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Synthesis and Antitumor Activity of Novel Water Soluble Derivatives of Camptothecin as Specific Inhibitors of Topoisomerase I

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The synthesis and antitumor activities of the novel water soluble camptothecin derivatives 7-[(4-methylpiperazino)methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**6**) and 7-[(4-methylpiperazino)methyl]-10,11-(ethylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**7**) are described. The solubilities of compounds **6** and **7** were measured to be 4.5 and 5.8 mg/mL, respectively, in pH 5 acetate buffer in contrast to <0.003 mg/mL for camptothecin in the same buffer. In the purified topoisomerase I cleavable complex enzyme assay, compounds **6** and **7** demonstrated potent inhibition of topoisomerase I with IC_{50}'s of 300 and 416 nM, respectively, in comparison to 679 nM for camptothecin and 1028 nM for topotecan. In human tumor cell cytotoxicity assays, compounds **6** and **7** demonstrated potent antitumor activity against ovarian (SKOV3), ovarian with upregulated MDRp-glycoprotein (SKVLB), melanoma (LOX), breast (T47D), and colon (HT29) with IC_{50}'s ranging from 0.5 to 102 nM. Compounds **6** and **7** induced tumor regressions in the HT29 human colon tumor xenograft model and demonstrated similar rank order of potency compared to *in vitro* assay results.

Introduction

Eukaryotic DNA topoisomerases I and II are essential nuclear enzymes responsible for the organization and modulation of the topological features of DNA so that a cell may replicate, transcribe, and repair genetic information.[1-3] Topoisomerase I functions by creating transient single-stranded nicks in DNA supercoils relieving torsional strain that has accumulated during DNA replication and transcription.[2] 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 target for the development of new cancer chemotherapeutic agents against a number of solid human tumors.

The pentacyclic alkaloid natural product camptothecin (**1**) was first reported by Wall and co-workers (Figure 1) to have potent anticancer activity and, upon the basis of its preclinical antitumor activity, was progressed into humans for clinical evaluation.[6-12] Because of the poor solubility of camptothecin in aqueous systems, **1** was evaluated in the clinic as the water soluble sodium salt of the lactone hydrolysate **2** (Figure 1). Objective tumor responses were observed in clinical studies; however, severe hemorrhagic cystitis and unpredictable myelosuppression were observed as the major dose-limiting toxicities, and thus further clinical development of camptothecin was halted.[11,12] At the

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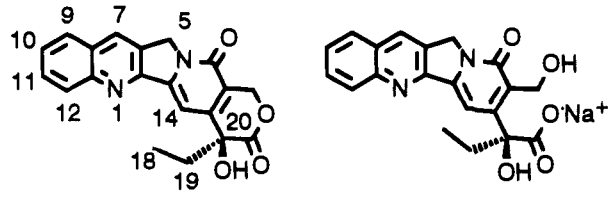


1 2

Figure 1. Camptothecin (1) and the sodium salt (**2**) of camptothecin.

time of the clinical studies, the cellular mechanism by which camptothecin caused cytotoxic events was unknown. Subsequent to the clinical studies of the sodium salt of camptothecin, seminal biochemical studies by Hsiang and co-workers identified topoisomerase I as the specific intracellular target of camptothecin.[13] In a purified enzyme assay, camptothecin was shown to bind to the complex formed from the single-stranded cleavage of DNA by topoisomerase I.[14] Camptothecin appears to stabilize this "cleavable complex" by interfering with the religation of DNA. Enzymology studies have demonstrated that the closed lactone of camptothecin was necessary for topoisomerase I inhibition.[13-16] However, the sequence of events following the inhibition of topoisomerase I, which leads to cell death, has not been fully elucidated. A number of research groups, including our own, have speculated that the efficacy and toxicity profile of camptothecin may be improved by producing a water soluble variant while maintaining the intact lactone.[17-19] The solubility of camptothecin and its analogs is complicated by a pH-dependant equilibrium hydrolysis and lactonization reaction. The reactivity of the lactone ring to chemical as well as biological hydrolysis and the reversibility of this reaction have been well studied. In general, the lactone predominates at pH ≤ 5, whereas the carboxylate is favored at pH >7.5. Studies have demonstrated that the solubility of camptothecin is less than 0.003 mg/mL in a variety of buffer systems at pH's necessary to maintain the closed lactone.[19,20]

Synthetic work by a number of research groups have contributed to the understanding of the structure–activity relationships of numerous structural modifications to the camptothecin nucleus with the intent of obtaining derivatives with an improved antitumor profile.[17-19,21] Due to the synthetic difficulty in obtaining useful amounts of camptothecin and derivatives thereof, most chemical efforts to obtain efficacious and water soluble derivatives of camptothecin have focused on the semisynthetic modification of plant-derived camptothecin.[22] This work led to the identification of Irinotecan (CPT-11), an aqueous soluble inactive prodrug of 7-ethyl-10-hydroxycamptothecin (SN-38), and topotecan, both of which are currently in clinical evaluation.[23] We wish to report a novel class of specific and potent inhibitors of topoisomerase I, based upon camptothecin, which demonstrate enhanced aqueous solubility and efficacious antitumor activity both *in vitro* and *in vivo*.

Several derivatives of 10,11-(methylenedioxy)-(20*S*)-camptothecin (**3**) were synthesized with water-solubilizing amines linked by a single methylene group at the C-7 position (**4**) to improve aqueous solubility and pharmacologically map this region of the molecule (Figure 2).[24-26] The choice of 10,11-(methylenedioxy)-(20*S*)-camptothecin (**3**) as the template for water soluble derivatives was based upon the outstanding *in vitro* and *in vivo* antitumor activity reported for 10,11-(methylenedioxy)-(20*RS*)-camptothecin.[17,27,28] However, the limited solubility characteristics of camptothecin were found to exist for 10,11-(methylenedioxy)-(20*S*)-camptothecin (Table 1). Further, it was anticipated that the dioxolane ring of **3** may be metabolically cleaved to the corresponding catechol by cytochrome P-450.[29] Wall and co-workers have reported that 10,11-dihydroxy-(20*RS*)-camptothecin is an inhibitor of topoisomerase I; however, this compound did not demonstrate significant antitumor efficacy when evaluated *in vivo*.[30] The 10,11-(ethylenedioxy)-(20*S*)-camptothecin series **5** was investigated in parallel to circumvent anticipated metabolism problems that may be encountered for the 10,-11-(methylenedioxy)camptothecin series. Synthetic efforts focused on the preparation of a diverse set of aminomethyl derivatives of the structural types **4** and **5**.[24,25] In particular, it was found that 7-[(4-methylpiperazino)methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**6**) and 7-[(4-methylpiperazino)methyl]-10,11-(ethylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**7**) (Figure 3) demonstrated *in vitro* inhibitory potencies against topoisomerase I greater than camptothecin, aqueous solubilities greater than 1 mg/mL, *ex vivo* potencies in a panel of tumor cell lines equal to or greater than camptothecin, and tumor regression in human tumor xenograft models.

Chemistry

The synthesis of **6** and **7** was accomplished using a Friedlander condensation between the respective functionalized A ring precursors **8** and **9** and the known tricyclic keto lactone **10** to afford the corresponding 7-(chloromethyl)camptothecin derivatives **11** and **12** as versatile advanced intermediates which were converted to the corresponding amines by displacement of the chloride (see Scheme 1).[31-33] The A ring precursor 2′-amino-2-chloro-4′,5′-(methylenedioxy)acetophenone (**8**) was obtained in three steps by the following synthetic route. N-Acetylation of 3,4-(methylenedioxy)aniline was carried out with acetic anhydride followed by a directed zinc chloride-catalyzed Friedel Crafts acylation with chloroacetyl chloride on the anilide to provide the expected α-chloroacetophenone. Hydrolysis of the acetamide group, using aqueous acid, produced the requisite 2′-amino-2-chloro-4′,5′-(methylenedioxy)acetophenone (**8**). In the ethylenedioxy series, 1,4-benzodioxan-6-amine was reacted with chloroacetonitrile in the presence of boron trichloride and catalytic anhydrous aluminum chloride producing the corresponding 2′-amino-2-chloro-4′,5′-(ethylenedioxy)acetophenone (**9**) in one step. The 2′-amino-2-chloro-4′,5′-(methylenedioxy)acetophenone (**8**) and 2′-amino-2-chloro-4′,5′-(ethylenedioxy)acetophenone (**9**) were reacted with the known tricyclic keto lactone **10** under pTSA catalyzed Friedlander condensation conditions to yield the corresponding 7-(chloromethyl)-10,11-(methylenedioxy)-(20*S*)-camptothecin (**11**) and 7-(chloromethyl)-10,11-(ethylenedioxy)-(20*S*)-camptothecin (**12**), respectively. The respective chlorides were displaced with 4-methylpiperazine to provide the corresponding tertiary amines. The final compounds were purified by HPLC and isolated as their trifluoroacetate salts.

Table 1. *In Vitro* Comparison of Camptothecin and Derivatives in the Topoisomerase I Cleavable Complex Assay, Solubility Assay, and Human Tumor Cell Cytotoxicity Assay (MTT)

compd	Topo I IC_{50} (nM)[a]	solubility, pH = 5.0 (mg/mL)	HT29 IC_{50} (nM)[b]	LOX IC_{50} (nM)[b]	T47D IC_{50} (nM)[b]	SKOV3 IC_{50} (nM)[b]	SKVLB IC_{50} (nM)[b]	MDR ratio[c]
compd 1	679 (±92)	0.003	46 (±15)	48	207	37 (±8)	41 (±15)	1
topotecan	1028 (±135)	3.1	25 (±2)	5	104	44 (±7)	149 (±36)	3
compd 3	78 (±35)	<0.003	ND	ND	ND	ND	ND	ND
compd **6**	300 (±63)	4.5	2 (±2)	0.5	30	4 (±3)	17 (±11)	4
compd **7**	416 (±95)	5.8	6 (±1)	1	38	16 (±4)	102 (±48)	6
compd 11	15 (±3)	<0.003	ND	ND	ND	ND	ND	ND
compd 12	11 (±6)	<0.003	13	ND	ND	4 (±2)	8	2
doxorubicin		>2.000	331 (±36)	42 (±8)	72 (±12)	36 (±7)	5650 (±940)	160

[a] Average concentration of compound to cause 50% inhibition of topoisomerase I as determined using the cleavable complex assay (see refs 17 and 34). [b] Human tumor cell cytotoxicity assay was performed using MTT (see ref 35) and the concentration of compound causing 50% cell kill (IC_{50}) determined. [c] MDR ratio is defined as the quotient of SKVLB IC_{50}/SKOV3 IC_{50}. For a and b, where three or more determinations were made, standard errors of the mean are shown in parentheses. ND indicates not determined.

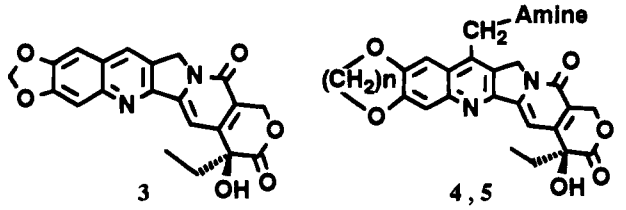


Figure 2. Structure of 10,11-(methylenedioxy)-(20*S*)-camptothecin (**3**) and the C-7-modified structures: n = 1, 7-aminomethyl-substituted 10,11-(methylenedioxy)-(20*S*)-camptothecin series **4**, and n = 2, 7-aminomethyl-substituted 10,11-(ethylenedioxy)-(20*S*)-camptothecin series **5**.

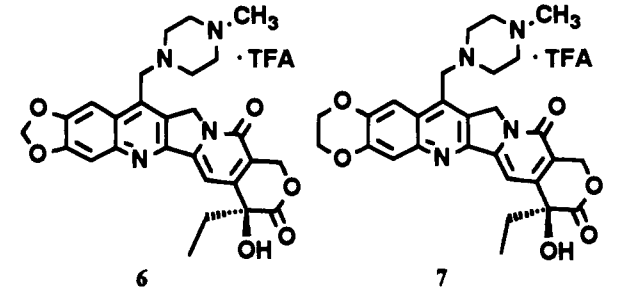


Figure 3. Water soluble topoisomerase I inhibitors 7-[(4-methylpiperazino)methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**6**) and 7-[(4-methylpiperazino)-methyl]-10,11-(ethylenedioxy)-(20*S*)-camptothecin trifluoroacetate (**7**).

Results and Discussion

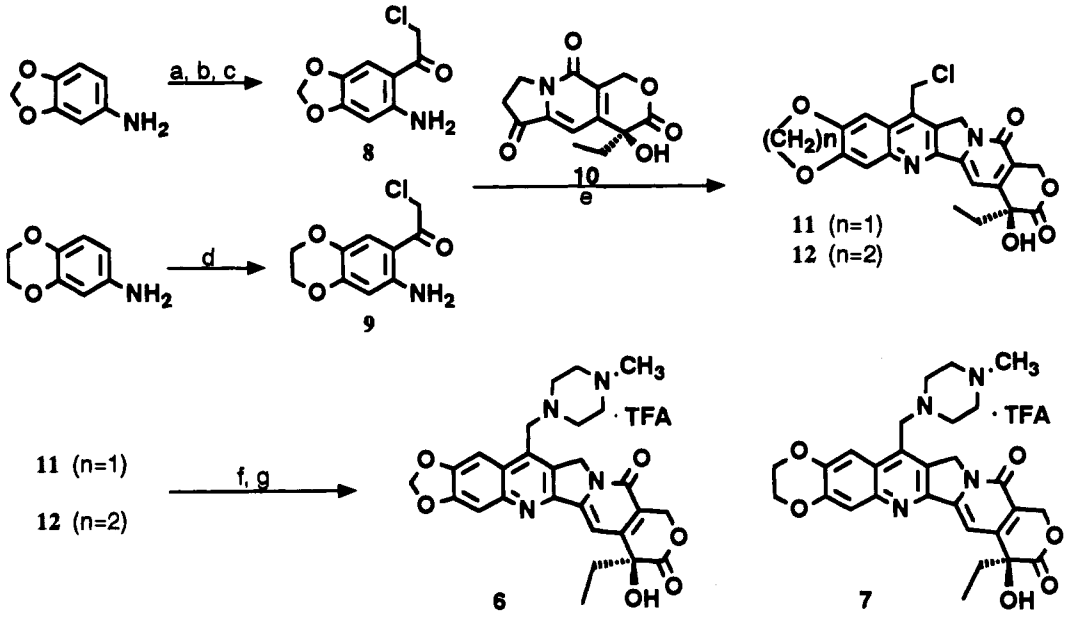
The solubilities of compounds **1**, **3**, **6**, **7**, **11**, and **12** and topotecan were measured in 0.1 M acetate buffer at pH 5.[20] Under these conditions, all compounds evaluated were found to possess the lactone intact. As can be seen in Table **1**, compounds **6** and **7** are highly soluble in pH 5 acetate buffer, demonstrating greater than 3 orders of magnitude improvement in solubility relative to camptothecin. At this pH, the 7-(4-methylpiperazino)methyl substituent is protonated, solubilizing the camptothecin analog in the buffer solution while maintaining the lactone in the closed and biologically active form. It may be speculated that the position of the equilibrium for the closed and open lactone forms of **6** and **7** would be similar to that of topotecan or CPT-11 upon *in vivo* administration.[20d] These studies are ongoing and will be reported in due course. As anticipated, the aqueous solubility profile of **11** and **12** was similar to that of **1** and **3**.

Compounds **6** and **7** were evaluated in the topoisomerase I cleavable complex enzyme assay and the *ex vivo* tumor cell cytotoxicity assays using camptothecin as an internal standard (see Table 1).[34,35] In the cleavable complex assay, 7-[(4-methylpiperazino)methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin (**6**) was a significantly more potent inhibitor of topoisomerase I (IC_{50} = 300 nM) than camptothecin (**1**) (IC_{50} = 679 nM); however, the inhibitory activity was decreased relative to 10,11-(methylenedioxy)-(20*S*)-camptothecin (**3**) (IC_{50} = 78 nM) and 7-(chloromethyl)-10,11-(methylenedioxy)-(20*S*)-camptothecin (**11**) (IC_{50} = 15 nM). The 7-[(4-methylpiperazino)methyl]-10,11-(ethylenedioxy)-(20*S*)-camptothecin (**7**) (IC_{50} = 416 nM) was a more potent inhibitor than camptothecin (**1**) but was about 2-fold less potent as an inhibitor than 7-[(4-methylpiperazino)-methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin (**6**) (IC_{50} = 300 nM). The 2-fold difference in the topoisomerase inhibitory activity of compounds **6** and **7** was not observed in the activity of the respective 7-chloromethyl precursors. The respective topoisomerase I inhibitory activity of compounds **11** and **12** was approximately 20 and 38 times that of **6** and **7**, and they were 5–7 times more potent than **3**. Compounds **6** and **7** were more cytotoxic than camptothecin or topotecan in the cellular cytotoxicity assays against the following human tumor cell lines: SKOV3 (ovarian), SKVLB (ovarian with upregulated MDRp-glycoprotein), LOX (melanoma), T47D (breast), and HT29 (colon). The relative ranking of the IC_{50}'s observed for **6** and **7** in the human tumor cell cytotoxicity assays parallels the IC_{50}'s observed for the inhibition of purified topoisomerase I. Further, the ratio of the IC_{50}'s of SKVLB (ovarian with upregulated MDRp-glycoprotein)/SKOV3 (ovarian) reflects the relative insensitivity of **6** and **7** to multidrug resistance mediated by the MDRp-glycoprotein when compared to doxorubicin.

The *in vivo* antitumor activities of 7-[(4-methylpiperazino)methyl]-10,11-(methylenedioxy)-(20*S*)-camptothecin (**6**) and 7-[(4-methylpiperazino)methyl]-10,11-(ethylenedioxy)-(20*S*)-camptothecin (**7**) were evaluated in direct comparison with topotecan in the HT29 human colon tumor xenograft model using the athymic nu/nu mouse (see Table 2). Compounds **6** and **7** were titrated to their maximally tolerated dose levels as determined by acute body weight loss and death. Compound efficacy was then determined using a 5 week dose schedule of twice weekly subcutaneous injection of compound, similar to previous studies reported for other camptothecin derivatives.[4]

Table 2 shows that both **6** and **7** produced dose-dependent responses and demonstrated significant antitumor activity *in vivo*. Tumor regression was observed

Scheme 1[a]



[a] Reagents: (a) AcCl, Na_2CO_3; (b) $ClCH_2COCl$, $ZnCl_2$, CH_3NO_2, 100 °C; (c) concd HCl, ethanol, 80 °C; (d) $ClCH_2CN$, BCl_3, CH_2Cl_2, 40 °C; (e) pTSA, tol, 100 °C; (f) 1-methylpiperazine, DMF, rt; (g) CF_3CO_2H.

Table 2. *In Vivo* Efficacy Study of Camptothecin Derivatives **6** and **7** and Topotecan Using the HT29 Human Colon Xenograft Model

compd	dose (mg/kg)	efficacy (T/B)[a]	BW loss (%)	deaths
compd **6**	1	6.4	11	0
	2	1.7	8	0
	3	0.9	12	0
	4	0.6	20	0
compd **7**	3	5.5	8	0
	6	1.6	5	0
	9	0.8	7	0
	12	0.4	22	2
topotecan	5	8.6	15	0
	7	7.0	11	0
	9	4.3	17	2
	11	2.9	23	0
vehicle		12.1	11% gain	0

[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. BW loss indicates mean body weight loss for animals in all experimental groups. $N = 6$ in all experimental groups.

at both the second highest and maximally tolerated dose (MTD) levels where **6** and **7** were able to reduce tumor load to 60% or less of the initial tumor burden. Topotecan, at its MTD, was able to significantly reduce tumor growth relative to control but was not able to demonstrate regression of tumor burden in this model. It is interesting to note that compound **12** demonstrated potent *in vitro* enzyme inhibition and was highly cytotoxic to HT29 colon tumor cells in the MTT assay, but when evaluated *in vivo*, **12** did not demonstrate any inhibition of tumor growth (data not shown). Thus, a potent inhibitor of topoisomerase I *in vitro* which demonstrates potent cytotoxicity against tumor cell lines does not always translate to a potent antitumor agent *in vivo*.

In conclusion, aminomethyl substituents at the C-7 position of camptothecin in combination with either 10,11-methylenedioxy or 10,11-ethylenedioxy substitution provides novel, potent, water soluble topoisomerase I inhibitors with significant antitumor activity. Compounds **6** and **7** are highly soluble in aqueous buffer and demonstrate potent antitumor activity against a number of tumor cell lines derived from human solid tumors. Tumor regression was observed in the *in vivo* evaluation of **6** and **7** demonstrating promising preclinical activity. Compound **7** was further evaluated in more detailed preclinical studies and has been progressed into phase 1 clinical studies.[36,37] Finally, compounds **6** and **7** represent a promising new class of camptothecin-based topoisomerase I inhibitors worthy of future anticancer drug research.

Experimental Section

Chemistry. Melting points were determined using a Thomas-Hoover melting point apparatus and are uncorrected. Unless stated otherwise, reagents were obtained from commercial sources and used directly. Reactions involving air- or moisture-sensitive reagents were carried out under a nitrogen atmosphere. Anhydrous solvents were obtained from Aldrich (Sure Seal) except CH_2Cl_2 which was distilled from CaH_2 prior to use. ^1H NMR spectra were recorded on a Varian 300 MHz spectrometer; chemical shifts are reported in parts per million (ppm) relative to TMS. The following abbreviations are used to describe peak patterns when appropriate: b = broad, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. High-performance liquid chromatography (HPLC) was performed on a Beckman 126 instrument with a Beckman 168 diode array detector or a Waters 3000 Delta Prep instrument with a Rainin Dynamax-60A column and a Waters Lamda-Max 481 UV detector (monitoring at 254 nm). Elemental analyses, performed by Atlantic Microlab, Inc., Norcross, GA, were within 0.4% of the theoretical values calculated for C, H, and N.

3,4-(Methylenedioxy)acetanilide. A three-neck 500 mL round bottom flask, equipped with a mechanical stirrer, a 20 mL dropping funnel, and a condenser, was charged with sodium bicarbonate (11.5 g, 136 mmol), 90 mL of chloroform, 50 mL of water, and 3,4-(methylenedioxy)aniline (17 g, 124 mmol). The mixture was stirred and cooled to 0 °C. The dropping funnel was charged with acetyl chloride (9.7 g, 124 mmol) which was then added dropwise to the cooled reaction mixture with stirring. After all the acid chloride was added, the reaction mixture was allowed to warm to room temperature and stirred until no aniline remained. The reaction mixture was poured into a separatory funnel and the aqueous phase removed. The organic layer was washed with brine. The

aqueous layers were combined and washed with chloroform. The organic extracts were combined, dried over magnesium sulfate, filtered, and concentrated to 18.1 g of a brown solid. The brown solid was recrystallized from water and gave 9.34 g, 42%, of 3,4-(methylenedioxy)acetanilide as pale tan crystals. ^{1}H NMR ($CDCl_3$): δ 7.2 (s, 1H), 6.75 (m, 2H), 5.95 (s, 2H), 2.15 (s, 3H). Nominal mass spectrum (CI mode): m/z 180 (M + H).

2′-(Acetylamino)-2-chloro-4′,5′-(methylenedioxy)acetophenone. A dry 250 mL three-neck round bottom flask was fitted with a mechanical stirrer, a condenser with a nitrogen inlet, and a dropping funnel, was charged with a mixture of anhydrous zinc chloride (23.4 g, 178.3 mmol), chloroacetyl chloride (16.1 ml, 202.1 mmol), and nitromethane (85 mL) under a nitrogen atmosphere, and was stirred at room temperature. A solution of 3,4-(methylenedioxy)acetanilide (8.96 g, 50 mmol) in 15 mL of nitromethane was added dropwise with stirring. After addition, the resultant solution was refluxed for 1.5 h. The dark red solution was cooled, poured onto ice/water, and extracted thoroughly with methylene chloride. The organic layers were combined, dried over magnesium sulfate, filtered, and concentrated to give a brown solid. The resulting solid was dissolved in methanol (50 mL), heated on a steam bath for about 5 min, and then allowed to cool. The resulting solid was collected, redissolved in ethyl acetate, decolorized with carbon, and filtered followed by addition of hexanes to crystallize the acetophenone. Recrystallization of the resulting solid from ethyl acetate/hexanes gave 0.83 g, 6.5%, of yellow crystals. ^{1}H NMR ($CDCl_3$): δ 8.45 (s, 1H), 7.2 (s, 2H), 6.09 (s, 2H), 4.65 (s, 2H), 2.25 (s, 3H). Nominal mass spectrum (CI mode): m/z 256 (M + H).

2′-Amino-2-chloro-4′,5′-(methylenedioxy)acetophenone (8). A 100 mL three-neck round bottom flask was equipped with a stir bar, a reflux condenser, and a dropping funnel. The reaction vessel was charged with 2′-(acetylamino)-2-chloro-4′,5′-(methylenedioxy)acetophenone (0.9 g, 3.53 mmol) and 60 mL of ethanol. The resulting solution was cooled to 0 °C followed by dropwise addition of concentrated hydrochloric acid (12.5 mL). The reaction mixture was heated to reflux for 1 h, cooled, poured onto ice, and neutralized with 2 N sodium hydroxide. The aqueous solution was extracted thoroughly with ethyl acetate. The organic layers were combined, dried over magnesium sulfate, filtered, decolorized with carbon, filtered, and concentrated to a greenish yellow solid. The solid was recrystallized from ethyl acetate/2-propanol/hexanes yielding, after drying, 0.39 g (52%) of pale yellow crystals. Mp = 151–152 °C. ^{1}H NMR ($CHCl_3$): δ 6.99 (s, 1H), 6.55 (b s, 1H), 6.16 (s, 1H), 5.93 (s, 2H), 4.52 (s, 2H). Nominal mass spectrum (CI mode): m/z 214 (M + H$^+$).

2′-Amino-2-chloro-4′,5′-(ethylenedioxy)acetophenone (9). A 1 L three-neck round bottom flask was fitted with a magnetic stir bar, a thermometer, a reflux condenser with a calcium chloride filled drying tube, and a nitrogen inlet. The reaction vessel was charged with 100 mL of dry methylene chloride and 1,4-benzodioxan-6-amine (15.12 g, 100 mmol). The reaction vessel was cooled to 0 °C followed by slow addition of 400 mL of a 1 M solution of boron trichloride in methylene chloride solution while maintaining an internal temperature at or below 10 °C. Aluminum chloride (13.34 g, 100 mmol) was added quickly in three portions followed by addition of chloroacetonitrile (7 mL, 110 mmol). The reaction mixture was stirred for 30 min at 0 °C and then heated to 40 °C for 16 h. The reaction mixture was removed from heat and allowed to cool to room temperature, and then the reaction was quenched into a mixture of 1 kg of ice/500 mL of 1 N HCl. The mixture was stirred until no solids were observed. The methylene chloride layer was removed, and the aqueous layer was extracted twice. The organic layers were combined, washed with brine, dried over magnesium sulfate, filtered, treated with decolorizing carbon, filtered through a pad of Celite, and concentrated to a solid residue. The solid was recrystallized from ethyl acetate/hexanes to give 8.3 g (36.5%) of 2′-amino-2-chloro-4′,5′-(ethylenedioxy)acetophenone. ^{1}H NMR ($CDCl_3$): δ 7.14 (s, 1H), 6.15 (s, 1H), 4.57 (s, 2H), 4.3 (m, 2H), 4.2 (m, 2H), 1.6 (s, b, 2H). Nominal mass spectrum (CI mode): m/z 228 (M + H).

7-(Chloromethyl)-10,11-(methylenedioxy)-(20S)-camptothecin (11). Into a 250 mL round bottom flask equipped with a stir bar were added 531.3 mg (2.49 mmol) of 2′-amino-2-chloro-4′,5′-(methylenedioxy)acetophenone (**8**), 50 mL of dry toluene, and 656.2 mg (2.49 mmol) of tricyclic keto lactone **10**. The reaction vessel was fitted with a reflux condenser and a Dean Stark trap, stirred under nitrogen, and refluxed for 0.5 h. The reaction mixture was allowed to cool followed by addition of 59 mg (0.31 mmol) of *p*-toluenesulfonic acid. The reaction mixture was then heated to reflux for 36 h. The reaction mixture was cooled, and the solids were collected by filtration and washings with toluene followed by thorough washings of the solids by anhydrous ethanol. The remaining solid was dried under vacuum at room temperature yielding 846.9 mg (77.2%) of >98% pure material. Mp > 250 °C. ^{1}H NMR (DMSO-d_6): δ 7.72 (s, 1H), 7.55 (s, 1H), 7.2 (s, 1H), 6.53 (s, 1H), 6.34 (s, 2H), 5.42 (s, 2H), 5.32 (s, 2H), 5.24 (s, 2H), 1.85 (s, 2H), 0.88 (m, 3H). Nominal mass spectrum (FAB mode): m/z 441 (M + H). High-resolution mass spectrum (M + H): calcd, 441.0853; found, 441.0857.

7-(Chloromethyl)-10,11-(ethylenedioxy)-(20S)-camptothecin (12). Into a 250 mL round bottom flask equipped with a stir bar were added 1.82 g (8.0 mmol) of 2′-amino-2-chloro-4′,5′-(ethylenedioxy)acetophenone (**9**), 40 mL of dry toluene, and 2.0 g (7.6 mmol) of tricyclic keto lactone **10**. The reaction vessel was fitted with a reflux condenser and a Dean Stark trap, stirred under nitrogen, and refluxed for 0.5 h. The reaction mixture was allowed to cool followed by addition of 100 mg (0.53 mmol) of *p*-toluenesulfonic acid. The reaction mixture was then heated to reflux for 36 h. The reaction mixture was cooled, and the solids were collected by filtration and washings with toluene followed by thorough washings of the solids by anhydrous ethanol. The remaining greenish-brown solid was dried under vacuum at room temperature yielding 2.67 g (77.2%) of >97% pure material. ^{1}H NMR (DMSO-d_6): δ 7.47 (s, 1H), 7.32 (s, 1H), 6.98 (s, 1H), 5.15 (s, 1H), 5.06 (s, 1H), 4.97 (s, 1H), 4.19 (s, 4H), 2.23 (s, 2H), 1.6 (m, 2H), 0.68 (m, 3H). Nominal mass spectrum (FAB mode): m/z 455 (M + H). High-resolution mass spectrum (M + H): calcd, 455.1009; found, 455.1005.

7-[(4-Methylpiperazino)methyl]-10,11-(methylenedioxy)-(20S)-camptothecin Trifluoroacetate (6). To a dry 100 mL round bottom flask fitted with a magnetic stir bar was added 220 mg (0.454 mmol) of **11** and 20 mL of dry dimethyl sulfoxide. The mixture was allowed to stir until all solids were dissolved. To the resultant solution was added a solution of 2 mL of 1-methylpiperazine in 20 mL of dry toluene. The reaction mixture was allowed to stir for 2 h after which the reaction mixture was concentrated to a thick residue under high vacuum followed by purification by reverse phase HPLC. The 7-(chloromethyl)-10,11-(methylenedioxy)-(20*S*)-camptothecin (**6**) was purified to homogeneity by reverse phase HPLC using a C-8 dynamax column with a mobile phase of an 80:20 mixture of 2% aqueous trifluoroacetic acid to (4:1 acetonitrile: THF). The relevant fractions were collected, and the solvents were removed in vacuo. The residue obtained was dissolved in water and lyophilized to yield 68 mg (24%) of **6** as a light fluffy yellowish-green solid. ^{1}H NMR (DMSO-d_6): δ 7.82 (s, 1H), 7.56 (s, 1H), 7.28 (s, 1H), 6.32 (s, 2H), 5.45 (s, 2H), 5.32 (s, 2H), 4.09 (s, 2H), 3.37 (m, 2H), 3.05 (m, 4H), 2.8 (m, 2H), 2.55 (s, 3H), 1.89 (m, 2H), 0.93 (t, 3H). Nominal mass spectrum (FAB mode): m/z 505 (M + H). High-resolution mass spectrum (M + H): calcd, 505.2083; found, 505.2087.

7-[(4-Methylpiperazino)methyl]-10,11-(ethylenedioxy)-(20S)-camptothecin Trifluoroacetate (7). A 15 mL round bottom flask equipped with a magnetic stir bar was charged with 0.2 g (0.44 mmol) of **12** and 5 mL of anhydrous *N,N*-dimethylformamide under dry nitrogen. The dark brown solution was stirred and cooled to −78 °C, and the mixture was then treated with 1-methylpiperazine (0.11 mL, 0.97 mmol). The cooling bath was removed, and the mixture was allowed to stir for 2.5 h. The solvent was removed by rotary evaporator at high vacuum and the resulting residue purified by silica gel chromatography using a sequential elution of ethyl acetate followed by 6:5:1 ethyl acetate:chloroform:methanol and then 5:1 chloroform:methanol to yield 74.9 mg (33% yield).

Mp = 261–264 °C. The trifluoroacetate salt was formed by the following method. Of the corresponding free base, 73.9 mg was dissolved in 10 mL of 3% trifluoroacetic acid/glass-distilled water. The resultant solution was filtered through a fine fritted glass funnel and lyophilized to afford 59 mg of fine yellow solid. The compound (**7**) was shown to be >97% pure by reverse phase HPLC. ^{1}H NMR (DMSO-d_6): δ 7.72 (s, 1H), 7.64 (s, 1H), 7.58 (s, 1H), 5.76 (d, 1H), 5.31 (s, 2H), 5.30 (d, 1H), 4.43 (s, 4H), 3.94 (s, 2H), 3.72 (s, 1H), 2.40–2.65 (m, 8H), 2.28 (s, 3H), 1.89 (m, 2H), 1.03 (t, 3H). Nominal mass spectrum (FAB mode): m/z 519 (M + H). High-resolution mass spectrum (M + H): calcd, 519.2236; found, 519.2246.

Solubility Determination (HPLC Method). Compounds were assayed for solubility using an HPLC method which separated the lactone and carboxylate forms. A BDS Hypersil C-18 column, 250 × 4.6 mm, 5 mM particle size, was used with a mobile phase of 40% organic/60% aqueous with 0.005 M tetrabutylammonium phosphate added as a competing base. The solubilities were determined at pH 5.0 in 0.1 M sodium acetate buffer, by equilibrating an excess of solid compound in 0.25 mL of buffer at 25 °C for 24 h. The samples were filtered through a 0.22 mM Millipore (Millex GV4) filter unit and injected on the column. Calibration curves were obtained by plotting the peak areas of standards as a function of drug concentration.

Topoisomerase I Cleavable Complex Assay. The ability of camptothecin analogs to inhibit topoisomerase I was quantified in the cleavable complex assay as previously described.[13,34] Topoisomerase I was isolated from calf thymus to a high degree of purity and was devoid of topoisomerase II.[38] 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 μg/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 the reaction 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}'s for each drug were determined from three separate topoisomerase-mediated cleavable complex experiments, and values are expressed as a mean ± the standard error.

Cell Culture Cytotoxicity Assays. The cytotoxicity of compounds was determined using a microculture tetrazolium assay.[35] 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, and nonessential amino acids (GibcoBRL, Grand Island, NY) at 37 °C in an atmosphere of 5% CO_2 in air. Tumor cells were plated in 100 μL of culture medium in 96-well microtiter plates at a density of 1500–4000 cells/well and allowed to adhere overnight. Cells were incubated with compound for 48 h at 37 °C and then with fresh medium for 48 h. The compounds were tested over a 0.17 nM–10 mM range, in quadruplicate at each concentration tested. Following a 4 h incubation of treated cells with MTT, the reduced dye product was extracted from the cells with 0.2 mL of DMSO followed by 50 μL of Sorensen's buffer. The plates were shaken briefly, and the absorbance at 570 nm was read and quantitated spectrophotometrically using a Molecular Devices UVmax plate reader. Curves were fitted to the MTT assay data for each compound using a four-parameter logistic equation.

Xenograft Studies (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 twice a week for 5 weeks and their body weights were 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 10^6 harvested HT29 tumor cells in a single subcutaneous site, on the flank of the mice in the left axillary region. The mice were then sorted according to body weight, grouped into 6 mice/dose group/cage, and tattooed on the tail for permanent identification. Within a treatment group, a narrow range in body weight ± 1 g and an average palpable tumor mass of 150 mm^2 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 computer-interfaced vernier calipers and calculated using the formula: tumor volume = (length × width^2)/2. The data was 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 treatment (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. Compounds **6** and **7** and topotecan were dissolved in 0.1 M sodium acetate buffer, pH 5, and injected subcutaneously in a 0.2 mL volume. For dosing of compound **12**, the vehicle used was 10% DMSO. The dose schedule was twice weekly injections (Tuesday and Friday) for 5 weeks of treatment.

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