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Synthesis and antitumor activity evaluation of a novel series of camptothecin analogs

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A series of novel 10-substituted camptothecin analogs (3-10) with a carbamate linker were synthesized, and their biological activities were evaluated. The amino acid-linked carbamate derivatives (8-10) of the camptothecin-type natural product not only possessed good to excellent inhibitory activity against three human tumor cell lines K562, HepG2, and HT-29, but also showed significantly less cytotoxicity against normal human cell HEK293 (half maximal inhibiting concentration >10 μ M). The selectivity of compound 9 toward tumor cells relative to normal cells is at least 250 times better than that of camptothecin. The preliminary testing result indicated that the solubility of these compounds was also improved compared to that of 10-hydroxy camptothecin.

Keywords: anticancer; solubility; alkaloid

1. Introduction

Camptothecin (CPT) and 10-hydroxycamptothecin (HCPT) are two pentacyclic alkaloids isolated from a natural plant [1], and both of them have been found to possess good inhibitory activities against a broad spectrum of tumors. Interest in CPT and HCPT derivatives was revitalized in 1985 by the discovery that the camptothecins exhibited a unique mechanism of action, characterized by their ability to bind to the transient topoisomerase I-DNA complex during DNA replication and induce double-strand breaks and cell death [2–5].

The clinical applications of CPT and HCPT were limited by their side effects [6,7] and poor solubility [8]. Early attempts to form a water-soluble sodium salt by opening the lactone ring with sodium hydroxide resulted in the loss of their antitumor activities and increased toxicities [9-11]. It was later reported that the closed lactone form is a prerequisite for antitumor activity of CPT-type drug [12,13]. Subsequent effort to develop water-soluble camptothecin derivatives was focused on the quinoline portion of the molecule, which led to the discovery of topotecan and irinotecan (Figure 1). Topotecan introduces a nitrogenous group at the 7-position, and irinotecan is considered as a prodrug formed between 7-ethyl HCPT and [1,4'-bipiperidine]-1'-carboxylic acid. Both topotecan and irinotecan possess a much improved solubility, and have been approved for the treatment of several types of cancer patients [14,15]. However, they still have some drawbacks, such as severe neutrophilic granulocytopenia and diarrhea [16]. Therefore, it is very important to further

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Figure 1. Structures of HCPT, SN38, irinotecan, and topotecan.

study the origin of the side effects and develop new generation of less toxic CPT/HCPT derivatives that could extend the application of these very potent natural product anticancer drugs.

Previous studies indicated that the substitution at 7 and 9 positions of CPT derivatives could enhance their antitumor activities and improve their physicochemical properties [17]. In this study, we aim to address the possibility that 10-hydroxy group of HCPT could be a key position responsible for the excellent potency as well as a potential cause of its toxicity. Irinotecan is a 10-bipiperidine-substituted carbamate of 7-ethyl HCPT, but the side chain causes a 1000 times decrease in activity when compared to its parent molecule SN38 [18]. Li and coworkers reported the conversion of HCPT into CPT quaternary ammonium salts bearing several water-solubilizing groups with an ether linker at the 10-position of CPT, and these salts showed good water solubility and different cytotoxicities in vitro [19,20]. They also reported a series of HCPT and SN-38 derivatives containing nitrogenous groups at the 10-position, and the water solubility of these compounds was indeed enhanced [21], with some compounds showing cytotoxic activity similar to that of CPT in vitro. However, their toxicities to normal cells were not evaluated. Appropriate modification at the 10-position of CPT could not only be beneficial for its antitumor activity and physicochemical property, but may also reduce its toxic effect. Carbamate was shown to be a promising linker to form CPT prodrugs modified at the 20-position [22-25], but 10-amino acid-linked carbamate CPTs have not previously been reported.

In order to clarify whether the 10hydroxy group is the major cause of the toxic effect of HCPT and to find novel CPT anticancer drugs with improved solubility and low toxicity, we report in this study the design, synthesis, and antitumor activities of a series of novel 10-position nitrogenous group-linked carbamate CPTs as well as a preliminary *in vitro* toxic evaluation by comparing their cell toxicity difference between normal human cell HEK293 and cancer cells K562, HepG2, and HT29.

2. Results and discussion

A series of novel 10-substituted camptothecin analogs (3-10) were synthesized by a convenient two-step approach. As illustrated in Scheme 1, HCPT was first reacted with 4-nitrophenyl chloroformate in the presence of a base to form the key intermediate 4-nitrophenyl camptothecin-10-yl carbonate (2), which was then treated with different nitrogenous reagents to afford the target compounds 3-10(Scheme 1).

The *in vitro* antitumor activities of the target compounds 3-10 and intermediate 2

against three human tumor cells, K562, HepG2, and HT-29, were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with CPT as the positive control. As can be seen from Table 1, all the compounds exhibited inhibitory activities against tumor cells, among which five compounds (2 and 6-9) have very comparable half maximal inhibiting concentration (IC₅₀) values with CPT, especially against K562 cell line.

In order to evaluate their toxicities, the inhibition of compounds 2 and 6-10 against normal human cell HEK293 was also tested (Table 2). The result showed that compounds 2, 6, and 8-10 exhibited much less toxicities against HEK293 than CPT. Especially, compounds 8-10 were



Scheme 1. Synthesis of compounds 3-10. (a) 4-Nitrophenyl chloroformate (4.0 equiv.), CH₂Cl₂, triethylamine (10.0 equiv.), 0°C to rt, 9 h, 64%. (b) Nitrogenous reagent (2.0 equiv.), DMF, 0°C to rt, 2 h, 40–60%.

	<i>In vitro</i> cytotoxicity (IC ₅₀ , μM)				
Compound	K562	HepG2	HT-29		
CPT	0.07	0.06	0.06		
2	0.07	0.33	0.22		
3	0.89	0.48	1.11		
4	0.41	0.69	0.56		
5	1.72	0.59	1.06		
6	0.06	0.17	0.15		
7	0.04	0.28	0.25		
8	0.06	0.31	0.26		
9	0.04	0.27	0.19		
10	0.11	0.30	0.30		

Table 1. Antitumor activities of compounds 2-10 in vitro.

found to be at least 250 times less toxic against HEK293 (IC₅₀ > 10 μ M). Considering the good to excellent antitumor activities of 8-10, it could be expected that they might have a much larger safety window. Interestingly, the cytotoxicity of compound 7 against HEK293 was identical to that of CPT. One possible reason is that compound 7 with a sterically less hindered glycine-linked carbamate could be very quickly hydrolyzed back into the active natural drug HCPT, which itself also has the similar toxic effect to that of CPT. These results also indicated that the 10-hydroxy group may be responsible for the toxicity of HCPT to some extent. Compounds 8-10 all contain sterically hindered groups next to carbamates so that they are difficult to turn back into HCPT, and therefore could avoid the toxic effect caused by the free 10-hydroxy group of HCPT. Taken together, compounds 8-10possess both good to excellent antitumor activities and much less toxic effect against human normal cell HEK293. The

structural modifications made on these compounds have, therefore, resulted in significantly higher selectivity toward tumor cells than normal cells.

To further evaluate the drug-like properties of these potent compounds, the predicted theoretical $\log P$ and $c \log P$ values of 7-10 were analyzed (Table 3) and their solubilities in DMSO, acetone, and deionized water were also briefly tested (Table 4). The data in Tables 3 and 4 showed that compounds 9 and 10 have better properties and compounds 7-10 have much better solubility in organic solvents (DMSO and acetone) than HCPT. The water solubility of only 7 and 8 has been slightly improved. The compound 9, which is 11 and 25 times more soluble in DMSO and acetone than that of HCPT, possessed excellent antitumor activities, low toxicity, and a relatively ideal $c \log P$ value and should deserve further investigation.

3. Experimental

3.1 General experimental procedures

¹H NMR spectra were recorded on a Bruker AM-400 NMR spectrometer (Billerica, Middlesex, MA, USA) in CDCl₃ or DMSO- d_6 . The chemical shifts are reported in δ (ppm) relative to tetramethylsilane as an internal standard. Mass spectra were obtained on a Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA). All reagents and solvents used in this study were of reagent grade. Thin layer chromatography was carried out using E. Merck silica gel 60 GF₂₅₄ precoated plates (Darmstadt, Germany) and visualized using a combination of UV₂₅₄ and UV₃₆₅. Silica gel (particle size 200-400 mesh, Marine Chemical Group

Table 2. Toxicities against HEK293 of compounds CPT, 2, and 6-10.

Compound	CPT	2	6	7	8	9	10
Toxicity (IC ₅₀ , µM)	0.01	1.71	0.25	0.009	>10	>10	>10

Compound	$\log P$	$c \log P$
СРТ	1.33	0.897
HCPT	0.94	0.9458
7	0.35	0.21
8	0.84	0.5217
9	1.73	1.4497
10	2.07	1.9787

Table 3. $\log P$ and $c \log P$ of compounds CPT, HCPT, **2**, and **6**–10.

Note: Predicted via Chemdraw Ultra 8.0.

Co., Qingdao, China) was used for flash chromatography.

3.2 Cytotoxicity assay

The cytotoxicities of the target compounds were evaluated by MTT assay. Cells were seeded at a density of $5 \times$ 10^4 cells/ml in 96-well microplate (100 µl/well). After 2h for K562 cells and 24 h for HEK293, HepG2, and HT-29 cells, media containing tested compounds were added in triplicate. After 48-h incubation, the media were replaced by phosphate-buffered saline medium containing 0.5 mg/ml MTT and incubated for another 4 h. Then, the medium was removed and 100 µl DMSO was added in each well to dissolve formazan. The absorbances at 570/630 nm were measured for K562 cells, and the absorbances at 490/630 nm were measured for HEK293, HepG2, and HT-29 cells using Thermo microplate reader. The untreated

Table 4. Solubilities of compounds 7–10.

controls were calculated as a cell viability value of 100%. The IC_{50} values were obtained by nonlinear regression using GraphPad Prism 4.0. IC_{50} measurements for each compound were done three times.

3.3 Synthesis of compounds 2–10

3.3.1 4-Nitrophenyl camptothecin-10-yl carbonate (2)

To a stirred solution of 10-hydroxy camptothecin (5.00 g, 13.7 mmol) in dry CH₂Cl₂ (500 ml) were added triethylamine (19.2 ml, 137.2 mmol) and then 4-nitrophenyl chloroformate (11.06 g, 54.9 mmol) at 0°C. The reaction mixture was stirred at 0°C for 15 min, before warming to room temperature. After stirring at room temperature for another 9h, the mixture was washed with brine $(3 \times 500 \text{ ml})$ and the organic layer was dried with anhydrous sodium sulfate. Removal of solvent under vacuum gave a brownish residue that was purified by column chromatography (200 mesh silica gel, CH₂Cl₂/CH₃OH, 200:1) to afford compound 2 (4.65 g, 64% yield) as a pale solid.

¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.89–0.92 (m, 3H, H-18), 1.85– 1.92 (m, 2H, H-19), 5.32 (s, 2H, H-5), 5.44 (s, 2H, H-17), 6.54 (s, 1H, 20-OH), 7.37 (s, 1H, H-14), 7.78 (d, 2H, J = 9.2 Hz, Hnitrophenyl), 7.97–7.80 (m, 1H, H-11), 8.20 (d, J = 2.4 Hz, 1H, H-9), 8.30 (d, J = 9.2 Hz, 1H, H-12), 8.40 (d, J = 9.2 Hz, 2H, H-nitrophenyl), 8.74 (s, 1H, H-7).

Compound		Solubility (mg/ml)
	DMSO	Acetone	Deionized water
HCPT ^a	11.1 ^a	0.23 ^a	< 0.1 ^a
7	24	2.4	0.14
8	27.14	0.56	0.12
9	135	6	< 0.1
10	45	4.17	< 0.1

Note: 2.0 mg of each compound was weighed, and the solvent was then carefully added until compound was dissolved.

^a The test result of HCPT is consistent with the literature report [23].

3.3.2 *Camptothecin-10-yl pyrrolidine-1carboxylate* (3)

Compound 2 (0.5 g, 0.9 mmol) was dissolved in dry N,N-Dimethylformamide (DMF) (3 ml), and pyrrolidine (0.16 ml, 1.9 mmol) was then added at 0°C. The reaction mixture was stirred for 2 h at room temperature before quenched with 15 ml icy water. The mixture was extracted with CH₂Cl₂ (3×25 ml). The organic layer was combined and dried with anhydrous sodium sulfate and concentrated under reduced pressure to give a crude product. Compound 3 (0.19 g, 45% yield) was then obtained by column chromatography (200 mesh silica gel, CH₂Cl₂/CH₃OH, 175:1) as a pale solid.

¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 1.86–1.97 (m, 2H, H-19), 3.33 (overlapped with H₂O in d_6 -DMSO, 4H, Hpyrrolidine), 3.34–3.45 (m, 2H, H-pyrrolidine), 3.57–3.60 (m, 2H, H-pyrrolidine), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.35 (s, 1H, H-14), 7.67–7.70 (dd, J = 9.2 and 2.4 Hz, 1H, H-11), 7.90 (d, J = 2.4 Hz, 1H, H-9), 8.18 (d, J = 9.2 Hz, 1H, H-12), 8.66 (s, 1H, H-7). ESI-MS (m/z): 462.2 [M + H]⁺.

3.3.3 Compounds 4-10

Similar procedures as for the preparation of compound **3** were used for the synthesis of compounds 4-10.

3.3.3.1 Camptothecin-10-yl morpholine-4-carboxylate (4). ¹H NMR (DMSO-d₆, 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 1.84–1.91 (m, 2H, H-19), 3.47 (brs, 2H, H-morpholine), 3.69 (brs, 6H, Hmorpholine), 5.29 (s, 2H, H-5), 5.42 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.34 (s, 1H, H-14), 7.67–7.70 (dd, J = 9.2 and 2.4 Hz, 1H, H-11), 7.92 (d, J = 2.4 Hz, 1H, H-9), 8.17 (d, J = 9.2 Hz, 1H, H-12), 8.65 (s, 1H, H-7). ESI-MS (m/z): 478.2 [M + H]⁺. 3.3.3.2 Camptothecin-10-yl diethylcarbamate (5). ¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 1.14–1.17 (m, 3H, CH₃diethylamine), 1.25–1.28 (m, 3H, CH₃diethylamine), 1.84–1.91 (m, 2H, H-19), 3.32–3.49 (m, 2 × CH₂-diethylamine), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.34 (s, 1H, H-14), 7.65–7.68 (dd, J = 9.2, 2.4 Hz, 1H, H-11), 7.91 (d, J = 2.4 Hz, 1H, H-9), 8.17 (d, J = 9.2 Hz, 1H, H-12), 8.66 (s, 1H, H-7). ESI-MS (m/z): 464.3 [M + H]⁺.

3.3.3. Camptothecin-10-yl butylcarbamate (6). ¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.87–0.94 (m, 6H, H-18 and CH₃butylamine), 1.33–1.54 (m, 4H, CH₃CH₂-CH₂CH₂NH—), 1.84–1.93 (m, 2H, H-19), 3.10–3.15 (m, 2H, CH₃CH₂CH₂CH₂CH₂NH—), 5.28 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.33 (s, 1H, H-14), 7.62–7.65 (dd, J = 9.2 and 2.4 Hz, 1H, H-11), 7.87 (d, J = 2.4 Hz, 1H, H-9), 7.95 (t, J = 5.6 Hz, 1H, CH₃CH₂CH₂CH₂NH—), 8.15 (d, J = 9.2 Hz, 1H, H-12), 8.65 (s, 1H, H-7). ESI-MS (m/z): 464.3 [M + H]⁺.

3.3.3.4 *N*-[(10-Camptothecinyloxy) carbonyl]-glycine methyl ester (7). ¹H NMR (DMSO-*d*₆, 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 1.84–1.91 (m, 2H, H-19), 3.70 (s, 3H, CH₃O—), 3.93 (d, J = 5.8 Hz, 2H, CH₃OCO<u>CH₂NHCOO</u>—), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.35 (s, 1H, H-14), 7.64–7.66 (m, 1H, H-11), 7.92 (s, 1H, H-9), 8.19 (d, J = 9.2 Hz, 1H, H-12), 8.42 (t, J = 5.8 Hz, 1H, CH₃OCOCH₂NHCOO—), 8.68 (s, 1H, H-7). ESI-MS (*m*/*z*): 480.2 [M + H]⁺.

3.3.3.5 *N*-[(10-Camptothecinyloxy) carbonyl]-alanine methyl ester (8). ¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 1.40 (d, J = 7.6 Hz, 3H, CH₃CH—), 1.84–1.91 (m, 2H, H-19),

3.70 (s, 3H, CH₃O⁻), 4.22–4.25 (m, 1H, CH₃<u>CH</u>⁻), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.34 (s, 1H, H-14), 7.63–7.65 (dd, J = 9.2, 2.4 Hz, 1H, H-11), 7.90 (d, J = 2.4 Hz, 1H, H-9), 8.18 (d, J = 9.2 Hz, 1H, H-12), 8.49 (d, J = 7.2 Hz, 1H, -<u>NHCOO</u>⁻), 8.68 (s, 1H, H-7). ESI-MS (m/z): 494.2 [M + H]⁺.

3.3.3.6 N-[(10-Camptothecinyloxy)carbonyl]-valine methyl ester (9). ¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.89 (t, J = 7.2 Hz, 3H, H-18), 0.98 (t, J = 6.4 Hz, 6H, (CH₃)₂CHCH—), 1.84–1.91 (m, 2H, H-19), 2.12–2.18 (m, 1H, (CH₃)₂-CHCH—), 3.70 (s, 3H, CH₃O—), 4.02– 4.06 (m, 1H, (CH₃)₂CHCH—), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.35 (s, 1H, H-14), 7.63-7.66 (dd, J = 9.2 and 2.8 Hz, 1H, H-11), 7.92 (d, J = 2.8 Hz, 1H, H-9), 8.19 (d, J = 9.2 Hz, 1H, H-12), 8.44 (d, J = 8.0 Hz, 1H, -NHCOO—), 8.69 (s, 1H, H-7). ESI-MS (m/z): 522.2 [M + H]⁺.

3.3.3.7 *N*-[(10-Camptothecinyloxy) carbonyl]-leucine methyl ester (10). ¹H NMR (DMSO- d_6 , 400 MHz) δ (ppm): 0.87–0.96 (m, 9H, H-18 and (CH₃)₂CHCH₂CH—), 1.57–1.60 (m, 1H, (CH₃)₂CHCH₂CH—), 1.65–1.78 (m, 2H, (CH₃)₂CHCH₂CH—), 1.84–1.89 (m, 2H, H-19), 3.70 (s, 3H, CH₃O—), 4.16–4.20 (m, 1H, (CH₃)₂CHCH₂CH—), 5.30 (s, 2H, H-5), 5.43 (s, 2H, H-17), 6.52 (s, 1H, 20-OH), 7.34 (s, 1H, H-14), 7.62–7.65 (dd, J = 9.2 and 2.8 Hz, 1H, H-11), 7.91 (d, J = 2.8 Hz, 1H, H-9), 8.18 (d, J = 9.2 Hz, 1H, H-12), 8.47 (d, J = 8.0 Hz, 1H, -NHCOO—), 8.68 (s, 1H, H-7). ESI-MS (m/z): 536.4 [M + H]⁺.

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