Novel A,B,E-Ring-Modified Camptothecins Displaying High Lipophilicity and Markedly Improved Human Blood Stabilities

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Camptothecins are DNA topoisomerase I inhibitors that have recently emerged as a prominent class of anticancer agent.[1−3] Topotecan and CPT-11 are water-soluble analogues of the natural product camptothecin and in 1996 were the first two members within the family to gain FDA approval (topotecan as second-line therapy for advanced epithelial ovarian cancer and CPT-11 as first-line therapy for colon cancer). Other camptothecin congeners currently under clinical evaluation include Lurtotecan (GI147211), 9-aminocamptothecin, and DX-8951f, all of which display improved water solubility over camptothecin, and 9-nitrocamptothecin, which is a lipophilic analogue. Although widely used, camptothecins are known to undergo relatively rapid hydrolysis in the bloodstream resulting in a marked loss of therapeutic potential. It is the key α-hydroxy-*δ*-lactone pharmacophore within the clinically relevant camptothecins that undergoes facile acyl cleavage at physiological pH[4] to yield a biologically inactive[5−7] carboxylate form. In this report we describe the rational design and total synthesis of highly lipophilic A,B,E-ring-modified camptothecins that are the most blood-stable camptothecins displaying intrinsic anticancer activity yet to be identified.

Our approach to the design of more blood-stable camptothecin-class topoisomerase I inhibitors was based upon three general considerations. First, structural modifications that eliminated the highly preferential binding of the carboxylate over the lactone form by human serum albumin (HSA)[8−12] were sought. Previous research efforts in our laboratories have shown that 9-aminocamptothecin and camptothecin display extremely poor stabilities in human blood due to the high-affinity, noncovalent binding interactions of their carboxylate forms with HSA.[11,12] For instance, frequency-domain lifetime fluorometry reveals that HSA preferentially binds camptothecin carboxylate with over a 100-fold higher affinity than camptothecin lactone;[11] this selective binding of carboxylate over lactone results in a shifting of the equilibrium in favor of the carboxylate. In this manner, camptothecin opens more rapidly and completely in the presence of HSA than in the absence of the protein. In a solution containing HSA and in human plasma, pH 7.4 and 37 °C, camptothecin and 9-aminocamptothecin open rapidly and essentially completely, such that negligible 0.2% lactone levels remain at equilibrium.[11,12] Time-resolved fluorescence spectroscopic investigations show that topotecan contains structural features which reduce binding of its carboxylate form to HSA;[13] as a result, topotecan displays improved stabilities in human blood and plasma relative to camptothecin and 9-aminocamptothecin.

Second, our finding that lactone stabilization is achieved through lipid bilayer partitioning[10,14,15] led us to pursue the design of more lipophilic camptothecin analogues. We have shown previously that lipid bilayer vesicles,[14,15] as well as erythrocytes,[10] promote camptothecin drug stability by preferentially binding electroneutral lactone over negatively charged carboxylate. Thus, the design of more lipophilic camptothecins would promote the reversible partitioning of the new agents into the lipid bilayers of erythrocytes, thereby protecting the active lactone forms from hydrolysis. Initial concerns about the loss of antitopoisomerase I activity through enhancing compound lipophilicity were lessened by the work of Pommier et al.,[16] who in 1990 noted that several more lipophilic camptothecin analogues such as 10,11-(methylenedioxy)camptothecin display superior intrinsic potencies against topoisomerase I.

Last, recent studies have shown that expansion of the camptothecin E-ring to a seven-membered system (by insertion of a methylene spacer between the 20-OH functionality and the carboxyl moiety) enhances the solution stability of the agent while maintaining anticancer activity.[17,18] Whereas the 20-OH functionality in conventional camptothecins is thought to interact with the carbonyl oxygen and facilitate ring opening, inclusion of a methylene spacer decreases the interactions between the *β*-OH and carbonyl oxygen. This change diminishes hydrogen-bonding interactions between the two groups and is thought to result in a slower rate of lactone hydrolysis. Since our total synthetic approach allows for the E-ring to be readily modified, we included the expanded *β*-hydroxy-*δ*-lactone E-ring functionality in our drug design strategy.

The new agents, which we refer to as homosilatecan derivatives, were prepared using the cascade radical annulation approach, as summarized in Scheme 1. Enol ether **2**, an intermediate in the synthesis of standard E-ring camptothecin analogues,[19,20] was oxidatively cleaved to keto formate **3** by treatment with OsO_4 followed by $Pb(OAc)_4$. Chain extension by Reformatsky reaction followed by treatment with TFA provided an expanded lactone, which was then treated with ICl followed by TMSI to generate the pyridone lactone **4**. This was then *N*-propargylated with trimethylsilyl- and *tert*-butyldimethylsilyl-substituted propargyl bromide to provide **5a**,**b**. In the key cascade radical annulation,

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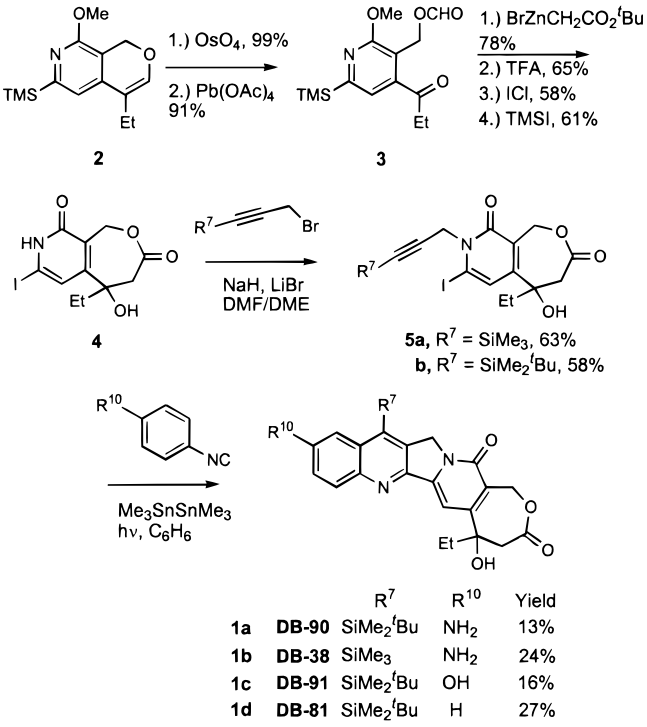
Scheme 1



		R^7	R^{10}	Yield
1a	DB-90	$SiMe_2{}^tBu$	NH_2	13%
1b	DB-38	$SiMe_3$	NH_2	24%
1c	DB-91	$SiMe_2{}^tBu$	OH	16%
1d	DB-81	$SiMe_2{}^tBu$	H	27%

these precursors were reacted under standard conditions[19,20] with phenyl isonitrile or with *p*-NHBoc- and *p*-OAc-substituted phenyl isonitrile (followed by cleavage of the respective protecting groups) to provide four new homosilatecan analogues (**1a**−**d**) in unoptimized yields of 13−27%. The new agents include 7-(trimethylsilyl)-10-aminohomocamptothecin (DB-38), 7-(*tert*-butyldimethylsilyl)homocamptothecin (DB-81), 7-(*tert*-butyldimethylsilyl)-10-aminohomocamptothecin (DB-90), and 7-(*tert*-butyldimethylsilyl)-10-hydroxyhomocamptothecin (DB-91). In addition to the expanded β-hydroxy-δ-lactone E-ring functionality, each of the new homosilatecans also contains a silylalkyl functionality at the 7-position. The silylalkyl functionality provides a convenient means of varying lipophilicity while concomitantly reducing the strength of carboxylate interactions with HSA. The DB-90 and DB-91 agents also contain amino and hydroxyl groups at the 10-position, respectively. These changes at position 10 were undertaken since previous studies had shown that 10-substitution in combination with 7-substitution decreased the binding of camptothecin carboxylate to HSA.[9] In some cases (i.e. SN-38), the combination of 7- and 10-position substituents decreases carboxylate interactions while promoting lactone associations.[9]

A variety of analytical and biophysical methods were employed to compare the blood component interactions and blood stabilities of the new homosilatecans with those of camptothecin and its clinically relevant analogues. The equilibrium associations of the new A,B,E-ring- and B,E-ring-modified homosilatecans with lipid bilayers were characterized by fluorescence spectroscopic methods. The intrinsic fluorescence from the new agents allowed us to directly monitor their interactions with small unilamellar vesicles (SUVs) composed of electroneutral L-α-dimyristoylphosphatidylcholine (DMPC) and negatively charged L-α-dimyristoylphosphatidylglycerol (DMPG). In the presence of model membranes,

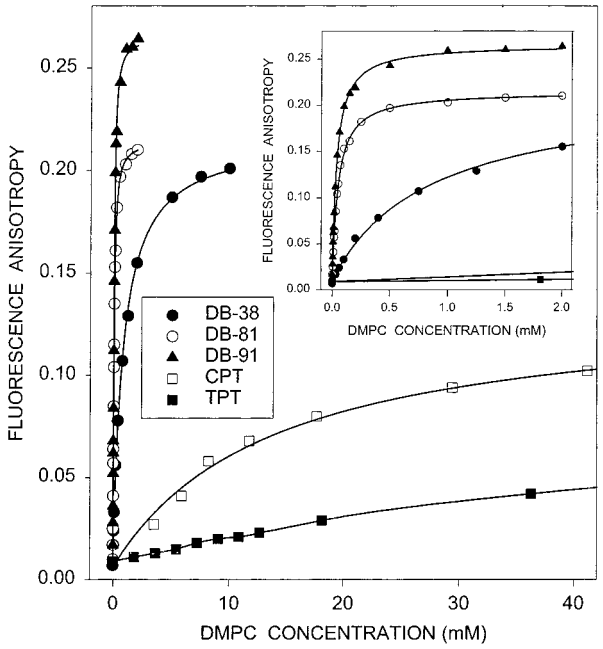


Figure 1. Comparison of the equilibrium binding of four novel homosilatecans to SUVs composed of electroneutral DMPC in PBS with data acquired for camptothecin (CPT) and topotecan (TPT) as well. The method of fluorescence anisotropy titration was used to construct the adsorption isotherms. Experiments were conducted at drug concentrations of 1 μM in PBS buffer (37 °C). Note how the anisotropy values of DB-38, DB-90, and DB-91 titrate much more rapidly than those of camptothecin or topotecan, indicating that the novel homosilatecans have much stronger interactions with these membranes relative to camptothecin and topotecan. Because of the potential of the lactone ring of the homosilatecans and camptothecins to hydrolyze in PBS, anisotropy values at each lipid concentration were determined immediately (approximately 1 min) following the addition of the lactone form of each agent to the liposome. *K* values are summarized in Table 1.

the λ_{MAX} of each homosilatecan agent was found to shift to lower wavelength, or blue shift, in the range of 5−20 nm (data not shown). While these spectral changes provide qualitative evidence of drug−membrane interactions, fluorescence anisotropy titration (Figure 1) offers the most sensitive means for comparing quantitatively the strength of membrane binding exhibited by camptothecin, the water-soluble analogue topotecan, and the new homosilatecans. The sensitivity of the membrane binding assay is exemplified by the 19-fold enhancement in the steady-state anisotropy value of camptothecin following its association with DMPC vesicles;[15] the newly synthesized DB-38 exhibits an even greater 30-fold change in anisotropy (Figure 1) upon associating with DMPC SUVs.

Analysis of the fluorescence anisotropy data using double-reciprocal plots gave identical overall association constants (*K* values) of 100 M^{-1} for camptothecin interacting with DMPC and DMPG bilayers[15] (Table 1). The association constants for water-soluble topotecan (K_{DMPC} = 10 M^{-1} and K_{DMPG} = 50 M^{-1}) are less than the values noted for camptothecin.[15] The data contained in Figure 1 and summarized in Table 2 indicate a marked enhancement of the membrane partition coefficient can be achieved through the creation of A,B,E-ring-modified camptothecins (i.e. DB-38, DB-90, and DB-91) or the B,E-ring-modified camptothecin (DB-81). DB-38, which contains a hydrophobic trimethylsilyl

Table 1. Overall Association Constants for Camptothecin Analogues Interacting with Unilamellar Vesicles of Electroneutral DMPC and Negatively Charged DMPG in PBS Buffer at pH 7.4 and 37 °C[a]

compound	K_{DMPC} (M^{-1})	K_{DMPG} (M^{-1})
DB-38	1400	800
DB-81	14000	19000
DB-90	9000	9000
DB-91	8000	4000
DB-90 carboxylate form	800	80
DB-91 carboxylate form	700	100
topotecan	10	50
camptothecin	100	100

[a] Equilibrium association constants were determined using the method of fluorescence anisotropy titration as described previously for camptothecins.[15] Electroneutral, fluid-phase lipid bilayers were represented by SUVs comprised of DMPC. Negatively charged, fluid-phase bilayers were represented by DMPC SUVs. Binding isotherms were constructed by the method of fluorescence anisotropy titration, and *K* values were determined from the slopes of double-reciprocal plots. The *K* values are subject to 10% uncertainty.

Table 2. Summary of the Stability Parameters for Homosilatecans in Human Blood, Plasma, PBS, PBS with HSA, and PBS with RBCs[a]

drug name and fluid	% lactone after 3 h
DB-38	
whole blood	56.4 ± 0.6
HSA	81.4 ± 0.3
PBS	82.8 ± 0.7
plasma	40.3 ± 2.1
RBC	84.8 ± 1.5
DB-81	
whole blood	86.6 ± 0.5
HSA	88.1 ± 0.2
PBS	84.9 ± 0.3
plasma	85.0 ± 4.3
RBC	92.0 ± 0.0
DB-90	
whole blood	85.2 ± 0.7
HSA	86.8 ± 0.2
PBS	83.7 ± 0.5
plasma	71.1 ± 3.5
RBC	85.5 ± 0.4
DB-91	
whole blood	84.9 ± 0.3
HSA	82.9 ± 0.3
PBS	83.1 ± 0.3
plasma	61.5 ± 3.9
RBC	88.5 ± 0.2

[a] Hydrolysis parameters were determined using HPLC assays. Drug concentrations of 1 μM were employed. Drug samples were incubated in blood, plasma, or PBS (pH 7.4) either with or without physiologically relevant concentrations of HSA or RBC. Plasma samples were continuously aerated with "blood gas" (MEDIBLEND) in order to maintain constant pH (7.5 ± 0.1). All experiments were conducted at 37 °C.

functionality at position 7 and an amino group at position 10, displays a 14-fold enhancement in the K_{DMPC} value relative to camptothecin. The presence of an even more hydrophobic *tert*-butyldimethylsilyl functionality at the 7-position (contained in DB-81, DB-90, and DB-91) elevates the membrane association constants further, with DB-81 exhibiting a K_{DMPC} value of 14 000 M^{-1}. This value represents an impressive 140-fold enhancement relative to the K_{DMPC} value noted for camptothecin. Overall, our data indicate that modification of the silylalkyl functionality provides a convenient and effective means of varying the lipophilicity of the silatecans over a wide range.

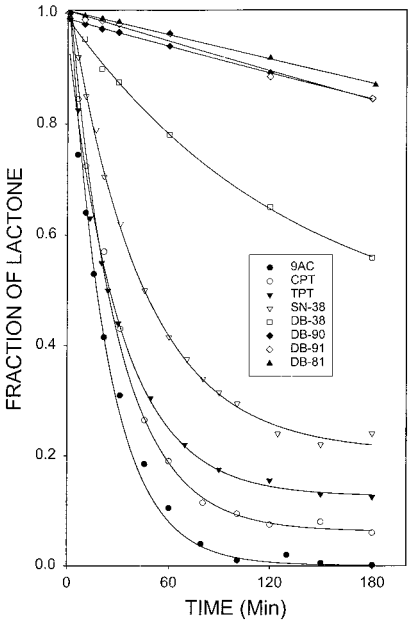


Figure 2. Depiction of the improved human blood stabilities of four novel homosilatecans relative to clinically relevant agents including 9-aminocamptothecin (9AC), camptothecin (CPT), topotecan (TPT), and SN-38 (SN-38). Stability profiles were determined using HPLC methods. All experiments were conducted at pH 7.4 and 37 °C. In all cases, the agents containing the expanded E-ring or homosilatecan structures displayed markedly enhanced human blood stabilities over the clinically relevant compounds. The stability parameters for the novel homosilatecans are summarized in Table 2.

In addition to the homosilatecans displaying markedly higher lipophilicities relative to the conventional camptothecins (i.e. camptothecin, topotecan), they exhibit improved aqueous stabilities as was observed previously for homocamptothecin.[17,18] Table 2 summarizes the stabilities of 1 μM solutions of DB-38, DB-81, DB-90, and DB-91 in solutions of phosphate-buffered saline (PBS) at a pH value of 7.4. Whereas clinically relevant camptothecins typically show approximately 10−15% lactone remaining at equilibrium following 3 h of incubation in PBS, greater than 80% lactone remains for each of the homosilatecans under identical incubation conditions.

The most distinguishing stability considerations for our new homosilatecan agents are observed when they are incubated in whole human blood. Figure 2 depicts the improved human blood stabilities of our four novel homosilatecans, and the stability parameters are summarized in Table 2. In all cases, the DB-38, DB-81, DB-90, and DB-91 structures display markedly enhanced human blood stabilities relative to camptothecin analogues such as topotecan and SN-38; the human blood stability values noted for DB-81, DB-90, and DB-91 are the highest yet to be measured for an intrinsically potent camptothecin analogue. The greater than 80% lactone values following 3 h of incubation compare very favorably to the corresponding percent lactone levels in whole human blood for 9-aminocamptothecin (approximately 1%), camptothecin (approximately 7%), topotecan (approximately 12%), CPT-11 (approximately 21.0%), and SN-38 (approximately 20%).

Insight into the superior human blood stabilities of DB-81, DB-90, and DB-91 relative to DB-38 can be

Table 3. Comparison of the Marked Interspecies Variations in Blood Stabilities for Camptothecin and 9-Aminocamptothecin Versus the Relatively Minor Differences Observed for Novel, Highly Lipophilic Camptothecin Analogues[a]

compound	% lactone after 3 h of incubation: in mouse blood	% lactone after 3 h of incubation: in human blood	ratio of lactone level mouse/human
9-aminocamptothecin	38	1	38
camptothecin	20	7	3
DB-38	72	56	1.3
DB-81	80	87	0.9
DB-90	61	85	0.7
DB-91	70	85	0.8

[a] Experiments were conducted at pH 7.4 and 37 °C and lactone levels determined using HPLC methods. Blood samples were drawn and kept at 5 °C prior to the initiation of an experiment.

gained through comparison of the data contained in Tables 1 and 2. DB-81, DB-90, and DB-91 all display significantly enhanced lipophilicities relative to DB-38. The increased lipophilicities of these agents correlate with the increased stabilities in solutions containing physiologically relevant concentrations [$(5 \pm 1) \times 10^6$ cell/μL] of albumin-free red blood cells (RBCs). This is consistent with the notion that the lactone form of these agents readily partitions into erythrocyte membranes and is thereby protected from hydrolysis. DB-81, the most lipophilic of the agents studied, is protected the greatest degree by the presence of RBCs and displays the greatest stabilities in PBS containing HSA and in plasma. In contrast, DB-38 is the least lipophilic of the new homosilatecans and displays the poorest stabilities both in the presence of RBCs and in plasma. Homocamptothecin has unsubstituted A,B-rings and, as a result, will have markedly reduced lipophilicity relative to our new homosilatecans; its *K* value is anticipated to be only slightly higher than that of camptothecin. The reported stability value for homocamptothecin in plasma following 3 h of incubation is approximately 60%;[17] this is 25% less than the observed value for DB-81. Although we have not studied the stability of homocamptothecin in whole blood, we anticipate that the reduced lipophilicity of the agent will translate to significantly reduced human blood stability relative to the stability parameters observed for lipophilic agents such as DB-81, DB-90, and DB-91.

Another attractive feature of the novel homosilatecans is that they exhibit similar stabilities in human versus mouse blood (Table 3), which contrasts with the marked interspecies variations in blood stabilities observed for camptothecin and 9-aminocamptothecin.[11] As discussed earlier, the hydrolysis problem for camptothecin and 9-aminocamptothecin is exacerbated in human blood by the fact that HSA preferentially binds to the carboxylate forms of these agents. In mouse blood the binding of carboxylate forms by serum albumin is markedly reduced, and thus no prominent shift in the lactone/carboxylate equilibrium occurs. The interspecies variations in blood stability for camptothecin and 9-aminocamptothecin may, at least in part, explain variations in the biological activity of the agents in mice versus humans. For instance, 9-aminocamptothecin is highly efficacious in curing mice of a variety of human cancers[3] yet was ineffective (1 partial response in 99 treated patients in need of urgent care) in treating human brain cancer in a recently completed clinical trial.[21] Differences in drug−serum albumin binding may likely contribute to the interspecies differences in activities: murine serum albumin displays a significantly reduced binding affinity for 9-AC, while HSA binds the 9-AC carboxylate tightly and, thereby, promotes the hydrolysis of the active drug to the inactive drug. Tight protein binding of the carboxylate form and low lipophilicity are factors that accordingly may limit 9-aminocamptothecin transport across the blood−brain barrier. Our novel, highly lipophilic homosilatecans may be of interest as they do not display marked interspecies variations in blood. Thus, successful treatment strategies and preclinical animal modeling and efficacy studies with the homosilatecans may be more readily translated to a clinical setting.

The cytotoxic potency of each homosilatecan against MDA-MB-435 breast carcinoma cells was determined for 72-h exposure periods.[22] Overall, the IC_{50} cytotoxicity values were in the 20−100 nM range, with DB-38 exhibiting the greatest potency of the four homosilatecans studied. As racemic solutions of each homosilatecan were used in these assays, it is expected that at least a doubling in activity will be observed using the optically pure active enantiomer. The potency of camptothecin against these cells (IC_{50} of 10 nM) is slightly better, but it is important to note that the potency of camptothecin against cancer cells can be diminished by several orders of magnitude when HSA is included in the incubation media.[13] Data presented in Table 2 indicate the homosilatecans have altered interactions with human blood proteins relative to camptothecin. We now intend to employ cytotoxicity assays containing HSA in an effort to identify homosilatecans with high potencies (in the 1−20 nM range) that are unaffected by the presence of the protein.

In summary, the novel homosilatecans described here are potent topoisomerase I inhibitors that are stable not only in mouse blood but human blood as well. Three of the first four homosilatecans to be synthesized are the most blood-stable camptothecins yet to be identified that display intrinsic potency against the topoisomerase I target. Given the demonstrated scope and generality of the cascade radical annulation approach,[19,20,23] the synthesis of homosilatecans described in Scheme 1 will prove useful for the future generation of a broad assortment of blood-stable and intriniscally potent homosilatecans.

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