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Water-Soluble Camptothecin Derivatives that Are Intrinsic Topoisomerase I Poisons

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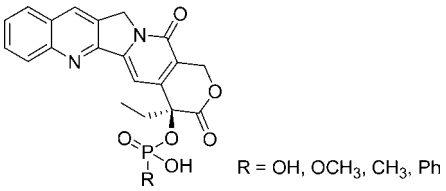
ABSTRACT



In an effort to improve the water solubility of camptothecin, four 20-*O*-phosphate and phosphonate analogues have been prepared. These analogues are freely water soluble, stable at physiological pH, and stabilize the human topoisomerase I-DNA covalent binary complex with the same sequence selectivity as camptothecin itself. All four compounds inhibited the growth of yeast expressing human topoisomerase I in an enzyme-dependent fashion.

Camptothecin (CPT, **1**, Figure 1) was isolated by Wall et al. from *Camptothecin acuminata*.[1] Testing as an antitumor agent in animal models produced promising results and led to the evaluation of camptothecin in the clinic.[2] The initial trials were complicated by the poor solubility of CPT, which was administered as the water-soluble sodium salt of the ring-opened carboxylate form of CPT (**2**). Severe and unpredictable toxicity led to suspension of the clinical trials.

The discovery that the primary cellular target for CPT is DNA topoisomerase I[3] has created renewed interest in the compound and led to the successful identification and development of the antitumor agents topotecan (Hycamtin, **3**) and irinotecan (Camptosar, **4**).[4] These agents are used clinically in several countries for the treatment of ovarian and small-cell lung cancers and for colorectal cancer, respectively.[5] At least 10 additional CPT analogues are in various stages of clinical trials.

A number of approaches are being used to improve the antitumor efficacy of the CPT family. This includes the development of prodrugs (conjugates and polymer-bound camptothecins), new formulations (liposomes or microparticulate carriers), and the synthesis of lipophilic camptothecins.[6] The identification of new water-soluble CPT analogues also continues to be of great interest.

Another possible limiting factor in camptothecin efficacy is that the E-ring lactone, at physiological pH, exists in an

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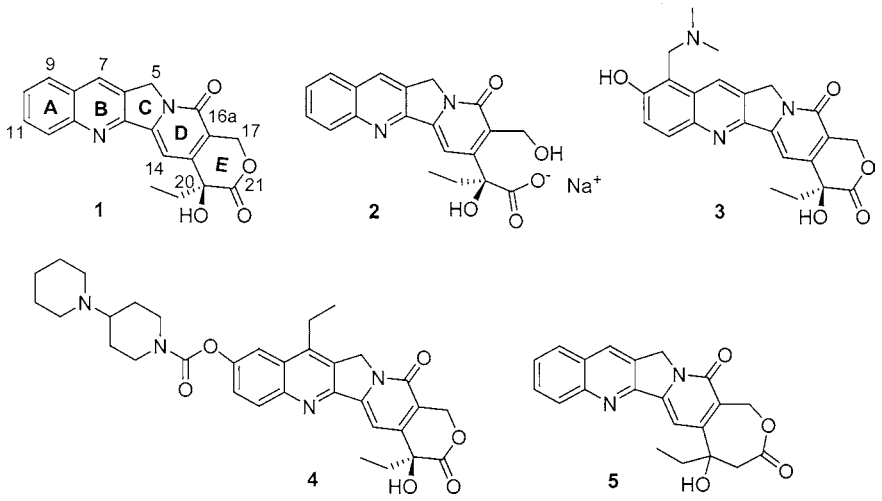
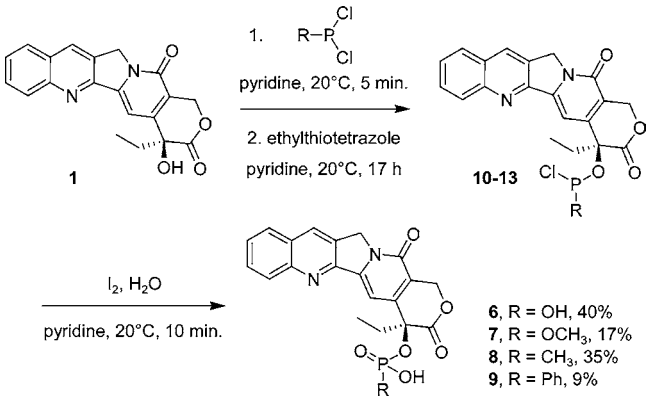


Figure 1. Structures of camptothecin (**1**), its carboxylate form (**2**), topotecan (**3**), irinotecan (**4**), and homocamptothecin (**5**).

equilibrium (between **1** and **2**) that favors the open carboxylate form, which is believed to be inactive as an antitumor agent. This may be exacerbated by the preferential binding of the carboxylate form to human serum albumin.[7] Several strategies have been employed to overcome this drawback.[7] The stability of CPTs in human plasma and blood may be enhanced through A- and B-ring substitution. For example, a relatively large percentage of the intact lactone form of irinotecan (**4**) persisted in the plasma of patients after administration, attributable to the preferential binding of the lactone form to serum albumin.[8] Homologation of the lactone ring has also been used to enhance the plasma stability of CPT.[9] In homoCPT (**5**), the presumed hydrogen bonding interaction between the C_{20}−OH and carbonyl groups in the E ring of CPT can no longer be significant, thus reducing the hydrolysis rate of the lactone.[10] Additionally, water-insoluble alkyl esters of varying size at the C_{20}−OH of CPT were reported to improve lactone ring stability in plasma and reduce cytoxicity. These esters act as prodrugs and liberate CPT slowly following administration.[11]

To improve the water solubility and the stability of CPT, we have prepared 20-*O*-phosphate and phosphonate esters of CPT and studied their DNA cleavage activity. The synthesis of CPT derivatives **6**−**9** is outlined in Scheme 1. First, the requisite camptothecin chlorophosphite intermediates **10**−**13** were synthesized. This was accomplished by

Scheme 1



treating anhydrous pyridine solutions of CPT with substituted dichlorophosphines ($RPCl_2$, R = Cl, OCH_3, CH_3, Ph). Activation of the dichlorophosphines was effected using ethylthiotetrazole.[12] This activation facilitated the substitution of the hindered C_{20} tertiary alcohol of CPT. Treatment of the formed CPT phosphines with iodine in aqueous pyridine[13] (20 °C, 10 min) then provided the desired CPTs **6**−**9**. Ethylthiotetrazole was removed by silica gel open-column chromatography, and the CPTs were purified by C_{18} reversed-

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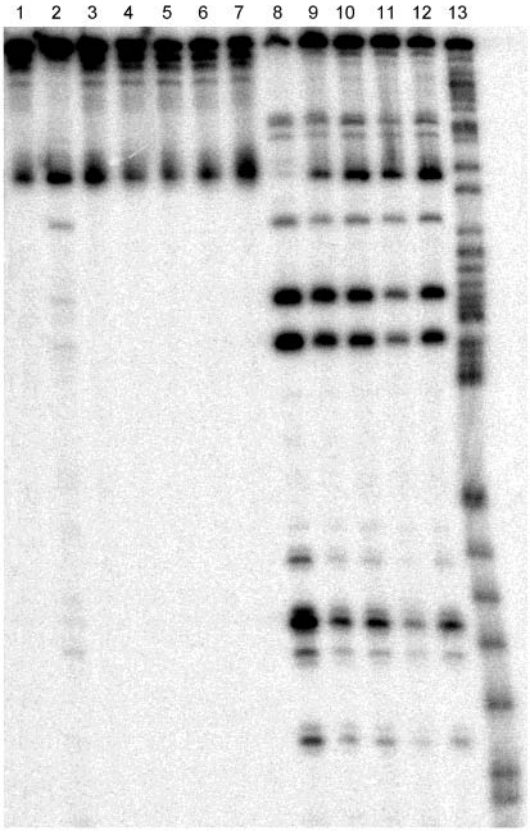

Figure 2. Autoradiogram of a 10% denaturing polyacrylamide gel showing the effect of camptothecin derivatives on human topoisomerase I-mediated cleavage of the *Hin*dIII−*Pvu*II restriction fragment of pSP64 plasmid DNA. The DNA substrate was 3′-^{32}P end labeled on the scissile strand. Human topoisomerase I-mediated cleavage reactions were incubated at 37 °C for 1 h and then digested with proteinase K. Lane 1, DNA alone; lane 2, 36 ng of topoisomerase I; lane 3, 50 μM camptothecin (**1**); lane 4, 50 μM **6**; lane 5, 50 μM **7**; lane 6, 50 μM **8**; lane 7, 50 μM **9**; lane 8, topoisomerase I + 50 μM camptothecin (**1**); lane 9, topoisomerase I + 50 μM **6**; lane 10, topoisomerase I + 50 μM **7**; lane 11, topoisomerase I + 50 μM **8**; lane 12, topoisomerase I + 50 μM **9**; lane 13, Maxam−Gilbert G-lane.

phase HPLC. The sensitivity of the initial substitution reaction to steric hindrance is clear from the inverse relationship between the size of the substituted dichlorophosphite and the yield of the resulting CPT derivative. Initial attempts to form compounds by treatment of CPT with substituted phosphoryl chlorides led to very low yields of the target compounds.

The ability of CPT derivatives **6**−**9** to stabilize the topoisomerase I−DNA covalent binary complex[3] was evaluated using a ^{32}P-end-labeled 222 base pair DNA duplex. Figure 2 shows the effect of CPT and CPT analogues **6**−**9** on the stabilization of topoisomerase I-mediated cleavage sites in this duplex. Like CPT, water-soluble derivatives **6**−**9** effected stabilization of the enzyme−DNA covalent binary complex. While the derivatives were less potent than CPT, stabilization was observed at the same sites as for CPT itself. It may be noted that **8** was the least potent of the four CPT

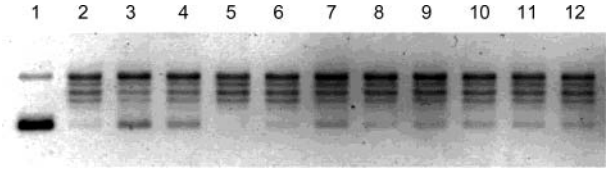

Figure 3. Effect of campthothecin derivatives on human topoisomerase I-mediated DNA relaxation. Supercoiled pSP64 plasmid DNA was incubated at 37 °C for 15 min. Lane 1, supercoiled DNA alone; lane 2, 0.1 ng of topoisomerase I; lanes 3 and 4, topoisomerase I + 500 and 100 μM camptothecin (**1**), respectively; lanes 5 and 6, topoisomerase I + 500 and 100 μM **6**, respectively; lanes 7 and 8, topoisomerase I + 500 and 100 μM **7**, respectively; lanes 9 and 10, topoisomerase I + 500 and 100 μM **8**, respectively; lanes 11 and 12, topoisomerase I + 500 and 100 μM **9**, respectively.

analogues. Neither CPT nor CPT analogues **6**−**9** had any effect on DNA in the absence of topoisomerase I.

While it is not thought that inhibition of DNA relaxation contributes to the antitumor activity of CPT, CPT does inhibit topoisomerase I-mediated relaxation of supercoiled DNA when present at high concentrations. This is illustrated in Figure 3 at 100 and 500 μM CPT concentrations in the presence of 0.1 ng of human topoisomerase I. As shown in Figure 3, the CPT derivatives **7**−**9** inhibited topoisomerase I-mediated relaxation of supercoiled DNA to almost the same extent as CPT. In contrast, CPT derivative **6** did not inhibit DNA relaxation.

To evaluate the possible cytotoxic effects resulting from stabilization of the enzyme−DNA binary complex, CPTs **6**−**9** were evaluated in a strain of *Saccharomyces cerevisiae* lacking yeast topoisomerase I but harboring a plasmid having the human topoisomerase I gene under the control of a galactose promoter.[14] As shown in Table 1, 1 μM CPT had only a slight effect when this yeast strain was grown on raffinose. However, when human topoisomerase I synthesis was induced by growth on galactose, the same strain showed 88% growth inhibition in the presence of 1 μM CPT. While CPT was more potent than analogues **6**−**9**, analogues **6**, **7**, and **9** seemed to be more topoisomerase I dependent at higher concentrations than CPT.

Among the derivatives, CPT phosphate monoester **6** was the most cytotoxic, although its potency was much lower than that of CPT itself. Only slightly less active than **6** were CPT derivatives **7** and **9**. CPT derivative **8**, having a 20-*O*-methylphosphonate substituent, was significantly less active than the other three CPT analogues and also exhibited significant toxicity when the yeast was grown on raffinose. This parallels the behavior observed for the CPT analogues in stabilizing the topoisomerase I−DNA covalent binary complex. Compounds **6**, **7**, and **9** had comparable potencies, while **8** was significantly less potent (Figure 2, cf. lanes 9, 10, and 12 vs lane 11). While entirely in the realm of speculation, the greater potency of phenylphosphonate

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Table 1. Topoisomerase I-Dependent Cytotoxicity of CPTs in *S. cerevisiae*[a]

compound	concentration (μM)	% inhibition on growth medium: raffinose	% inhibition on growth medium: galactose	IC_{50} TOP1−Gal (μM)
CPT (**1**)	2	40	88	0.9
	1	12	88	
6	50	7	75	20
	25	29	69	
7	50	12	66	33
	25	15	48	
8	50	25	36	57
	25	17	40	
9	50	5	67	37
	25	1	27	

[a] Inhibition of RS321Nph-TOP1 grown in minimal medium containing 3% raffinose or galactose for 2 days at 30 °C.

derivative **9** as compared with that of methylphosphonate **8** could relate to the ability of the former to participate in stacking interactions believed[15] to be important in stabilizing the formed ternary complex between CPT and the topoisomerase I−DNA covalent binary complex.

As anticipated, these new CPT derivatives proved to be much more soluble in water than CPT; this was readily apparent during the bioassays, which were performed in aqueous media. The greater water solubility of CPT analogues **6**−**9** is fully consistent with the observation of Yaegashi et al. for 7-ethyl-10-hydroxycamptothecins bearing phosphate groups at the 10-position.[16]

To determine whether these CPT derivatives act as prodrugs of CPT, we incubated each compound at 37 °C for 48 h in the media used for the growth of the RS321Nph-TOP1 yeast strain and for 30 min in the buffer used to measure human topoisomerase I-mediated DNA cleavage. No degradation of the molecules or transformation into CPT was observed under either set of conditions, as judged by HPLC analysis. These observations are consistent with the interpretation that CPT analogues **6**−**9** have intrinsic activity and do not act as prodrugs of CPT. Further evidence was obtained by monitoring topoisomerase I-mediated DNA cleavage of CPT and analogues **6**−**9** over a wide concentration range. The analogues exhibited activity at concentrations as low as 400 nM. At least 3.2 nM CPT would have been required to produce comparable effects. In contrast, no significant conversion of **6**−**9** to CPT was observed under the conditions of the assay; <0.01% conversion would have been readily detectable by HPLC analysis, as verified by intentional admixture of authentic CPT. Thus, the activity observed for the analogues could not have been due to their adventitious conversion to CPT in the assay medium.

While the much greater differences in cytotoxic potency between CPT and analogues **6**−**9** make it difficult to exclude the possibility that these derivatives act as CPT prodrugs in yeast, it may be noted that some 10-phosphate derivatives of 7-ethyl-10-OH CPT have been shown to have antitumor activity superior to that of the parent CPT itself.[16]

CPT and derivatives **6**−**9** showed similar effects in stabilizating the topoisomerase I−DNA covalent binary complex and in producing human topoisomerase I-dependent cytotoxicity in yeast, suggesting that the new analogues function in the same fashion as CPT. These derivatives are easily obtained by semi-synthesis starting from CPT and represent an attractive strategy for the design of water-soluble CPT derivatives.

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