Water Soluble Inhibitors of Topoisomerase I: Quaternary Salt Derivatives of Camptothecin

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Eleven water soluble 7-substituted quaternary ammonium salt derivatives of 10,11-(methylenedioxy)- and 10,11-(ethylenedioxy)-(20*S*)-camptothecin were synthesized via the Friedlander reaction followed by nucleophilic displacement with an aromatic amine. All of these compounds were more potent than camptothecin in the *in vitro* cleavable complex assay. These inherently charged camptothecin derivatives were cytotoxic against three different human tumor cell lines (SKOV3, an ovarian adenocarcinoma; SKVLB a multidrug resistant ovarian adenocarcinoma; and HT-29, a colon carcinoma). A selected group of five compounds was evaluated in the nude mouse HT-29 xenograft model. Two of these quaternary salts (**17** and **18**) were more efficacious than Topotecan in delaying tumor growth. In an extended *in vivo* model, **18** demonstrated tumor regression.

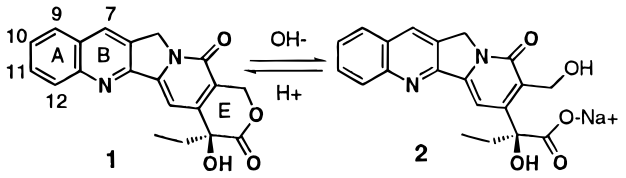
Introduction

Topoisomerase I is a nuclear enzyme that is required for topological manipulation of DNA during cellular events such as replication and transcription.[1,2] Topoisomerase I functions by causing transient single-strand breaks allowing for the swiveling and relaxation of supercoiled DNA.[3] Intracellular levels of topoisomerase I are elevated in a number of human solid tumors, relative to the respective normal tissues, suggesting that variations in topoisomerase I levels are tumor type specific.[4,5] Thus, topoisomerase I represents a promising molecular target for the development of new cancer chemotherapeutic agents for the treatment of solid tumors.

Researchers have shown that camptothecin (**1**), an alkaloid originally isolated by Wani and Wall in 1966, is an antitumor agent due to the inhibition of topoisomerase I.[6,7] Camptothecin stabilizes the complex between DNA and topoisomerase I (cleavable complex), in which the DNA is covalently linked to a tyrosine residue on topoisomerase I via the backbone 3′-phosphodiester. The formation of this stabilized cleavable complex is readily reversible *in vitro* by increasing the concentration of NaCl which facilitates religation of the 5′-backbone phosphate.[8] Presumably, this complex, when present in high enough concentrations within the cell, leads to an S phase specific cell death through interference with the replication fork.[9] Studies have shown a correlation between the ability to cause stabilization of a DNA−topoisomerase I intermediate, DNA strand breaks, and antitumor effects of several camptothecin analogs.[10]

The clinical utility of camptothecin as an anticancer agent was limited due to its nonmechanism-related toxicity and an extremely poor solubility profile.[11] In an attempt to circumvent the solubility problem, researchers evaluated the soluble sodium salt of the hydroxy acid form (**2**) in the clinic (Scheme 1).[12] The severe toxicities that were observed resulted in the discontinuation of the clinical development of camptothecin. Biochemical studies have subsequently demonstrated that the closed lactone form of camptothecin possesses the inhibitory activity against topoisomerase I, whereas the ring-opened lactone form is inactive.[7,13] Further, the equilibrium between the lactone and hydroxy acid is pH dependent, and at physiological pH, the carboxylate form (**2**) predominates.[14]

Scheme 1[a]



[a] **1**, active form: closed lactone. **2**, inactive form: soluble salt of the hydroxy acid.

Several strategies have been used to render camptothecin or its analogs more water soluble and, hence, more promising oncolytic agents. For example, researchers at Smith Kline Beecham attached a water soluble (dimethylamino)methyl group to the naturally occurring 10-hydroxycamptothecin to produce the water soluble drug[15] Topotecan (**3**), which is currently undergoing human clinical studies in the United States (Figure 1).[16,17] While this compound is somewhat less active than camptothecin as a cytotoxic agent, it has a reported solubility of 1 mg/mL compared with the <0.003 mg/mL solubility of camptothecin.[14] In an alternate approach, Yakult-Honsha researchers synthesized the water soluble prodrug **4** which is also undergoing human clinical trials in the United States under the name Irinotecan.[18] This compound is inactive *in vitro*, but upon hydrolysis of the carbamate in plasma, the active component 10-hydroxy-7-ethyl-(20*S*)-camptothecin is liberated. Glaxo researchers have recently reported B-ring-substituted camptothecin derivatives, one of which was selected for further development.[19,20] This compound, 10,11-(ethylenedioxy)-7-[(*N*-methylpiperazino)methyl]-(20*S*)-camptothecin (**5**) is currently undergoing phase II clinical trials in the United States and Europe.

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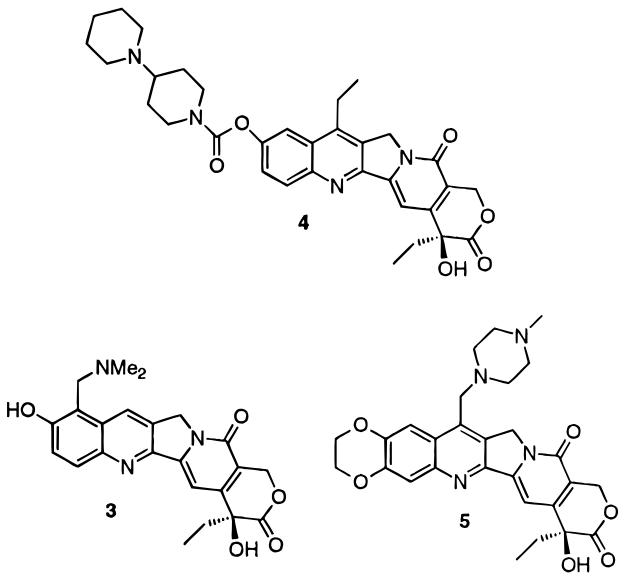


Figure 1. **3**, Topotecan; **4**, Irinotecan; **5**, 10,11-(ethylenedioxy)-7-[(*N*-methylpiperazino)methyl]-(20*S*)-camptothecin.

The structure−activity relationships (SAR) of camptothecin have been reviewed.[21−23] As mentioned previously, the intact E-ring lactone is essential for antitumor activity. A great deal is known about the substitution of the A-ring and, more recently, B-ring substitutions. Substitution of camptothecin is tolerated at the 7-, 9-, and 10-positions but not at the 12-position. Substitutions with groups (e.g., CN) at the 11-position are also tolerated provided the groups are small. In addition, it has been noted that substitution of the camptothecin A-ring with a 10,11-methylenedioxy group greatly enhances topoisomerase I inhibitory activity. For this reason, we explored a series of compounds which retained the 10,11-methylenedioxy and 10,11-ethylenedioxy substitution and placed the water-solubilizing groups in the 7-position of camptothecin as aromatic quaternary ammonium salts. Our goal was to discover water soluble camptothecin analogs with enhanced *in vivo* efficacy against human tumors.

Chemistry

The synthesis of these aromatic quaternary ammonium salt derivatives utilized an acid-catalyzed Friedlander condensation between the highly functionalized A-ring precursor **6** or **7** and the known tricyclic keto lactone[24] **8** to afford the corresponding 7-(chloromethyl)-(20*S*)-camptothecin derivatives **9** and **10**.[20] Quaternization was carried out neat with aromatic amines as exemplified in Scheme 2 for pyridine. The product precipitated from the reaction mixture in yields ranging from 75% to 94%. Generally, the reactions were carried out at room temperature or at the melting point of the solid aromatic amine. All of the quaternary amine analogs shown in Tables 1 and 2 were synthesized by this route.

When the reaction was carried out using a cosolvent such as DMF, the major reaction product was identified as the 7-methylcamptothecin derivative **13** or **14** (Scheme 3). While the formation of **13** (or **14**) is a net reduction, no oxidized products were isolated from the reaction mixture. This curious reduction also occurred when imidazole was used as both the nucleophile and the solvent. Treatment of **9** with neat 1-(trimethylsilyl)-imidazole followed by aqueous acid hydrolysis of the silylated C-20 hydroxyl group afforded a much higher yield of **15**. The reduction side product was also produced when the Friedlander reaction described in Scheme 2 was performed with aqueous acid catalysis, implying that a proton source or water may be required for the side reaction.

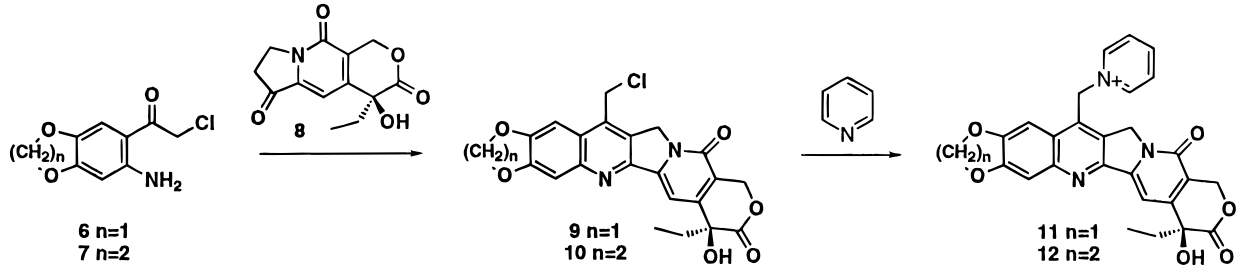
The solubilities of the target compounds were measured in D_2O using ^1H NMR integration of the C-20 ethyl group against an internal standard, 1,4-dioxane (Tables 1 and 2). These compounds were diluted as salts (except **15** and **16**) without additional buffers. The pH of the solutions was measured to be between 6.6 and 6.8.[25] Under these conditions, the lactone ring remains closed for at least 1 week at room temperature. To be sure that we could monitor the formation of the hydroxy acid by ^1H NMR, a sample of compound **12** was allowed to stand in NaOD/D_2O. Not only did this treament open the lactone, it also exchanged the C-7 methylene protons as well as the 2- and 6-protons on the pyridinium ring. The NMR method for solubility determinations was selected because HPLC methods gave varying results due to the specific conditions selected. For example, the buffer system, electrolyte concentrations, and the pH of the sample solution and mobile phase all affect the measured water solubility of a camptothecin compound.

In general, the quaternary salts of 10,11-methylenedioxy-substituted camptothecin were more soluble than their 10,11-ethylenedioxy counterparts (compare **11**, >4.5 mg/mL, and **12**, 1.8 mg/mL). The solubilities of the uncharged compounds **15** and **16** were below the detection limit of the ^1H NMR method. Topotecan had a solubility of about 2 mg/mL (as measured by our NMR method), while our target camptothecin derivatives had solubilities ranging from 0.9 mg/mL to >4.4 mg/mL. All of the quaternary salts met our solubility criteria for *in vivo* evaluation.

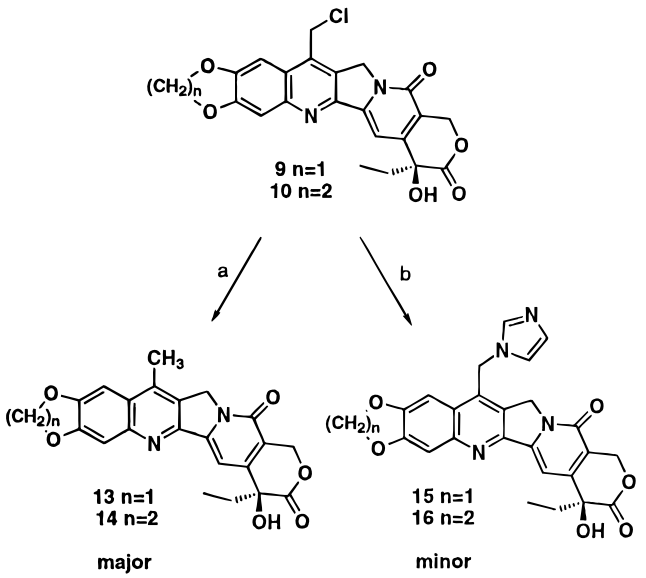
Biological Studies and Results

The results of the topoisomerase I cleavable complex enzyme assay are reported in Tables 1 and 2 as IC_{50}

Scheme 2

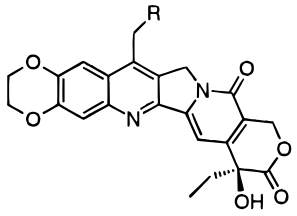


Scheme 3[a]



[a] (a) 1. TMS-imidazole, 2. H_3O^+; (b) DMF or imidazole.

Table 1. *In Vitro* Comparison of 10,11-(Ethylenedioxy)-(20*S*)-camptothecin Derivatives

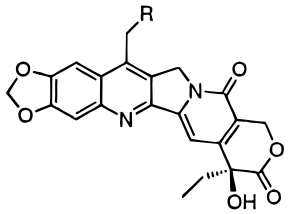


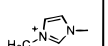
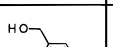
Cmpd	R	Solubility (mg/mL)[a]	Topo I IC_{50} (nM)[b]	HT-29 IC_{50} (nM)[c]	SKOV3 IC_{50} (nM)[c]	SKVLB IC_{50} (nM)[c]	MDR ratio[d]
3	Topotecan	2.1	1100	25	44	150	3
12		1.8	190	84	85	84	1
16		<0.5	200	ND	ND	ND	ND
18		1.4	180	16	11	870	80
22		0.91	250	ND	69	94	1
24		1.2	600	ND	ND	ND	ND
25		1.3	520	ND	ND	ND	ND
26		1.1	340	ND	ND	ND	ND

[a] Determined using ^1H NMR method. [b] Average concentration of compound to cause 50% inhibition of topoisomerase I as determined using the cleavable complex assay (see ref 12 and 22). [c] Human tumor cell cytotoxicity assay was performed using MTT (see ref 29) and the concentration of compound causing 50% cell kill (IC_{50}) determined. [d] MDR ratio is defined as the quotient of SKVLB IC_{50}/SKOV3 IC_{50}. ND indicates not determined.

values. Using a method described by Hsiang and coworkers,[7] the IC_{50} values are defined as the concentration of the test compound needed to produce ca. 50% fragmentation of end-labeled DNA in the presence of calf thymus topoisomerase I using camptothecin as an internal standard. The quaternary salts were at least

Table 2. *In Vitro* Comparison of 10,11-(Methylenedioxy)-(20*S*)-camptothecin Derivatives



Cmpd	R	Solubility (mg/mL)[a]	Topo I IC_{50} (nM)[b]	HT-29 IC_{50} (nM)[c]	SKOV3 IC_{50} (nM)[c]	SKVLB IC_{50} (nM)[c]	MDR ratio[d]
3	Topotecan	2.1	1100	25	44	150	3
	Doxorubicin	>2.0		330	36	5600	160
11		>4.5	210	70	74	72	1
15		<0.5	160	ND	ND	ND	ND
17		>3.5	220	75	110	1000	9
19		1.4	140	46	6	23	4
21		>4.4	100	13	11	23	2
23		>3.1	190	ND	83	130	2

[a] Determined using ^1H NMR method. [b] Average concentration of compound to cause 50% inhibition of topoisomerase I as determined using the cleavable complex assay (see ref 12 and 22). [c] Human tumor cell cytotoxicity assay was performed using MTT (see ref 29) and the concentration of compound causing 50% cell kill (IC_{50}) determined. [d] MDR ratio is defined as the quotient of SKVLB IC_{50}/SKOV3 IC_{50}. ND indicates not determined.

2 times as potent (IC_{50} values ranging from 100 to 600 nM) as Topotecan (IC_{50} = 1028 nM) against topoisomerase I as measured in this assay.[26] For reference, camptothecin had an IC_{50} of 700 nM and 10,11-(methylenedioxy)-(20*S*)-camptothecin had an IC_{50} of 27 nM in this assay.

Several of the water soluble camptothecin analogs in Tables 1 and 2 were assayed for cytotoxicity against three different human tumor cell lines which also served to demonstrate the cell permeability of these permanently charged salts. The data show that these salts were very cytotoxic to the following human cell lines evaluated: SKOV3, an ovarian adenocarcinoma; SKVLB, ovarian adenocarcinoma with upregulated multidrug resistant (MDR) *p*-glycoprotein; and HT-29, a colon adenocarcinoma. The ratio of the IC_{50} values of SKVLB/SKOV3 reflects the relative insensitivity of this series of compounds to multidrug resistance mediated by the MDR *p*-glycoprotein when compared to doxorubicin. There is a decrease in potency against the MDR cell line for compounds **17** and **18** indicating that these compounds may be slightly susceptible to extrusion by the multidrug transporter. It is interesting to note that while **18** and **19** have the same 7-substitution, same solubilities, and similar potency against topoisomerase I, the susceptibility to MDR is significantly different.

Five of these compounds (**11**, **17**, **18**, **19**, and **21**) were selected for evaluation in 2-week tumor growth delay *in vivo* assay using nude mice implanted with HT-29 (human colon) xenografts (Table 3). Four of the compounds were in the 10,11-methylenedioxy series, and

Table 3. *In Vivo* Efficacy Study: Tumor Growth Delay

compd	dose (mg/kg)	T/C[a] (days)	MTD[b] (mg/kg)
3, Topotecan	54	10	54
11	100	4	>100
17	100	14	100
18	80	14	100
19	48	11	80
21	100	9	>100

[a] T/C is defined as the delay in tumor growth, measured in days, required for the tumor to reach 500% of the initial volume in drug-treated animals (T) compared to controls (C) at or below the MTD. [b] MTD, maximally tolerated dose, is defined as the highest dose which caused ≤30% body weight loss.

Table 4. *In Vivo* Study: HT-29 Human Colon Xenograft 5-Week Assay

compd	dose (mg/kg)	efficacy[a] (T/B)	BW loss[b] (%)	deaths
Topotecan	5	8.6	15	0
	7	7.0	11	0
	9	4.3	17	2
	11	2.9	23	0
17	9.5	6.74	4	0
	10.5	3.84	12	0
	11	2.85	16	0
18	8	2.1	21	0
	12	0.70	27	0
	14	0.32	13	0
vehicle		12.1	(11%) gain	

[a] T/B, tumor growth ratio, is defined as the group mean volume of the tumor at the end of treatment divided by the initial mean volume at the beginning of treatment. [b] BW loss indicates mean body weight loss for animals in all experimental groups. $N = 6$ in all experimental groups.

only one was in the 10,11-ethylenedioxy class due to the superior solubility characteristics of the former series. The drug was administered subcutaneously to xenograft-bearing mice ($N = 6$) on the first day of the study. The mice were monitored for delay in tumor growth at several doses close to the maximum tolerated dose (MTD). The tumor growth delay (T/C) is defined as the difference in days required for the tumors to reach a volume of 5 times their starting volume in the treated mice vs the control mice. Overall, the T/C values ranged from 4 days for the 10,11-methylenedioxypyridinium salt **11** to 14 days for both the 10,11-ethylenedioxy *N*-methylimidazolium salt **17** and the 10,11-ethylenedioxy pyrazinium salt **18**, compared to 10 days for Topotecan at the MTD.

The abilities of **17** and **18** to affect the growth of HT-29 human colon xenografts were further assessed by monitoring tumor growth kinetics dosing subcutaneously twice a week over a 5-week period. The results are reported in Table 4 as the ratio (T/B) of the tumor volume in the treated animals at the end of the 5 weeks (T) over the initial tumor volume of the animals at the beginning of the experiment (B). The limit of toxicity was determined by the body weight loss approaching 30%. Tumor regression was seen in a dose dependent manner for compound **18** which had a T/B ratio of 0.7 when dosed at 12 mg/kg. The efficacy of **17** was essentially equivalent to that of Topotecan which had a ratio of 2.9 at a dose of 11 mg/kg in the same protocol, while **5** afforded a T/B ratio of 0.6 at 4 mg/kg.[20]

Conclusion

Quaternary aromatic aminomethyl-substituted camptothecin analogs can be readily synthesized from 10,11-(methylenedioxy)- and 10,11-(ethylenedioxy)-7-(chloromethyl)-(20*S*)-camptothecin and nitrogen-containing heterocycles. These water soluble salts are all potent topoisomerase I inhibitors in the cleavable complex assay described by Hsiang and co-workers.[7] These analogs demonstrate potent cytotoxicity and cell permeability in whole cell assays several human tumor cell lines. Five of these compounds were evaluated in the mouse HT-29 xenograft tumor growth delay model, where **17** and **18** emerged as the most promising compounds in this series. Finally, tumor regression was observed for **18** in an extended *in vivo* evaluation.

Experimental Section

Chemistry. Melting points were taken on a Mel-Temp II apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA. ^{1}H NMR spectra were aquired using a Varian Unity 300-MHz spectrometer in DMSO-d_6 and are reported in parts per million (ppm) internally referenced to residual DMSO (2.49 ppm) unless otherwise noted. Mass spectra were recorded on either a Perkin-Elmer Sciex API III or a JEOL SX102 mass spectrometer.

Method a: 10,11-(Methylenedioxy)-7-(pyridiniumylmethyl)-(20*S*)-camptothecin Chloride (11). 10,11-(Methylenedioxy)-7-(chloromethyl)-(20*S*)-camptothecin (110 mg, 0.25 mmol) was added to anhydrous pyridine (Aldrich No. 27,097-0; 2.0 mL) under a blanket of nitrogen at room temperature. This was stirred for 4.0 h before the addition of diethyl ether (1.0 mL) which precipitated the desired product. The yellow solid was collected by filtration and washed once with ethanol (absolute, 2 mL) and twice with diethyl ether (5 mL each). The compound was dried under high vacuum to afford a yellow solid (116 mg, 84%): mp 285 °C dec. ^{1}H NMR (DMSO): δ 0.92 (t, 3H), 1.84 (m, 2H), 5.35 (s, 2H), 5.41 (s, 2H), 6.27 (s, 2H), 6.42 (s, 2H), 6.42 (s, 2H), 6.56 (s, 1H), 7.29 (s, 1H), 7.56 (s, 1H), 7.61 (s, 1H), 8.09 (m, 2H), 8.59 (m, 1H), 9.01 (d, 2H). Electrospray MS: *m*/*z* 484 (M^+). Anal. ($C_{27}H_{22}N_3O_6Cl\cdot 2H_2O$) C, H, N.

Method b: 7-(Imidazolylmethyl)-10,11-(methylenedioxy)-(20*S*)-camptothecin (15). Imidazole (150 mg, excess) was melted under a blanket of nitrogen at 95 °C. To this was added 10,11-(methylenedioxy)-7-(chloromethyl)-(20*S*)-camptothecin (50 mg, 0.11 mmol), and the mixture was stirred for 1.5 h. Upon cooling the reaction mixture to room temperature, a mixture of $CHCl_3$/MeOH (9:1, 3 mL) was added. A black solid was removed by filtration, and the filtrate was concentrated to 0.5 mL and chromatographed using $CHCl_3$/MeOH (9:1) on silica gel (15 g). Two major components were isolated. 7-Methyl-10,11-(methylenedioxy)-(20*S*)-camptothecin (**13**) (11.4 mg, 25%, $R_f = 0.82$): mp 249 °C dec. ^{1}H NMR (DMSO): δ 0.87 (t, 3H), 1.82 (m, 2H), 2.68 (s, 3H), 5.22 (s, 2H), 5.41 (s, 2H), 6.30 (s, 2H), 6.48 (bs, 1H), 7.22 (s, 1H), 7.46 (s, 1H), 7.61 (s, 1H). Electrospray MS: *m*/*z* 407 (MH^+). Anal. ($C_{22}H_{18}N_2O_6\cdot 1H_2O$) C, H, N.

The desired product (24.5 mg, 47%, $R_f = 0.45$): mp 222 °C dec. ^{1}H NMR (DMSO): δ 0.93 (t, 3H), 1.85 (m, 2H), 5.11 (s, 2H), 5.40 (s, 2H), 5.80 (s, 2H), 6.28 (s, 2H), 6.52 (s, 1H), 6.86 (s, 1H), 7.20 (s, 1H), 7.22 (s, 1H), 7.55 (s, 1H), 7.65 (s, 1H), 7.88 (s, 1H). Electrospray MS: *m*/*z* 473 (MH^+). HR FAB MS: calcd for $C_{25}H_{20}N_4O_6$, 473.1461; found, 473.1459.

Method c: 10,11-(Ethylenedioxy)-7-pyraziniumyl-(20*S*)-camptothecin Chloride (18). Pyrazine (350 mg, 4.38 mmol) was melted at 75 °C under nitrogen. To this was added 7-(chloromethyl)-10,11-(ethylenedioxy)-(20*S*)-camptothecin (275 mg, 0.60 mmol), and the entire reaction mixture (including the oil bath) was wrapped in aluminum foil. The reaction mixture was stirred in this manner for 40 h and then cooled to room temperature. Ethanol (10 mL) was added, and a gray-yellow solid was collected by filtration. The solid was washed with ethyl acetate (twice, 5 mL each) and diethyl ether (twice, 5 mL each) and dried under vacuum to afford a pale yellow solid dihydrate (318 mg, 92%): mp 298 °C dec. ^{1}H NMR (DMSO): δ 0.93 (t, 3H), 1.92 (m, 2H), 4.42 (s, 4H), 5.39 (s, 2H), 5.42 (s, 2H), 6.50 (s, 1H), 7.31 (s, 1H), 7.66 (s, 2H), 9.04

(d, 2H), 9.46 (d, 2H). FAB MS: *m/z* 499 (M^+). HR FAB MS: calcd for $C_{27}H_{23}N_4O_6$, 499.1618; found, 499.1606.

10,11-(Ethylenedioxy)-7-(pyridiniumylmethyl)-(20*S*)-camptothecin Chloride (12). Title compound was prepared as in method a to afford the desired product as a pale yellow solid (122 mg, 92%): mp >300 °C. ^{1}H NMR (DMSO): δ 0.89 (t, 3H), 1.90 (m, 2H), 4.41 (s, 4H), 5.38 (s, 2H), 5.41 (s, 2H), 6.43 (s, 1H), 6.56 (bs, 1H), 7.30 (s, 1H), 7.58 (s 1H), 7.65 (s, 1H), 8.09 (t, 2H), 8.60 (t, 1H), 9.03 (d, 2H). HR FAB MS: calcd for $C_{28}H_{24}N_3O_6$, 498.1665; found, 498.1653.

10,11-(Ethylenedioxy)-7-(imidazolylmethyl)-(20*S*)-camptothecin (16). Title compound was prepared as in method b to afford the desired product as a pale yellow-green solid (38.1 mg, 32%, $R_f = 0.56$): mp >325 °C. ^{1}H NMR (DMSO): δ 0.94 (t, 3H), 1.83 (m, 2H), 4.42 (s, 4H), 5.13 (s, 2H), 5.40 (s, 2H), 5.80 (s, 2H), 6.48 (s, 1H), 6.88 (s, 1H), 7.21 (s, 1H), 7.24 (s, 1H), 7.59 (s, 1H), 7.63 (s, 1H), 7.84 (s, 1H). FAB MS: *m/z* 487 (MH^+). Anal. $C_{26}H_{22}N_4O_6 \cdot 1H_2O$) C, H, N. Alternatively, 16 can be synthesized via the following precursor.

10,11-(Ethylenedioxy)-7-(imidazolylmethyl)-(20*S*)-camptothecin Trimethylsilyl Ether. 7-(Chloromethyl)-10,11-(ethylenedioxy)-(20*S*)-camptothecin (50 mg, 0.11 mmol) was added to 1-(trimethylsilyl)imidazole (1.0 mL) under a blanket of nitrogen at room temperature. This was stirred for 1.5 h before the addition of diethyl ether (1.0 mL) which precipitated the desired product. The pale yellow solid was collected by filtration and washed twice with diethyl ether (5 mL each). The compound was dried under high vacuum to afford a yellow solid (56 mg, 91%): mp 266 °C dec. ^{1}H NMR (DMSO): δ 0.24 (s, 9H), 0.98 (t, 3H), 1.98 (m, 2H), 4.49 (s, 4H), 5.40 (s, 2H), 5.53 (s, 2H), 5.88 (s, 2H), 6.98 (s, 1H), 7.43 (s, 1H), 7.46 (s, 1H), 7.62 (s, 1H), 7.25 (s, 2H), 7.91 (s, 1H). Electrospray MS: *m/z* 559 (MH^+). HR FAB MS: calcd for $C_{29}H_{31}N_4O_6Si$, 559.2013; found, 559.2008. It was necessary to heat the title compound in 1 N HCl (aqueous) at 60 °C for 1 h in order to hydrolyze the trimethylsilyl ether to afford **16**.

10,11-(Ethylenedioxy)-7-[(3′-methylimidazoliumyl)-methyl]-(20*S*)-camptothecin Chloride (25). Title compound was prepared as described in method a with a reaction time of 2.25 h, 90% yield: mp >303 °C. ^{1}H NMR (DMSO): δ 0.95 (t, 3H), 1.93 (m, 2H), 3.63 (s, 3H), 4.41 (s, 4H), 5.38 (s, 2H), 5.42 (s, 2H), 6.0 (s, 2H), 6.55 (s, 1H), 7.18 (s, 1H), 7.61 (s, 1H), 7.70 (d, 1H), 7.73 (s, 1H), 7.90 (s, 1H), 9.01 (s, 1H). Electrospray MS: *m/z* 501 (M^+). HR FAB MS: calcd for $C_{27}H_{25}N_4O_6$, 501.1774; found, 501.1769.

10,11-(Methylenedioxy)-7-[(3′-methylimidazoliumyl)-methyl]-(20*S*)-camptothecin Chloride (17). Title compound was prepared as in method a with a reaction time of <4.0 h, 82% yield: mp 242 °C dec (froths). ^{1}H NMR (DMSO: δ 0.93 (t, 3H), 1.93 (m, 2H), 3.77 (s, 3H), 5.38 (s, 2H), 5.42 (s, 2H), 6.00 (s, 2H), 6.31 (s, 2H), 6.57 (s, 1H), 7.30 (s, 1H), 7.60 (s, 1H), 7.68 (s, 2H), 7.88 (s, 1H), 9.03 (s, 1H). Electrospray MS: *m/z* 487 (M^+). HR FAB MS: calcd for $C_{26}H_{23}N_4O_6$, 487.1618; found, 487.1607. Anal. ($C_{26}H_{23}N_4O_6Cl \cdot 1H_2O$) C, H. N.

10,11-(Methylenedioxy)-7-(pyridaziniumylmethyl)-(20*S*)-camptothecin Chloride (23). Title compound was prepared as in method a with a reaction time of 1.5 h, 85% yield: mp >275 °C dec. ^{1}H NMR (DMSO): δ 0.95 (t, 3H), 1.93 (m, 2H), 5.41 (s, 2H), 5.43 (s, 2H), 6.31 (s, 2H), 6.52 (s, 1H), 6.66 (s, 2H), 7.26 (s, 1H), 7.60 (s, 1H), 7.86 (s, 1H), 8.58 (m, 1H), 8.75 (m, 1H), 9.22 (m, 1H), 9.48 (m, 1H), 10.20 (m, 1H). FAB MS: *m/z* 486 (MH^+). HR FAB MS: calcd for $C_{26}H_{21}N_4O_6$, 485.1461; found, 485.1454. Anal. ($C_{26}H_{21}N_4O_6Cl \cdot 2H_2O$) C, H, N.

10,11-(Ethylenedioxy)-7-pyridaziniumyl-(20*S*)-camptothecin Chloride (22). Title compound was prepared as in method a with a reaction time of 1.25 h, 92% yield: mp >325 °C dec. ^{1}H NMR (DMSO): δ 0.93 (t, 3H), 1.95 (m, 2H), 4.43 (s, 4H), 5.41 (s, 4H), 6.62 (bs, 1H), 6.68 (s, 2H), 7.28 (s, 1H), 7.63 (s, 1H), 7.86 (s, 1H), 8.58 (m, 1H), 8.76 (m, 1H), 9.49 (m, 1H), 10.24 (m, 1H). FAB MS: *m/z* 499 (M^+). HR FAB MS: calcd for $C_{27}H_{23}N_4O_6$, 499.1618; found, 499.1611. Anal. ($C_{27}H_{23}N_4O_6Cl \cdot 2H_2O$) C, H, N.

10,11-(Methylenedioxy)-7-pyraziniumyl-(20*S*)-camptothecin Chloride (19). Title compound was prepared as in method b with a reaction time of 18 h, 89% yield: mp 260 °C dec. ^{1}H NMR (DMSO): δ 0.92 (t, 3H), 1.90 (m, 2H), 5.40 (s, 2H), 5.41 (s, 2H), 6.34 (s, 2H), 6.48 (s, 1H), 7.28 (s, 1H), 7.63 (s, 2H), 9.03 (d, 2H), 9.44 (d, 2H). Electrospray MS: *m/z* 485 (M^+). HR FAB MS: calcd for $C_{26}H_{21}N_4O_6$, 485.1461; found, 485.1463.

7-[[3-(Hydroxymethyl)pyridiniumyl]methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin Chloride (21). Title compound was prepared as in method a with a reaction time of 16 h, 99% yield: mp 230 °C dec. ^{1}H NMR (DMSO): δ 0.92 (t, 3H), 1.90 (m, 2H), 4.67 (s, 2H), 5.50 (s, 2H), 5.61 (s, 2H), 5.80 (s, 2H), 6.28 (s, 2H), 6.42 (s, 1H), 7.18 (s, 1H), 7.27 (s, 1H), 7.51 (s, 1H), 7.74 (s, 1H), 8.02 (m, 1H), 8.49 (m, 1H), 8.85 (m, 1H), 8.93 (s, 1H). Electrospray MS: *m/z* 514 (M^+). HR FAB MS: calcd for $C_{28}H_{24}N_3O_7$, 514.1614; found, 514.1623.

10,11-(Ethylenedioxy)-7-[[3-(hydroxymethyl)pyridiniumyl]methyl]-(20*S*)-camptothecin Chloride (24). Title compound was prepared as in method a with a reaction time of 16 h, 92% yield: mp 250 °C dec. ^{1}H NMR (DMSO): δ 0.96 (t, 3H), 1.91 (m, 2H), 4.33 (s, 4H), 4.67 (s, 2H), 5.34 (s, 2H), 5.41 (s, 2H), 5.87 (bs, 1H), 6.44 (s, 2H), 6.54 (s, 1H), 7.31 (s, 1H), 7.54 (s, 1H), 7.66 (s, 1H), 8.06 (t, 1H), 8.52 (d, 1H), 8.91 (d, 1H), 8.99 (s, 1H). Electrospray MS: *m/z* 528 (M^+). HR FAB MS: calcd for $C_{29}H_{26}N_3O_7$, 528.1771; found, 528.1771.

10,11-(Ethylenedioxy)-7-[[4-(hydroxymethyl)pyridiniumyl]methyl]-(20*S*)-camptothecin Chloride (26). Title compound was prepared as in method c with a reaction time of 16 h, light red-orange solid, 93% yield: >260 °C dec. ^{1}H NMR (DMSO): δ 0.96 (t, 3H), 1.91 (m, 2H), 4.41 (s, 4H), 4.67 (s, 2H), 5.35 (s, 2H), 5.44 (s, 2H), 6.41 (bs, 1H), 6.49 (s, 2H), 7.31 (s, 1H), 7.53 (s, 1H), 7.61 (s, 1H), 7.68 (d, 2H), 8.69 (d, 2H), 9.06 (s, 1H). Electrospray MS: *m/z* 527 (M^+). HR FAB MS: calcd for $C_{29}H_{26}N_3O_7$, 528.1771; found, 528.1766.

Solubility Determinations. The compound was equilibrated for at least 30 min in 1.0 mL of deuterium oxide containing a known amount of 1,4-dioxane (0.015 M standardized solution) as an internal standard. The mixture was filtered, and a 300-MHz spectrum was taken of the filtrate. The concentration of the drug was determined by comparing the 1,4-dioxane integral at 3.75 ppm with the integral of the camptothecin analog C-18 methyl triplet at 0.8−0.9 ppm.

Biology. Gel-Based Cleavable Complex Assay for Topoisomerase I Inhibition. The ability of camptothecin analogs to inhibit topoisomerase I was quantified in the cleavable complex assay as previously described.[12,27,28] Topoisomerase I was isolated from calf thymus to a high degree of purity and was devoid of topoisomerase II.[29] All reactions were carried out in 10-mL volumes of reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 30 mg/mL BSA) in microtiter plates. The camptothecin analogs were dissolved in DMSO at 10 mg/mL and serially diluted in 96-well microtiter plates to which the ^{32}P-end-labeled pBR322 DNA and topoisomerase enzyme were added. The reaction mixture was incubated at room temperature for 30 min and then stopped by adding 2 mL of a mixture of sodium dodecyl sulfate and proteinase K (Boehringer Mannheim, Indianapolis, IN) (1.6% and 0.14 mg/mL final concentrations, respectively). The plates were heated at 50 °C for 30 min, 10 mL of standard stop mixture containing 0.45 N NaOH was added in order to generate single-stranded DNA, and the samples were electrophoresed in 1.5% agarose gels in TBE buffer. Gels were blotted on nitrocellulose paper (BioRad, Richmond, CA), dried, and exposed to X-ray film. The units of cleavage were calculated from the autoradiographs and plotted against the log drug concentration using the Nonlin84 software package from SCI Software (Lexington, KY). The IC_{50} values for each drug were determined as an average of multiple runs.

Cell Culture Cytotoxicity Assays. The cytotoxicity of compounds was determined using a microculture tetrazolium assay.[30] All cell lines used for the cytotoxicity assay were grown under identical conditions in α-MEM medium containing 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 U/mL insulin, nonessential amino acids, and 0.5 mg/mL gentamycin (GibcoBRL, Grand Island, NY) at 37 °C in an atmosphere of 5% CO_2 in air. Tumor cells were plated in 96-well microtiter plates and

allowed to adhere overnight. Cells were incubated with compound for 48 h and then with fresh medium for 48 h. The drugs were tested over a 0.17 nM−10 mM range, in quadruplicate at each concentration tested. Following a 4-h incubation of treated cells to the yellow water soluble dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the reduced dye product was extracted from the cells with DMSO and quantitated spectrophotometrically. Assay data for each compound were fitted using a four-parameter logistic equation to obtain IC_{50} values.

***In Vivo* Protocol: Xenograph Study (5-Week Assay).** Female nu/nu mice (18−24 g, 10−14 weeks old) were housed in microisolator filtration racks and maintained with filtered acidified water and sterile lab chow ad libitum. Mice were allowed to acclimate for 1 week prior to testing. Before treating tumor-bearing animals with test compounds, a dose-ranging study of compound was performed in naive mice to determine the highest dose for the 5-week schedule. For this purpose, mice were dosed subcutaneously twice a week for 5 weeks and their body weight was monitored twice weekly. The loss of 30% body weight or greater was considered lethal, and the highest dose was defined as that dose which caused sufficient morbidity as determined by body weight loss. Tumors were established by injecting harvested HT-29 tumor cells in a single subcutaneous site, on the flank of the mice in the left auxillary region. The mice were then sorted according to body weight, grouped 4 mice/cage, and tatooed on the tail for permanent identification. Within a treatment group, a narrow range in body weight ± 1 g and tumor size was established. Efficacy studies were performed over a dose range which included the highest dose. The tumor volume for each mouse was determined by measuring two dimensions with vernier calipers and calculated using the formula: tumor volume = (length × width^2)/2. The data were plotted as the percent change in mean values of tumor volume and body weight for each group. The overall growth of tumors was expressed as a ratio of T/B where the tumor volume at the end of treatment (T) was divided by the initial volume at the beginning of the experiment (B). Thus, any tumor group which did not respond to treatment and grew over the course of the experiment displayed a T/B ratio of >1, and treatment groups in which tumors regressed displayed T/B ratios of <1.

***In Vivo* Protocol: Tumor Growth Delay (2-Week Assay).** This protocol was carried out as above with the following modification in the dosing schedule. The maximum tolerated dose for the compound was first determined by evaluation of body weight loss in nude mice (without xenografts) upon administration of a single bolus dose of drug subcutaneously. This dose was then divided into three portions which were administered to xenograft-bearing mice ($N = 6$) over the course of a single day. The delay in tumor growth, the time in days required for the tumor to reach 500% of initial volume in drug treated animals (T) compared to controls (C), was determined and expressed as (T/C).

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Supporting Information Available: Copies of ^{1}H NMR and representative graphs for the *in vivo* experiments (6 pages). Ordering information is given on any current masthead page.

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