 8% 2-propanol, and 1% 0.1 M sodium dodecyl sulfate. The detector was set to an excitation wavelength of 370 nm and an emission wavelength of 438 nm for camptothecin; for topotecan the λ_{EX} and λ_{EM} values were 400 and 537 nm, respectively. Analysis of CMC utilized excitation and emission wavelengths of 370 and 440 nm, respectively. Flow rates of 1 mL/min were employed, and retention times for parent drugs typically ranged between 7 and 9 min, while carboxylate forms rapidly eluted from the columns with short retention times of approximately 2–3 min. Solutions of drug at a concentration of 1 μM in PBS (pH 7.4) with and without lipid were prepared and incubated at 37 °C. Immediately after the drug stock solution was mixed with PBS and at intervals of 5 min thereafter, 50-μL aliquots were taken out of the various samples and mixed with 150 μL of methanol solution containing internal standard (9-toluoylamidocamptothecin or acridine for topotecan analysis) and either analyzed immediately or kept cold under dry ice. The mixture was then diluted with 1 mL of water immediately prior to injection (90-μL injection volumes were used). The fraction of intact lactone (f) vs time (t) data for camptothecin and related congeners were fit to eq 1 by the method of nonlinear least-squares analysis using SigmaPlot 4.1 (Jandel Scientific, Corte Madera, CA):

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

where the adjustable parameter k_1 is the 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).

Quenching of Camptothecin Fluorescence by Iodide. Using methodologies employed previously (Burke & Tritton, 1985b), iodide quenching experiments conducted at constant ionic strength were used to compare the accessibility of camptothecin when free in solution versus situations where drug was associated with membrane. Aqueous drug stock solutions were added to aliquots of liposome preparations such that drug, lipid, and salt concentrations were 5 μM, 0.29 M, and 0.5 M, respectively. Solutions also contained 2 mM sodium thiosulfate to prevent the oxidation of iodide. Iodide concentration was varied from 0 to 0.5 M. The DMPC lipid concentration of 0.29 M utilized in these experiments assured a bound drug fraction in excess of 97%. A modified form of the Stern–Volmer relationship was employed to analyze the static and

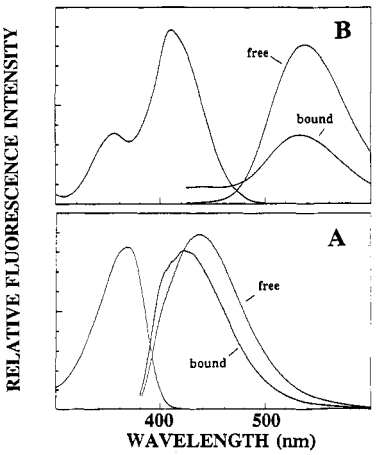

FIGURE 1: Fluorescence excitation and emission spectra of camptothecin (bottom panel) and topotecan (top panel). The relative fluorescence intensities of 1 μM drug in PBS buffer and in DMPG SUVs at 37 °C are shown. The emission spectra of camptothecin were recorded using exciting light of 360 nm, while exciting light of 390 nm was used to record the emission spectra of topotecan. Shown in each panel are emission spectra of drug free in solution as well as that for drug bound to DMPG SUVs at a lipid concentration of 0.29 M. Excitation spectra were recorded using samples of drug free in PBS solution.

dynamic quenching processes (Eftink & Ghiron, 1976):

$$F_0/Fe^{v[Q]} = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher concentration ([Q]), K_{SV} is the collisional or dynamic quenching constant, and V is the static quenching constant. Quenching data were fit to this equation by determining the value of V which yielded the line with the best correlation coefficient for the plot of $F_0/Fe^{V[Q]}$ vs [Q]. Under the experimental conditions described above, camptothecin's excited-state lifetime (τ) value for drug free in solution was determined using a 200-MHz frequency-domain fluorometer (Laczko et al., 1990) to be 4.2 ns, similar to the average τ value for membrane-bound drug (3.7 ns). Since τ remains relatively constant upon drug association with membrane, and $K_{SV} = \tau k_q$, where k_q is the collisional rate constant, the observed differences in K_{SV} between free and membrane-bound species (as depicted in Figure 7) are dominated by differences in the collision rate constant (k_q).

RESULTS

Spectral Characteristics of Camptothecin Drugs Free in Solution and Bound to Membranes. The fluorescence excitation and emission spectra of camptothecin and topotecan in PBS (pH 7.4) and the emission spectra of these drugs when bound to fluid-phase DMPG SUVs are shown in Figure 1. A decrease in the emission intensity is observed for both camptothecin and topotecan upon binding the DMPG bilayers. While the emission spectrum of camptothecin is shifted to lower wavelength (blue-shifted) upon associating with the membrane (the change in emission λ_{MAX} between the free and membrane-bound forms of camptothecin is 16 nm), only slight shifting (approximately 4 nm) in the emission spectrum of topotecan is observed upon association with DMPG membranes. Since blue shifting of a fluorophore upon membrane binding is thought to involve relocation to a microenvironment with a lower dielectric constant such as the acyl chain region of the membrane, our spectral data suggest that camptothecin penetrates more deeply into DMPG bilayers relative to its positively-charged analogue topotecan.

Table II: Fluorescence Spectral Parameters for Camptothecin Drugs Free in Solution and Bound to DMPC and DMPG SUVs[a]

compound	pH	λ_{MAX} excitation (nm)	λ_{MAX} emission (nm)			relative fluorescence[b]
			PBS	DMPC	DMPG	
camptothecin	7.4	366	438	433	422	1
camptothecin carboxylate	7.4	377	443	437	435	1
10-hydroxycamptothecin	7.4	392	561	558	550	0.5
topotecan	7.4	410	537	538	533	1
9-aminocamptothecin	7.4	381	439	442	436	0.01
9-aminocamptothecin	3.0	381	464	477		0.1
9-nitrocamptothecin	7.4	378	456			0.001
10,11-methylenedioxycamptothecin	7.4	395	410	408	408	1
9-amino-10,11-methylenedioxycamptothecin	7.4	404	550			0.002
9-amino-10,11-methylenedioxycamptothecin	1.5	373	430			0.06
9-chloro-10,11-methylenedioxycamptothecin	7.4	394	410		417	1

[a] All spectra were recorded as described in the Materials and Methods section using drug concentrations of 1 μM and DMPC and DMPG concentrations of 0.29 M. Experiments were conducted in PBS buffer at 37 °C. [b] These are approximate values, determined in PBS at each drug's excitation and emission maxima, while the voltage to the phototube was held constant. Available samples were often too small (<1 mg) to allow for elaborate efforts to remove residual moisture prior to weighing.

Table II summarizes the fluorescence spectral parameters for camptothecin, toptecan, and seven other camptothecin analogues both free in solution and bound to DMPC and DMPG SUV preparations. The table compares the excitation and emission λ_{MAX} for drugs free in solution and bound to both DMPC and DMPG membranes. Also found in Table II is a relative index of the emission intensity levels from these analogues in PBS at their respective excitation and emission maxima (λ_{MAX}).

Camptothecin, topotecan, camptothecin carboxylate, 10-hydroxycamptothecin, 10,11-methylenedioxycamptothecin, and CMC all exhibit strong fluorescence emission in PBS at pH 7.4. In contrast, 9-amino- and 9-nitro-substituted derivatives do not fluoresce as efficiently. 9-Nitro substitution of camptothecin results in an approximate 1000-fold reduction in the emission intensity level at pH 7.4 relative to the parent drug molecule (Table II), while 9-amino substitution of camptothecin or 10,11-methylenedioxycamptothecin reduces the fluorescence emission (pH 7.4) at least 100-fold in each case.

9-Amino substitution of camptothecin drugs results in congeners that display very pH-sensitive emission spectra. For example, Figure 2 depicts the strong dependence on pH observed in the fluorescence emission spectra of the 9-amino derivative of camptothecin. Panel B shows spectra for drug at pH 7.4 (PBS, 37 °C), while panel A shows data taken under identical experimental and instrumental conditions, with the exception that the pH of the buffer has been reduced to a value of 3.0. Note that the fluorescence intensity scale in panel A is 10 times greater than that in panel B. A similar pH dependency was observed for the 9-amino derivative of 10,11-methylenedioxycamptothecin. Thus, apparent protonation of the 9-amino groups of camptothecin derivatives upon lowering the pH to values between 2 and 3 results in strong increases in fluorescence quantum yields. In contrast with the strong blue-shifting of the emission spectrum of the 9-amino-10,11-methylenedioxy analogue upon protonation (data not shown), the spectrum of 9-aminocamptothecin in PBS red-shifts some 25 nm in λ_{MAX} value upon protonation of the 9-amino functionality (Figure 2).

Figure 2 also displays how associations with lipid bilayers composed of DMPC, both at pH 7.4 and at pH 3.0, modulate the spectral characteristics of 9-aminocamptothecin. Shown in each panel are the emission spectra of identical concentrations of drug, both free and membrane-bound. Both spectra show a reduction in emission intensity upon association with membrane. At pH 7.4 there is a slight red-shifting of the

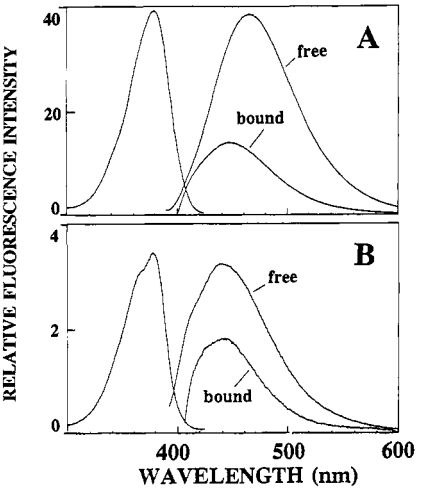

FIGURE 2: Fluorescence excitation and emission spectra of 9-aminocamptothecin at pH 3.0 (panel A) and pH 7.4 (panel B). Note that the relative fluorescence intensity scale for each panel is different, the ionized form of the drug (panel A, pH 3) being approximately 10-fold more fluorescent than the unionized form (panel B, pH 7.4). Drug and DMPC lipid concentrations used in the experiments were 1 μM and 0.29 M, respectively. An excitation wavelength of 380 nm was employed.

spectrum upon binding (the emission λ_{MAX} value changes from 439 nm in PBS to 442 nm in membrane), while at pH 3.0 a more significant spectral shift of 13 nm occurs upon membrane binding, but the shift is toward the blue region of the spectrum.

Comparison of the data presented in Table II thus clearly indicates that substitution of the quinoline residue of camptothecin at the 9 and 10 positions strongly modulates the drug's fluorescence emission characteristics. Conversion of camptothecin to the 9-amino derivative makes this agent 100-fold less fluorescent at pH 7.4, although the loss of fluorescence intensity can be regained partially by lowering solution pH. Table II also shows that substitution of a hydroxy substituent into camptothecin at the 10 position (10-hydroxycamptothecin) strongly red-shifts the fluorescence emission maximum of camptothecin over 120 nm. Topotecan, which also contains a 10-hydroxy moiety, also emits at longer wavelengths relative to the other compounds; the emission λ_{MAX} value for topotecan in PBS is approximately 100 nm higher than that for camptothecin.

Of the drugs studied, camptothecin's emission is the most sensitive to associations with membranes, its spectrum blue-shifting 16 nm upon association with DMPG and 5 nm for

DMPC. The spectral shifts observed during membrane association for other drugs such as 10,11-methylenedioxycamptothecin and topotecan were less sensitive relative to the changes observed for camptothecin.

Fluorescence Anisotropy Titration as a Method for Determining the Equilibrium Association Constants of Camptothecin Drugs for Lipid Vesicles. A steady-state fluorescence anisotropy (a) measurement is related to the rotational rate of the fluorescent molecule through the Perrin equation:

$$a_0/a = 1 + (\tau/\phi) \quad (3)$$

where a_0 is the limiting fluorescence anisotropy in the absence of depolarizing rotations, τ is the excited-state lifetime, and ϕ is the rotational correlation time of the fluorophore. Equation 3 states that changes in either the τ or ϕ values of a fluorescent compound can modulate its steady-state anisotropy.

We have examined the excited-state liftime values of camptothecin in PBS, glycerol, and methanol at 37 °C. The lifetime values were determined to be 4.7, 3.8, and 3.5 ns, respectively. Similarly, we measured camptothecin's lifetime value when associated with DMPC bilayers at 37 °C, and the average value for membrane-bound drug was found to be 3.7 ns.

Thus the lifetime measurements described above indicate that camptothecin's excited-state lifetime is relatively insensitive to alterations in microenvironment (e.g., a change in solvent or fluorophore relocation from an aqueous milieu to a phospholipid membrane). For a fluorophore whose τ value remains relatively constant during a transition which strongly impacts on its rotational motion (such as a change in solvent viscosity or fluorophore binding to large macromolecular assemblies such as liposomal particles), the Perrin equation indicates a direct correlation between a and ϕ values will exist (i.e., as the ϕ value of the fluorescent compound increases, then so too does its steady-state anisotropy value).

As shown in Figures 3 and 4 and summarized in Table III, the steady-state fluorescence anisotropy values of the camptothecin analogues are highly sensitive to solvent viscosity and to associations with small unilamellar lipid vesicles. For example, topotecan has an a value of 0.008 in PBS, but its a value increases 9- and 40-fold in the viscous solvents octanol and glycerol, respectively. A 21-fold enhancement in the a value of camptothecin is observed upon binding of drug to vesicles composed of either DMPC or DMPG (Figure 4).

We have also made measurements on the limiting fluorescence anisotropy (a_0) values of the camptothecin drugs as well. These maximum values of a were obtained by dissolving the compounds in the viscous solvent glycerol and cooling the samples to temperatures below 0 °C. The data from these experiments are presented in Figure 4 and summarized in Table III. Under such experimental conditions where solvent viscosity is high and temperature is low, depolarizing rotations are at a minimum. By extrapolating these a values to −25 °C, a_0 values for the camptothecin drugs have been estimated, and these parameters are summarized in Table III.

Because of the sensitivity of a of the camptothecin drugs to membrane associations (Figure 3), we employed the method of fluorescence anisotropy titration to study the equilibrium binding of camptothecin analogues with lipid bilayers. As described both in the legend to Figure 3 and in the Materials and Methods section, the experiments consisted of determining the a values for a set of samples where the drug concentration in each was held constant (typically 2 μM), while the lipid concentration among the members of a set was varied from 0 to 0.29 M.

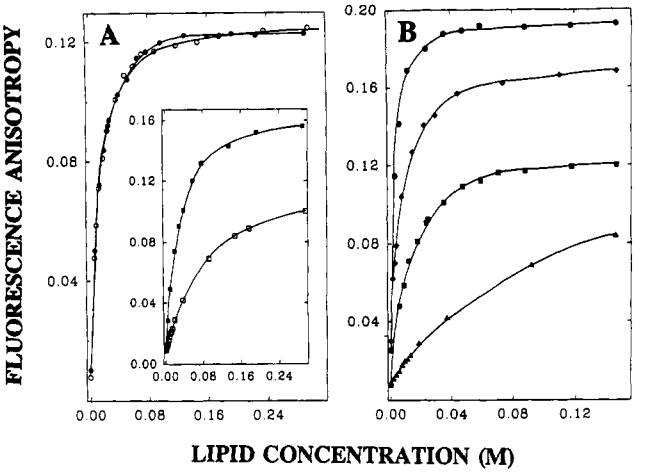

FIGURE 3: Equilibrium binding of camptothecin analogues to SUVs. The method of fluorescence anisotropy titration was used to construct the adsorption isotherms. Panel A compares the binding of camptothecin to DMPG (solid symbols) and DMPC (open symbols) with that of topotecan (inset). Note that topotecan preferentially binds negatively-charged DMPG (solid symbols) relative to DMPC (open symbols), while camptothecin binds the two types of bilayers equally well. Panel B shows how modifications to ring A modulate drug binding to DMPG bilayers. Data are shown for CMC (●), 10,11-methylenedioxycamptothecin (◆), camptothecin (■), and topotecan (▲). Experiments were conducted at drug concentrations of 2 μM in PBS buffer 937 °C). Because of the instability of the lactone ring of the drugs under study, anisotropy values at each lipid concentration were determined immediately (approximately 30 s) following drug addition to the liposome suspension. Binding constants were determined using double-reciprocal plots (Figure 5) and the K values are summarized in Table IV.

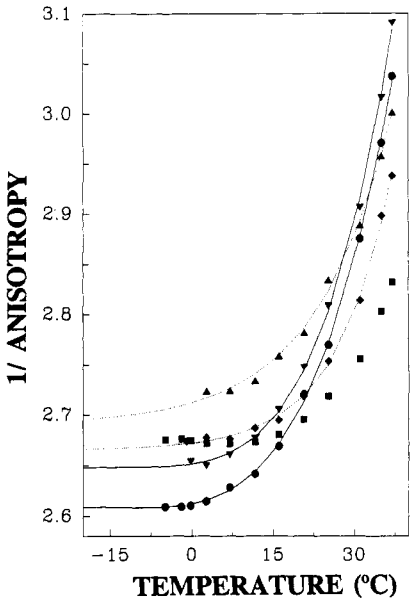

FIGURE 4: Plots of 1/anisotropy vs temperature for camptothecin (●), topotecan (▲), 10,11-methylenedioxycamptothecin (■), 10-hydroxycamptothecin (◆), and camptothecin carboxylate (▼) in the viscous solvent glycerol. By extrapolating to low temperature, the limiting anisotropy values (*i.e.*, anisotropy values in the absence of depolarizing rotations) were determined. These values are summarized in Table III.

From a spectroscopic viewpoint, this approach to constructing a binding isotherm is highly advantageous over its alternative: holding the lipid concentration constant and varying the drug concentration. The latter approach is complicated by self-association phenomena which occur for

Table III: Steady-State Anisotropy Values and Limiting Anisotropies of Camptothecin Analogues in Solvents and in Phospholipid Bilayers[a]

compound	solvents at 37 °C			membranes at 37 °C			
	PBS	octanol	glycerol	electroneutral	negatively-charged	solid-phase	a_0
camptothecin	0.007	0.051	0.334	0.127	0.122	0.087	0.383
camptothecin carboxylate	0.008	0.073	0.327	0.102			0.377
10-hydroxycamptothecin	0.021	0.127	0.331	0.191	0.201		0.372
topotecan	0.008	0.071	0.320	0.125	0.170	0.059	0.367
10,11-methylenedioxycamptothecin	0.021	0.089	0.357	0.168	0.206		0.374
9-aminocamptothecin	$0.009^{(pH3)}$			$0.190^{(pH3)}$			
9-amino-10,11-methylenedioxycamptothecin	$0.049^{(pH3)}$			$0.160^{(pH2)}$			
9-chloro-10,11-methylenedioxycamptothecin	0.025	0.098	0.363	0.202	0.207		0.367

[a] Steady-state anisotropy (a) values were determined on samples containing 1 μM drug at 37 °C, and the a values have been corrected for background scatter (<1% in most cases). Camptothecin, camptothecin carboxylate, and 10,11-methylenedioxycamptothecin were studied using λ_{EX} = 370 nm and 418-nm long-wave-pass filters on each emission channel. Topotecan samples were studied using λ_{EX} = 390 nm and 500-nm long-wave-pass filters on the emission channels. For camptothecin, the fluorescence intensity level from drug in PBS buffer exceeded 99.98% (or 0.02% scatter), while the fluorescence signal from the most concentrated membrane preparations (200 mg/mL lipid) ranged from 99.5% to 99%. Electroneutral, fluid-phase lipid bilayers were represented by small unilamellar vesicles comprised of dimyristoylphosphatidylcholine. Negatively-charged, fluid-phase bilayers were represented by dimyristoylphosphatidylglycerol SUVs. Electroneutral but solid-phase bilayers were represented by SUVs composed of distearoylphosphatidylcholine. Preparation of SUVs composed of the longer chain DSPC lipid required extensive sonication time in order to prepare; thus we limited our studies with these membranes to the camptothecin and topotecan analogues. Anisotropy values for membrane-bound drugs were determined by plotting $1/a$ vs 1[lipid] and extrapolating to infinite lipid concentration. Unless specified otherwise, measurements were made at pH 7.4. Anisotropy values have an uncertainty of ±5%.

the camptothecin drugs at concentrations in excess of 10 μM (Burke et al., unpublished results). Another complication which occurs using fluorescent material at high optical densities is the well-studied phenomenon known as inner filter effect (Lakowicz et al., 1983).

As a consequence of the brilliant fluorescence emission from camptothecin, camptothecin carboxylate, 10-hydroxycamptothecin, topotecan, 10,11-methylenedioxycamptothecin, and 9-chloro-10,11-methylenedioxycamptothecin, the adsorption isotherms presented in Figure 4 are relatively free from any background signal. Using drug concentrations of 2 μM and long-pass filters to isolate emitted light from background signal (i.e., scattered exciting light and extraneous fluorescence signal due to the possible presence of impurities), signal levels from drugs dissolved in PBS buffer were typically 99.97% in the absence of membrane and 99.3% in the presence of membrane.

Adsorption isotherms like those shown in Figure 4 were used to determine overall association constants for the camptothecin drugs. Overall association constants are defined as

$$K = [A_B]/[A_F][L] \quad (4)$$

where $[A_B]$ represents the concentration of bound drug, $[A_F]$ represents the concentration of free drug, and [L] represents the total lipid concentration in the vesicle suspension. This equation is valid when the concentration of free lipid is approximately equal to the concentration of total lipid (i.e., the concentration of free lipid is in great excess over the concentration of bound drug). Provided this condition is satisfied, K may be determined from the inverse of the slope of a double-reciprocal plot. In such a plot, 1/fraction of the total drug bound is plotted vs 1/lipid concentration, with a y-intercept value of 1 (for a system displaying binding-site homogeneity).

Such double-reciprocal plots for the associations of four camptothecin analogues with DMPC SUV preparations are shown in Figure 5. The linearity of these plots, we well as the corresponding plots for drug associations with other types of membrane preparations (data not shown), indicates that fluorophore binding at these lipid concentrations is adequately described by eq 2.

Modifications to Camptothecin's A Ring Strongly Modulate Lipid Bilayer Partitioning. The contents of Table IV examine the structural basis of camptothecin associations for

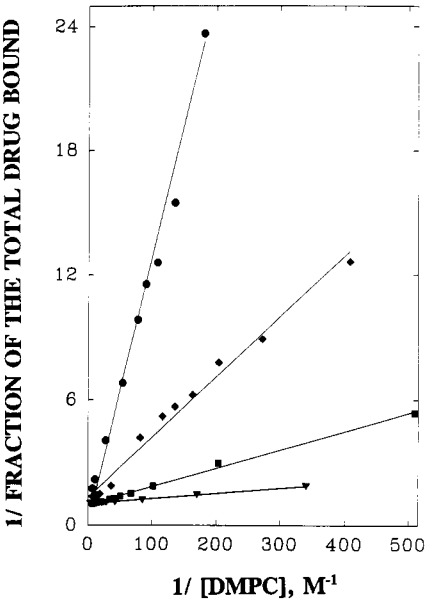

FIGURE 5: Double-reciprocal plots for the binding of camptothecin drugs to DMPC SUVs at 37 °C. Data are shown for CMC (▼), camptothecin (■), camptothecin sodium salt (◆), and topotecan (●). For additional experimental details, see the Materials and Methods section and the legend to Figure 3.

lipid bilayers. Three types of membrane were included in our studies which were conducted under near physiological conditions of pH and temperature; these membranes include fluid-phase and electroneutral L-α-dimyristoylphosphatidylcholine (DMPC), fluid-phase and negatively-charged L-α-dimyristoylphosphatidylglycerol (DMPG), and solid-phase and electroneutral L-α-distearoylphosphatidylcholine (DSPC). While DMPC and DMPG have identical chain length, the charges on their head groups differ. DMPC and DSPC have identical head groups, but the former has acyl chains which are four carbons shorter than the latter (14 carbons per chain versus 18 carbons per chain). This difference of 4-carbon chain length results in DMPC being fluid-phase at 37 °C while DSPC is solid-phase at this temperature.

Of the seven compounds studied, the new compound 9-chloro-10,11-methylenedioxycamptothecin (CMC) synthesized recently by Wall et al. (unpublished methods) was found to display the highest membrane affinities (K_{DMPC} = 400 M^{-1}, K_{DMPG} = 320 M^{-1}), followed by 10,11-methylenedioxycamp-

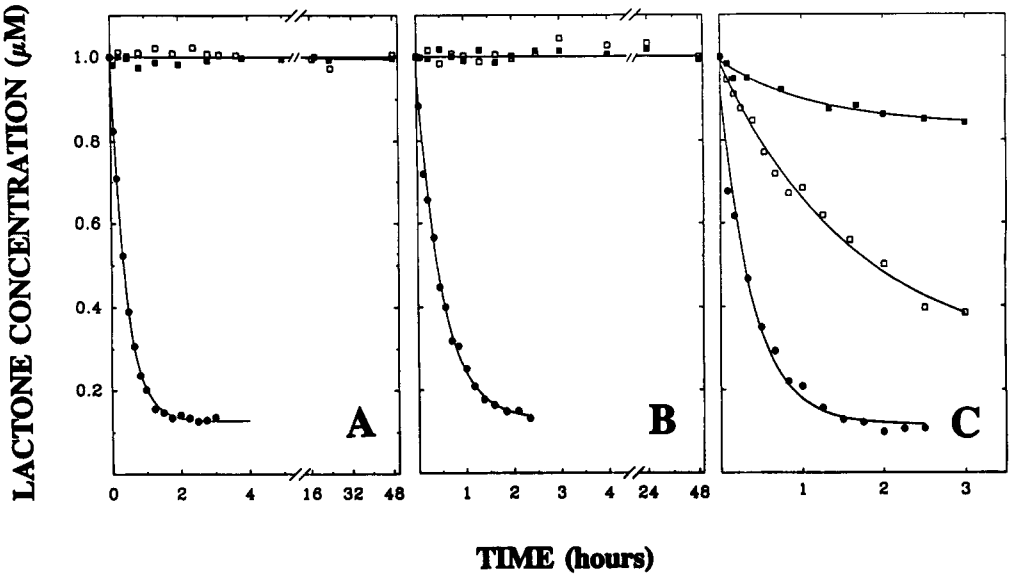

FIGURE 6: Kinetic evaluation of the rate of lactone ring opening for camptothecin drugs free in PBS solution and in the presence of DMPC or DMPG SUVs at 37 °C. Lactone concentration vs time profiles are shown for camptothecin (panel A), CMC (panel B), and topotecan (panel C). For each panel, solid circles represent data for drug free in solution, while the open and solid squares represent data for drug in the presence of DMPC and DMPG liposomes, respectively. The $t_{1/2}$ values for the hydrolysis of free drugs are summarized in Table V. Drug and lipid concentrations used in all experiments were 1 μM and 0.29 M, respectively. Under these conditions, the lipophilic drugs camptothecin and CMC were stable in the presence of membrane. In contrast, topotecan (93% bound in the presence of DMPG, 74% bound in the presence of DMPC) shows some hydrolysis at lipid concentrations of 0.29 M.

Table IV: Overall Association Constants for Camptothecin Analogues Interacting with Unilamellar Vesicles Composed of Electroneutral DMPC, Negatively-Charged DMPG, and Solid-Phase DSPC in PBS Buffer at pH 7.4 and 37 °C[a]

	K (M^{-1})		
compound	electro-neutral	negatively-charged	solid-phase
9-chloro-10,11-methylenedioxycamptothecin	400	320	
10,11-methylenedioxycamptothecin	190	130	
camptothecin	100	100	300
10-hydroxycamptothecin	75	75	
camptothecin carboxylate	40		
9-aminocamptothecin	25(pH3)		
topotecan	10	50	50

[a] Electroneutral membranes are represented by DMPC SUVs, negatively-charged are represented by DMPG SUVs, while solid-phase bilayers are represented by DSPC SUVs. Binding isotherms were constructed using the method of fluorescence anisotropy titration, and K values determined from the slopes of double-reciprocal plots as described in the Materials and Methods section. The K values are subject to 10% uncertainty. 9-Aminocamptothecin was studied at pH 3, where the agent is more fluorescent than at pH 7.4.

tothecin and camptothecin, which gave K_{DMPC} and K_{DMPG} values of 100 M^{-1} or greater. Conversion of camptothecin to its carboxylate form (opened ring structure) was found to result in a greater than 2-fold reduction in binding for electroneutral DMPC membranes. Topotecan, a positively-charged analogue, was found to display a significant 6-fold reduced ability to bind negatively-charged bilayers relative to CMC (topotecan K_{DMPG} = 50 M^{-1}). An even greater disparity (approximately 40-fold) was noted between topotecan and CMC in their association constants for electroneutral DMPC bilayers (topotecan K_{DMPC} = 10 M^{-1}). Of the camptothecin compounds included in this study, topotecan was the only agent found to display a strong binding preference for negatively-charged membranes relative to electroneutral membranes (Table IV). The binding preference of topotecan for negatively-charged membranes was determined to be 5-fold.

Drug associations with solid-phase DSPC bilayers were also studied for two of the camptothecin drugs, specifically camptothecin and topotecan, and the K values are listed in Table IV. Both of these drugs display binding preferences (5- and 3-fold, respectively) for solid-phase DSPC bilayers over fluid-phase DMPC bilayers. Similar binding preferences for solid-phase bilayers have been observed previously for several members of the anthracycline anticancer drug family (Burke and Tritton, 1985a).

Overall, the most striking feature of the data contained in Table IV is the strong modulation which can be achieved through substitution at the 9 and 10 positions of ring A. For the seven compounds studied, the range in K_{DMPC} values span a 40-fold range, while a greater than 6-fold span is observed in the K_{DMPG} values.

Enhanced Stabilities of Camptothecin Drugs in Lipid Bilayers. To study the consequences of membrane binding on the stabilities of the camptothecin drugs, HPLC assays were developed for each drug in order to separate the lactone form from the carboxylate form. In this manner stability profiles were determined for drugs both free in solution and bound to DMPC and DMPG bilayers. Experiments were conducted under near-physiological conditions of pH, temperature, and ionic strength. Samples were monitored at time points from 0 min (immediately following dissolution) to 48 h.

The kinetics of lactone ring hydrolysis for camptothecin, CMC, and topotecan are shown in panels A–C of Figure 6, and half-lives and equilibrium carboxylate:lactone ratios are summarized in Table V. Analysis of the decay profile for each analogue free in PBS was accomplished using a nonlinear least-squares program (as described in the Materials and Methods section). Accordingly, the half-life ($t_{1/2}$ value) of camptothecin in PBS (pH 7.4) was determined to be 17 min, with a final equilibrium for the carboxylate:lactone ratio of 87:13 being determined. The CMC analogue, as depicted in Figure 6, panel B, displays a $t_{1/2}$ value of 19 min and an equilibrium carboxylate:lactone ratio of 86:16. In comparison with the data obtained for camptothecin and CMC, the hydrolysis of topotecan in PBS (shown in panel C of Figure 6) is somewhat slower. Topotecan's $t_{1/2}$ value in PBS (pH

Table V: Summary of the Kinetic and Equilibrium Parameters for the Hydrolysis of Camptothecin Drugs in PBS Buffer and Bound to DMPC and DMPG Membranes[a]

compound	solvent/ suspension	$t_{1/2}$	% lactone form at equilibrium
camptothecin	PBS	17 min	13
	DMPC	>3 days	~100
	DMPG	>3 days	~100
9-chloro-10,11-methylenedioxycamptothecin	PBS	19 min	14
	DMPC	>3 days	~100
	DMPG	>3 days	~100
topotecan	PBS	21 min	14
	DMPC	80 min	21
	DMPG	130 min	65

[a] The hydrolysis of drug was monitored using HPLC assays as described in the Materials and Methods section. The $t_{1/2}$ and % lactone form at equilibrium values were determined from decay profiles by the method of nonlinear least-squares (as described in the Materials and Methods section). Drug and lipid concentrations of 1 μM and 0.29 M, respectively, were employed in these studies. The $t_{1/2}$ values are subject to 3% uncertainty. All experiments were conducted in PBS at 37 °C.

7.4) is 21 min, with a final equilibrium carboxylate:lactone ratio of 86:14. Since CMC is the most hydrophobic of the agents analyzed (as determined by the degree of retention on a reversed-phase HPLC column using identical mobile-phase conditions for each drug; CMC is also a lipophilic drug as shown in Table IV), followed by camptothecin and then topotecan, no physical correlation is apparent between the relative hydrophobicities of the compounds of interest and their half-lives.

Also depicted in Figure 6 and summarized in Table V are the $t_{1/2}$ values and final equilibrium carboxylate:lactone ratios for camptothecin, CMC, and topotecan when associated with DMPC and DMPG SUVs at 37 °C. Lipid concentrations of 0.29 M DMPC were employed, concentrations which resulted in bound drug levels for CMC, camptothecin, and topotecan of 99%, 97%, and 74%, respectively (values of 99%, 97%, and 93%, respectively, for drugs associated with 0.29 M DMPG). The lipophilic and essentially completely bound analogues CMC and camptothecin were found to be stable, even at observation times as long as 72 h.

Samples containing the less lipophilic drug topotecan did show detectable drug hydrolysis in the presence of 0.29 M lipid. At these lipid concentrations of 0.29 M, and using the K_{DMPC} and K_{DMPG} values for topotecan of 10 M^{-1} and 50 M^{-1}, respectively (Table VI), the bound drug levels in these experiments for topotecan are estimated to be 74% for DMPC and 93% for DMPG. Consistent with these predicted bound drug levels is the observation that topotecan is stabilized to a greater degree in DMPG suspensions vs DMPC suspensions (see Figure 6, panel C).

The membrane stabilities of each of the other drugs listed in Table IV were examined in an identical manner to the experiments described in Figure 6. In the presence of 0.29 M DMPC or DMPG, the lipophilic analogue 10,11-methylenedioxycamptothecin was found to be stable, with no evidence of hydrolysis in observation times extending out to 72 h (data not shown). The presence of membrane was also found to stabilize the lactone ring of 9-aminocamptothecin. However, with this less lipophilic analogue, some hydrolysis was observed upon standing at 37 °C in suspensions containing 0.29 M lipid. In the presence of DMPC and DMPG, 9-aminocamptothecin displayed half-lives of 56 and 50 min, respectively, with equilibrium carboxylate:lactate ratios of 14:86 and 29:71, respectively.

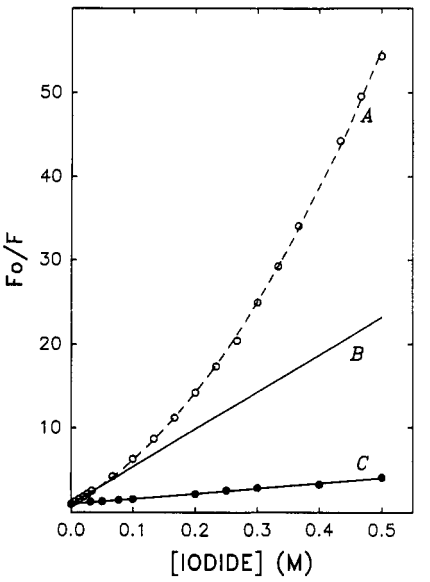

FIGURE 7: Iodide quenching of camptothecin free in solution (A) and bound to DMPC bilayers (C). Experiments were conducted at 37 °C in PBS buffer maintained under a constant halide concentration of 0.5 M. The dashed line (plot A) represents the static and dynamic quenching components for drug free in solution as determined by the F/F_0 values, while plot B represents only the dynamic component for drug free in solution (K_{SV} = 44.3 M^{-1}, where V = 1.4 M^{-1}). Plot C shows the quenching profile of membrane-bound drug, where V was assumed to be 0 and the K_{SV} was calculated to be 6.2 M^{-1}.

To assess the impact of raising drug levels on the stabilization process for camptothecin, both MLV and SUV suspensions were prepared containing the same 0.29 M lipid concentration as used in previous experiments, but the camptothecin drug concentration was raised to 2 mM. Despite the 1000-fold increase in drug concentration, predicted bound drug levels for these experiments remain at 97% since the 0.29 M lipid remains in great excess and dominates the binding equilibrium. At the higher drug concentrations (lipid:drug ratios of 150), no evidence of lactone ring opening was observed for camptothecin (data not shown).

Quenching Studies on the Binding Site Location of Camptothecin in Lipid Bilayers. The fact that liposome-associated camptothecin is stable suggests that the drug's lactone ring penetrates into the bilayer where it has significantly reduced contact with water molecules. Two types of spectroscopic data are available which indeed reinforce the notion that camptothecin's chromophore locates deep within the lipid bilayer. The first type of evidence comes from Figure 1, where a blue-shifting of the drug's emission spectrum is observed upon association with membrane. Such a spectral shift is indicative of a change in the dielectric constant of the medium surrounding the fluorophore (Lakowicz et al., 1983), as when a compound leaves an aqueous environment and intercalates between the acyl chains of a phospholipid membrane.

Additional evidence that camptothecin's fluorochrome penetrates into the lipid bilayer and is thus removed from the aqueous environment at the membrane/water interface comes from iodide quenching data (shown in Figure 7). These experiments were conducted under conditions of constant ionic strength as described previously (Burke & Tritton, 1985b). Iodide has an immeasurably small permeation of the bilayer (Jendrasiak, 1972; Cranney et al., 1983) and its thus able to discriminate between drug molecules free in solution and those located in the hydrophobic interior of the membrane. As described in the Materials and Methods section, a modified

form of the Stern–Volmer relationship was used to analyze the quenching data in order to ascertain the static (V) and dynamic (K_{SV}) quench components. While camptothecin free in solution was quenched readily by iodide (V = 1.8 M^{-1}; K_{SV} = 44.3 M^{-1}), drug bound to DMPC membranes was much more difficult to quench (V = 0 M^{-1}; K_{SV} = 6.2 M^{-1}). Our data suggest that iodide can form a static complex with drug only when the fluorophore is free in solution. In a similar manner, the dynamic quenching effects of iodide on camptothecin emission are more efficient for drug free in solution rather than membrane-bound. It can thus be concluded that membrane-bound drug is much less accessible to both types of quenching by iodide, presumably because the fluorochrome locates deep with the bilayer.

DISCUSSION

Relationship between Drug Structure and Membrane Affinity. Camptothecin is an electroneutral compound, relatively planar in nature, consisting of five ring systems. It possesses few polar substituents other than an α-hydroxy substituent on ring E, and the poor water solubility of the compound has been well documented. In our studies we find that camptothecin in the presence of 0.29 M lipid SUV suspensions partitions efficiently from aqueous solution into lipid bilayer.

The data summarized in Table IV indicate that camptothecin displays an identical overall association constant of 100 M^{-1} for small unilamellar vesicles composed of either electroneutral DMPC or negatively-charged DMPG. The fluorescence methodologies utilized in this study to characterize the equilibrium associations of camptothecin with membrane vesicles have been used previously to study the membrane interactions of other intrinsically fluorescent drug molecules. For the sake of comparison, the measured affinities of camptothecin for DMPC and DMPG in PBS at 37 °C exceed the values observed for the fluoroquinolone antimicrobial agent ciprofloxacin (K_{DMPC} = 10 M^{-1}, K_{DMPG} = 80 M^{-1}; Burke et al., 1992), but camptothecin's membrane affinity values do not exceed the K_{DMPC} and K_{DMPG} values of 200 M^{-1} and 3400 M^{-1}, respectively, displayed by the anthracycline anticancer drug doxorubicin (Burke & Tritton, 1985; Burke et al., 1988). Both ciprofloxacin and doxorubicin contain positively-charged amine moieties at physiological pH, resulting in both agents displaying marked binding preferences for negatively-charged phospholipids (i.e., 8-fold preference exhibited by ciprofloxacin and 17-fold preference exhibited by doxorubicin). No such binding preference for negatively-charged bilayers is observed for camptothecin, as well as the other electroneutral congeners of the parent drug (Table IV); each agent partitions with approximately equal affinity into electroneutral and negatively-charged bilayers.

Topotecan, however, is a camptothecin congener which displays a significant 5-fold binding preference for negatively-charged membrane. This agent contains a positively-charged dimethylaminomethyl group which promotes drug interactions with negatively-charged membranes through favorable electrostatic interactions. It is interesting to note that the binding preference of topotecan for negatively-charged membranes is 5-fold, not 17-fold as observed in the case of doxorubicin but closer to the 8-fold binding preference exhibited by ciprofloxacin. This can be explained by the fact that topotecan displays a disubstituted amine functionality, while doxorubicin displays an unsubstituted primary amino group. Like topotecan, ciprofloxacin also displays a substituted amine group. The K_{DMPC} and K_{DMPG} values for the dimethylamino analogue of doxorubicin have been shown previously to be 90 M^{-1} and 600 M^{-1}, respectively; thus dimethylation of the amino group of doxorubicin results in an attenuated binding preference (6.7-fold from 17-fold) for negatively-charged membrane, more akin to the 5-fold binding preference displayed by topotecan. These data concerning a reduction in doxorubicin's affinity for negatively-charged membrane indicate unfavorable steric factors caused by the substitution of two methyl moieties onto the amine nitrogen counteract the favorable electrostatic interactions between the positively-charged nitrogen and the negatively-charged headgroup of the phospholipid. The biological significance of topotecan's binding preference to negatively-charged membrane on the drug's spectrum of biological activities remains to be determined.

Examination of the data presented in Table IV indicates that substitutions in ring A of camptothecin strongly modulate lipid bilayer associations. For drug interactions with electroneutral membranes, addition of the 10,11-methylenedioxy functionality enhances binding 2-fold relative to camptothecin; the presence of a 9-chloro substituent together with the 10,-11-methylenedioxy functionality enhances binding 4-fold. Addition of the 10-hydroxy functionality plus a 9-dimethylaminomethyl functionality (i.e., topotecan) results in a dramatic 10-fold reduction in the compound's K_{DMPC} value. Overall, our data indicate that through substitution of the A ring of camptothecin membrane binding can be modulated over a 40-fold range. Comparison of the K values between camptothecin and camptothecin carboxylate indicates that there is a considerable loss of affinity for membranes upon ring opening (camptothecin's K_{DMPC} = 100 M^{-1}; camptothecin carboxylate's K_{DMPC} value is 40 M^{-1}).

It is also apparent from Table IV that a binding preference for solid-phase bilayers exists for the camptothecins. In our studies DMPC bilayers were chosen to represent fluid-phase bilayers in that at 37 °C this lipid is above its gel-to-liquid crystalline phase transition temperature. In contrast, DSPC lipid is below its phase transition temperature at 37 °C and thus represents solid-phase bilayers. For the two drugs we chose to study, camptothecin and topotecan, strong binding preferences of 3- and 5-fold, respectively, were noted for drug associations with solid-phase bilayer. A similar 2–3-fold binding preference for solid-phase bilayers over fluid-phase bilayers has been noted previously for the anthracycline drugs (Burke & Tritton, 1985a). Accordingly, like the anthracyclines, the camptothecin drugs can be expected to display enhanced binding to the more ordered lipid bilayer domains of biomembranes.

Relationship between Membrane Affinity and Lactone Ring Stability. In this report we have demonstrated that membrane associations of the camptothecins act to conserve the biologically-active, lactone form of the drug. Iodide quenching studies were useful in demonstrating that membrane-bound drug inserts deeply in the bilayer. The results of the iodide quenching studies thereby provide a physical explanation (i.e., location of the drug in the acyl chain region of the bilayer, in a nonaqueous milieu well removed from the aqueous surface) for the observed stabilities of the lactone ring of membrane-bound camptothecins.

As shown in Figure 3, high concentrations of lipid (0.29 M) are required to achieve a greater than 97% bound drug fraction for camptothecin. For the more lipophilic analogues such as 10,11-methylendioxycamptothecin and 9-chloro-10,11-methylenedioxycamptothecin, the 97% bound drug level is attainable using lower lipid concentrations; for other less lipophilic agents, such as 9-aminocamptothecin and topotecan,

greater lipid concentrations are required to reach this level of binding. While the lipophilic drugs camptothecin, 10,11-methylenedioxycamptothecin, and 9-chloro-10,11-methylenedioxycamptothecin all exhibit stability in the presence of 0.29 M lipid, the other less lipophilic agents hydrolyze at this lipid concentration.

With respect to the instability of the less lipophilic topotecan analogue in the presence of membranes (Table V), this agent is not completely bound at lipid levels of 0.29 M (74% of the drug is bound in the experiments using DMPC, while 93% of the drug is bound for the experiments utilizing DMPG). The consequences of the incomplete binding of topotecan in the DMPC and DMPG membrane systems are clearly evident in the data shown in Figure 6, with substantial conversion of topotecan to its carboxylate form, especially in the DMPC system. The instability of topotecan under these experimental conditions is apparently due to its reduced level of lipophilicity. Preparation of SUV suspensions at lipid concentrations in substantial excess of the 0.29 level is difficult, if not unfeasible using our existing equipment, and thus we did not attempt to conduct stability studies at higher lipid concentrations.

From a physiological viewpoint, consideration of the stability of camptothecin, topotecan, and related analogues at lipid concentrations in the 4–8 mg/mL range is instructive in that these are the total lipid levels normally found in blood (Burke, 1980). Using the K_{DMPC} values (Table IV) for camptothecin, CMC, and topotecan and the blood lipid values given above, we calculate that the bound drug fractions will range from 38% to 55% for camptothecin, from 71% to 83% for CMC, and from 6% to 11% for topotecan. Although in blood the situation is clearly more complex due to the presence of many other types of macromolecules to which the camptothecins can bind, our results do predict that the levels of lipid bilayer interactions among the members of the family will be very different. Since lipid bilayer interactions result in a stabilization of the lactone ring, exploiting the lipophilic character of the camptothecins may be a potentially important means of enhancing the half-lives of these medications in circulation. A systematic study of the half-lives of the camptothecin drugs in blood is presently in progress in this laboratory.

Currently we are also utilizing the fluorescence properties of the camptothecins to characterize their interactions with purified blood components such as albumin and globulin proteins. In contrast to the stabilization of the lactone ring in the presence of lipid bilayers, HPLC analyses have shown that the presence of human serum albumin (HSA) at physiologically relevant concentrations (20–50 mg/mL) significantly shorten the half-lives of camptothecin ($t_{1/2}$ = 12 min), 10,11-methylendioxycamptothecin ($t_{1/2}$ = 13 min), and 9-chloro-10,11-methylendioxycamptothecin ($t_{1/2}$ = 13 min) relative to half-lives of approximately 18–20 min in the absence of HSA; HSA also shifts the final equilibrium for camptothecin, 10,11-methylendioxycamptothecin, and CMC almost completely (>99%) in favor of the carboxylate form (Burke et al., 1993). Spectroscopic investigations have shown that the carboxylate form of these drugs preferentially binds the HSA protein in a dramatic manner (e.g., camptothecin carboxylate's apparent affinity for HSA is approximately 150-fold greater than the K value observed for its lactone form), thereby providing a physical explanation for the rapid conversion of lactone to carboxylate which occurs in the presence of HSA. Albumin has long been known to selectively bind fatty acids, and our findings concerning the selective binding of carboxylate forms of camptothecins to HSA parallel the results concerning the preferential binding of fatty acids to this important blood protein. Consistent with stability assays completed in the presence of purified albumin, camptothecin's half-life in blood is considerably shorter than the $t_{1/2}$ value observed in aqueous buffers (Scott et al., 1992).

Quite remarkably, one structural analogue of camptothecin, topotecan, does not exhibit enhanced destabilization in the presence of HSA (T. Burke, unpublished results). Neither topotecan nor its carboxylate form binds HSA as do camptothecin or camptothecin carboxylate according to excited-state lifetime titration experiments. Thus our results concerning the interactions of various analogues with HSA indicate that structural modification of camptothecin can limit drug interaction with HSA.

In summary, two clear paths directed toward extending the half-lives of camptothecins in blood seem to exist. The first of these, as described in this report, is to promote the lipophilic nature of the drugs, thereby favoring drug interactions with lipid bilayers which stabilize the lactone ring structure. Camptothecin is poorly water-soluble, and hence delivery systems such as liposomes may need to be considered for even more hydrophobic analogues. Another approach to extending the half-life of camptothecin is to include structural moieties such as those displayed by topotecan which limit drug interactions with HSA, thereby limiting the shift in the lactone $\rightleftharpoons$ carboxylate equilibrium due to the presence of HSA and resulting in longer half-lives.

Either electrostatic considerations due to the presence of the positively-charged 9-dimethylaminomethyl group, or steric considerations due to the presence of the amino group or the 10-hydroxy functionality or both, are responsible for topotecan's stability in the presence of HSA (relative to that of camptothecin). It is possible that lipophilic analogues of topotecan can be developed which retain either or both of these functionalities. These agents may, like topotecan, display limited interactions with HSA, but due to more favorable interactions with lipid bilayers greater overall stability in blood may be displayed. The topoisomerase II poison doxorubicin, like topotecan, also contains a positively-charged amino group; substitution of doxorubicin's amino group with a benzyl moiety enhances the drug's affinity for DMPC bilayers some 7-fold while conserving the drug's positively-charged nature (Burke et al., 1989). Such a finding suggests that topotecan's affinity for lipid bilayers can be enhanced without loss of its positive charge. A benzyl derivative of topotecan may result in enhanced stability due to favorable interactions with lipid bilayers but limited destabilization by the presence of HSA since the congener still displays both the dimethylaminomethyl and 10-hydroxy functionalities. It is important to note, however, that drug interactions with the topoisomerase target may be negatively or positively affected through such structural substitutions (Jaxel et al., 1989).

Interrelationship of Membrane Affinity, Cellular Uptake, and Cytotoxic Potency. Camptothecins, like the majority of other small drug molecules, are thought to accumulate in cells by passive diffusion. Thus, in order for a drug to access the cytoplasm the agent must initially bind the outer lipid bilayer leaflet of the plasma membrane. Following the binding event, the agent must diffuse through the lipid bilayer acyl chain region of low dielectric constant and by doing so relocate to the inner leaflet of the membrane. The drug is then able to diffuse from the inner leaflet of the plasma membrane into the cytosol and subsequently interact with its target protein, topoisomerase I.

The consequences of lactone ring opening prior to drug interactions with the topoisomerase I target will greatly affect

camptothecin's cytotoxic potency. A closed lactone ring is widely regarded as being an essential pharmacophore for activity against cancer cells, and ring opening for camptothecin is thought to result in a loss of potency for the following three reasons. First of all, the carboxylate form displays decreased membrane associations as demonstrated in this report. Comparison of the membrane affinity of camptothecin with that of its carboxylate form (Table IV) indicates that ring opening results in diminished membrane associations by a factor greater than 2-fold. Second, ring opening results in a charged drug species, and charged species exhibit limited diffusibility through lipid bilayer domains of low dielectric constant; hence, ring opening of camptothecins results in agents with altered diffusibility characteristics. Last, evidence from cell-free experiments which examined directly the structural basis of drug potency against the topoisomerase I target indicates that ring opening results in significantly attenuated activity (Jaxel et al., 1989). In summary, decreased membrane binding, decreased membrane diffusibility, and decreased intrinsic potency against the topoisomerase target all contribute to explain the reduction in cytotoxic activity which accompanies lactone ring opening for a camptothecin drug.

Because camptothecins are accumulated in cancer cells by passive diffusion, it follows that the lipid bilayer affinities of the camptothecins should be important determinants of net cellular accumulation and cytotoxicity. Comparison of our membrane binding data (Table IV) for the camptothecins with cellular uptake data available from the literature suggest that lipid bilayer affinities are indeed important factors influencing the biological activities of the camptothecins. Recent studies by Costin et al. (1992) have compared the cellular accumulation of 10,11-methylenedioxycamptothecin and 9-aminocamptothecin with that of camptothecin in CASE, SW-48, and HT-29 human colon cancer cells. They have shown that the lipophilic analogue 10,11-methylenedioxycamptothecin (K_{DMPC} = 190 M^{-1}, K_{DMPG} = 130 M^{-1}) displays significantly enhanced uptake levels relative to the less lipophilic analogues 9-aminocamptothecin and camptothecin (K_{DMPC} = K_{DMPG} = 100 M^{-1}). They also found that the 10,11-methylenedioxy analogue was the most potent analogue in the series, a finding most likely due to the following three factors: (1) enhanced cellular uptake due to enhanced lipophilicity (Table IV), (2) enhanced stability of the lactone due to its more favorable interactions with lipid bilayers, and (3) higher intrinsic potency of this congener against the topoisomerase I target (Jaxel et al., 1989).

In addition, Chen et al. (1991) have compared the potencies of several camptothecins against human KB carcinoma cells. Studies were conducted in a drug-sensitive parental cell line KB 3-1 as well as a multidrug-resistant phenotype KB V1. The order of potency of the agents in both cell lines was found to be 10,11-methylenedioxycamptothecin > camptothecin > 10-hydroxycamptothecin > topotecan. According to the data found in Table IV, the membrane affinities of these compounds appear in the same order. Hence a good correlation between membrane affinity and cytotoxic potency appears to exist, suggesting that lipid bilayer partitioning of the camptothecins is indeed an important parameter in determining relative cytotoxic potencies.

Whereas camptothecin, and other analogues which are electroneutral at physiological pH, display approximately equivalent potencies between the drug-sensitive KB 3-1 and the drug-resistant KB V1 cell lines, the positively-charged analogue topotecan displays a significant reduction in potency against the drug-resistant KB V1 cell line (Chen et al., 1991). A similar reduction in topotecan's potencies against multidrug-resistant phenotypes of human and hamster cell lines was observed by Hendricks et al. (1992). The inactivity of topotecan against the multidrug-resistant phenotypes has been attributed to the overexpression of the P-glycoprotein (PGP) transport protein in these cell lines and the finding that this agent apparently serves as substrate for the transport protein (Chen et al., 1991; Hendricks et al., 1992). Chen et al. (1991) speculate that the reduced net cellular accumulation of topotecan in multidrug-resistant cancer cells relative to camptothecin may be accounted for by two factors: (1) reduced drug influx due to the former's reduced lipophilic nature and (2) topotecan's positively-charged amino group [a positively-charged amino group is a structural feature of many of the agents thought to be removed from cancer cells by the PGP protein (Zamora et al., 1988)]. In our study we have determined that the lipophilic nature of topotecan, relative to camptothecin, is reduced 10-fold for DMPC lipid, thereby providing supporting evidence for the reduced drug influx theory.

However, differences in the lipid bilayer partitioning between these two agents alone cannot fully explain the biological data. Whereas topotecan's accumulation levels in MDR cells are sensitive to the presence of reversal agents such as verapamil and quinidine (Chen et al., 1991; Hendricks et al., 1992), camptothecin's accumulation is not (Chen et al., 1991). The reversal agents are thought to bind and inhibit PGP function (Zamora et al., 1988), and thus a role for PGP in topotecan's reduced cellular accumulation in MDR cells seems apparent (Chen et al., 1991; Hendricks et al., 1992).

Related to this point, differences between the lipid bilayer interactions of camptothecin and its positively-charged analogue topotecan are evident in spectroscopic data contained in this report. From Figure 1 it is clear that the fluorescence spectrum of camptothecin blue-shifts significantly upon membrane binding, whereas this is not observed in the case of topotecan. Such spectral shifting in the case of camptothecin is suggestive of drug relocation to a more hydrophobic environment, and our iodide quenching studies conducted on membrane-bound drug corroborate deep penetration into the bilayer for the camptothecin analogue. The diminished spectral shifting in the case of topotecan suggests that this agent does not penetrate as deeply into the bilayer. Perhaps the presence or absence of a positively-charged moiety translates into a difference in the degree of penetration of a drug into the lipid bilayer domains of PGP-containing membranes. Such a difference in lipid bilayer location may potentially, at least in part, explain the observed differences between the two analogues in their degrees of transport by the protein. Clearly a need exists to determine the molecular-level details of how anticancer drugs, as well as reversal agents, come in contact with the PGP protein and whether or not drug location in the lipid bilayer domains of PGP-containing membranes can modulate the efficiency by which the transport protein removes these agents from the cells.

Thus, for the analogues which have been compared in the literature, lipid bilayer affinities appear to correlate reasonably well with intracellular accumulation as well as cytotoxic potency. A more detailed and systematic study of the interrelationships of lipid partitioning, net cellular accumulation, intrinsic potency of the drugs against the topoisomerase I target, and *in vitro* cytotoxic activity against cancer cells is now in progress in our laboratories.

Relationship between Membrane Affinity and Biological Activity. For several reasons the lipophilic nature of the

camptothecins imparts favorable characteristics on the biological activities of these drugs observed *in vivo*. As we have mentioned previously in this report, enhanced lipophilic character of the camptothecins allows for more extensive membrane transport, thereby allowing for greater levels of accessibility and greater levels of drug accumulation by the cancer cells at the tumor site. In recent years more water-soluble camptothecin drugs such as topotecan and CPT-11 have been developed as an approach to improving formulation characteristics, but typically these water-soluble compounds display reduced potencies in *in vivo* test models (Wani et al., 1980, 1986, 1987a,b; Wall et al., unpublished results).

We have also discussed earlier in this paper that camptothecins in circulation can bind either membrane or protein components of blood, with the former type of interactions resulting in stabilization of the lactone ring moiety, while the latter interactions can positively or negatively affect stability, depending upon drug structure. Accordingly, structural modifications of camptothecin which promote membrane interactions and/or inhibit drug binding to albumin in the carboxylate form should be expected to display enhanced half-lives in blood. With respect to the substitution of a 10,11-methylenedioxy functionality into the camptothecin molecule, we see that this change results in an agent with a greater propensity to interact with lipid bilayers relative to camptothecin (Table IV), thereby enhancing stability characteristics. Another positive attribute to including this functionality is that it dramatically improves potency against the topoisomerase target (Jaxel et al., 1989; Wall et al., unpublished results), with improved potency against cancer cells being noted as well (Jaxel et al., 1989; Chen et al., 1991; Wall et al., unpublished results). Unfortunately, the presence of human serum albumin destabilizes 10,11-methylenedioxycamptothecin by preferentially binding the agent in its carboxylate form (T. Burke, unpublished results). Thus there will be a competition in blood between drug binding in lactone form to lipid bilayers and drug binding in the carboxylate form to HSA. Being that, on a weight-to-volume basis, the albumin in blood is 5–10 times more abundant than lipid and that the association of 10,11-methylenedioxycamptothecin carboxylate to HSA has a significantly (>10-fold) higher association constant relative to lipid binding (Burke, unpublished results), drug interactions with HSA will most likely predominate in human blood, thereby resulting in rapid ring opening (relative to drug in solution in the absence of HSA). As stated previously, the overall consequences of ring opening are obviously undesirable with respect to biological activity.

The problems with drug inactivation described above may potentially be reduced by stably packaging the camptothecins within lipid microstructures (Burke et al., 1992). Such an approach may be of some utility in extending the circulatory half-life of the biologically-active lactone form of the encapsulated agent.

Yet another approach, and a logical extension of the work presented in this report, is to better understand the binding specificities of camptothecin drugs to HSA and topoisomerase I as well as lipid bilayers, with the specific goal of identifying topoisomerase I-inhibitory camptothecin analogues which display the following: favorable interactions with the topoisomerase I target, reduced HSA affinities, or enhanced lipid bilayer affinities, or most preferably all of these properties. Research efforts aimed at further elucidating the structural basis of drug binding to the various macromolecules of interest are presently underway.

ACKNOWLEDGMENT

We thank Dr. Henryk Malak of The University of Maryland School of Medicine for his assistance with lifetime determinations. Lori Jo Latus of the Molecular and Cellular Developmental Biology Program of The Ohio State University is gratefully acknowledged for her critical reading of the manuscript, and Edward Burke is thanked for his contribution of artwork.

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