Novel 7-Substituted Camptothecins with Potent Antitumor Activity

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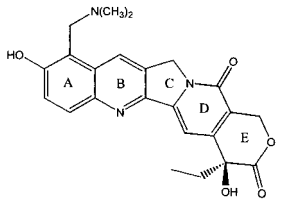
The natural alkaloid camptothecin is the lead compound of a new class of antitumor agents with a unique mechanism of action (i.e. inhibition of DNA topoisomerase I). The pharmacological interest of these agents has generated a large number of derivatives and analogues endowed with potent cytotoxic activity, two of them being in clinical use as antitumor drugs. We have synthesized a new series of camptothecins substituted in position 7 with an alkyl or alkenyl chain bearing cyano and/or carbethoxy groups. These compounds showed potent cytotoxic activity in vitro against the human non-small-cell lung carcinoma H460 cell line, most of them exhibiting IC_{50} values in the 0.05−1 μM range, more active than topotecan used as a reference compound. In particular 7-cyano-20*S*-camptothecin (**5a**) showed high in vitro cytotoxicity against a topotecan-resistant H460 cell subline (H460/TPT) and a cisplatin-resistant ovarian carcinoma subline (IGROV-1/Pt 1). In an in vivo evaluation of the antitumor activity, **5a** appeared significantly more effective than topotecan in the H460 tumor model and comparable with topotecan in a small-cell lung carcinoma model and a colon carcinoma model. The efficacy and good tolerability of this compound increase interest for further preclinical development.

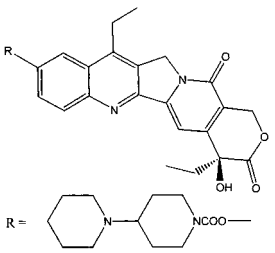
Introduction

Camptothecin (**1a**, CPT), an alkaloid isolated from *Camptotheca acuminata* by Wall and Wani in 1966,[1] exhibits cytotoxic activity through a unique mechanism. The cellular target of the drug is DNA topoisomerase I,[2] an enzyme essential for relaxation of DNA during a number of critical cellular processes, including replication, transcription, and repair.[3] CPT induces topoisomerase I-mediated DNA breaks by preventing DNA religation. The intermediate of the enzymatic process resulting in topoisomerase I-linked DNA single-strand breaks is referred to as a "cleavable complex".[4]

Intensive efforts in medicinal chemistry over the past decade[5] have provided a large number of derivatives of CPT, of which topotecan (Hycamtin) and irinotecan (Camptosar) are now used in clinical practice. Other



Topotecan



Irinotecan

analogues are in various stages of clinical development.[6] Much effort, including that leading to the two above-cited drugs, has been spent toward increasing the water solubility of CPT, to obtain compounds with an improved pharmacological profile and/or enhanced efficacy against human tumors. Although poorly soluble in water, potent CPT derivatives can be administered per os without reduction of activity and even demonstrating pharmacokinetic advantages.[7] In fact, highly lipophilic compounds are reported to be in an advanced stage of investigation.[8]

From structure−activity (SAR) studies[9] it appears that the ring E lactone and the natural 20*S*-configuration of CPTs are essential for antitumor activity. Whereas activity of compounds with substitutions in the C[9,10] and D[9] rings is critically dependent on the size and type of substituents, most structural modifications have concerned the A and B rings, in which wide possibilities of variation exist, especially in positions 7, 9, 10, and 11.[9]

Recently, the structure of the topoisomerase I covalent and noncovalent complexes with a 22-base pair DNA duplex has been solved by X-ray analysis. On the basis of these observations and on SARs, a binding mode for CPT has been proposed.[11] In this and in an analogous model,[12] there is wide space for substitutions in positions 7 and 9 of CPT without steric compromise.

Therefore we envisaged the possibility of the synthesis of new CPTs substituted in position 7 with a lipophilic chain containing, however, polar groups, these latter having the function of possible probes for more specific interactions than a simple hydrocarbon chain. For example, Pommier et al.[13] used a 7-chloromethyl group as an alkylating group. As a starting material we chose the easily accessible camptothecin-7-aldehyde. We report here on the synthesis and the in vitro and in vivo antitumor evaluation of this new series of compounds.

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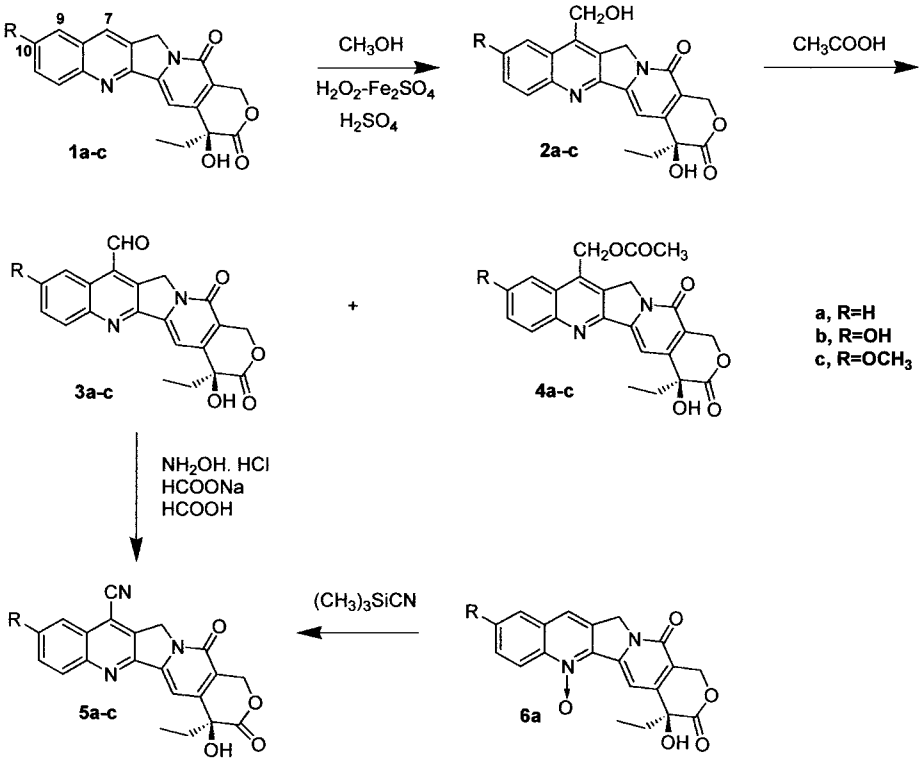
Scheme 1



Chemistry

A key intermediate for the synthesis of the whole series of compounds was 20*S*-camptothecin-7-aldehyde (**3a**), which was prepared according to Sawada et al.[14] Thus, free radical Minisci hydroxymethylation of natural camptothecin (**1**) gave the alcohol **2a**, which when treated with acetic acid gave a mixture of **3a** and **2a** acetate (**4a**), easily separable by chromatography (Scheme 1). The mechanism of the conversion of **2a** to **3a** and **4a** has been discussed.[15] Alternatively, direct oxidation of **2a** to **3a**, so far not reported and difficult to perform because of the sensitivity of **2a** to Brönsted and Lewis acids,[14] was achieved using iodoxybenzoic acid in DMSO[16] as an oxidant.

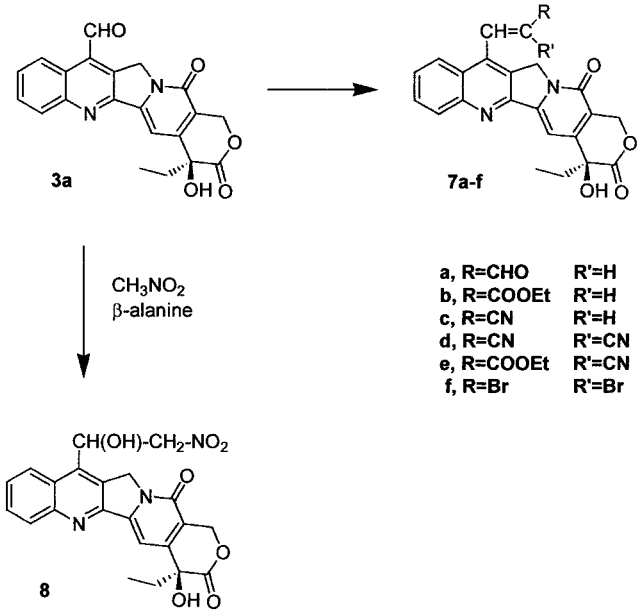
Reaction of the aldehyde **3a** with cyanide gave a very unstable cyanohydrin, which immediately reverted to the starting compound, and the same occurred when the adduct with trimethylsilyl cyanide **10** was chromatographed on silica gel. On the contrary, conversion of **3a** to 7-cyanocamptothecin **(5a)** was easily obtained by dehydration of the oxime or, better, by one-pot reaction with $NH_2OH{\cdot}HCl$, formic acid, and sodium formate.[17] The same nitrile **5a** was prepared by a different pathway, via treatment of camptothecin *N*-oxide[18] (**6a**) with trimethylsilyl cyanide in the presence of benzoyl chloride.[19] (Scheme 1).

SAR studies[9] reported that substitution at positions 10 and 11 with *O*-alkyl groups increases cytotoxic activity with respect to CPT itself. Whereas substitution at C-11 is usually achieved only by total synthesis, 10-substituted derivatives can be obtained by ring A functionalization (e.g. nitration, although in low yield,[20] and hydroxylation). In fact, 10-hydroxycamptothecin (**1b**) is available via oxidation of a ring A-reduced derivative[21] or via photooxidation of camptothecin *N*-oxide (**6a**).[18] Due to the interesting activity shown by **5a**, we subjected **1b** and 10-methoxycamptothecin (**1c**), obtained from **1b** with diazomethane,[18] to the same sequence of reactions that led from CPT to the nitrile **5a**, thus obtaining 10-hydroxy- and 10-methoxy-substituted camptothecin-7-methanols **2b,c**, their acetates **4b,c**, the 7-aldehydes **3b,c**, and the 7-nitriles **5b,c**.

To prepare new derivatives in position 7 starting from the aldehyde **3a**, the Wittig and Knoevenagel reactions were the obvious choice. However, attempts to react **3a** with unstabilized Wittig ylides in the presence of strong bases failed to give the expected alkenes, giving instead an inactive dimeric compound, derived from an intermolecular aldol condensation.[22] Due to the sensitivity of **3a** to strongly basic conditions, the Wittig reactions were then conducted with neutral, stabilized ylides, which allowed to prepare derivatives **7a**−**c**, as mixtures of *E* and *Z* isomers, which were not separated. Also the dibromoethenyl derivative **7f** was obtained by reacting the aldehyde **3a** with CBr_4 in the presence of Ph_3P.[23] To obtain information about the relevance of the conjugated double bond for cytotoxic activity, the ester **7b** was hydrogenated to the corresponding propionate **9**.

On the basis of the preceding experience, conditions were sought to run Knoevenagel reactions avoiding strong bases. Thus, the dicyanoethenyl derivative **7d** was obtained by condensation of **3a** with malononitrile in the presence of LiBr,[24] whereas β-alanine was used as a catalyst[25] in the reaction with ethyl cyanoacetate to obtain compound **7e**. The same catalyst appeared the best one to condense nitromethane with **3a** to afford the nitroaldol **8**. Surprisingly, no nitroalkene was formed in the reaction, and attempts to dehydrate **8** with a variety of acidic reagents failed (Scheme 2).

Scheme 2

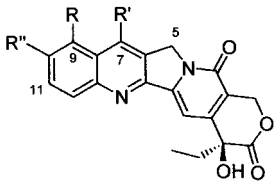


Results and Discussion

Cytotoxicity Studies. Cytotoxicities of the novel CPTs were evaluated against a human non-small-cell lung carcinoma cell line, H460. Topotecan was used as a reference compound since it is a clinically effective CPT and does not require metabolic activation. This cell model was chosen for its sensitivity to topoisomerase I inhibitors, likely related to overexpression of the target enzyme.[26] The H460 cell line is also a useful model for in vivo studies of antitumor efficacy for its reproducible growth in athymic mice. The results of the cytotoxicity studies are summarized in Table 1. With few exceptions (**4b, 5b, 7f**), the tested compounds exhibited potent cytotoxic activity. CPT itself exhibited a remarkable cytotoxic effect in this cell system. The introduction of the hydroxyl group at position 10 (compounds **3b, 4b, 5b**), a structural feature present in topotecan, substantially reduced the cytotoxic potency. A similar change of cellular response was not found in the case of topotecan, since the removal of the hydroxyl group (i.e. 9-dimethylaminocamptothecin) did not influence cytotoxic activity.[27]

Methylation of the 10-OH (compounds **3c, 4c, 5c**) restored high potency, indicating that whereas in position 10 the H-bonding polar group OH is detrimental, a small alkoxy group, possibly an H-bond acceptor, is more than tolerated. On the other hand, the 10,11-methylenedioxy substitution has a well-known positive effect on cytotoxicity.[9,28] However, such a substitution was not possible in our series of compounds because it would have required a total synthesis. In the series of compounds modified only at position 7 (**3a, 4a, 5a**, **7a**−**f, 8, 9**), the presence of a linear chain usually resulted in a significant cytotoxicity. The nature of the substituent likely influences the cytotoxicity. The presence of a polar group in position 3 of the chain, such as a CO or CN, was apparently favorable. The steric rigidity conferred by the presence of the conjugated double bond does not seem to be relevant (see compound **7b** vs **9**). A bulky nonpolar substituent (e.g. in **7f**) was not favorable.

Table 1. In Vitro Cytotoxic Activity of CPT Derivatives on H460 Cell Lines



compd	R	R′	R″	IC_{50} (μM ± SEM) H460
topotecan	$CH_2N(CH_3)_2$	H	OH	1.38 ± 0.95
CPT	H	H	H	0.33 ± 0.05
3a	H	CHO	H	0.39 ± 0.3
3b	H	CHO	OH	1.78 ± 0.5
3c	H	CHO	OCH_3	0.18 ± 0.05
4a	H	CH_2OCOCH_3	H	0.15 ± 0.05
4b	H	CH_2OCOCH_3	OH	7 ± 2.6
4c	H	CH_2OCOCH_3	OCH_3	0.04 ± 0.01
5a	H	CN	H	1.04 ± 0.5
5b	H	CN	OH	5.65 ± 2.5
5c	H	CN	OCH_3	0.66 ± 0.02
7a	H	CH=CHCHO	H	0.17 ± 0.02
7b	H	CH=CHCOOEt	H	0.04 ± 0.006
7c	H	CH=CHCN	H	0.13 ± 0.06
7d	H	CH=C(CN)CN	H	0.30 ± 0.14
7e	H	CH=C(CN)COOEt	H	0.25 ± 0.014
7f	H	CH=C(Br)Br	H	4.6 ± 0.5
8	H	$CH(OH)CH_2NO_2$	H	1.23 ± 0.34
9	H	CH_2CH_2COOEt	H	0.06 ± 0.04

Table 2. Pattern of Cross-Resistance of Topotecan and **5a** in Human Tumor Cell Lines with Acquired Resistance

	IC_{50} (μM)			
compd	H460	H460/TPT	IGROV-1	IGROV-1/Pt1
topotecan	1.37 ± 0.9	7.1 ± 3.5 (5.2)[a]	0.3 ± 0.09	0.5 ± 0.2 (1.7)[a]
5a	1.04 ± 0.5	0.37 ± 0.2 (0.36)[a]	1.2 ± 0.5	0.13 ± 0.008 (0.11)[a]

[a] In parentheses: resistance index, i.e., ratio between IC_{50} value in resistant cells and IC_{50} value in sensitive line.

Among the prepared compounds, compound **5a** was selected for further antitumor activity studies, on the basis of easy synthesis, high yield of the preparation, and expected stability. Moreover, the nitrile **5a** maintained its in vitro efficacy against cell systems with acquired resistance to topotecan or cisplatin (Table 2). Indeed, a collateral sensitivity of resistant cells to **5a** was evident in either cell systems. In addition, preliminary experiments of topoisomerase I-mediated stimulation of DNA cleavage indicated an increased enzyme poisoning activity of **5a** (data not shown).

In Vivo Antitumor Efficacy Studies. In vivo evaluation of antitumor activity of **5a** was performed on two human lung tumor models, the non-small-cell lung carcinoma H460 and the small-cell lung carcinoma (POVD), and a colon carcinoma model (COCF). The tested tumor models exhibited appreciable responsiveness to topotecan. As a consequence of low water solubility, the compound was administered only by oral route. Topotecan, used for comparison, was administered using either intravenous or oral route maintaining the same schedule (q4dx4). The maximum tolerated dose (MTD) of **5a** was somewhat higher than that of topotecan, thus indicating that the analogue was better tolerated than the reference compound, despite a com-

Table 3. Comparison of Antitumor Activity of Topotecan and **5a** against Human Tumor Xenografts

		H460			POVD			COCF		
compd	dose[a] (mg/kg)	TVI (%)	LCK	toxic deaths	TVI (%)	LCK	toxic deaths	TVI (%)	LCK	toxic deaths
topotecan	15 (iv)	80	1.3	0/5	87	1.1	0/6	91	1.2	0/5
	15 (po)	87	1.7	0/5	98	2.5	0/6	91	1.1	0/5
5a	25 (po)	98*	2.1	0/5	99*	2.7	0/6	91	1.2	0/5

[a] Treatment schedule: q4dx4. *$p < 0.05$ vs topotecan (po).

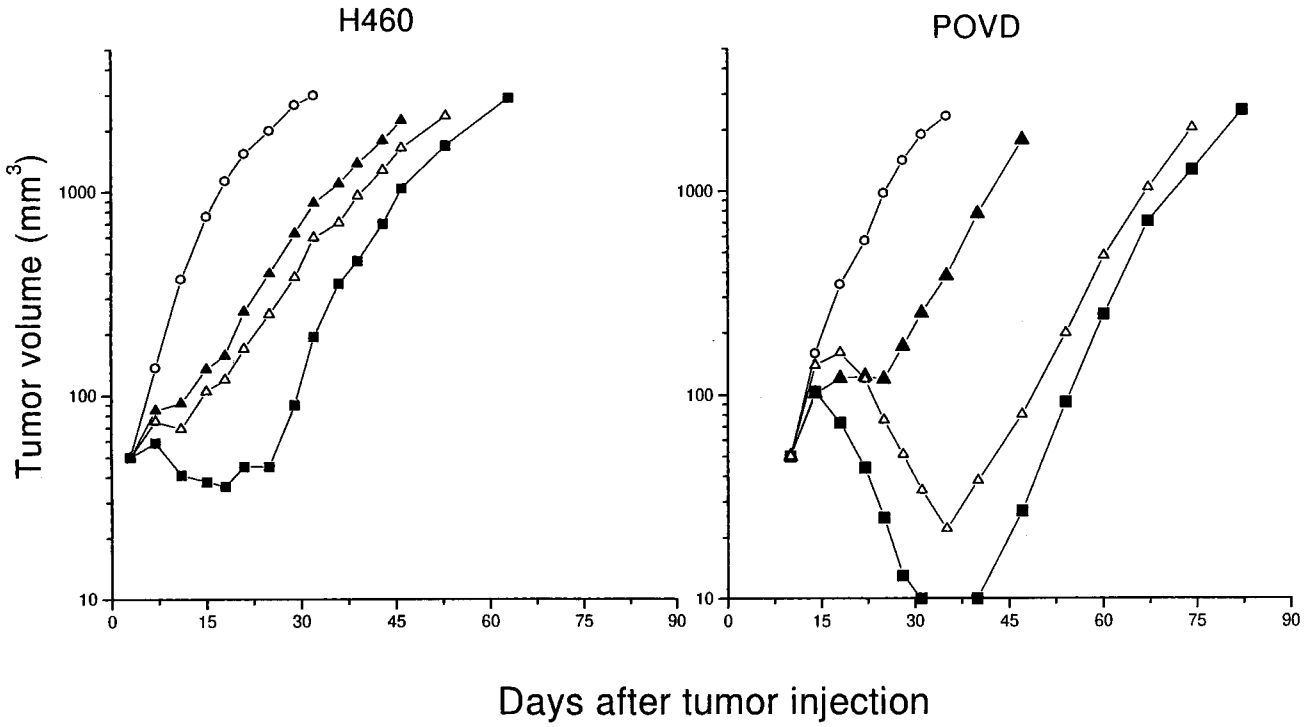


Figure 1. Growth curves of human lung tumor xenografts (H460, non-small-cell lung carcinoma; POVD, small-cell lung carcinoma) after treatment with topotecan or **5a** (schedule, q4dx4): (○) control; (▲) topotecan (iv), 15 mg/kg; (△) topotecan (po), 15 mg/kg; (■) **5a** (po), 25 mg/kg.

parable or increased cytotoxic potency (Table 1). Thus, at their MTD, **5a** was significantly more effective than topotecan in the treatment of the H460 tumor, in terms of both tumor growth inhibition and delay of tumor regrowth (as indicated by LCK values) (Table 3 and Figure 1). The schedule used allows an early comparison of drug efficacy in terms of tumor growth inhibition. However, in the tumor models used, the tested agents did not achieve persistent tumor regression. Thus, under these conditions, usually 7−14 days after the end of treatment, regrowth of residual tumor was observed with a doubling time comparable to that of the control tumor, allowing the determination of LCK. The improvement of activity was also evident if the antitumor effect of topotecan following iv administration (i.e. the standard route of administration) is considered. The pattern of tumor response observed in the treatment of the small-cell lung carcinoma (POVD) (Figure 1) indicated an increased efficacy of **5a** compared to iv topotecan. However, in this model, oral topotecan was appreciably more effective than by parenteral drug administration. In the colon carcinoma model **5a** and topotecan exhibited comparable efficacy.

Conclusions

In conclusion, taken together, the results presented in this study clearly indicate that C-7 of CPT is a favorable position for introduction of a lipophilic group, since with proper substituents the activity is maintained or improved. A substantial increase of cytotoxic potency was afforded with a linear chain containing a CO function at position 3 (e.g., **4a, 7b, 9**). It is likely that the improvement of cytotoxic activity is the result of multiple favorable events, including increased drug uptake (as expected on the basis of increased lipophilicity) and increased stability (as documented also for 7-silyl-modified CPTs[8]). However, since the cytotoxic activity is markedly dependent on the nature of the liphophilic chain, it is possible that the chain at the 7 position is involved in drug interaction with the target enzyme. A possible pharmacological advantage of 7-modified CPTs is the ability to overcome mechanisms of resistance to conventional hydrophilic CPTs (e.g., topotecan). The mechanism of the lack of cross-resistance is still unknown. A better understanding of this feature could provide insights for optimization of drug design. The improved pharmacological profile of a compound of this series suggests that modification at the 7 position is a promising approach in the development of more effective topoisomerase I inhibitors.

Experimental Section

General Methods. All reagents and solvents were reagent grade or were purified by standard methods before use. Melting points were determined in open capillaries on a Büchi melting point apparatus and are uncorrected. Column chro-

matography was carried out on flash silica gel (Merck 230−400 mesh). TLC analysis was conducted on silica gel plates (Merck 60F_{254}). NMR spectra were recorded at 300 MHz with a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. Mass spectra were recorded at an ionizing voltage of 70 eV on a Finnigan TQ70 spectrometer. The relative intensities of mass spectrum peaks are listed in parentheses.

Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium benzophenone ketyl; dry methylene chloride was obtained by distillation from phosphorus pentoxide. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware was oven-dried and/or flame-dried.

10-Hydroxycamptothecin,[18] 10-methoxycamptothecin,[18] 7-hydroxymethylcamptothecin,[14] 7-acetoxymethylcamptothecin[14] and camptothecin *N*-oxide[18] were synthesized according to the literature. The physical data were in agreement with those given in the literature.

7-Hydroxymethyl-10-hydroxycamptothecin (2b). 10-Hydroxycamptothecin (**1b**) (100 mg, 0.27 mmol) was suspended in a mixture of MeOH (3 mL) and water (2.5 mL). To the ice-cooled suspension 96% H_2SO_4 (1.3 mL) was added dropwise, followed by $FeSO_4 \cdot 7H_2O$ (80 mg, 0.27 mmol). The resulting solution was cooled at −10 °C and then 30% H_2O_2 (240 μL) was dropped. The mixture was stirred for 4 days at room temperature, then 5 mL of water was added; the precipitate was filtered and used without further purification: yield 90%.

7-Hydroxymethyl-10-methoxycamptothecin (2c). 10-Methoxycamptothecin (**1c**) (80 mg, 0.21 mmol) was treated as described above to give **2c,** which was used without further purification: yield 93%.

Camptothecin-7-aldehyde (3a). (A) Synthesized according to the literature.[14]

(B) To a solution of 2-iodoxybenzoic acid (2.23 g, 7.96 mmol) in dimethyl sulfoxide (17 mL), 7-hydroxymethylcamptothecin (1 g, 2.65 mmol) was added. The reaction mixture was vigorously stirred for 24 h, diluted with H_2O (100 mL) and continuously extracted with CH_2Cl_2. The organic layer was dried and evaporated. The product was purified by flash chromatography (eluent: CH_2Cl_2:MeOH 97:3): yield 45%; mp 254−258 °C, lit.[14] mp 256−260 °C. The physical data were in agreement with those given in the literature.

10-Hydroxycamptothecin-7-aldehyde (3b) and 7-Acetoxymethyl-10-hydroxycamptothecin (4b). A solution of 50 mg (0.12 mmol) of 7-hydroxymethyl-10-hydroxycamptothecin (**2b**) in 30 mL of glacial acetic acid was refluxed for 5 h. The solvent was evaporated under reduced pressure and the products were purified by flash chromatography (eluent: CH_2Cl_2:MeOH 95:5) to give 10-hydroxycamptothecin-7-aldehyde: yellow powder, yield 50%; mp > 250 °C; ^{1}H NMR (DMSO-d_6) δ 0.83 (t, J = 7 Hz, H_3-18) 1.75−1.93 (m, H_2-19) 5.42 (s, H_2-17) 5.48 (s, H_2-5) 6.50 (s, −OH) 7.32 (s, H-14) 7.48 (dd, J = 9 Hz; J = 1.84 Hz, H-11) 8.18 (d, J = 9 Hz, H-12) 8.22 (d, J = 1.84 Hz, H-9) 10.75 (s, −OH) 10.9 (s, −CHO). Anal. ($C_{21}H_{16}N_2O_6$) C, H, N. 7-Acetoxymethyl-10-hydroxycamptothecin: yellow powder, yield 20%; mp > 250 °C; ^{1}H NMR (DMSO-d_6) δ 0.83 (t, J = 7 Hz, H_3-18) 1.78−1.93 (m, H_2-19) 5.38 (s, H_2-17) 5.42 (s, H_2-5) 5.60 (s, CH_2 Ac) 6.45 (s, −OH) 7.2−7.5 (m, H-14 + H-9 + H-11) 8.05 (d, J = 9 Hz, Hz, H-12) 10.45 (s, −OH). Anal. ($C_{23}H_{20}N_2O_7$) C, H, N.

10-Methoxycamptothecin-7-aldehyde (3c) and 7-Acetoxymethyl-10-methoxycamptothecin (4c). 7-Hydroxymethyl-10-methoxycamptothecin (**2c**) (80 mg, 0.20 mmol) was treated as described above and the products purified by flash chromatography (eluent: CH_2Cl_2:MeOH 98:2) to give 10-methoxycamptothecin-7-aldehyde: yellow powder, yield 40%; mp 273 °C dec; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, J = 7 Hz, H_3-18) 1.78−1.93 (m, H_2-19) 4.02 (s, $-OCH_3$) 5.43 (s, H_2-17) 5.52 (s, H_2-5) 6.52 (s, −OH) 7.31 (s, H-14) 7.64 (dd, J = 9.56; J = 1.84 Hz, H-11) 8.22 (d, J = 9.56, H-12) 8.38 (d, J = 1.84 Hz, H-9) 11.06 (s, −CHO); MS m/z 406 (100 M^+) 378 (47) 363 (49) 349 (57). Anal. ($C_{22}H_{18}N_2O_6$) C, H, N. 7-Acetoxymethyl-10-methoxycamptothecin: yellow powder, yield 20%; mp 215 °C dec; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, H_3-18 J = 7 Hz) 1.78−1.93 (m, H_2-19) 2.11 (s, CH_3CO) 3.97 (s, $-OCH_3$) 5.31−5.40 (m, H_2-17; CH_2−Ac) 5.71 (s, H_2-5) 6.50 (s, −OH) 7.27 (s, H-14) 7.44−7.59 (m, H-9; H-12) 8.10 (dd, H-11); MS m/z 450 (35 M^+) 390 (100) 346 (36) 318 (18). Anal. ($C_{24}H_{22}N_2O_7$) C, H, N.

7-Cyanocamptothecin (5a). (A) To a solution of 400 mg (1.06 mmol) of camptothecin-7-aldehyde (**3a**) in 30 mL of formic acid, 190 mg (2.73 mmol) of hydroxylamine hydrochloride and 960 mg (14.1 mmol) of sodium formate were added. The resulting solution was refluxed for 5 h. The solvent was evaporated under reduced pressure, then 20 mL of water was added and the undissolved solid was filtered. The aqueous layer was extracted with CH_2Cl_2 (20 mL ×3). The extracts were dried, concentrated in vacuo and combined with the filtered solid. Purification by flash chromatography (CH_2Cl_2:MeOH 99:1) afforded 200 mg of **5a** as a yellow solid: yield 50%; mp 235 °C dec; $[\alpha]_D$ +30° (c 0.4, $CHCl_3$:MeOH 8:2); IR (Nujol) 2310 cm^{-1} (CN); ^{1}H NMR (DMSO-d_6) : 0.86 (t, H_3-18, J = 7 Hz) 1.86 (m, H_2-19, J = 7 Hz) 5.45 (s, H_2-5) 5.55 (s, H_2-17) 6.6 (s, −OH) 7.4 (s, H-14) 7.9−8.15 (m, H-10; H-11) 8.2 (dd, H-9) 8.4 (dd, H-12); MS m/z 373 (M^+ 100) 344(30) 314 (44) 273 (99) 243 (47). Anal. ($C_{21}H_{15}N_3O_4$) C, H, N.

(B) 300 mg (0.82 mmol) of camptothecin *N*-oxide and 400 mg (4 mmol) of trimethylsilylcyanide were dissolved in 30 mL of tetrachloroethane. The solution was stirred for 10 min at room temperature, than 230 mg (1.65 mmol) of benzoyl chloride was added. The reaction mixture was refluxed for 40 h. The solvent was evaporated under reduced pressure and the product was purified by flash chromatography using as eluent a solution of hexane:ethyl acetate 7:3: yield 46%.

7-Cyano-10-hydroxycamptothecin (5b). 50 mg (0.13 mmol) of **3b** was dissolved in 5 mL of formic acid. After the addition of 23 mg (0.33 mmol) of hydroxylamine hydrochloride and 116 mg (1.7 mmol) of sodium formate the solution was refluxed for 4 h. The solvent was evaporated under reduced pressure, then 3 mL of water was added and the undissolved solid was filtered. The aqueous layer was extracted with CH_2Cl_2 (2 mL ×3). The extracts were dried, concentrated in vacuo and combined with the filtered solid. Purification by flash chromatography (CH_2Cl_2:MeOH 98:2) afforded 40 mg of **5b** as a yellow solid: yield 79%; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, H_3-18, J = 7 Hz) 1.86 (m, H_2-19, J = 7 Hz) 5.45 (s, H_2-5) 5.55 (s, H_2-17) 6.45 (s, −OH) 7.3 (s, H-14) 7.45 (d, J = 1.8 Hz, H-9) 7.6 (dd, J = 1.8 J = 9 Hz, H-11) 8.23 (d, J = 9 Hz H-12); MS m/z 389 (M^+ 43) 345(100) 289 (81) 259 (70) 215 (33) 149 (28) 90(28). Anal. ($C_{21}H_{15}N_3O_5$) C, H, N.

7-Cyano-10-methoxycamptothecin (5c). 10 mg (0.025 mmol) of 10-methoxycamptothecin-7-aldehyde (**3c**) was treated as described above to give **5c**: yellow powder; yield 35%; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, J = 7 Hz H_3-18) 1.81−1.94 (m, H_2-19) 4.05 (s, OCH_3) 5.44 (s, H_2-17) 5.50 (s, H_2-5) 6.56 (s, −OH) 7.31 (s, H-14) 7.45 (d, J = 2.56 Hz H-9) 7.71 (dd, J = 2.56; J = 9.56 Hz H-12) 8.26 (dd, J = 2.56 Hz, J = 9.56 Hz H-11). Anal. ($C_{22}H_{17}N_3O_5$) C, H, N.

7-Formylethenylcamptothecin (7a). A solution of camptothecin-7-aldehyde (**3a**) (500 mg, 1.33 mmol) and triphenylphosphoranylideneacetaldehyde (400 mg, 1.33 mmol) in 50 mL of $CHCl_3$ was refluxed for 3 h. The product was purified by flash chromatography using as eluent hexane:ethyl acetate 1:1 and it was isolated (54 mg, 10%) as a mixture of *Z* and *E* isomers: mp 180−185 dec; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, J = 7 Hz H_3-18) 1.81−1.94 (m, H_2-19) 5.25−5.55 (m, H_2-17; H_2-5) 6.5−6.65 (m, −OH;-*CH*-CHO (Z)) 7.03 (dd, J = 16.18 Hz, J = 7.72 Hz, -*CH*-CHO (E)) 7.38 (s, H-14) 7.72−7.8 (m, H-10; H-11; *CH*═CH−CHO (Z)) 8.19−8.29 (m, H-12) 8.46 (dd, J = 8.46 Hz; J = 1.47 Hz, H-9) 8.67 (d, J = 16.18 Hz, -*CH*CH−CHO (E)) 9.48 (d, J = 8.09 Hz, CHO (Z)) 9.98 (d, J = 7.72 Hz, CHO (E)).

Anal. ($C_{23}H_{18}N_2O_5$) C, H, N.

***E*-7-Ethoxycarbonylethenylcamptothecin (7b).** A solution of (ethoxycarbonylmethylene)triphenylphosphorane (460 mg, 1.3 mmol) and camptothecin-7-aldehyde (**3a**) (500 mg, 1.3 mmol) in 50 mL of $CHCl_3$ was refluxed for 2 h. The solvent was evaporated in vacuo and the residual slurry was purified

by flash chromatography (CH_2Cl_2:MeOH 98:2). Extensive column chromatography was required to separate most of the Ph_3PO from the desired product. The byproduct was completely removed washing the solid with cold ethanol and then with benzene. 300 mg of a yellow powder was obtained: yield 52%; ^{1}H NMR (DMSO-d_6) δ 0.88 (t, H_3-18, J = 7 Hz) 1.35 (t, H_3-ethyl, J = 7 Hz) 1.88 (m, H_2-19, J = 7 Hz) 4.32 (q, H_2-ethyl J = 7 Hz) 5.44 (s, H_2-5) 5.49 (s, H_2-17) 6.55 (s, −OH) 6.86 (d, CH═, J = 16 Hz) 7.4 (s, H-14) 7.8 (ddd, H-11) 7.93 (ddd, H-10) 8.18−8.30 (m, H-12 + H-9) 8.34 (d, CH═, J = 16 Hz). Anal. ($C_{25}H_{22}N_2O_6$) C, H, N.

7-Cyanoethenylcamptothecin (7c). A solution of **3a** (500 mg, 1.33 mmol) and (cyanomethylene)triphenylphosphorane (45 mg, 1.33 mmol) in 50 mL of $CHCl_3$ was refluxed for 4 h. The solvent was evaporated in vacuo and the residual slurry was partially purified by flash chromatography (CH_2Cl_2:MeOH 98:2). The appropriate fractions were pooled and the residual Ph_3PO was removed washing the solid with cold ethanol to afford, after filtration, **7c** as a mixture of *Z* and *E* isomers in a 1:2 ratio: yellow powder, yield 45%; mp 250 dec; ^{1}H NMR (DMSO-d_6) δ 0.9 (t, H_3-18, J = 7 Hz) 1.85 (m, H_2-19, J = 7 Hz) 5.2−5.5 (m, H_2-5 + H_2-17) 6.55 (s, −OH) 6.6 (d, CH═ (Z), J = 12 Hz) 6.75 (d, CH═ (E), J = 17 Hz) 7.4 (s, H-14) 7.7−8 (m, H-11 + H-10) 8.15 (dd, H-12 (Z)) 8.2−8.35 (m, H-12 (E) + H-9 (Z)) 8.2 (d, CH═ (Z), J = 12 Hz) 8.4 (dd, H-9 (E)) 8.55 (d, CH═ (E), J = 17 Hz). Anal. ($C_{23}H_{17}N_3O_4$) C, H, N.

7-(2,2-Dicyanoethenyl)camptothecin (7d). To a mixture of camptothecin-7-aldehyde (**3a**) (600 mg, 1.6 mmol), and malononitrile (105 mg, 1.6 mmol) in 80 mL of tetrachloroethane in the presence of 4 Å molecular sieves, commercial grade lithium bromide (28 mg, 0.32 mmol) was added at room temperature. After being stirred for 5 min, the resulting mixture was heated at 80 °C for 3 h. The solvent was evaporated under vacuum. The crude product was purified by flash chromatography with ethyl acetate to afford 150 mg of a pale yellow solid: yield 22%; mp 185−190 °C dec; ^{1}H NMR (DMSO-d_6) δ 0.85 (t, H_3-18, J = 7 Hz) 1.85 (m, H_2-19, J = 7 Hz) 5.38 (s, H_2-5) 5.45 (s, H_2-17) 6.58 (s, −OH) 7.37 (s, H-14) 7.82 (ddd, H-11) 7.98 (ddd, H-10) 8.2 (dd, H-12) 8.3 (dd, H-9) 9.3 (s, CH═); MS m/z 424 (M^+ 10) 380 (45) 351 (10) 324 (13) 295 (20) 266 (10) 217 (10) 144(14) 81 (10) 44(30) 32 (100). Anal. ($C_{24}H_{16}N_4O_4$) C, H, N.

7-Ethoxycarbonyl-7-cyanoethenylcamptothecin (7e). To a mixture of **3a** (100 mg, 0.26 mmol) and ethyl cyanoacetate (850 μL, 7.8 mmol) in 20 mL of 95% ethanol, β-alanine (92 mg, 1.04 mmol) was added. After being stirred for 3 h at room temperature, the solution was concentrated to dryness under vacuum. The residue was dissolved in CH_2Cl_2 (20 mL) and washed with water (30 mL × 3). The organic layers were dried with magnesium sulfate, filtered and evaporated. The residual solid was washed with diethyl ether to remove the excess of ethyl cyanoacetate, filtered and purified by flash chromatography (CH_2Cl_2:MeOH 98:2) to give 60 mg of a yellow solid: yield 48%; ^{1}H NMR (DMSO-d_6) δ: 0.87 (t, H_3-18, J = 7 Hz) 1.38 (t, H_3-ester, J = 7 Hz) 1.85 (m, H_2-19, J = 7 Hz) 4.42 (q, H_2-ester, J = 7 Hz) 5.37 (s, H_2-5) 5.47 (s, H_2-17) 6.58 (s, −OH) 7.4 (s, H-14) 7.8 (ddd, H-11) 7.95 (ddd, H-10) 8.22 (dd, H-12) 8.30 (dd, H-9) 9.0 (s, CH═). Anal. ($C_{26}H_{21}N_3O_6$) C, H, N.

7-(2,2-Dibromoethenyl)camptothecin (7f). To a solution of CBr_4 (166 mg, 0.5 mmol) in dry CH_2Cl_2 (2 mL) under nitrogen, PPh_3 (262 mg, 1 mmol) was added at 0 °C. An intense yellow color developed. After 15 min NEt_3 (0.26 mmol, 26 mg) was added. The reaction mixture was stirred for 5 min at 0 °C, then a solution of **3a** (100 mg, 0.26 mmol) in 1 mL of dry CH_2Cl_2 was added. The resulting red mixture was refluxed for 8 h then it was poured into 3 mL of water. The two phases were separated and the aqueous layer was extracted three times with CH_2Cl_2. The combined organic extracts were dried, concentrated in vacuo and purified by flash chromatography (hexane:ethyl acetate 4:6) to afford 20 mg of **7f** as a yellow powder: yield 15%; mp 142 °C; ^{1}H NMR (DMSO-d_6) δ 0.85 (t, H_3-18, J = 8 Hz) 1.9 (m, H_2-19, J = 8 Hz) 5.25 (s, H_2-5) 5.45 (s, H_2-17) 6.55 (s, −OH) 7.35 (s, H-14) 7.8 (ddd, H-11) 7.92 (ddd, H-10) 8.07 (dd, H-12) 8.25 (dd, H-9) 8.35 (s, CH═). Anal. ($C_{22}H_{16}N_2O_4Br$) C, H, N.

α-Nitromethylcamptothecin-7-methanol (8). To a solution containing 50 mg (0.13 mmol) of **3a** in 10 mL of 95% ethanol, 46 mg (0.52 mmol) of β-alanine and 210 μL (3.8 mmol) of nitromethane were added. The solution was refluxed for 7 h. The solvent was evaporated and the residual slurry was then suspended in 10 mL of water and extracted with CH_2Cl_2. The combined organic extracts were dried, concentrated in vacuo and purified by flash chromatography (eluent CH_2Cl_2: MeOH 98:2) to afford 20 mg of **8** as a yellow powder: yield 34%; ^{1}H NMR (DMSO-d_6) δ 0.9 (t, H_3-18, J = 7 Hz) 1.85 (m, H_2-19, J = 7 Hz) 5 (m, CH_2−NO_2) 5.45 (s, H_2-5) 5.5 (s, H_2-17) 6.35 (m, C*H*-OH) 6.52 (s, −OH) 6.86 (d, J = 4 Hz CH−O*H*) 7.35 (s, H-14) 7.8 (ddd, H-11) 7.92 (ddd, H-10) 8.2−8.35 (m, H-9 + H-12). Anal. ($C_{22}H_{19}N_3O_7$) C, H, N.

7-Ethoxycarbonylethylcamptothecin (9). To a solution of **7b** (250 mg, 0.56 mmol) in 300 mL of methanol 150 mg of 10% Pd/C was added. After being stirred for 3 h under hydrogen atmosphere at room temperature, the reaction mixture was filtered, concentrated and purified by flash chromatography (CH_2Cl_2:MeOH 98:2) to give 50 mg of the desired product as a yellow powder: yield 20%; ^{1}H NMR (DMSO-d_6) δ 0.85 (t, H_3-18, J = 7 Hz) 1.1 (t, −OCH_2CH_3, J = 7 Hz) 1.85 (m, H_2-19, J = 7 Hz) 2.8 (t, J = 7.7 Hz, −CH_2-) 3.48 (t, J = 7.7 Hz, −CH_2-) 4.05 (q, J = 7 Hz, −OCH_2CH$_3$) 5.41 (s, H_2-5) 5.44 (s, H_2-17) 6.53 (s, −OH) 7.4 (s, H-14) 7.74 (m, H-11) 7.87 (m, H-10) 8.18 (dd, H-12) 8.28 (dd, H-9). Anal. ($C_{25}H_{24}N_2O_6$) C, H, N.

7-(1-Trimethylsilyloxy-1-cyano)methylenecamptothecin (10). 50 mg (0.133 mmol) of **3a** in methylene chloride (25 mL) was allowed to react with 15.8 mg (0.160 mmol) of trimethylsilyl cyanide in the presence of a catalytic amount of trimethylsilyl triflate. The mixture was refluxed for 1 h, the solvent was evaporated under reduced pressure to yield **11** which easily reverted to the starting compound when chromatographed.

In Vitro Studies. The human tumor cell lines used in this study included H460, a human lung large-cell carcinoma cell line (ATCC HTB 177) and a topotecan-resistant subline (H460/TPT); IGROV-1, an ovarian carcinoma cell line,[29] and its cisplatin-resistant variant IGROV-1/Pt1. The resistant cell lines were selected in our laboratory after exposure to increasing drug concentrations; their growth characteristics were similar to those of the correspondent parental cell lines. All the cell lines were cultured in RPMI-1640 containing 10% fetal calf serum. Cytotoxicity was assessed by growth inhibition assay after 1 h of drug exposure. Briefly, cells in the logarithmic phase of growth were harvested and seeded in duplicates into 6-well plates. Twenty-four hours after seeding, cells were exposed to the drug; they were harvested 72 h after exposure and counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control. All compounds are insoluble in water and were dissolved in DMSO prior to dilution into the biological assay.

In Vivo Studies. Nude athymic CD1 mice (Charles River Laboratory), 8−10 weeks old, were used for the studies. Animals were maintained in laminar flow rooms and experimental protocols were approved by the Ethical Committee for Animal Experimentation of our Institute. The human lung carcinoma NCI-H460, the small-cell lung cancer POVD and the colon carcinoma COCF were used. Tumor lines were maintained sc by serial passages. For chemotherapy studies mice were xenografted sc in both flanks with tumor fragments as already described.[29] Tumor growth was monitored by diameters measurement and tumor volume (TV) was calculated as: TV = $d2 \times D/2$, where d and D represent the shortest and the longest diameter, respectively. Drugs were delivered in a volume of 10 mL/kg body weight, starting treatment when tumors were visible but not measurable. The effects of drug treatment were assessed as: TV inhibition percent in treated versus control tumors, 7−10 days after the last treatment; $\log_{10}$ cell kill (LCK) induced by the treatment and calculated as: T

− C/3.32 × tumor doubling time, where T and C represent days in treated and control tumors to reach a mean TV of 1000 mm^3. Student's *t*-test was used to compared tumor volumes of treated mice. *P* values < 0.05 were considered significant.

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