Synthesis and Biological Evaluation of Novel A-Ring Modified Hexacyclic Camptothecin Analogues

Dae-Kee Kim,* Do Hyun Ryu, Ju Young Lee, Namkyu Lee, Young-Woo Kim, Jae-Sun Kim, Kieyoung Chang, Guang-Jin Im, Tae-Kon Kim, and Won-Son Choi

Life Science Research Center, SK Chemicals, 600 Jungja-Dong, Changan-Ku, Suwon-Si, Kyungki-Do 440-745, Korea

Received November 7, 2000

Eleven A-ring modified hexacyclic analogues of camptothecin (CPT) containing a 1,4-oxazine ring were synthesized from 10-hydroxycamptothecin (**11a**) and 7-ethyl-10-hydroxycamptothecin (**3**) (SN-38) in four to five steps and were subjected to the biological tests such as cytotoxicity, topoisomerase I (Topo I) inhibitory activity, acetylcholinesterase (AChE) inhibition, and stability in human plasma. Four compounds **15a**, **15b**, **16a**, and **16c** were about 2-fold more potent than topotecan and as potent as CPT toward human cancer cell lines A549, H128, WiDr, MKN45, SK-OV-3, and SK-BR-3 in vitro, even though the most active compound **15b** was slightly less potent than SN-38. The potency of Topo I inhibition of these compounds showed relatively good correlation with their cytotoxicity. Most of the compounds exhibited AChE inhibitory activity weaker (9 ± 2 to 20 ± 3%) than CPT (23 ± 5%) or topotecan (20 ± 4%) and similar to SN-38 (13 ± 2%), indicating that they might have little effect on causing early diarrhea. The stability of lactone forms of these compounds in human plasma seemed to be much higher than that of CPT and similar to that of topotecan but lower than that of SN-38. Among the new hexacyclic CPT analogues, compound **15b** showed higher antitumor activity against human tumor xenograft, WiDr, in nude mice compared to that of SN-38. The most promising compound **15b** has been selected for further development.

Introduction

Camptothecin (CPT, **1**), a pentacyclic alkaloid isolated by Wall in 1966 from the Chinese tree *Camptotheca acuminata* (Nyssaceae) which has been used for many years as a traditional Chinese medicine, was reported to possess potent antitumor activity.[1] Clinical trials of the water-soluble sodium salt **2** were discontinued in the early 1970s because of severe and unpredictable toxicity, in particular hemorrhagic cystitis, although objective antitumor responses were observed among patients with gastric and colon cancers.[2,3] In 1985, however, it was reported by Liu and co-workers that the cytotoxic activity of CPT was attributed to a novel mechanism of action involving the nuclear enzyme topoisomerase I (Topo I),[4] and this discovery of unique mechanism of action revived interest in CPT and its analogues as anticancer agents.

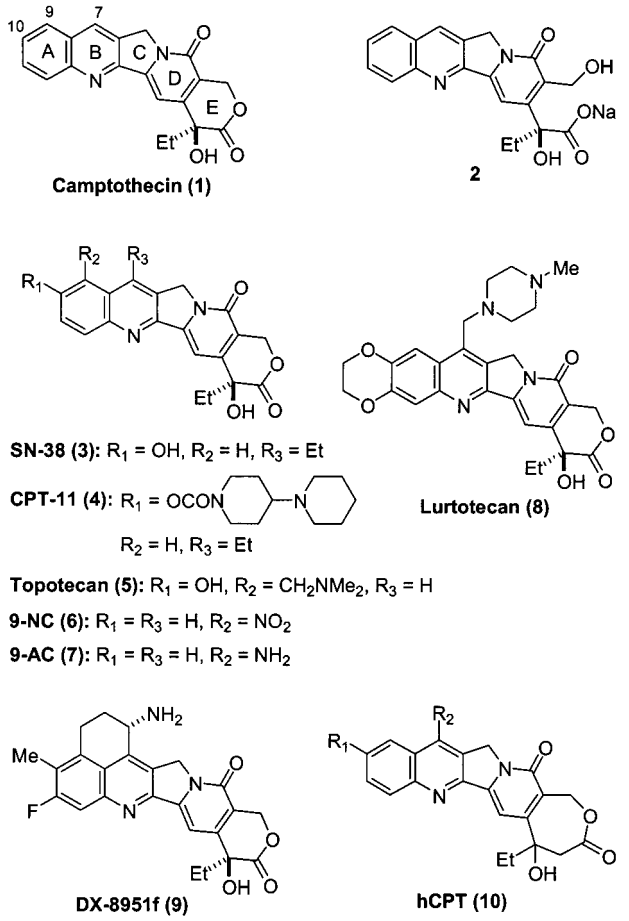
Topo I is a ubiquitous protein which possesses the capacity to relax supercoiled DNA during a number of critical cellular processes, including replication, transcription, and repair.[5] It could be a very attractive target for developing selective cancer chemotherapy since intracellular levels of Topo I are elevated in a number of human solid tumors, relative to the respective normal tissues.[5,6] Enzymology studies have revealed a rather intriguing mechanism in that CPT does not interact with Topo I alone, nor does it bind to DNA,[7−9] and exerts its cytotoxic effect by binding and stabilizing the cleavable complex, a transient species where the hydroxyl group on tyrosine 723 of Topo I binds covalently to DNA via its phosphodiester backbone and causes a single-strand break. The formation of a stable ternary complex between CPT, Topo I, and the cleaved DNA leads to the S-phase specific arrest of replication at the single strand level, causing irreversible DNA damage and eventually cell death.[10] Contrary to these initial reports, recent studies have suggested that CPT analogues may interact directly with double-stranded DNA prior to the action of Topo I, and the DNA-associated drugs are likely to be involved in the subsequent formation of a ternary complex.[11] Although the exact structure of the ternary complex remains to be determined,[12,13] this mechanism accounts for the good correlation found between the ability to induce stabilized cleavable complexes and the cytotoxicity of various CPT analogues.[14]

Subsequently, CPT analogues, classified as DNA Topo I inhibitors, have recently emerged as a prominent class of anticancer agents with a novel mechanism of action, potent antiproliferative activity on a wide spectrum of tumor cells including multidrug-resistant lines, and impressive activity in xenograft models.[15] Because of the intrinsic poor solubility of CPT in aqueous system, numerous research efforts have been focused on water-soluble derivatives, leading to the launching of irinotecan, the prodrug of SN-38 (**3**), (CPT-11, Camptosar or Campto, **4**; Chart 1)[16] and topotecan (Hycamtin, **5**)[17] and the clinical evaluation of lurtotecan (GG-211, **8**)[18] and DX-8951f (**9**).[19] Clinical studies of non-water-soluble CPT analogues, oral 9-nitrocamptothecin (9-NC, **6**),[20] and intravenous 9-aminocamptothecin (9-AC, **7**)[21] are also under investigation. Whereas most CPT analogues

* To whom requests for reprints and correspondence should be addressed. Dae-Kee Kim, Ph.D., Life Science Research Center, SK Chemicals, 600 Jungja-Dong, Changan-Ku, Suwon-Si, Kyungki-Do 440-745, Korea. Tel: (82)-31-240-8400. Fax: (82)-31-252-1379. E-mail: dkkim@skchemicals.com.

10.1021/jm0004751 CCC: $20.00
Published on Web 04/18/2001

Chart 1



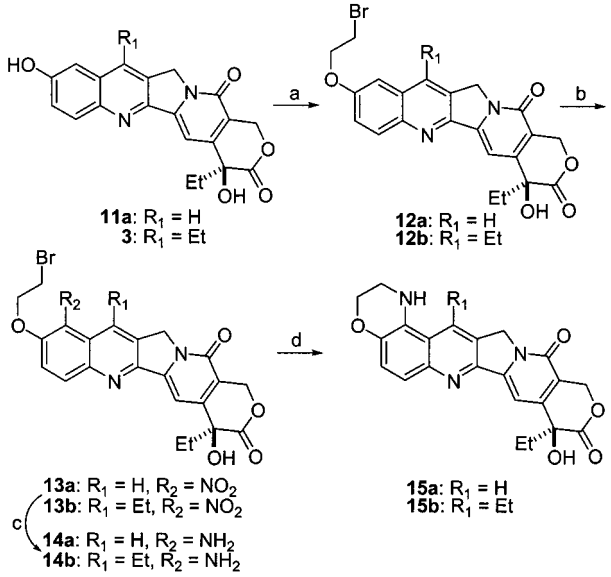
share the pentacyclic skeleton possessing the highly electrophilic six-membered α-hydroxylactone ring, somewhat interesting and informative studies on homocamptothecins (hCPTs, **10**), in which the lactone ring was modified to the seven-membered *β*-hydroxylactone ring, have been reported recently as well.[22]

It was claimed that the hexacyclic CPT analogues exhibited antitumor activities superior to those of the original pentacyclic ring system, probably due to the increased planarity exerted by an additional ring.[23] Based on these findings, our CPT research has been focused to further explore the potential of new hexacyclic compounds. We decided to develop new hexacyclic CPT analogues which have a 1,4-oxazine ring fused with positions 9 and 10 of the ring A, since it is well established that substitution at positions C-9 or C-10 of the ring A frequently increases activity and potency.[15,24] Herein, we now describe the preparation of various new hexacyclic CPT analogues and the biological results obtained for this novel series of compounds.

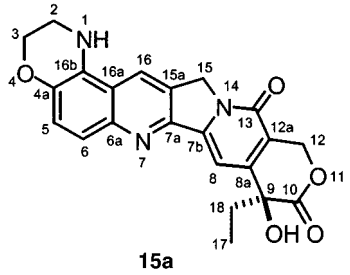
Results and Discussion

Two hexacyclic derivatives of CPT, **15a** and **15b**, were synthesized as shown in Scheme 1 starting from the known CPT derivatives, **11a** and **3**, which were prepared according to the literature procedures.[25] First, the 10-hydroxy CPT **11a** was treated with excess amounts of 1,2-dibromoethane in *N*,*N*-dimethylformamide (DMF) in the presence of anhydrous potassium carbonate (K_2CO_3) at 80 °C for 10 h to afford the 10-(2-bromoethoxy) CPT **12a** in 61% yield. Nitration of **12a** under typical

Scheme 1[a]



[a] (a) $Br(CH_2)_2Br$, K_2CO_3, DMF, 80 °C; (b) HNO_3, H_2SO_4, 0 °C; (c) $SnCl_2$, HCl, rt; (d) method A; NaI, K_2CO_3, acetone, reflux or method B; DMSO, rt.

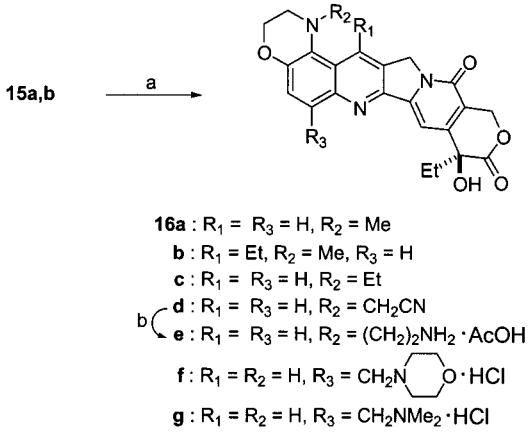


15a

reaction conditions provided only the desired 9-nitro compound **13a** in 85% yield.[26] Chemoselective reduction of the nitro group in **13a** was attempted under several reaction conditions. Unfortunately, the typical reactions using catalytic hydrogenation or Zn turned out to be unsuccessful due to the concomitant reduction of the bromo group in **13a**. In contrast, treatment of **13a** with tin(II) chloride in concentrated HCl solution provided the amine **14a** in an excellent yield of 94%. Since the amine **14a** was found to be rather unstable under chromatographic condition on SiO_2, the crude product was treated with sodium iodide in the presence of anhydrous K_2CO_3 in anhydrous acetone under reflux to afford the desired hexacyclic CPT **15a** in 93% overall yield (method A). In the meantime, it was observed that the amine **14a** was also unstable in solution, and it slowly underwent cyclization in an NMR solvent (DMSO-d_6) at room temperature to give the cyclized product **15a**. On the basis of these findings, the solution of **14a** in DMSO was just stirred for 20 h at room temperature to furnish **15a** in 92% overall yield (method B). The 7-ethyl hexacyclic analogue **15b** was obtained from **3** by using the same reaction sequence as described earlier.

Further transformations of **15a** and **15b** to compounds **16a**−**g** were carried out as shown in Scheme 2. Treatment of **15a** with an excess amount of iodomethane using anhydrous K_2CO_3 as a base in DMF afforded the *N*-methyl CPT **16a** in a reasonable yield of 60%. However, the methylation of **15a** in the absence of K_2-

Scheme 2[a]



[a] (a) MeI, DMF, 50 °C for **16a** or 120 °C for **16b**; EtI, DMF, 85 °C for **16c**; $BrCH_2CN$, DMF, 80 °C for **16d**; CH_2O, morpholine, AcOH, rt for **16f**; *N*,*N*-dimethylmethyleneammonium chloride, DMF, rt for **16g**; (b) H_2, Raney-nickel, AcOH, rt.

CO_3 gave **16a** in higher yield of 83%. Other N-alkylated analogues **16b**−**d** were obtained by using similar reaction conditions without using any base in 65%, 70%, and 98% yield, respectively. Water-soluble hexacyclic CPT derivatives **16e**−**g** were prepared either by hydrogenation of **16d** or the Mannich type reactions of **15a**. While the catalytic hydrogenation of **16d** using 10% palladium on carbon gave only the N-dealkylated product **15a**, the reaction with Raney-nickel in acetic acid instead of 10% palladium on carbon produced the desired **16e** as an acetic acid salt albeit in a rather low yield of 31% along with a substantial amount (64%) of **15a**. Formation of the major byproduct **15a** might be explained by invoking an iminium intermediate that can be generated from extrusion of the cyano group in **16d**. This reactive iminium species would react readily with ethanol to form an amino acetal derivative, and then the acid labile group could fall apart to produce the N-dealkylated **15a** upon exposure to water under acidic conditions. When **15a** was reacted with morpholine and paraformaldehyde in glacial acetic acid at room temperature, the 6-morpholinomethyl hexacyclic CPT was isolated as an acetic acid salt and subsequently treated with 0.1 N HCl to afford the HCl salt of **16f** in 63% yield. In a similar manner, treatment of **15a** with *N*,*N*-dimethylmethyleneammonium chloride in DMF provided the 6-dimethylaminomethyl CPT **16g** as a HCl salt in 89% yield after MPLC purification on C_{18} reversed-phase silica gel using an acidic eluent.

The biological test results, in vitro cytotoxicity, Topo I inhibition, and AChE inhibition for the new CPT analogues are presented in Table 1. Cytotoxicity of compounds **15a**,**b** and **16a**−**g** was evaluated in comparison with CPT, topotecan, and SN-38, an active metabolite of CPT-11, in the following six different human cancer cell lines: lung cancer (A549, H128), colon cancer (WiDr), stomach cancer (MKN45), ovarian cancer (SK-OV-3), and breast cancer (SK-BR-3) cell lines. Compounds **15a**, **15b**, **16a**, and **16c** were about 2-fold more potent than topotecan and as potent as CPT toward cancer cell lines tested, while compounds **16b** and **16e**−**g** were much less cytotoxic than CPT. These results indicated that the substitution at position C-6 decreased cytotoxicity dramatically and are in good agreement with those reported previously.[15] Interestingly, the most active compound **15b**, although slightly less potent than SN-38, was about 2-fold more potent than CPT in some of the human cancer cell lines such as SK-OV-3, H128, and SK-BR-3 cell lines.

The inhibitory effect of compounds **15a**,**b** and **16a**−**e** on Topo I was examined by the Topo I cleavable complex enzyme assay using agarose gel electrophoresis. The addition of compounds **15a**,**b** and **16a**−**e** resulted in a stabilization of the nicked DNA intermediate in a concentration-dependent manner (data not shown). The proportion of nicked DNA intermediate was then determined by an analysis imaging system, and the concentration of each compound that resulted in 50% formation of nicked DNA intermediate was measured. As shown in Table 1, the Topo I inhibitory activity of compounds **15b**, **16a**, **16c**, and **16d** was significantly higher than that of CPT, while compounds **16b** and **16e** were less potent than CPT (Table 1). The most active compound **15b** ($IC_{50} = 5 \pm 2\ \mu M$) was 8-fold more potent than CPT ($IC_{50} = 40 \pm 8\ \mu M$), 4-fold more potent than topotecan ($IC_{50} = 20 \pm 2\ \mu M$), and as potent as SN-38 ($IC_{50} = 4 \pm 1\ \mu M$). Relatively good correlation between cytotoxic potency and Topo I inhibition was established in the test compounds, except for compounds **16b** and **16e**. While compound **16b** was much less potent than compound **16e** as an inhibitor of Topo I, it showed higher potency than compound **16e** with respect to in

Table 1. In Vitro Cytotoxicity, Topo I Inhibition, and AChE Inhibition of Hexacyclic CPT Derivatives **15a**,**b** and **16a**−**g**

	in vitro cytotoxicity (IC_{50}, nM)[a]						Topo I	%AChE
compd	WiDr	A549	MKN45	SK-OV-3	H128	SK-BR-3	IC_{50} (μM)[b]	inhibition[c]
15a	23 ± 6	23 ± 7	20 ± 8	28 ± 5	13 ± 5	19 ± 5	41 ± 8	13 ± 2
15b	16 ± 3	14 ± 6	20 ± 7	14 ± 2	7 ± 2	13 ± 6	5 ± 2	12 ± 1
16a	23 ± 3	21 ± 6	19 ± 2	26 ± 5	10 ± 3	25 ± 5	21 ± 6	14 ± 2
16b	135 ± 9	85± 21	152 ± 37	142 ± 11	76 ± 9	107 ± 21	1600 ± 264	19 ± 3
16c	21 ± 3	17 ± 6	19 ± 4	19 ± 3	13 ± 1	16 ± 3	12 ± 1	9 ± 2
16d	30 ± 5	37 ± 10	40 ± 4	32 ± 6	14 ± 3	17 ± 6	16 ± 6	20 ± 3
16e	415 ± 30	507 ± 165	536 ± 90	533 ± 81	174 ± 39	417 ± 118	133 ± 52	17 ± 2
16f	5339 ± 355	9593 ± 652	15414 ± 883	10585 ± 907	7439 ± 363	ND	ND	ND
16g	3190 ± 243	8073 ± 332	9571 ± 749	8629 ± 121	8500 ± 528	ND	ND	ND
CPT	17 ± 3	14 ± 3	17 ± 5	20 ± 4	18 ± 4	20 ± 8	40 ± 8	23 ± 5
SN-38	14 ± 4	16 ± 3	8 ± 1	11 ± 4	6 ± 1	4 ± 1	4 ± 1	13 ± 2
topotecan	56 ± 4	54 ± 13	38 ± 17	48 ± 7	31 ± 6	26 ± 8	20 ± 2	20 ± 4

[a] Human cancer cell cytotoxicity assay was performed using MTT, and the concentration of compound causing 50% cell death (IC_{50}) was determined. [b] Average concentration of compound to cause 50% inhibition of topo I using the cleavable complex assay. [c] AChE activity was determined by spectrophotometry using ATChI as a substrate. For *a*, *b*, and *c*, three or more determinations were made. Data represent the mean ± SD. ND indicates not determined.

Table 2. Antitumor Activity of Compounds **15b** and SN-38 against WiDr Xenografts in Nude Mice[a]

compd	dose (μmol/kg)	MTW_0[b] (mg)	MTW_{28}[b] (mg)	RTG[b]	IR (%)	PR[c] (%)	CR[d] (%)
control		179 ± 56	1485 ± 751	8.30 ± 2.89		0	0
15b	10	176 ± 53	21 ± 38	0.12 ± 0.17	98.6	25	75
SN-38	100	177 ± 59	27 ± 54	0.15 ± 0.24	98.2	8	75

[a] Twelve mice were used for treatment groups and the control group, respectively. Compounds **15b** and SN-38 were given i.p. on a q4d×8 schedule. Relative tumor growth (RTG) was calculated by dividing the tumor weight on day 28 (MTW_{28}) by that on day 0 (MTW_0). Inhibition rate (IR) was obtained from the equation (1 − RTG in treated/RTG in control) × 100. [b] Data represent the mean ± SD. [c] PR means 50% or greater regression. [d] CR means complete regression.

vitro cytotoxicity. It is likely that cellular uptake may play an important role in determining cytotoxic potency in vitro. On the other hand, the compound **15b**, the most potent Topo I inhibitor among the test compounds, also exhibited the highest cytotoxicity against the human cancer cell lines tested.

Although CPT-11 was effective in clinical trials, it caused early diarrhea during or immediately after intravenous infusion of CPT-11 at high doses, a dose-limiting factor for this drug.[27] Kawato et al. reported that the early diarrhea was related to the inhibition of AChE by CPT-11, which causes an increase in acetylcholine level and induces cholinergic action including diarrhea. Therefore, we investigated the AChE inhibitory activity (%) of compounds **15a**,**b** and **16a**−**e** at a concentration of 2 μM, since it is known that CPT-11 inhibits AChE by more than 90% at 1.7 μM.[28] Most of the test compounds exhibited activity weaker (9 ± 2 to 20 ± 3%) than CPT (23 ± 5%) or topotecan (20 ± 4%) and similar to SN-38 (13 ± 2%) (Table 1). These results indicate that all the new hexacyclic CPT analogues **15a**,**b** and **16a**−**e** might have little effect on causing early diarrhea.

It has been well established that CPT analogues with intact lactone rings showed significantly greater antitumor activities over the carboxylate forms or analogues with modified lactone rings.[29] Although still controversial, the position of equilibrium between the lactone and carboxylate form under physiological conditions has been investigated as an important factor for the antitumor activities of CPT analogues. In the present study, therefore, the in vitro lactone levels in human plasma for compounds **15a**,**b** and **16a**−**d** were determined (see Table S1, Supporting Information). It was observed that the percent lactone of CPT was only 2.1 ± 0.1% after 60 min and 1.5 ± 0.1% after 120 min, indicating that most of the lactone form of CPT was lost in a very short time period in human plasma. The stability of lactone forms of compounds **15a**,**b** and **16a**−**d** in human plasma seemed to be much higher than that of CPT and similar to that of topotecan.[29d] These results were consistent with the earlier report that specific modifications at the 7- and 9-positions of the quinoline nucleus enhanced drug stability in the presence of human plasma.[29d] However, their stability was lower than that of SN-38,[29d] which was detected in a significant amount (31.0 ± 2.5%) as the lactone form even after 120 min.

Human tumor xenograft models have been used to predict the chemotherapeutic response of tumors to anticancer agents.[30] Since compound **15b** showed the most promising biological data, the in vivo antitumor activity of **15b** was evaluated against human tumor xenograft, WiDr, in nude mice and compared with that of SN-38 (Table 2). Maximum tolerated dose (MTD) of compound **15b** for i.p. administration q4d×8 was determined to be 10 μmol/kg per injection while that of SN-38 was 100 μmol/kg per injection (see Table S2, Supporting Information). Mice in each group treated with compound **15b** and SN-38 at MTD dose lost 9.8 ± 1.6% and 9.0 ± 3.3% of their initial body weight, respectively, after treatment. Concerning the in vivo antitumor activity shown in Table 2, the inhibition rate (IR) values at MTD dose were 98.6% and 98.2% in compound **15b** and the SN-38 treated group, respectively, on day 28. In addition, the other parameters used to evaluate the in vivo antitumor activity were also similar between the compound **15b** and the SN-38 treated group, although the administered dose of compound **15b** was 10-fold lower than that of SN-38. The higher in vivo antitumor activity of compound **15b** compared to that of SN-38 could be ascribed to its prolonged intratumor retention.

In conclusion, a new hexacyclic CPT analogue **15b** demonstrated potent in vitro cytotoxicity against a number of human cancer cell lines, high Topo I inhibitory activity, weak inhibition of AChE, and human plasma stability comparable to topotecan. On the basis of these promising results, combined with its high in vivo antitumor activity, further preclinical evaluations of compound **15b** are currently underway in our laboratory.

Experimental Section

Caution: *Camptothecin and all structurally related compounds must be considered potential mutagens and potential reproductive hazards for both males and females. Appropriate precautions (use of respirators, gloves, and fumehood) should be taken when handling these compounds and any waste streams generated from their use.*

Chemistry. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were recorded on a Magna 750 FTIR spectrophotometer. ^{1}H NMR spectra were recorded on either a Varian Unity 300 or a JEOL Lambda 300 spectrometer. The chemical shifts are reported in parts per million (ppm) relative to internal solvent (2.50 ppm in DMSO-d_6). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a VG Quattro mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60F-254 glass plates. Medium-pressure chromatography (MPLC) was performed using Merck silica gel 60 (230−400 mesh) with a VSP-2200 ceramic pump (Eyela). Elemental analyses were performed on a Carlo Erba 1106 elemental analyzer. Where indicated by the symbols of the elements, analyses were within ±0.4% of theoretical values.

10-(2-Bromoethoxy)-(20*S*)-camptothecin (12a). A mixture of 10-hydroxy-(20*S*)-camptothecin (**11a**) (30 g, 82.3 mmol), anhydrous 1,2-dibromoethane (310 g, 1.648 mol, 142 mL), and anhydrous K_2CO_3 (56.9 g, 412 mmol) in anhydrous DMF (800 mL) was mechanically stirred at 80 °C for 10 h under N_2 atmosphere. The reaction mixture was filtered through a pad of Celite, and the filtered residue was washed well with DMF. The combined filtrate and washings were evaporated to dryness in vacuo to afford a dark residue. The residue was

dissolved in 10% MeOH/$CHCl_3$ (1.5 L), and the insoluble materials were filtered off. The filtrate was concentrated under reduced pressure, and the residue was purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (3:97 to 5:95, v/v) as eluent to afford 23.55 g (61%) of **12a**, which was crystallized from MeOH/$CHCl_3$: mp 237−238 °C; IR (neat) 3334, 1752 (lactone), 1660 (pyridone), 1603, 1556, 1505, 1239, 1156 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.89 (t, J = 7.2 Hz, 3 H, H-18), 1.87 (m, 2 H, H-19), 3.92 (t, J = 5.5 Hz, 2 H, CH_2Br), 4.53 (t, J = 5.5 Hz, 2 H, CH_2CH_2Br), 5.28 (s, 2 H, H-5), 5.42 (s, 2 H, H-17), 6.49 (br s, 1 H, OH), 7.30 (s, 1 H, H-14), 7.53−7.59 (m, 2 H, H-9 and H-11), 8.10 (d, J = 9.0 Hz, 1 H, H-12), 8.55 (s, 1 H, H-7). Anal. ($C_{22}H_{19}Br\ N_2O_5$) C, H, N.

10-(2-Bromoethoxy)-7-ethyl-(20*S*)-camptothecin (12b). The titled compound was prepared according to procedures identical to those for **12a** using 7.87 g (20.1 mmol) of 7-ethyl-10-hydroxy-(20*S*)-camptothecin (**3**). Purification by MPLC on SiO_2 using MeOH/$CHCl_3$ (1:99 to 3:97, v/v) as eluent provided 5.79 g (58%) of **12b**, which was crystallized from MeOH/$CHCl_3$: mp 236.9−237.2 °C; IR (neat) 3326, 1755 (lactone), 1658 (pyridone), 1602, 1516, 1254 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.89 (t, J = 7.4 Hz, 3 H, H-18), 1.30 (t, J = 7.5 Hz, 3 H, CH_2CH_3), 1.87 (m, 2 H, H-19), 3.17 (q, J = 7.5 Hz, 2 H, CH_2-CH_3), 3.93 (t, J = 5.3 Hz, 2 H, CH_2Br), 4.58 (t, J = 5.3 Hz, 2 H, CH_2CH_2Br), 5.25 (s, 2 H, H-5), 5.42 (s, 2 H, H-17), 7.26 (s, 1 H, H-14), 7.50 (s, 1 H, H-9), 7.52 (d, J = 9.0 Hz, 1 H, H-11), 8.06 (d, J = 9.0 Hz, 1 H, H-12). Anal. ($C_{24}H_{23}Br\ N_2O_5$) C, H, N.

10-(2-Bromoethoxy)-9-nitro-(20*S*)-camptothecin (13a). To a stirred concentrated H_2SO_4 (175 mL) at 0 °C was added 10-(2-bromoethoxy)-(20*S*)-camptothecin (**12a**) (12.55 g, 26.6 mmol) in small portions over 1 h, and the resulting clear solution was cooled to −10 °C. A mixture of concentrated H_2SO_4 (5 mL) and fuming HNO_3 (5 mL), pre-cooled to −10 °C, was added dropwise over 30 min to the cooled reaction mixture at −10 °C. The reaction mixture was allowed to warm to 0 °C, stirred for an additional 1 h, and then poured slowly onto the ice chips (800 g). The yellow precipitate was filtered and washed with H_2O, cold EtOH, and Et_2O. The aqueous filtrate was filtered again through a pad of Celite, and the Celite filtercake was extracted with 30% MeOH/$CHCl_3$ (500 mL). Evaporation of the organic solvent afforded an additional yellow solid. Trituration of the combined yellow solid with EtOH afforded 11.68 g (85%) of **13a**, which was crystallized from MeOH/$CHCl_3$: mp 251.5 °C (dec); IR (neat) 3309, 1751 (lactone), 1656 (pyridone), 1598, 1531, 1257, 1166 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, J = 7.4 Hz, 3 H, H-18), 1.87 (m, 2 H, H-19), 3.86 (t, J = 5.4 Hz, 2 H, CH_2Br), 4.75 (t, J = 5.4 Hz, 2 H, CH_2CH_2Br), 5.27 (s, 2 H, H-5), 5.43 (s, 2 H, H-17), 6.53 (br s, 1 H, OH), 7.34 (s, 1 H, H-14), 8.04 (d, J = 9.6 Hz, 1 H, H-11), 8.43 (d, J = 9.6 Hz, 1 H, H-12), 8.47 (s, 1 H, H-7). Anal. ($C_{22}H_{18}BrN_3O_7$) C, H, N.

10-(2-Bromoethoxy)-7-ethyl-9-nitro-(20*S*)-camptothecin (13b). The titled compound was prepared according to procedures identical to those for **13a** using 8.60 g (17.2 mmol) of 10-(2-bromoethoxy)-7-ethyl-(20*S*)-camptothecin (**12b**). Trituration with Et_2O (200 mL) provided 8.58 g (92%) of **13b** as a yellow solid, which was crystallized from MeOH/$CHCl_3$: mp 268 °C (dec); IR (neat) 3236, 1751 (lactone), 1659 (pyridone), 1597, 1538, 1263, 1162 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, J = 7.4 Hz, 3 H, H-18), 1.21 (t, J = 7.4 Hz, 3 H, CH_2CH_3), 1.88 (m, 2 H, H-19), 2.88 (q, J = 7.4 Hz, 2 H, CH_2CH_3), 3.83 (t, J = 5.3 Hz, 2 H, CH_2Br), 4.72 (t, J = 5.3 Hz, 2 H, CH_2CH_2-Br), 5.34 (s, 2 H, H-5), 5.44 (s, 2 H, H-17), 7.31 (s, 1 H, H-14), 8.03 (d, J = 9.3 Hz, 1 H, H-11), 8.40 (d, J = 9.3 Hz, 1 H, H-12). Anal. ($C_{24}H_{22}BrN_3O_7$) C, H, N.

9-Amino-10-(2-bromoethoxy)-(20*S*)-camptothecin (14a). To a stirred concentrated HCl solution (18 mL) at 0 °C was added 10-(2-bromoethoxy)-9-nitro-(20*S*)-camptothecin (**13a**) (815 mg, 1.579 mmol) in small portions over 10 min, and the resulting clear solution was cooled to −10 °C after 15 min. To the reaction mixture was added $SnCl_2$ (1.08 g, 5.696 mmol) in small portions over 6 min. The reaction mixture was allowed to warm to room temperature, stirred for 1.5 h, and then poured slowly onto the ice chips (150 g). The dark brown precipitate was filtered and washed with EtOH and Et_2O, and the aqueous filtrate was extracted with 10% MeOH/$CHCl_3$ (50 mL × 4). The filtered precipitate was dissolved in 30% MeOH/$CHCl_3$ (350 mL), and the combined organic solution was passed through a short SiO_2 pad using MeOH/$CHCl_3$ (3:7, v/v) as eluent. The organic solvent was evaporated to dryness to afford 722 mg (94%) of **14a**, which was used in the next step without further purification: IR (neat) 3363 and 3257 (NH_2), 1755 (lactone), 1653 (pyridone), 1589, 1450, 1258, 1161 cm^{-1}; ^1H NMR (DMS0-d_6) δ 0.89 (t, J = 7.4 Hz, 3 H, H-18), 1.87 (m, 2 H, H-19), 3.89 (t, J = 5.6 Hz, CH_2Br), 4.46 (t, J = 5.6 Hz, CH_2-CH_2Br), 5.28 (s, 2 H, H-5), 5.42 (s, 2 H, H-17), 6.47 (br s, 1 H, OH), 7.29 (s, 1 H, H-14), 7.45 (d, J = 9.0 Hz, 1 H, H-11), 7.63 (d, J = 9.0 Hz, 1 H, H-12), 8.85 (s, 1 H, H-7).

9-Amino-10-(2-bromoethoxy)-7-ethyl-(20*S*)-camptothecin (14b). The titled compound was prepared according to procedures identical to those for **14a** using 8.78 g (16.13 mmol) of 10-(2-bromoethoxy)-7-ethyl-9-nitro-(20*S*)-camptothecin (**13b**). Purification by passing through a short SiO_2 pad using MeOH/$CHCl_3$ (1:9, v/v) as eluent provided 7.55 g (91%) of **14b**, which was used in the next step without further purification: IR (neat) 3460 and 3388 (NH_2), 1751 (lactone), 1658 (pyridone), 1605, 1278, 1165 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, J = 7.0 Hz, 3 H, H-18), 1.34 (t, J = 7.4 Hz, 3 H, CH_2CH_3), 1.85 (m, 2 H, H-19), 3.35 (q, J = 7.4 Hz, 2 H, CH_2CH_3), 3.92 (t, J = 5.2 Hz, 2 H, CH_2Br), 4.48 (t, J = 5.2 Hz, 2 H, CH_2CH_2Br), 5.28 (s, 2 H, H-5), 5.41 (s, 2 H, H-17), 7.23 (s, 1 H, H-14), 7.52 (d, J = 9.3 Hz, 1 H, H-11), 7.63 (d, J = 9.3 Hz, 1 H, H-12).

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino-[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13-(9*H*,15*H*)-dione (15a): Procedures for Method A. A stirred mixture of 9-amino-10-(2-bromoethoxy)-(20*S*)-camptothecin (**14a**) (3.20 g, 6.58 mmol), anhydrous K_2CO_3 (1.80 g, 13.02 mmol), and NaI (2.00 g, 13.34 mmol) in anhydrous acetone (600 mL) was heated under reflux for 16 h under N_2 atmosphere. The reaction mixture was filtered through a pad of Celite, and the filtered residue was washed well with hot acetone. The filtrate was evaporated to dryness, and the resulting orange solid was purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (3:97, 5:95 to 1:9, v/v) as eluent to afford 2.63 g (99%) of **15a**, which was crystallized from 10% MeOH/$CHCl_3$: mp >300 °C; IR (neat) 3375 (NH), 1755 (lactone), 1658 (pyridone), 1591, 1341, 1242, 1160 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.89 (t, J = 7.4 Hz, 3 H, H-17), 1.87 (m, 2 H, H-18), 3.48 (m, 2 H, H-2), 4.24 (m, 2 H, H-3), 5.28 (s, 2 H, H-15), 5.42 (s, 2 H, H-12), 6.48 (br s, 2 H, OH and NH), 7.27 (s, 1 H, H-8), 7.34 (d, J = 9.2 Hz, 1 H, H-5), 7.41 (d, J = 9.2 Hz, 1 H, H-6), 8.67 (s, 1 H, H-16); FAB-MS m/z 406 (MH^+). Anal. ($C_{22}H_{19}N_3O_5$) C, H, N.

Procedures for Method B. A solution of 9-amino-10-(2-bromoethoxy)-(20*S*)-camptothecin (**14a**) (70 mg, 0.144 mmol) in DMSO (4 mL) was stirred at room temperature for 20 h, and transferred dropwise into H_2O (40 mL). The precipitated orange solid was filtered, dissolved in a small volume of 30% MeOH/$CHCl_3$, and purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (5:95, v/v) as eluent to afford 57 mg (98%) of **15a**.

(9*S*)-9,16-Diethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1, 2-*b*]quinoline-10,-13(9*H*,15*H*)-dione (15b): Procedures for Method A. The titled compound was prepared according to procedures identical to those for **15a** using 7.55 g (14.68 mmol) of 9-amino-10-(2-bromoethoxy)-7-ethyl-(20*S*)-camptothecin (**14b**). Purification by passing through a short SiO_2 pad using MeOH/$CHCl_3$ (1:9, v/v) as eluent followed by triturating with Et_2O (200 mL) provided 6.17 g (97%) of **15b** as an orange solid, which was crystallized from EtOH/$CHCl_3$: mp 249−250 °C (dec); IR (neat) 3318, 1756 (lactone), 1659 (pyridone), 1598, 1236, 1163 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, J = 7.2 Hz, 3 H, H-17), 1.33 (t, J = 7.5 Hz, 3 H, CH_2CH_3), 1.86 (m, 2 H, H-18), 3.30−3.45 (m, 4 H, CH_2CH_3 and H-2), 4.20 (m, 2 H, H-3), 5.27 (s, 2 H, H-15), 5.34 (br s, 1 H, NH), 5.42 (s, 2 H, H-12), 6.46 (br s, 1 H, OH),

7.22 (s, 1 H, H-8), 7.32 (d, J = 9.3 Hz, 1 H, H-5), 7.52 (d, J = 9.3 Hz, 1 H, H-6); FAB-MS m/z 434 (MH^+). Anal. ($C_{24}H_{23}N_3O_5$) C, H, N.

Procedures for Method B. The titled compound was prepared according to procedures identical to those for **15a** using 70 mg (0.136 mmol) of 9-amino-10-(2-bromoethoxy)-7-ethyl-(20*S*)-camptothecin (**14b**). Purification by MPLC on SiO_2 using MeOH/$CHCl_3$ (1:99, v/v) as eluent provided 57 mg (97%) of **15b**.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-1-methyl-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (16a). To a stirred suspension of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15a**) (200 mg, 0.49 mmol) in anhydrous DMF (5 mL) was added MeI (210 mg, 1.48 mmol, 92 μL), and the mixture was heated at 50 °C for 21 h under N_2 atmosphere. Additional MeI (490 mg, 3.45 mmol, 215 μL) was added to the reaction mixture, and it was heated at the same temperature for an additional 48 h. The reaction mixture was cooled to room temperature and poured into hexane (100 mL). The brown liquid was separated and poured into H_2O (800 mL), and it was extracted with $CHCl_3$ (60 mL × 3). The organic solution was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The residue was purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (1:99, v/v) as eluent to afford 172 mg (83%) of **16a**, which was crystallized from MeOH: mp 262 °C (dec); IR (neat) 3103, 1746 (lactone), 1665 (pyridone), 1608, 1573, 1505, 1363, 1240, 1116 cm^{-1}; ^1H NMR (DMS0-d_6) δ 0.89 (t, J = 7.4 Hz, 3 H, H-17), 1.87 (m, 2 H, H-18), 2.92 (s, 3 H, NCH_3), 3.22 (m, 2 H, H-2), 4.31 (m, 2 H, H-3), 5.23 (s, 2 H, H-15), 5.41 (s, 2 H, H-12), 6.48 (br s, 1 H, OH), 7.25 (s, 1 H, H-8), 7.37 (d, J = 9.3 Hz, 1 H, H-5), 7.73 (d, J = 9.3 Hz, 1 H, H-6), 8.69 (s, 1 H, H-16); FAB-MS m/z 420 (MH^+). Anal. ($C_{23}H_{21}N_3O_5$) C, H, N.

(9*S*)-9,16-Diethyl-2,3-dihydro-9-hydroxy-1-methyl-12*H*-1,4-oxazino[3,2-*f*]-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (16b). To a stirred solution of (9*S*)-9,16-diethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15b**) (3.03 g, 6.99 mmol) in anhydrous DMF (100 mL) was added MeI (29.80 g, 210 mmol, 9.2 mL), and the mixture was heated at 120 °C for 18 h under N_2 atmosphere. The reaction mixture was cooled to room temperature, evaporated to dryness in vacuo, and purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (1:99, 2:98, to 3:97, v/v) as eluent to afford 2.037 g (65%) of **16b** as a yellow solid, which was crystallized from EtOH/$CHCl_3$: mp 250−251 °C (dec); IR (neat) 3286, 1745 (lactone), 1657 (pyridone), 1598, 1564, 1232, 1157 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.87 (t, J = 7.2 Hz, 1.5 H, 0.5 H-17), 0.88 (t, J = 7.2 Hz, 1.5 H, 0.5 H-17), 1.10 (t, J = 6.9 Hz, 1.5 H, 0.5 CH_2CH_3), 1.11 (t, J = 6.9 Hz, 1.5 H, 0.5 CH_2CH_3), 1.86 (m, 2 H, H-18), 2.68 (s, 3 H, NCH_3), 3.01 (m, 1 H, H-2), 3.20−3.42 (m, 2 H, CH_2CH_3), 3.96 (m, 1 H, H-2), 4.35 (m, 2 H, H-3), 5.24 (d, $J_{a,b}$ = 18.6 Hz, 1 H, H-15), 5.37 (d, $J_{a,b}$ = 18.6 Hz, 1 H, H-15), 5.43 (s, 2 H, H-12), 6.47 (br s, 1 H, OH), 7.24 (s, 1 H, H-8), 7.38 (d, J = 9.3 Hz, 1 H, H-5), 7.78 (d, J = 9.3 Hz, 1 H, H-6); FAB-MS m/z 448 (MH^+). Anal. ($C_{25}H_{25}N_3O_5$) C, H, N.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-1-ethyl-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino- [1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (16c). To a stirred suspension of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15a**) (865 mg, 2.134 mmol) in anhydrous DMF (21 mL) was added EtI (9.98 g, 64.00 mmol, 5.12 mL), and the mixture was heated at 85 °C for 48 h under N_2 atmosphere. The reaction mixture was cooled to room temperature, and the yellow precipitate was filtered and washed with DMF, Et_2O, and MeOH to afford 645 mg (70%) of **16c**, which was crystallized from MeOH/$CHCl_3$: mp 257−259 °C (dec); IR (neat) 3216. 1749 (lactone), 1671 (pyridone), 1610, 1576, 1237, 1117 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.87 (t, J = 6.9 Hz, H-17), 1.39 (t, J = 6.9 Hz, 3 H, NCH_2CH_3), 1.86 (m, 2 H, H-18), 3.01 (q, J = 6.9 Hz, NCH_2CH_3), 3.19 (m, 2 H, H-2), 4.27 (m, 2 H, H-3), 5.29 (s, 2 H, H-15), 5.42 (s, 2 H, H-12), 6.50 (s, 1 H, OH), 7.26 (s, 1 H, H-8), 7.39 (d, J = 9.3 Hz, 1 H, H-5), 7.73 (d, J = 9.3 Hz, 1 H, H-6) 8.55 (s, 1 H, H-16); FAB-MS m/z 434 (MH^+). Anal. ($C_{24}H_{23}N_3O_5$) C, H, N.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-1-(cyanomethyl)-12*H*-1,4-oxazino[3,2-*f*]-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]-quinoline-10,13(9*H*,15*H*)-dione (16d). To a stirred suspension of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15a**) (1.62 g, 3.996 mmol) in anhydrous DMF (38 mL) was added bromoacetonitrile (15.15 g, 126 mmol, 8.8 mL), and the mixture was heated at 80 °C for 20 h under N_2 atmosphere. The reaction mixture was evaporated to dryness in vacuo, and the residue was purified by MPLC on SiO_2 using MeOH/$CHCl_3$ (1:9 to 1:4, v/v) as eluent. The resulting solid was then crystallized from 20% MeOH/$CHCl_3$ to afford 1.74 g (98%) of **16d**: mp 248−249 °C (dec); IR (neat) 3318, 1749 (lactone), 1661 (pyridone), 1600, 1560, 1236, 1157 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.87 (t, J = 7.3 Hz, 3 H, H-17), 1.86 (m, 2 H, H-18), 3.43 (m, 2 H, H-2), 4.41 (s, 2 H, CH_2CN), 4.46 (m, 2 H, H-3), 5.24 (s, 2 H, H-15), 5.41 (s, 2 H, H-12), 6.51 (br s, 1 H, OH), 7.27 (s, 1 H, H-8), 7.45 (d, J = 9.3 Hz, 1 H, H-5), 7.86 (d, J = 9.3 Hz, 1 H, H-6), 8.59 (s, 1 H, H-16); FAB-MS m/z 445 (MH^+). Anal. ($C_{24}H_{20}N_4O_5$) C, H, N.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-1-(2-aminoethyl)-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]-indolizino[1,2-*b*]-quinoline-10,13(9*H*,15*H*)-dione acetic Acid (16e). A stirred suspension of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-1-(cyanomethyl)-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]-quinoline-10,13(9*H*,15*H*)-dione (**16d**) (1.00 g, 2.250 mmol) in glacial AcOH (180 mL) and Raney-nickel (50% slurry in H_2O, 32 mL) was purged with H_2 gas three times, and was stirred under H_2 atmosphere at room temperature for 12 h with a hydrogen-filled balloon. The resulting mixture was filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The reddish residue was treated with H_2O (250 mL) and filtered through a pad of Celite. The Celite filter-cake was extracted with 20% MeOH/$CHCl_3$ (100 mL) to afford 580 mg (64%) of **16e**. The filtrate was concentrated under reduced pressure, and the residue was purified by C_{18} reversed-phase MPLC (gradient elution: 20% MeOH/H_2O containing 1% AcOH, followed by 50% MeOH/H_2O containing 1% AcOH) to afford additional 350 mg (31%) of **16e** as a yellow solid: mp 238 °C (dec, darkened from 138 °C); IR (neat) 3101, 1747 (lactone), 1659 (pyridone), 1597, 1572, 1415, 1243, 1051 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.87 (t, J = 7.1 Hz, 3 H, H-17), 1.78 (s, 3 H, CH_3CO_2H), 1.84 (m, 2 H, H-18), 3.04 (m, 2 H, CH_2NH_2), 3.20 (m, 2 H, $CH_2CH_2NH_2$), 3.40 (m, 2 H, H-2), 4.26 (m, 2 H, H-3), 5.27 (s, 2 H, H-15), 5.41 (s, 2 H, H-12), 6.50 (br s, 1 H, OH), 7.26 (s, 1 H, H-8), 7.37 (d, J = 9.3 Hz, 1 H, H-5), 7.72 (d, J = 9.3 Hz, 1 H, H-6), 8.87 (s, 1 H, H-16); FAB-MS m/z 449 ($MH^+ - CH_3CO_2H$). Anal. ($C_{26}H_{28}N_4O_7$) C, H, N.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-6-(4-morpholino-methyl)-12*H*-1,4-oxazino[3,2-*f*]pyrano- [3′,4′:6,7]indolizino-[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione Hydrochloride (16f). To a stirred solution of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15a**) (420 mg, 1.04 mmol) in glacial AcOH (50 mL) were added morpholine (903 mg, 10.36 mmol) and formaldehyde (37 wt % solution in H_2O, 3.0 mL), and the mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated in vacuo, and the brown residue was dissolved in 30% MeOH/$CHCl_3$ (60 mL). After addition of 1 N aqueous HCl solution (7 mL) to the mixture, the methanolic $CHCl_3$ solution was evaporated to dryness. The resulting residue was purified by C_{18} reversed-phase MPLC (20% MeOH/H_2O containing 0.5% 1 N aqueous HCl solution) to afford 353 mg (63%) of **16f**, which was resolidified from EtOH/Et_2O to give a red-orange powder: mp 212 °C; IR (neat) 3213, 1752 (lactone), 1657 (pyridone), 1594, 1572, 1339 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.89 (t, J = 7.5 Hz, 3 H, H-17), 1.87 (m, 2 H, H-18), 3.31 (m, 4 H, 2 NCH_2CH_2O), 3.52 (m, 2 H, H-2), 3.71 (m, 2 H, NCH_2CH_2O), 3.93 (m, 2 H, NCH_2CH_2O), 4.25 (m, 2 H, H-3), 4.89 (m, 2 H, benzylic), 5.30 (s, 2 H, H-15), 5.42 (s, 2

H, H-12), 6.54 (br s, 1 H, OH), 7.01 (br s, 1 H, NH), 7.46 (s, 1 H, H-8), 7.71 (s, 1 H, H-5), 8.74 (s, 1 H, H-16), 10.28 (br s, 1 H, N^+H); FAB-MS *m*/*z* 505 ($M^+ - HCl$). Anal. ($C_{27}H_{29}ClN_4O_6$) C, H, N.

(9*S*)-9-Ethyl-2,3-dihydro-9-hydroxy-6-(dimethylaminomethyl)-12*H*-1,4-oxazino[3,2-*f*]pyrano-[3′,4′:6,7]indolizino-[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione Hydrochloride (16g). To a stirred suspension of (9*S*)-9-ethyl-2,3-dihydro-9-hydroxy-12*H*-1,4-oxazino[3,2-*f*]pyrano[3′,4′:6,7]indolizino-[1,2-*b*]quinoline-10,13(9*H*,15*H*)-dione (**15a**) (200 mg, 0.493 mmol) in anhydrous DMF (5 mL) was added *N*,*N*-dimethylmethyleneammonium chloride (230 mg, 2.458 mmol), and the mixture was stirred at room temperature for 19 h under N_2 atmosphere. The reaction mixture was evaporated to dryness in vacuo, and the residue was purified by C_{18} reversed-phase MPLC (20% MeOH/H_2O containing 0.5% 1 N aqueous HCl solution). The resulting solid was resolidified from EtOH/Et_2O to afford 219 mg (89%) of **16g**: mp 208 °C; IR (neat) 3361, 3287, 1744 (lactone), 1658 (pyridone), 1598, 1343, 1160 cm^{-1}; ^1H NMR (DMSO-d_6) δ 0.88 (t, $J = 7.5$ Hz, 3 H, H-17), 1.87 (m, 2 H, H-18), 2.75 (s, 6 H, 2 NCH_3), 3.53 (m, 2 H, H-2), 4.25 (m, 2 H, H-3), 4.79 (s, 2 H, benzylic), 5.26 (s, 2 H, H-15), 5.42 (s, 2 H, H-12), 6.54 (br s, 1 H, OH), 7.01 (br s, 1 H, NH), 7.44 (s, 1 H, H-8), 7.65 (s, 1 H, H-5), 8.77 (s, 1 H, H-16), 10.00 (br s, 1 H, N^+H); FAB-MS *m*/*z* 463 ($M^+ - HCl$). Anal. ($C_{25}H_{27}ClN_4O_5$) C, H, N.

Biology. CPT was purchased from Sigma, and topotecan and SN-38 were prepared by employing the reported procedures.[16,17]

In Vitro Cytotoxicity. Six human cancer cell lines (one colon, WiDr; one stomach, MKN45; one ovary, SK-OV-3; one breast, SK-BR-3; and two lung cancers, A549 and H128) were tested in MTT assay.[31] WiDr, A549, SK-OV-3, H128, and SK-BR-3 were obtained from the American Type Culture Collection (Bethesda, MD), and MKN45 was kindly provided by Dr. N. Saijo, National Cancer Center Hospital and Research Institute, Japan. These cell lines were grown in RPMI 1640 medium containing penicillin-streptomycin (100 units/mL) and 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. Single-cell suspensions were prepared by trypsinization and pipet disaggregation. The number of cells for each cell line plated in 96-well microtiter plates was determined from the growth curve obtained in MTT assay. Test compounds were diluted from stock solution in DMSO into fresh medium to a 10-fold concentration. Cells were inoculated into each well in 180 μL of medium, and eight different concentrations of 20 μL of test compounds were added to each well. The plates were then incubated for 4 days at 37 °C in an atmosphere of 5% CO_2. After 4 days of culture, 0.1 mg (20 μL of 5 mg/mL) of MTT was added to each well. The plates were then incubated at 37 °C for 4 h. After the plates were centrifuged at 1000 rpm for 10 min, the supernatant was aspirated. DMSO (150 μL) was added to each well to solubilize formazan crystals. The plates were read immediately at 550 nm on Elisa reader (Dynatech, MR 5000). The IC_{50} was defined as the concentration of compounds that produced a 50% reduction of surviving cells and calculated by quantal probit analysis of pharmacologic calculations with a computer program.[32]

Cleavable Complex Formation Assay for Topo I Inhibition. The cleavable complex formation assay was performed as previously described with some modification.[33] All reactions were carried out in reaction buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, and 5% glycerol. Each reaction mixture (20 μL total volume) contained 250 ng of pHOT1 supercoiled DNA, 1 unit of topo I, and test compound. The reaction mixture was incubated at 37 °C for 30 min and terminated by the addition of 5 μL of stop buffer/loading dye (2.5% SDS, 15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol). Then, 5 μL of proteinase K (0.75 mg/mL) was added and incubated at 37 °C for 60 min. The content of each reaction mixture was analyzed by electrophoresis on 1% agarose gel in 40 mM Tris-acetate and 1 mM EDTA (pH 8.0) at 40 V for 6 h. Both the gel and running buffer contained 0.5 μg/mL ethidium bromide. After electrophoresis, the gel was photographed, and the percentage of nicked DNA to the total DNA for each lane was quantified by BIO-1D analysis imaging system (Vilber Lourmat, France). The IC_{50} was defined as the concentration of compounds that produced 50% formation of nicked DNA and calculated by quantal probit analysis of pharmacologic calculations with a computer program.

Evaluation of AChE Inhibition. AChE activity was determined as previously described with minor modification.[34] The reaction mixture (1.3 mL total volume) contained 10 mM dithiobisnitrobenzoic acid (DTNB), 2 μM test compounds, and 0.05 unit AChE in potassium phosphate buffer (0.1 M, pH 8.0). The reaction mixture was preincubated at 37 °C for 15 min. Reactions were initiated by the addition of 85 μM acetylthiocholine iodide (ATChI), and the mixture was incubated at 37 °C for 15 min. Thiocholine, which was produced from ATChI by AChE, was reacted with DTNB, forming a yellow color. The amount of color production was measured by spectrophotometry at 412 nm and expressed as a percentage of the color production achieved in the absence of test compounds.

In Vitro Determination of Lactone Levels in Human Plasma. Forty microliters of 2.5 mM test compound in DMSO was added to 800 μL of human plasma. The mixture was incubated at 37 °C, and 100 μL aliquots were taken at 15, 30, 45, 60, 90, and 120 min. To precipitate plasma proteins, 400 μL of ice-cold MeOH was added, vortex-mixed for 10 s, and centrifuged at 12 000 rpm for 2 min. Supernatant (400 μL) was transferred to a glass vial and stored at −20 °C immediately until HPLC analysis. For the analysis, 20 μL of the sample was injected onto a Capcell-Pak C-18 reversed-phase column (Shiseido, Japan), 250 × 4.6 mm, 5 μm particle size, proceeded by a matching C-18 guard column. The mobile phase consisted of 325 mL MeOH, 215 mL H_2O, 20 mL of 0.25 M sodium dioctylsulfosuccinate (DOSS) solution, and 11.5 mL of 1.0 M phosphate buffer (pH 6.0). The flow rate was maintained at 0.8 mL/min. The lactone form of test compound was detected at 346 nm with a Waters 480 absorbance detector. The percent of lactone was determined by the ratio of lactone levels measured at different time points to the lactone level measured at the starting time point ($t = 0$ min).

Determination of Maximum Tolerated Dose (MTD). To discover the maximum effect of drugs on tumors in vivo, the appropriate dosage of antitumor drugs defined as the MTD was determined in WiDr xenografted nude mice, based on the nonlethality and weight loss rates not in excess of 10% after treatment. Each treatment and control group was consisted of five mice. Compound **15b** was suspended in 0.5% carboxymethylcellulose (CMC) aqueous solution and given i.p. on a q4d×8 schedule at doses of 10, 13.5, 18, and 24 μmol/kg per injection, while SN-38 was given at doses of 45, 67.5, 100, and 150 μmol/kg per injection. Body weight in each group was measured, and the MTD was determined.

In Vivo Antitumor Activity Test. WiDr was xenografted s.c. into nude mice (10^7 cells) and maintained by serial s.c. transplantation of 3 mm^3 fragments into the right subaxillary region of nude mice. Mice bearing the tumor xenograft of WiDr were randomized into treated and control groups, with 12 mice being used. Treatment was initiated at approximately 3 weeks after transplantation, when each tumor reached a weight of 100 to 200 mg. Compounds **15b** and SN-38 suspended in 0.5% CMC aqueous solution were given i.p. on a q4d×8 schedule at a dose of 10 μmol/kg and 100 μmol/kg per injection, respectively (previously determined as the MTD). Tumors were measured with Vernier calipers, and tumor volume (*V*) was calculated weekly using the equation $V = 1/2 \times a \times b^2$, where *a* and *b* represent the length and width (in millimeters). On day 28 after treatment, the mean tumor weight (MTW), relative tumor growth (RTG), and inhibition rate (IR) were determined. RTG was calculated by dividing the tumor weight on day 28 (MTW_{28}) by that on day 0 (MTW_0). IR was obtained from the equation (1 − RTG in treated/RTG in control) × 100.

Supporting Information Available: Tables S1 and S2, as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; McPhail, A. T.; Sim, G. A. Plant Antitumor Agents. I. The Isolation and Structure of Camptothecin, a Novel Alkaloidal Leukemia and Tumor Inhibitor from *Camptotheca Acuminata*. *J. Am. Chem. Soc.* **1966**, *88*, 3888−3890.

(2) Gottlieb, J. A.; Luce, J. K. Treatment of Malignant Melanoma with Camptothecin. *Cancer Chemother. Rep.* **1972**, *56*, 103−105.

(3) Moertel, C. G.; Schutt, A. J.; Reitemeier, R. J.; Hahn, R. G. Phase II Study of Camptothecin in the Treatment of Advanced Gastrointestinal Cancer. *Cancer Chemother. Rep.* **1972**, *56*, 95−101.

(4) Hsiang, Y. H.; Hertzberg, R.; Hecht, S.; Liu, L. F. Camptothecin Induces Protein-Linked DNA Breaks via Mammalian DNA Topoisomerase I. *J. Biol. Chem.* **1985**, *260*, 14873−14878.

(5) Husain, I.; Mohler, J. L.; Seigler, H. F.; Besterman, J. M. Elevation of Topoisomerase I Messenger RNA, Protein, and Catalytic Activity in Human Tumors: Demonstration of Tumor-Type Specificity and Implications for Cancer Chemotherapy. *Cancer Res.* **1994**, *54*, 539−546.

(6) Lima, C. D.; Wang, J. C.; Mondragon, A. Three-Dimensional Structure of the 67K N−Terminal Fragment of *E. Coli* DNA Topoisomerase I. *Nature* **1994**, *367*, 138−146.

(7) Hertzberg, R. P.; Caranfa, M. J.; Hecht, S. M. On the Mechanism of Topoisomerase I Inhibition by Camptothecin: Evidence for Binding to an Enzyme-DNA Complex. *Biochemistry* **1989**, *28*, 4629−4638.

(8) Li, L. H.; Fraser, T. J.; Olin, E. J.; Bhuyan, B. K. Action of Camptothecin on Mammalian Cells in Culture. *Cancer Res.* **1972**, *32*, 2643−2650.

(9) Fukada, M. Action of Camptothecin and Its Derivatives on Deoxyribonucleic Acid. *Biochem. Pharmacol.* **1985**, *34*, 1225−1230.

(10) Holm, C.; Covey, J. M.; Kerrigan, D.; Pommier, Y. Differential Requirement of DNA Replication for the Cytotoxicity of DNA Topoisomerase I and II Inhibitors in Chinese Hamster DC3F Cells. *Cancer Res.* **1989**, *49*, 6365−6368.

(11) Yang, D.; Strode, J. T.; Spielmann, H. P.; Wang, A. H.-J.; Burke, G. DNA Interactions of Two Clinical Camptothecin Drugs Stabilize Their Active Lactone Forms. *J. Am. Chem. Soc.* **1998**, *120*, 2979−2980.

(12) Redinbo, M. R.; Stewart, L.; Kuhn, P.; Champoux, J. J.; Hol, W. G. Crystal Structures of Human Topoisomerase I in Covalent and Noncovalent Complexes with DNA. *Science* **1998**, *279*, 1504−1513.

(13) Fan, Y.; Weinstein, J. N.; Kohn, K. W.; Shi, L. M.; Pommier, Y. Molecular Modeling Studies of the DNA-Topoisomerase I Ternary Cleavable Complex with Camptothecin. *J. Med. Chem.* **1998**, *41*, 2216−2226.

(14) Jaxel, C.; Kohn, K. W.; Wani, M. C.; Wall, M. E.; Pommier, Y. Structure−Activity Study of the Actions of Camptothecin Derivatives on Mammalian Topoisomerase I: Evidence for a Specific Receptor Site and a Relation to Antitumor Activity. *Cancer Res.* **1989**, *49*, 1465−1469.

(15) Kawato, Y.; Terasawa, H. 2 Recent Advances in the Medicinal Chemistry and Pharmacology of Camptothecin. *Prog. Med. Chem.* **1997**, *34*, 69−109.

(16) (a) Horowitz, R. W.; Wadler, S.; Wiernik, P. H. A Review of the Clinical Experience with Irinotecan (CPT-11). *Am. J. Ther.* **1997**, *4*, 203-210. (b) Sawada, S.; Matsuoka, S.; Nokata, K.; Nagata, H.; Furuta, T.; Yokokura, T.; Miyasaka, T. Synthesis and Antitumor Activity of 20(*S*)-Camptothecin Derivatives: A-Ring Modified and 7,10-Disubstituted Camptothecins. *Chem. Pharm. Bull.* **1991**, *39*, 3183−3188.

(17) (a) Relias, V.; Skirvin, J. A. Topoisomerase I Inhibitors: 1. Topotecan. *J. Oncol. Pharm. Practice* **1997**, *3*, 173−185. (b) Kingsbury, W. D.; Boehm, J. C.; Jakas, D. R.; Holden, K. G.; Hecht, S. M.; Gallagher, G.; Caranfa, M. J.; McCabe, F. L.; Faucette, L. F.; Johnson, R. K. Synthesis of Water-Soluble (Aminoalkyl)Camptothecin Analogues: Inhibition of Topoisomerase I and Antitumor Activity. *J. Med. Chem.* **1991**, *34*, 98−107.

(18) (a) Besterman, J. M. Topoisomerase I Inhibition by the Camptothecin Analog Gl147211C. From the Laboratory to the Clinic. *Ann. N. Y. Acad. Sci.* **1996**, *803*, 202−209. (b) Luzzio, M. J.; Besterman, J. M.; Emerson, D. L.; Evans, M. G.; Lackey, K.; Leitner, P. L.; McIntyre, G.; Morton, B.; Myers, P. L.; Peel, M.; Sisco, J. M.; Sternbach, D. D.; Tong, W.-Q.; Truesdale, A.; Uehling, D. E.; Vuong, A.; Yates, J. Synthesis and Antitumor Activity of Novel Water Soluble Derivatives of Camptothecin as Specific Inhibitors of Topoisomerase I. *J. Med. Chem.* **1995**, *38*, 395−401.

(19) Rowinsky, E. K.; Johnson, T. R.; Geyer, C. E.; Hammond, L. A.; Eckhardt, S. G.; Drengler, R.; Smetzer, L.; Coyle, J.; Rizzo, J.; Schwartz, G.; Tolcher, A.; Von Hoff, D. D.; De Jager, R. L. DX-8951f, a Hexacyclic Camptothecin Analog, on a Daily-times-five Schedule: a Phase I and Pharmacokinetic Study in Patients with Advanced Solid Malignancies. *J. Clin. Oncol.* **2000**, *18*, 3151−3163.

(20) Verschraegen, C. F.; Natelson, E. A.; Giovanella, B. C.; Kavanagh, J. J.; Kudelka, A. P.; Freedman, R. S.; Edwards, C. L.; Ende, K.; Stehlin, J. S. A Phase I Clinical and Pharmacological Study of Oral 9-Nitrocamptothecin, a Novel Water-Insoluble Topoisomerase I Inhibitor. *Anti-Cancer Drugs* **1998**, *9*, 36−44.

(21) Potmesil, M.; Arbuck, S. G.; Takimoto, C. H.; Liebes, L.; Hochster, H. 9-Aminocamptothecin and Beyond. Preclinical and Clinical Studies. *Ann. N. Y. Acad. Sci.* **1996**, *803*, 231−246.

(22) (a) Lavergne, O.; Demarquay, D.; Bailly, C.; Lanco, C.; Rolland, A.; Huchet, M.; Coulomb, H.; Muller, N.: Baroggi, N.; Camara, J.; Le Breton, C.; Manginot, E.; Cazaux, J.; Bigg, D. C. H. Topoisomerase I-Mediated Antiproliferative Activity of Enantiomerically Pure Fluorinated Homocamptothecins. *J. Med. Chem.* **2000**, *43*, 2285−2289. (b) Bom, D.; Curran, D. P.; Chavan, A. J.; Kruszewski, S.; Zimmer, S. G.; Fraley, K. A.; Burke, T. G. Novel A,B,E-Ring-Modified Camptothecins Displaying High Lipophilicity and Markedly Improved Human Blood Stabilities. *J. Med. Chem.* **1999**, *42*, 3018−3022. (c) Lavergne, O.; Lesueur-Ginot, L.; Pla Rodas, F.; Kasprzyk, P. G.: Pommier, J.; Demarquay, D.; Prévost, G.; Ulibarri, G.; Rolland, A.; Schiano-Liberatore, A.; Harnett, J.; Pons, D.; Camara, J.; Bigg, D. C. H. Homocamptothecins: Synthesis and Antitumor Activity of Novel E-Ring-Modified Camptothecin Analogues. *J. Med. Chem.* **1998**, *41*, 5410−5419.

(23) (a) Sugimori, M.; Ejima, A.; Ohsuki, S.; Uoto, K.; Mitsui, I.; Kawato, Y.; Hirota, Y.; Sato, K.; Terasawa, H. Synthesis and Antitumor Activity of Ring A- and F-Modified Hexacyclic Camptothecin Analogues. *J. Med. Chem.* **1998**, *41*, 2308−2318. (b) Sugimori, M.; Ejima, A.; Ohsuki, S.; Uoto, K.; Mitsui, I.; Matsumoto, K.; Kawato, Y.; Yasuoka, M.; Sato, K.; Tagawa, H.; Terasawa, H. Antitumor Agents. 7. Synthesis and Antitumor Activity of Novel Hexacyclic Camptothecin Analogues. *J. Med. Chem.* **1994**, *37*, 3033−3039.

(24) Jew, S.-S.; Kim, M. G.; Kim, H.-J.; Roh, E.-Y.; Park, H.-G. Synthesis and Antitumor Activity of Camptothecin Analogues. *Korean J. Med. Chem.* **1996**, *6*, 263−282.

(25) (a) Sawada, S.; Nokata, K.; Furuta, T.; Yokokura, T.; Miyasaka, T. Chemical Modification of an Antitumor Alkaloid Camptothecin: Synthesis and Antitumor Activity 7-C-Substituted Camptothecins. *Chem. Pharm. Bull.* **1991**, *39*, 2574−2580. (b) Wood, J. L.; Fortunak, J. M.; Mastrocola, A. R.; Mellinger, M.; Burk, P. L. An Efficient Conversion of Camptothecin to 10-Hydroxycamptothecin. *J. Org. Chem.* **1995**, *60*, 5739−5740.

(26) (a) Cabri, W.; Candiani, I.; Zarini, F.; Penco, S.; Bedeschi, A. A New High Yield Semisynthetic Approach to (20S)-9-NH_2-Camptothecin Based on a Sequence of Palladium-Cataylsed Reductions. *Tetrahedron Lett.* **1995**, *36*, 9197−9200. (b) Wani, M. C.; Nicholas, A. W.; Wall, M. E. Plant Antitumor Agents. 23. Synthesis and Antileukemic Activity of Camptothecin Analogues. *J. Med. Chem.* **1994**, *37*, 40−46.

(27) Negoro, S.; Fukuoka, M.; Masuda, N.; Takada, M.; Kusunoki, Y.; Matsui, K.; Takifuji, N.; Kudoh, S.; Niitani, H.; Taguchi, T. Phase I Study of Weekly Intravenous Infusions of CPT-11, a New Derivative of Camptothecin, in the Treatment of Advanced Non-Small-Cell Lung Cancer. *J. Natl. Cancer Inst.* **1991**, *83*, 1164−1168.

(28) Kawato, Y.; Sekiguchi, M.; Akahane, K.; Tsutomi, Y.; Hirota, Y.; Kuga, H.; Suzuki, W.; Hakusui, H.; Sato, K. Inhibitory Activity of Camptothecin Derivatives Against Acetylcholinesterase in Dogs and Their Binding Activity to Acetylcholine Receptors in Rats. *J. Pharm. Pharmacol.* **1993**, *45*, 444−448.

(29) (a) Giovanella, B. C.; Stehlin, J. S.; Wall, M. E.; Wani, M. C.; Nicholas, A. W.; Liu, L. F.; Silber, R.; Potmesil, M. DNA Topoisomerase I-Targeted Chemotherapy of Human Colon Cancer in Xenografts. *Science* **1989**, *246*, 1046−1048. (b) Hertzberg, R. P.; Caranfa, M. J.; Holden, K. G.; Jakas, D. R.; Gallagher, G.; Mattern, M. R.; Mong, S.-M.; Bartus, J. O.; Johnson, R. K.; Kingsbury, W. D. Modification of the Hydroxy Lactone Ring of Camptothecin: Inhibition of Mammalian Topoisomerase I and Biological Activity. *J. Med. Chem.* **1989**, *32*, 715−720. (c) Burke, T. G.; Mi, Z. Preferential Binding of the Carboxylate Form of Camptothecin by Human Serum Albumin. *Anal. Biochem.* **1993**, *212*, 285−287. (d) Burke, T. G.; Mi, Z. The Structural Basis of Camptothecin Interactions with Human Serum Albumin: Impact on Drug Stability. *J. Med. Chem.* **1994**, *37*, 40−46. (e) Mi, Z.; Burke, T. G. Differential Interactions of Camptothecin Lactone and Carboxylate Forms with Human Blood Components. *Biochemistry* **1994**, *33*, 10325−10336. (f) Mi, Z.; Burke, T. G. Marked Interspecies Variations Concerning the Interactions of Camptothecin with Serum Albumins: A Frequency-Domain Fluorescence Spectroscopic Study. *Biochemistry* **1994**,

33, 12540−12545. (g) Burke, T. G.; Mi, Z.; Jiang, Y.; Munshi, C. B. The Important Role of Albumin in Determining the Relative Human Blood Stabilities of the Camptothecin Anticancer Drugs. *J. Pharm. Sci.* **1995**, *84*, 518−519.

(30) Thompson, J.; Zamboni, W. C.; Cheshire, P. J.; Richmond, L.; Luo, X.; Houghton, J. A.; Stewart, C. F.; Houghton, P. J. Efficacy of Oral Irinotecan Against Neuroblastoma Xenografts. *Anti-Cancer Drugs* **1997**, *8*, 313−322.

(31) (a) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a Tetrazole-Based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity Testing. *Cancer Res.* **1987**, *47*, 936−942. (b) Mossman, T. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assay. *J. Immunol. Methods* **1983**, *65*, 55−63.

(32) Tallarida, R. J.; Murray, R. B. *Manual of Pharmacologic Calculations with Computer Programs*; Springer-Verlag: New York, 1987; pp 31−35.

(33) Hsiang, Y.-H.; Hertzberg, R.; Hecht, S.; Liu, L. F. Camptothecin Induces Protein-linked DNA Breaks via Mammalian DNA Topoisomerase I. *J. Biol. Chem*. **1985**, *260*, 14873−14878.

(34) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol*. **1961**, *7*, 88−95.

JM0004751