Synthesis and Preliminary Anticancer Evaluation of 10-Hydroxycamptothecin Analogs

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We have synthesized new 10-hydroxycamptothecin (HCPT) analogs and evaluated their anticancer activity in cell culture and in experimental animal tumor model. Although the new analogs were less potent against L1210 leukemia cells *in vitro*, some of them were more efficacious against L1210 leukemia *in vivo* compared to the parent HCPT.

Key words anticancer; 10-hydroxycamptothecin; analog

The natural product camptothecin (CPT) (Fig. 1) is a pentacyclic alkaloid, first isolated in 1966 from the extract of a Chinese plant, *Camptotheca accuminata*, by Wall *et al.*[1] CPT and its synthetic analogues are among the most promising new agents for the treatment of human cancers.[2,3] They act by a unique mechanism inhibiting DNA topoismerase. DNA topoisomerase I covalently binds to double-stranded DNA, forming a cleavable complex and producing a single-strand break.[4] This cleavable complex facilitates the relaxation of torsional strain of the supercoiled DNA. Once the torsional strain is relieved, the enzyme rejoins the cleaved strands of DNA and dissociates from the relaxed double helix.[5] CPT binds to and stabilizes the cleavable complex and inhibits the religation of DNA, leading to the accumulation of DNA single-stranded breaks.[6,7] The single-stranded breaks are not in themselves toxic to cell, because they readily religate upon drug removal; however, collision of the DNA replication fork with the drug–enzyme–DNA complex generates an irreversible double-strand break that ultimately leads to cell death.[8] CPT is S phase-specific, because ongoing DNA synthesis is needed to induce the above sequence of events leading to cytotoxicity. This mechanism of action has significant implications for the use of these agents. It suggests that a prolonged exposure of CPT to tumors is needed to ensure its optimal therapeutic efficacy.

In initial clinical trials, CPT was limited by its poor solubility in physiologically compatible media. Early attempts to form a water-soluble sodium salt of CPT by opening the lactone ring with sodium hydroxide led to poor antitumor activity.[9–11] Later research found that the closed lactone form is an absolute requirement for antitumor activity.[12] Intensive efforts in medicinal chemistry over the past several decades have provided a large number of camptothecin analogues, of which topotecan and irinotecan are among those clinically approved for the treatment of cancers (Fig. 1).

In irinotecan, a piperidino–piperidino carbonyl moiety was introduced at the 10-hydroxy position which greatly increases the compound water-solubility. Irinotecan is converted *in vivo* to its much higher active metabolite 7-ethyl-10-hydroxy camptothecin by carboxylesterases.[13] Structure–activity relationship (SAR) studies suggested that the intact lactone ring E of CPT is the most critical structural feature accounting for

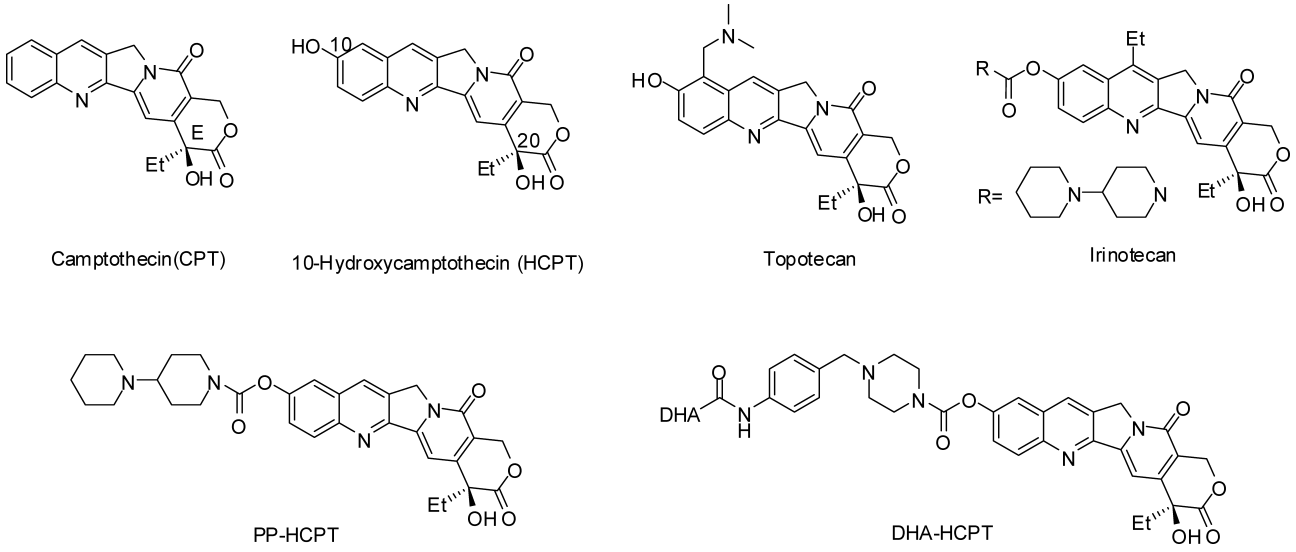

Fig. 1. Structures of Camptothecin, 10-Hydroxycamptothecin, Irinotecan, Topotecan, PP-HCPT and DHA-HCPT

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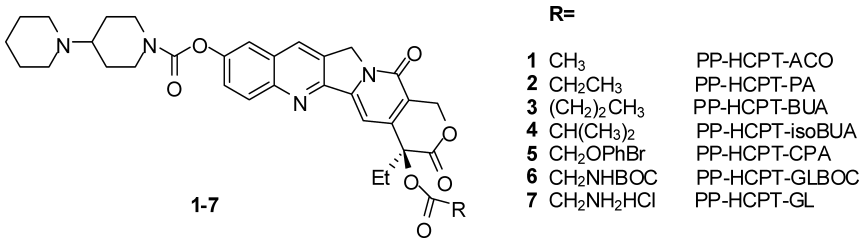

Fig. 2. Chemical Structures of New HCPT Analogs

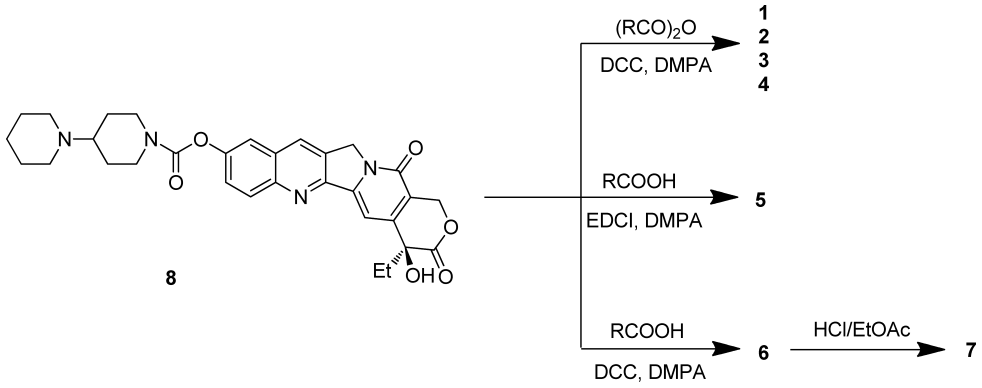

Chart 1. Synthesis of Compounds **1–7**

its antitumor activity. For these reasons, extensive efforts are made to modify at CPT's 10 and 20th positions to reduce its toxicity and increase its anticancer activity. A member of the CPT family, 10-hydroxycamptothecin (HCPT), is more potent and less toxic than CPT.[3] We herein report synthesis of a series of new derivatives of HCPT with esterifications on both the 10 and 20th-hydroxy groups. Their antitumor activity was evaluated against L1210 leukemia *in vitro* and in experimental animals.

Inspired by our previous work,[14] modification of 10-hydroxy with a piperazine moiety in HCPT was used, the tertiary nitrogen of piperazine (PP-HCPT) is expected to be protonated at physiologic pH, leading to increased aqueous solubility. In addition, the piperazine and HCPT were linked through a carbamoyl bond, which should provide certain stability to cleavage by carboxylate esterase. However, this carbamoyl bond will be cleaved by carboxylate esterase to release HCPT, leading to the killing of cancer cells.

The 20S-hydroxyl group is thought to participate in the enhanced rate of lactone hydrolysis of HCPT at neutral pH by shifting the lactone-carboxylate equilibrium in favor of the carboxylate form. There is an intramolecular hydrogen bonding in the E-ring of HCPT molecule, it would not only activate the lactone but also diminish the interaction with the enzyme. Esterification of 20S-hydroxyl group blocks this process. Although 20-*O*-acylated HCPTs possess no intrinsic Topo I (topoisomera- se I) inhibitory activity, they can function as prodrugs to release the HCPTs *in vivo*, and improve solubility, pharmacokinetics character and toxicity of HCPTs.[15] Therefore, the esterification of 20-hydroxyl group can either eliminate the intramolecular hydrogen bonding and increase the steric hindrance of carbonyl group of E-ring, so lactone ring stability was improved *in vivo*.[16]

Our approach to the above chemical stability/water solubility problems is to introduce the alkanoic acid esters and nitrogen-based esters into the molecule of PP-HCPT at its 20-hydroxyl *via* esterification produced 10-*O* and 20-*O*-double-acylated HCPT derivatives (Fig. 2). The new compounds have enhanced water solubility and as improved stability of the lactone ring and showed significant antitumor activity and low toxicity *in vivo*.

MATERIALS AND METHODS

Chemistry All reagent and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. Melting points were measured using a melting point detector and are uncorrected. ^{1}H-NMR spectra were recorded at ambient temperature on an NT-400 spectrometer. Mass spectra (electrospray ionization, EI) were recorded using a Micromass Q-Tof 1 mass spectrometer. Atlantic Microlab, Inc., Norcross, GA, U.S.A. performed the elemental analyses, and the results were within ±0.4% of the theoretical values unless otherwise noted. Analytical thin-layer chromatography was performed on silica-coated plastic plates (silica gel 60 F-254, Merck) and visualized under UV light. Preparative separations were performed by flash chromatography on silica gel (Qinghdao Haiyang, 200–300 mesh).

The general synthetic method for new HCPT analogs **1–7** is shown in Chart 1.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxycamptothecin (8) Compound **8** was synthesized by treatment of the precursor carbonate of HCPT with 4-piperidinopiperidine as reported in our previous work.[14]

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-acetylcamptothecin (1) PP-HCPT (100 mg, 0.18 mmol) was dissolved in tetrahydrofuran (THF) (20 mL), and acetic anhydride

(0.1 mL) was added, followed by the addition of 4-dimethylamino pyridine (DMAP) (12 mg, 0.10 mmol). The reaction was allowed to proceed overnight at room temperature. Solvent was removed under vacuo. The product was purified by column chromatography, eluting with trichloromethane and methanol (20 : 1, v/v) to afford **1** as a yellow solid (38 mg, 35%), mp 188–189.3°C. ^{1}H-NMR (DMSO-d_6) δ: 0.91 (3H, t, J=7.4 Hz), 1.24 (2H, s), 1.50 (6H, s), 1.91 (1H, s), 2.14 (4H, m), 2.22 (3H, s), 3.28 (4H, s), 4.27 (2H, m), 4.43 (2H, m), 5.31 (2H, s), 5.48 (2H, s), 7.06 (1H, s), 7.68 (1H, dd, J=2.5, 6.7 Hz), 7.91 (1H, d, J=2.6 Hz), 8.18 (1H, d, J=9.2 Hz), 8.66 (1H, s). MS m/z: 602.0 (M^++H). *Anal.* Calcd for $C_{33}H_{36}N_4O_7\cdot 2H_2O$: C, 62.25; H, 6.33; N, 8.80. Found: C, 62.57; H, 6.51; N, 8.57.

Compounds **2**–**4** were synthesized by using the similar synthetic procedure as for compound **1**.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-propionylcamptothecin (2) Yellow solid, yield: 21%. ^{1}H-NMR (DMSO-d_6) δ: 0.92 (3H, t, J=7.4 Hz), 0.99 (2H, m), 1.06 (3H, t, J=7.5 Hz), 1.50 (6H, s), 1.78 (1H, m), 2.20 (4H, m), 2.53 (2H, s), 3.17 (4H, m), 4.08 (2H, m), 4.09 (2H, m), 5.30 (2H, s), 5.48 (2H, s), 7.04 (1H, s), 7.67 (1H, dd, J=2.5, 6.6 Hz), 7.90 (1H, d, J=2.6 Hz), 8.16 (1H, d, J=9.2 Hz), 8.65 (1H, s). MS m/z: 615.5 (M^++H). *Anal.* Calcd for $C_{34}H_{38}N_4O_9\cdot 2H_2O$: C, 62.76; H, 6.51; N, 8.61. Found: C, 62.60; H, 6.24; N, 8.27.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-butyrylcamptothecin (3) Yellow solid, yield: 18%, mp 198.5–199.3°C. ^{1}H-NMR (DMSO-d_6) δ: 0.92 (6H, t, J=7.3 Hz), 1.41 (2H, t, J=2.4 Hz), 1.52 (4H, m), 1.59 (2H, q), 1.82 (1H, m), 2.15 (4H, m), 2.47 (2H, s), 3.17 (2H, m), 3.31 (4H, m), 4.08 (2H, s), 4.26 (2H, s), 5.31 (2H, s), 5.49 (2H, s), 7.04 (1H, s), 7.67 (1H, dd, J=2.3, 6.9 Hz), 7.91 (1H, d, J=2.3 Hz), 8.16 (1H, d, J=9.1 Hz), 8.66 (1H, s). MS m/z: 629.9 (M^++H). *Anal.* Calcd for $C_{35}H_{40}N_4O_7\cdot 2H_2O$: C, 63.24; H, 6.67; N, 8.43. Found: C, 63.44; H, 6.43; N, 8.65.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-isobutyrylcamptothecin (4) Yellow solid, yield: 44%, mp 183.7–185.0°C. ^{1}H-NMR (DMSO-d_6) δ: 0.93 (3H, t, J=7.4 Hz), 1.06 (2H, d, J=7.0 Hz), 1.17 (6H, dd, J=2.1, 4.9 Hz), 1.50 (6H, s), 1.80 (1H, m), 2.12 (4H, m), 2.79 (1H, m), 3.17 (4H, m), 4.11 (2H, m), 4.25 (2H, m), 5.30 (2H, s), 5.48 (2H, dd, J=2, 16.9 Hz), 7.01 (1H, s), 7.66 (1H, dd, J=2.6, 6.6 Hz), 7.91 (1H, d, J=2.6 Hz), 8.16 (1H, d, J=9.2 Hz), 8.65 (1H, s). MS m/z: 630.1 (M^++H). *Anal.* Calcd for $C_{35}H_{40}N_4O_7\cdot 2H_2O$: C, 63.24; H, 6.67; N, 8.43. Found: C, 63.53; H, 6.81; N, 8.12.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-(4-bromophenoxy)-acetoxycamptothecin (5) PP-HCPT (120 mg, 0.22 mmol) in THF (30 mL) was treated with 4-bromophenoxy acetic acid (120 mg, 0.52 mmol) in the presence of a coupling reagent 1-[3-(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride (EDCI, 240 mg, 1.25 mmol) and a catalyst 4-dimethylamino pyridine (DMAP, 20 mg, 0.16 mmol), and the reaction mixture was stirred at room temperature for 12 h. Solvent was removed *in vacuo* and the product was purified by column chromatography, eluting with chloroform and methanol (20 : 1, v/v) to afford **5** as a yellow solid (55 mg, 40%), mp 196.4–197.8°C. ^{1}H-NMR (DMSO-d_6) δ: 0.93 (3H, t, J=7.4 Hz), 1.08 (2H, t, J=7.2 Hz), 1.64 (6H, s), 1.95 (4H, s), 2.15 (1H, m), 3.16 (4H, m), 4.13 (2H, m), 4.35 (2H, m), 5.05 (2H, m), 5.31 (2H, s), 5.52 (2H, d, J=3.2 Hz), 6.86 (2H, dd, J=2, 6.8 Hz), 6.98 (2H, dd, J=2.4, 7.2 Hz), 7.21 (1H, s), 7.72 (1H, dd, J=2.8, 6.4 Hz), 7.94 (1H, d, J=2.4 Hz), 8.23 (1H, d, J=9.2 Hz), 8.68 (1H, s). MS m/z: 773.5 (M^++H). *Anal.* Calcd for $C_{39}H_{39}BrN_4O_8\cdot H_2O$: C, 59.32; H, 5.23; N, 7.09. Found: C, 59.14; H, 5.06; N, 7.31.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-(*N*-BOC)-glycinylcamptothecin (6) *N*-BOC-glycine (53 mg, 0.3 mmol) in THF (10 mL) was treated with triethylamine (0.25 mL, 1.80 mmol) and ethyl chloroformate (0.13 mL), and the reaction mixture was stirred at room temperature for 2 h. Then compound **8** (50 mg, 0.09 mmol) and DMAP (10 mg, 0.08 mmol) were added. The reaction was allowed to proceed at room temperature for 24 h. Solvent was removed *in vacuo*, and the product was purified by column chromatography, eluting with chloroform and methanol (10 : 1, v/v) to afford **6** as a yellow solid (15 mg, 23%). ^{1}H-NMR (MeOD) δ: 1.01 (3H, t, J=7.4 Hz), 1.43 (2H, m), 1.53 (9H, m), 1.68 (6H, m), 1.96 (4H, m), 2.70 (4H, m), 2.99 (1H, s), 3.08 (2H, s), 4.35 (4H, m), 5.27 (2H, s), 5.37 (1H, d, J=16.3 Hz), 5.56 (1H, d, J=16.3 Hz), 7.61 (2H, m), 7.75 (1H, d, J=2.4 Hz), 8.13 (1H, d, J=9.2 Hz), 8.53 (1H, s). MS m/z: 716.5 (M^++H). *Anal.* Calcd for $C_{38}H_{45}N_5O_9\cdot 2H_2O$: C, 60.71; H, 6.57; N, 9.32. Found: C, 60.35; H, 6.81; N, 9.05.

10-[4-(1-Piperidino)-1-piperidino]carbonyloxy-20-glycinylcamptothecin·HCl (7) Compound **6** (20 mg, 0.028 mmol) was dissolved in a mixture of THF and ethyl acetate. Then anhydrous HCl in ethyl acetate was added. The precipitate was filtered, and the solid was dried to afford **7** as a salt (13 mg, 71%). ^{1}H-NMR (MeOD) δ: 1.01 (3H, t, J=7.3 Hz), 1.22 (1H, s), 1.91 (6H, m), 1.96 (2H, m), 2.01 (2H, s), 3.10 (4H, m), 3.26 (4H, s), 3.58 (2H, m), 4.15 (4H, m), 5.25 (2H, m), 5.52 (2H, m), 6.99 (1H, d, J=7.6 Hz), 7.60 (1H, m), 7.79 (1H, d, J=2.3 Hz), 8.11 (1H, d, J=8.9 Hz), 8.63 (1H, s). MS m/z: 616.5 (M^++H). *Anal.* Calcd for $C_{33}H_{37}N_5O_7\cdot 3H_2O\cdot HCl$: C, 56.13; H, 6.28; N, 9.92. Found: C, 56.46; H, 6.19; N, 9.57.

PHARMACOLOGY

For the cytotoxicity study, drugs were dissolved in dimethyl sulfoxide (DMSO) to provide a stock solution of 1 mg/mL, which were stored at −20°C. For each experiment, drug solutions were freshly prepared from the stock solution by the addition of sterile water to afford concentrations suitable for the experiment. For animal experiments, drugs were first dissolved in DMSO and Tween 80 was then added. The solution was then diluted with sterile water.

L1210 mouse leukemia cells were cultured in RPMI-1640 plus 10% fetal bovine serum (FBS) with the addition of 100 U/mL penicillin and 100 μg/mL streptomycin. Cytotoxic effects of drugs were measured by inhibition of DNA synthesis. L1210 leukemia cells in RPMI-1640 plus 10% FBS medium were seeded at 5×10^4 cells/well in a 96-well plate. Drugs (10 μL) at increasing concentrations were added to each well, and the total volume was adjusted to 0.1 mL/well using the same medium. The plate was incubated for 24 h at 37°C followed by the addition of drugs. The plate was incubated for another 48 h. The cells were harvested and radioactivity was counted using the Packard Matrix 96 beta counter. The percentage growth inhibition was calculated as follows:

$$\%\text{growth inhibition} = \frac{[(\text{total cpm} - \text{experimental cpm})]}{\text{total cpm}} \times 100$$

For all experiments, tumors were implanted on day 0 to male BDF_1 mice, weighing 18–22 g (6/group for the L1210 model), and drugs (0.1 mL) were administered intraperitoneally (i.p.) on the dates as indicated in the experiment. Animals were monitored and weighed daily. L1210 leukemia cells (10^5 cells/mouse, 0.1 mL) were inoculated i.p. Antitumor activity was determined by comparing the median survival time of the treated groups (T) with that of the control group (C), and was expressed as a percentage of ILS [increase of life span, where $\%ILS=(T/C-1)\times100$]. The calculations considered dying animals only. The median number of days in the untreated group of mice (given the vehicle only) died was 7.5.

RESULTS AND DISCUSSION

The new compounds were first tested *in vitro* against L1210 leukemia cells, and the results are shown in Table 1. In agreement with what we have reported previously that adding a 4-(1-piperidino)-1-piperidino moiety to the 10-hydroxy increased HCPT's IC_{50} from 1.15 μM to 134 μM (PP-HCPT).[14] This data are also in agreement with what was reported for irinotecan, where the 4-(1-piperidino)-1-piperidino moiety significantly increased the free drug SN-38's *in vitro* potency.[17] When the 20-hydroxy of PP-HCPT was converted to its acetic ester, there were little changes in their potencies (IC_{50} values of 134 μM for PP-HCPT and 146 μM for PP-HCPT-ACO, compound **1**). Replacing the 20-hydroxy of PP-HCPT with a butyryl (compound **3**), isobutyryl (compound **4**) or BOC-glycinyl (compound **6**) had no significant effects on the IC_{50} values either. What was surprised was that replacing the 20-hydroxy of PP-HCPT with a propionyl group significantly increased the compound's potency from 134 μM for PP-HCPT to 25.6 μM for PP-HCPT-PA (compound **2**). What was more surprised was that replacing the 20-hydroxy of PP-HCPT with a bulky $OCOCH_2OPhBr$ (compound **5**) also significantly increased the compound's potency from 134 μM for PP-HCPT to 35 μM for PP-HCPT-CPA. The reasons for these results were not understood currently. The rationale to place a glycine moiety at the 20-hydroxy position was to further increase water-solubility, and the resulting compound PP-HCPT-GL·HCl (compound **7**) had an increased potency compared with its precursor PP-HCPT. The data in Table 1 did not reveal any obvious SAR when the 20-hydroxy group was replaced by different substituents.

Next, we tested selected new compounds against L1210 leukemia in mice, and the result was shown Table 2. When one dose was administered, PP-HCPT-PA showed the best efficacy and was more efficacious than its parent HCPT. For example, at the optimal dose (100 mg/kg), PP-HCPT-PA had a 128% increase in life span (ILS) while HCPT (at an optimal dose of 15 mg/kg) had an ILS of 71%. Furthermore, PP-HCPT-PA had a greatly decreased toxicity compared to HCPT (bodyweight loss of 13% for PP-HCPT-PA at a dose of 200 mg/kg while 14% for HCPT at a dose of 15 mg/kg). Except for PP-HCPT-ACO, all new compounds showed a higher therapeutic efficacy than HCPT. It is interesting to note that PP-HCPT-ACO was the least potent new compound against L1210 leukemia cells *in vitro* (Table 1).

After testing the compounds at the same dose levels, we then evaluated PP-HCPT-PA, the one with the best therapeutic index, the free drug HCPT and PP-HCPT at an equimolar dose. The result was shown in Table 3. PP-HCPT produced a 79% ILS, which is much better than that of its parent HCPT with an ILS of 64%. In addition, the former was much less toxic. This result is in agreement with what we had previously reported,[14] and once again demonstrated that modifying the 10-hydroxyl group with a 4-(1-piperidino)-1-piperidino moiety improved therapeutic efficacy. Modification of the 20-hydroxyl group of PP-HCPT with a propionyl moiety further improved the therapeutic index, increasing the ILS from 79% to 114%. These results demonstrate that di-esterification of the 10- and

Table 1. Antitumor Activity against L1210 Leukemia *in Vitro**

Drug	IC_{50} (μM)
HCPT	1.15±0.61
PP-HCPT	134±36
PP-HCPT-ACO (**1**)	146±20.1
PP-HCPT-PA (**2**)	25.6±16.7
PP-HCPT-BUA (**3**)	113±12.5
PP-HCPT-isoBUA (**4**)	93±12.3
PP-HCPT-CPA (**5**)	35.24±10.72
PP-HCPT-GL-BOC (**6**)	116±13
PP-HCPT-GL·HCl (**7**)	55.7±27.6

*Cytotoxicity was measured in a 48-h proliferation assay. The results were reported as the minimal drug concentration that inhibits uptake of MTT assay by 50% and were the mean values of at least three experiments.

Table 2. Antitumor Activity against L1210 Leukemia in Mice*

Drug	Dose (mg/kg)	%Weight change[a)]	%ILS
HCPT	15	−14	71
PP-HCPT-PA	200	−13	82
	100	−5	128
	50	−3	118
PP-HCPT-ACO	200	−8	67
	100	−6	53
	50	−5	40
PP-HCPT-BUA	200	−9	93
	100	−6	80
	50	−4	67
PP-HCPT- iso-BUA	200	−9	93
	100	−7	80
	50	−4	67
PP-HCPT-CPA	200	−12	93
	100	−9	93
	50	−4	80

*Male $C_{57}BL$ mice (6/group) were injected i.p. with 10^5 cells on day 0. Drugs were administered i.p. on day 1. *a*) Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight. The median number of days of survival of the untreated mice was 7.5.

Table 3. Antitumor Activity against L1210 Leukemia in Mice*

Drug	Dose (mg/kg)	%Weight change[a)]	%ILS
HCPT	10	−10	64
PP-HCPT	91	−4	79
PP-HCPT-PA	100	−3	114

*Male $C_{57}BL$ mice (6/group) were injected i.p. with 10^5 cells on day 0. Drugs were administered i.p. on day 1. *a*) Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight. The median number of days of survival of the untreated mice was 7.5.

20-hydroxy positions of HCPT is a valid strategy to increase its therapeutic efficacy. It will be very interesting to find if this strategy can be used to improve the therapeutic efficacy of irinotecan, the first CPT analog used to treat cancer.

In conclusion, we have found that the therapeutic efficacy of HCPT can be first improved by modifying the 10-hydroxy group with a 4-(1-piperidino)-1-piperidino moiety, and then further improved by modifying its 20-hydroxy group with a propionyl moiety. That is PP-HCPT-PA (compound **2**) showed the best efficacy and was more efficacious than its parent HCPT. The novel PP-HCPT-PA merits further investigation as an anticancer agent.

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