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Bioorganic & Medicinal Chemistry 13 (2005) 5592–5599
Bioorganic & Medicinal Chemistry

# Synthesis and evaluation of a DHA and 10-hydroxycamptothecin conjugate

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Received 28 February 2005; accepted 3 June 2005
Available online 3 August 2005

**Abstract**—We have synthesized a conjugate of *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA) and 10-hydroxycamptothecin (HCPT), DHA–HCPT. The antitumor activity of DHA–HCPT was evaluated in vitro against L1210 leukemia cells and in experimental animal tumor models including L1210 leukemia, Lewis lung carcinoma, and colon 38 adenocarcinoma. DHA–HCPT showed a greatly improved antitumor efficacy compared to HCPT.


## 1. Introduction

The natural product camptothecin (CPT) (Fig. 1) and its synthetic analogues are among the most promising new agents for the treatment of human cancers.[1,2] CPT is a pentacyclic alkaloid, which was first isolated in 1966 from the extract of a Chinese plant, *Camptotheca acuminata*, by Wall et al.[3] Initial clinical trials with CPT were 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 resulted in a compound with poor antitumor activity.[4–6] It was later reported that the closed lactone form is an absolute requisite for antitumor activity.[7] 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).

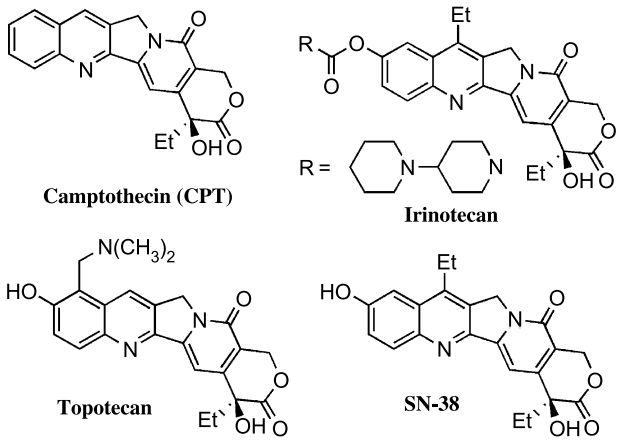


**Figure 1.** Structures of camptothecin, irinotecan, topotecan, and SN-38.

DNA topoisomerase I covalently binds to double-stranded DNA, forming a cleavable complex and producing a single-strand break.[8] This cleavable complex facilitates the relaxation of torsional strain of the supercoiled DNA. Once the torsional strain has been relieved, the enzyme rejoins the cleaved strand of DNA and dissociates from the relaxed double helix.[9] CPT binds to and stabilizes the cleavable complex and inhibits the religation of DNA, leading to the accumulation of DNA single-stranded breaks.[10,11] The single-stranded breaks are not in themselves toxic to the 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.[12] 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.

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doi:10.1016/j.bmc.2005.06.039

Sauer et al.[13–15] had studied the uptake of fatty acids and other metabolic precursors by tumor cells in tissue-isolated hepatomas with a single arterial inflow and a single venous outflow. In these systems, tumors avidly take up certain kinds of natural fatty acids from the arterial blood, presumably for use as biochemical precursors and energy sources. Based on these observations, a conjugate of *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA) and paclitaxel, DHA–paclitaxel, was synthesized (Fig. 2).[16] Pharmacokinetic studies of paclitaxel and DHA–paclitaxel in normal rats suggest that most of the DHA–paclitaxel is confined within the intravascular plasma volume, whereas paclitaxel is rapidly cleared from plasma and distributed into a large volume of peripheral tissue space.[16] When paclitaxel at 20 mg/kg, DHA–paclitaxel at 27.4 mg/kg (a dose equimolar with 20 mg/kg of paclitaxel), and DHA–paclitaxel at 120 mg/kg (a dose equitoxic with 20 mg/kg of paclitaxel) were injected through the tail vein of mice bearing M109 tumors weighing ~100 mg, paclitaxel remained >2 μM in tumors for only 16 h. In contrast, paclitaxel derived from DHA–paclitaxel at an equitoxic dose of 120 mg/kg remained >2 μM for 10 days after injection (tumors grow at concentration below 2 μM).[16] Although less potent than free paclitaxel, DHA–paclitaxel has a significantly higher therapeutic index than free paclitaxel in mice bearing tumors. In addition, DHA–paclitaxel has decreased side effects.

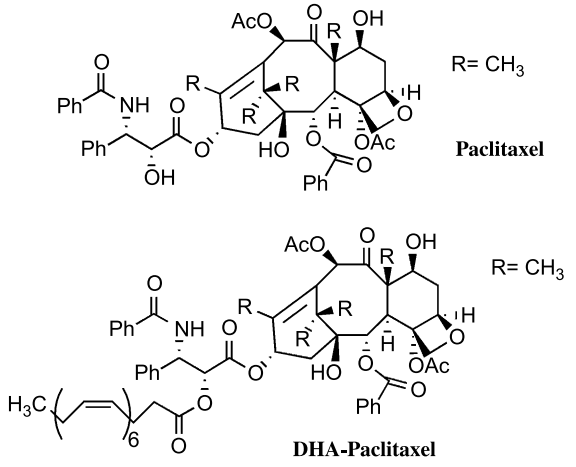



**Figure 2.** Structures of paclitaxel and DHA–paclitaxel.

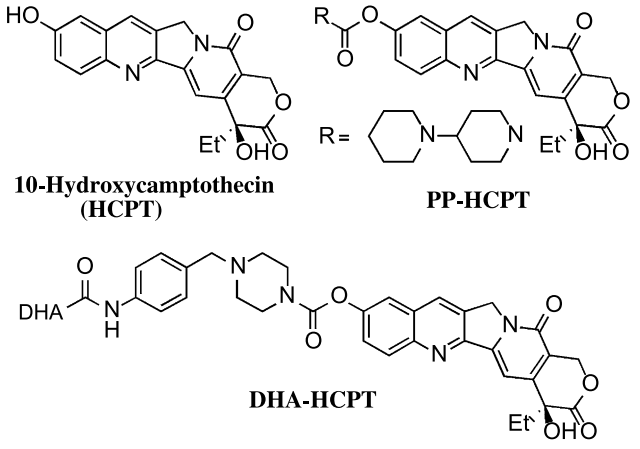



**Figure 3.** Structures of HCPT, PP–HCPT, and DHA–HCPT.

This data suggest that DHA may be used to enhance the therapeutic efficacy of other antitumor agents, especially the camptothecins because a prolonged exposure of the drug to tumor is particularly needed to ensure an optimal efficacy. Herein, we report the design, synthesis, and antitumor efficacy evaluations of DHA–HCPT, a conjugate of DHA and 10-hydroxycamptothecin (HCPT) (Fig. 3).

## 2. Chemistry

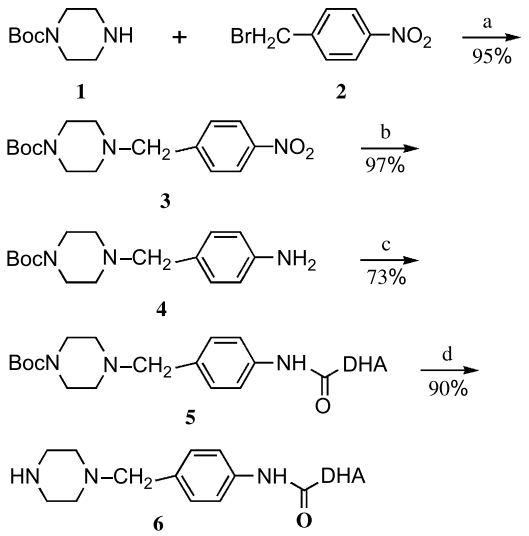
### 2.1. Rationale of drug design

Experiments demonstrate that tumor cells selectively take up certain fatty acids including DHA and DHA conjugate such as the DHA–paclitaxel, which had a greatly extended half-life. Consequently, DHA–paclitaxel has a superior therapeutic efficacy to free paclitaxel with reduced side effects. These data suggest that DHA may be used to improve the therapeutic index of other anticancer agents.

The commercially available HCPT is a member of the camptothecin class of compounds isolated from a Chinese tree, *Camptothecca acuminata*,[17] and has a wide spectrum of antitumor activity in vitro and in vivo through inhibition of topoisomerase I.[17] HCPT is more potent and less toxic than camptothecin. For example, in a Topo I inhibition assay, the $IC_{50}$ values of HCPT, CPT, and topotecan are 0.106, 0.677, and 1.11 μM, respectively.[18] HCPT has been approved for treatment of human cancers in China for more than 10 years. Synthesis of simple saturated long-chain fatty acid esters of HCPT has been reported.[19] The reported compounds are hydrolyzed by carboxylate esterase in vitro, but no in vivo antitumor activities are seen. Both CPT and HCPT have poor water solubility, leading to difficulty in formulation. Simply conjugating a fatty acid moiety to them will further decrease their water solubility. Furthermore, an ester bond is easily hydrolyzed by carboxylate esterase, which exists abundantly in serum. A carbamoyl bond is more stable than an ester bond to hydrolysis by carboxylate esterase. Consequently, compounds such as irinotecan have a longer half-life. Based on these considerations, we designed and synthesized DHA–HCPT. DHA is expected to provide a prolonged exposure of the drug to cancer cells, resulting in an enhanced therapeutic index. In DHA–HCPT, a piperazine linker was used. The tertiary nitrogen of piperazine 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.

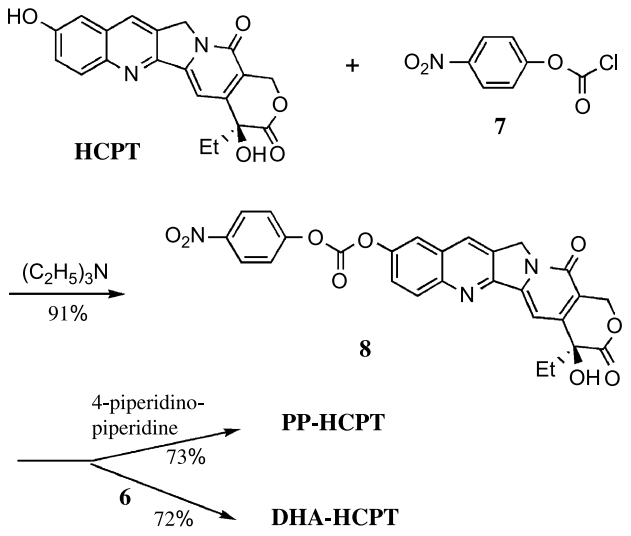
### 2.2. Chemical synthesis

The commercially available *tert*-butyl-1-piperazine carboxylate, **1**, and 4-nitrobenzyl bromide, **2**, were coupled in *N*,*N*-dimethylformamide catalyzed by potassium




**Scheme 1.** Synthesis of compound **6**. Reagents: (a) $K_2CO_3$, DMF; (b) $H_2$, Pd/c; (c) DHA, HBTU; (d) HCl/EtOAc.

carbonate to afford compound **3** in 95% yield. The nitro group of compound **3** was reduced by hydrogenation catalyzed by 10% Pd/C, cleanly affording amine **4**. The latter was then coupled with the commercially available DHA in the presence of 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), producing compound **5** in good yield. Removal of the Boc protecting group of **5** with saturated anhydrous HCl in ethyl acetate produced the required amine **6** (Scheme 1). The commercially available HCPT was first dissolved in tetrahydrofuran, and was treated with 4-nitrophenylchloroformate, affording carbonate **8**. The product was pure enough for the next reaction without further purification. Carbonate **8** in tetrahydrofuran was allowed to react with amine **6** to produce the target DHA–HCPT. To increase water solubility, DHA–HCPT was converted to the hydrochloride by treatment with saturated anhydrous HCl in ethyl acetate. Piperidinopiperidine (PP)–HCPT was synthesized by treatment of carbonate **8** with 4-piperidinopiperidine (Scheme 2).




**Scheme 2.** Synthesis of DHA–HCPT and PP–HCPT.

## 3. Results and discussion

### 3.1. Cytotoxicity

We tested the antitumor activity of DHA–HCPT against L1210 leukemia cells in vitro, and the results are shown in Table 1. DHA–HCPT was 8-fold less potent than its parent HCPT in this 48 h cell growth inhibition assay with an $IC_{50}$ value of 1.8 μM. PP–HCPT was 16- and 128-fold less potent than DHA–HCPT and HCPT, respectively, with an $IC_{50}$ value of 30 μM. Doxorubicin (Dox) was used as a positive control. This data correlate well with the previously reported data showing that irinotecan was 500-fold less potent than its free drug SN-38 against HL-60 leukemia cells in a 72 h assay in vitro.[20]

### 3.2. Antitumor activity in mouse tumor models

We tested DHA–HCPT in three different mouse tumor models including L1210 leukemia, Lewis lung carcinoma, and colon 38 adenocarcinoma. In the L1210 leukemia model, when one dose was administered, DHA–HCPT was much more efficacious than its parent HCPT (Table 2). For example, at the optimal dose (best therapeutic efficacy and bodyweight loss ⩽15% and/or toxic death ⩽15%), HCPT had a 77% increase in life span (ILS) with no long-term survivors. In contrast, DHA–HCPT had an ILS of 154% with two long-term survivors at a dose of 180 mg/kg. At an equimolar dose, DHA–HCPT (40 mg/kg) had a much higher therapeutic

**Table 1.** Antitumor activity against L1210 leukemia in vitro[a]

| Drug | $IC_{50}$ (μM) |
|---|---|
| HCPT | 0.23 ± 0.03 |
| DHA–HCPT | 1.8 ± 1.5 |
| PP–HCPT | 30 ± 0.6 |
| Dox | 0.18 ± 0.09 |

[a] Cytotoxicity was measured in a 48 h proliferation assay. The results were reported as the minimal drug concentration that inhibits uptake of [$^3$H]thymidine by 50% and were the mean values of at least three experiments.

**Table 2.** Antitumor activity against L1210 leukemia in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | %ILS | 30 day survivors |
|---|---|---|---|---|
| HCPT | 20 | −16 | 108 | 0 |
| | 15 | −10 | 77 | 0 |
| DHA–HCPT | 180 | −12 | 154 | 2 |
| | 60 | +1 | 138 | 0 |
| | 40 | +2 | 108 | 0 |
| PP–HCPT | 73 | +2 | 77 | 0 |
| | 37 | +5 | 46 | 0 |

[a] Male $BDF_1$ mice (6/group) were injected ip with $10^5$ cells on day 0. Drugs were administered ip on day 1.

[b] 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 6.5.

efficacy (ILS: 108%) than HCPT (15 mg/kg, ILS: 77%). Furthermore, DHA–HCPT had a greatly decreased toxicity compared to HCPT (bodyweight loss of 10% for HCPT versus a 2% gain for DHA–HCPT).

To find out if the enhanced therapeutic index of DHA–HCPT is simply due to the addition of a carbamate moiety to HCPT as in the case of irinotecan and its free drug SN-38, we synthesized PP–HCPT, a conjugate of piperidinopiperidine (the same group used in irinotecan) and HCPT (Fig. 3), and tested its antitumor activity. Clearly, PP–HCPT was better than HCPT. For example, although PP–HCPT at 73 mg/kg and HCPT at 15 mg/kg had the same ILS (77%), PP–HCPT was much less toxic (weight gain of 2%) to the animal than HCPT (weight loss of 10%). At an equimolar dose, the therapeutic efficacy of DHA–HCPT (60 mg/kg, ILS: 138%) was three-times higher than that of PP–HCPT (37 mg/kg, ILS: 46%). At an equitoxic dose, DHA–HCPT (40 mg/kg) had an ILS of 108% while the ILS produced by PP–HCPT (73 mg/kg) was only 77%. Furthermore, on a molar basis, 40 mg of DHA–HCPT was only 1/3 of 73 mg of PP–HCPT. These results demonstrate that DHA–HCPT is better than PP–HCPT, suggesting that conjugating DHA to HCPT leads to an increased therapeutic index.

We then tested DHA–HCPT using multiple doses (days 1, 5, and 9) (Table 3). At a dose of 120 mg/kg, DHA–HCPT produced an ILS of 323% with three cures. In contrast, PP–HCPT only produced an ILS of 123% with no cure. Once again, the therapeutic efficacy of DHA–HCPT is higher than that of PP–HCPT.

Next, the compound was tested in the mouse Lewis lung carcinoma model. When the drug was given one dose, ip DHA–HCPT showed significant activity in the mouse Lewis lung carcinoma model (Table 4). At a dose of 120 mg/kg, it inhibited tumor growth by 83% while the bodyweight loss was only 10%. In contrast, the parent HCPT inhibited tumor growth by 57% at a dose of 20 mg/kg with a 24% weight loss. The group treated with DHA–HCPT also lived longer.

In the multiple dose experiment (days 1, 5, and 9), at a dose of 100 mg/kg, DHA–HCPT produced a tumor growth inhibition (TGI) of 88% with a 9% bodyweight loss. In contrast, cisplatin, one of the most effective drugs for treating lung cancer, produced a TGI of 73% at a very toxic dose of 5 mg/kg (19% bodyweight loss). These results suggest that DHA–HCPT is a reasonable alternative to cisplatin and is less toxic (Table 5).

Irinotecan first received FDA approval for the treatment of human colorectal cancer. For this reason, we tested DHA–HCPT against colon cancer in mice. Colon 38 is a well-characterized mouse colon cancer and has been widely used in the primary anticancer activity screening of new agents. When the drug was given once, at a dose of 120 mg/kg, DHA–HCPT significantly inhibited the tumor growth with a TGI of 91% (Table 6). In contrast, cisplatin produced a TGI of 88%. However, DHA–HCPT produced less weight loss than cisplatin.

Next, the drug was evaluated on a multiple dose schedule. Two multiple dose experiments (days 1, 5, and 9) were performed. The first experiment was designed to evaluate the antitumor activity of the drugs against early tumor, that is, the tumors were transplanted on day 0, and the drugs were given beginning the next day. The second experiment was designed to evaluate the drug's activity against the established tumor, that is, the

**Table 3.** Antitumor activity against L1210 leukemia in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | %ILS | 30 day survivors |
|---|---|---|---|---|
| DHA–HCPT | 120 | −12 | 323 | 3 |
| PP–HCPT | 150 | −18 | 123 | 0 |

[a] Male $BDF_1$ mice (6/group) were injected ip with $10^5$ cells on day 0. Drugs were administered ip on days 1, 5, and 9.
[b] 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 6.5.

**Table 4.** Antitumor activity against Lewis lung carcinoma in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | Tumor weight (g, ±SD) | %TGI | %ILS | *P* value[c] |
|---|---|---|---|---|---|---|
| Control | — | — | 1.77 ± 0.89 | — | — | — |
| HCPT | 20 | −24 | 0.76 ± 0.59 | 57 | 16 | <0.01 |
| DHA–HCPT | 120 | −10 | 0.31 ± 0.18 | 83 | 33 | <0.01 |
| | 80 | −5 | 0.93 ± 0.53 | 48 | 23 | <0.01 |

[a] Male $BDF_1$ mice (8/group) were injected sc with $10^6$ cells on day 0. Drugs were administered ip on day 1.
[b] Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight.
[c] Comparing to the control.

**Table 5.** Antitumor activity against Lewis lung carcinoma in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | Tumor weight (g, ±SD) | %TGI | *P* value[c] |
|---|---|---|---|---|---|
| Control | — | — | 1.20 ± 0.61 | — | |
| DHA–HCPT | 100 | −9 | 0.15 ± 0.10 | 88 | <0.01 |
| Cisplatin | 5 | −19 | 0.32 ± 0.17 | 73 | <0.01 |

[a] Male $BDF_1$ mice (8/group) were injected sc with $10^6$ cells on day 0. Drugs were administered ip on days 1, 5, and 9.
[b] Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight.
[c] Comparing to the control.

**Table 6.** Antitumor activity against colon 38 carcinoma in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | Tumor weight (g, ±SD) | %TGI | *P* value[c] |
|---|---|---|---|---|---|
| Control | — | — | 1.20 ± 0.84 | — | — |
| Cisplatin | 10 | −20 | 0.14 ± 0.07 | 88 | <0.01 |
| DHA–HCPT | 120 | −16 | 0.11 ± 0.09 | 91 | <0.01 |

[a] Male $BDF_1$ mice (8/group) were injected sc with $10^6$ cells on day 0. Drugs were administered ip on day 1.
[b] Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight.
[c] Comparing to the control.

tumors were transplanted on day 0, and the drugs were given when the tumors grew to 3–4 mm in size. In the early stage tumor model, at a dose of 100 mg/kg, DHA–HCPT significantly inhibited the tumor growth with a TGI of 78% (Table 7). In the late stage tumor model, at a dose of 100 mg/kg, DHA–HCPT inhibited tumor growth by 80% (Table 8). These data suggest that DHA–HCPT is equally effective in both the early and late stage tumor models.

The CPT compounds have emerged as one of the most promising class of anticancer agents. Over the last two decades, intensive research has led to the development of improved analogues with increased solubility and impressive antitumor efficacy. Among the analogues synthesized, some are new compounds, and others are prodrugs of existing compounds. Efforts are made to address several important aspects of the CPT compounds. Because CPT failed clinical development mainly due to its low water solubility of the lactone form, an amino group has been incorporated into irinotecan, topotecan, 9-aminocamptothecin, GI-147211, and exatecan to improve their aqueous solubility.[1,2] At physiologic pH, the amino group is protonated, and thus, all of these compounds have improved solubility in aqueous media. In addition to an improved aqueous solubility, modification of the CPT structure also shifted the equilibrium between the lactone and carboxylate forms of the molecule to the favored lactone form in some of these compounds.[21] Because CPT is a S-phase-specific drug, prolonged exposure to cancer cells is especially important for achieving optimal efficacy. O'Leary and Muggia[22] reported that CPTs require a prolonged schedule of administration given continuously at low doses to achieve the optimal efficacy. One approach used to increase the half-life of CPT is to make a prodrug such as irinotecan or to conjugate it to a polymer. For example, the half-life of the lactone form of SN-38, an active metabolite released from irinotecan, was 11.5 h, which is much longer than that of topotecan, 2.4 h.[1] Zamai et al.[23] reported that camptothecin–polymer conjugate had a prolonged exposure to tumors, which led to a remarkably enhanced efficacy.

The improved therapeutic efficacy of DHA–paclitaxel over free paclitaxel was mainly attributed to its extensive binding to plasma proteins, which led to a small volume of distribution (~4 L) and slow systemic clearance (~0.11 L/h).[24] In experimental animals, the concentration of paclitaxel after DHA–paclitaxel administration was maintained at >2 μM for 10 days, while that after paclitaxel administration was only 16 h.[16] In this report, DHA–HCPT was designed as a prodrug, which incorporates a DHA moiety to prolong its half-life and to increase its accumulation in tumors. As expected, DHA–HCPT showed greatly improved therapeutic efficacies in all tumor models tested, compared to the free HCPT and PP–HCPT. At this time, we do not know

**Table 7.** Antitumor activity against early stage colon 38 carcinoma in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | Tumor weight (g, ±SD) | %TGI | *P* value[c] |
|---|---|---|---|---|---|
| Control | — | — | 1.13 ± 0.39 | — | — |
| DHA–HCPT | 150[d] | — | 0.18 ± 0.12 | 84 | <0.01 |
| | 100 | −12 | 0.25 ± 0.22 | 78 | <0.01 |
| | 67 | −10 | 0.64 ± 0.50 | 43 | <0.01 |

[a] Male $BDF_1$ mice (8/group) were injected sc with $10^6$ cells on day 0. Drugs were administered ip on days 1, 5, and 9.
[b] Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight.
[c] Comparing to the control.
[d] Drugs were given on days 1 and 5, and four mice died of toxicity.

**Table 8.** Antitumor activity against late stage colon 38 carcinoma in mice[a]

| Drug | Dose (mg/kg) | %Weight change[b] | Tumor weight (g, ±SD) | %TGI | *P* value[c] |
|---|---|---|---|---|---|
| Control | — | — | 1.13 ± 0.39 | — | — |
| DHA–HCPT | 150[d] | — | 0.24 ± 0.18 | 79 | <0.01 |
| | 100 | −14 | 0.22 ± 0.14 | 80 | <0.01 |

[a] Male $BDF_1$ mice (8/group) were injected sc with $10^6$ cells on day 0. Drugs were administered ip on days 9, 13, and 17.
[b] Group bodyweight change was between day 0 and the day at which time the group of mice had the lightest weight.
[c] Comparing to the control.
[d] Drugs were given on days 9 and 13, and three mice died of toxicity.

if the enhanced therapeutic efficacy of DHA–HCPT is due to a prolonged half-life in vivo and/or a selective accumulation by tumors. We will perform experiments to answer these and other questions related to the mechanism of action of DHA–HCPT and will report the results in due course.

## 4. Experimental

### 4.1. Chemistry

Melting points were measured using a Mel-Temp II instrument 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, 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 was visualized under UV light. Preparative separations were performed by flash chromatography on silica gel (Merck, 70–230 mesh).

**4.1.1. *tert*-Butyl 4-(4-nitrobenzyl)-1-piperazinecarboxylate (3).** To a solution of *tert*-butyl 1-piperazinecarboxylate, **1** (0.74 g, 4 mmol), and 4-nitrobenzyl bromide, **2** (0.86 g, 4 mmol), in *N*,*N*-dimethylformamide (DMF, 5 mL) was added potassium carbonate (3 g), and the reaction mixture was stirred at room temperature for 4 h. The product was extracted with ethyl acetate (50 mL), and the organic phase was washed with water (20 mL × 3). The solution was dried with sodium sulfate and the solvent was removed in vacuo. The product was purified by column chromatography, eluting with ethyl acetate to afford **3** as a solid (1.22 g, 95% yield), mp 99–100 °C. $^1$H NMR (DMSO-$d_6$): 8.21–8.19 (d, 2H, $J$ = 8.8 Hz, ArH), 7.61–7.59 (d, 2H, $J$ = 8.0 Hz, ArH), 3.63 (s, 2H, $CH_2$), 3.34–3.30 (m, 4H, partially obscured, 2× $CH_2$), 2.35–2.32 (m, 4H, 2× $CH_2$), 1.39 (s, 9H, 3× $CH_3$). Anal. ($C_{16}H_{23}N_3O_4$) C, H, N.

**4.1.2. *tert*-Butyl 4-[4-(*cis*-4,7,10,13,16,19-docosahexenoyl)amino]benzyl-1-piperazinecarboxylate (5).** To compound **3** (0.68 g, 2.1 mmol) in ethyl acetate (100 mL) was added 10% Pd/C (0.1 g), and the reaction mixture was hydrogenated for 2 h under a hydrogen pressure of 60 lb/in.$^2$. The product was filtered, and the solvent was removed in vacuo to afford amine **4** (0.6 g, 97% yield). Without further purification, to amine **4** (0.6 g, 2.06 mmol) dissolved in acetonitrile (90 mL) was added *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA, 0.67 g, 2 mmol), 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 0.94 g, 2.5 mmol), and *N*,*N*-diisopropylethylamine (2.1 mL). The reaction mixture was stirred overnight. The solvent was removed in vacuo, and the product was purified by column chromatography, eluting with ethyl acetate to afford **5** as an oil (0.91 g, 73% yield). $^1$H NMR (DMSO-$d_6$): 9.84 (s, 1H, NH), 7.53–7.51 (d, 2H, $J$ = 8.80 Hz, ArH), 7.19–7.17 (d, 2H, $J$ = 8.40 Hz, ArH), 5.35–5.31 (m, 12H, CH=CH), 3.40 (s, 2H, $CH_2$), 3.29 (br s, 4H, 2× $CH_2$), 2.83–2.75 (m, 10H, 5× $CH_2$), 2.35 (br s, 4H, 2× $CH_2$), 2.27 (t, 4H, $J$ = 4.80 Hz, 2× $CH_2$), 2.04–2.00 (m, 2H, $CH_2$), 1.38 (s, 9H, 3× $CH_3$), 0.91 (t, 3H, $J$ = 7.20 Hz, $CH_3$). Anal. Calcd for ($C_{38}H_{55}N_3O_3 \cdot 4.1H_2O$) C, 67.55; H, 9.42; N, 6.21. Found: C, 67.91; H, 9.42; N, 5.86.

**4.1.3. 10-[4-[[4-(*cis*-4,7,10,13,16,19-Docosahexenoyl)amino]benzyl]-1-piperazino]carbonyloxy-camptothecin.** Compound **5** (0.85 g, 1.4 mmol) was dissolved in ethyl acetate (10 mL), and saturated anhydrous HCl in ethyl acetate (7 mL) was added. The reaction mixture was stirred at room temperature for 30 min. The precipitate was filtered, and the solid was washed with ethyl ether to afford **6** (0.68 g, 90% yield). The product was used for the next reaction without further purification.

HCPT (1.0 g, 2.76 mmol) in tetrahydrofuran (THF, 200 mL) was treated with 4-nitrophenyl chloroformate, **7** (2.16 g, 10.8 mmol), and triethylamine (4.0 mL), and the reaction was allowed to proceed at room temperature for 1 h. The product was extracted with ethyl acetate (1 L) and the organic phase was washed with water (500 mL × 3). The solution was dried with sodium sulfate and the solvent was removed in vacuo. The product was purified by column chromatography, eluting with ethyl acetate to afford **8** as a yellow solid (1.32 g, 91% yield). The product was used for the next reaction without further purification. Carbonate **8** (0.66 g, 1.25 mmol) in THF (60 mL) was treated with amine **6** (0.85 g, 1.58 mmol) in the presence of triethylamine (2.4 mL), and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed in vacuo, and the product was purified by column chromatography, eluting with ethyl acetate and methanol (15:1, v/v) to afford DHA–HCPT as a yellow solid (0.8 g, 72% yield), mp 196–199 °C. MS $m/z$ ($M^+$+H): 893.0. The product was dissolved in a mixture of THF and ethyl acetate, and anhydrous HCl in ethyl acetate was then added. The precipitate was filtered and the solid was dried to afford DHA–HCPT as a HCl salt, mp 230 °C (dec). $^1$H NMR (DMSO-$d_6$): 9.89 (s, 1H, NH), 8.66–7.26 (m, 9H, ArH), 6.53 (s, 1H, OH), 5.43–5.30 (m, 16H), 3.68 (s, 1H, $CH_2$), 3.50 (br s, 4H, 2× $CH_2$), 3.21–3.18 (m, 2H, $CH_3CH_2$), 2.84–2.76 (m, 10H), 2.48 (br s, 4H, 2× $CH_2$), 2.35 (br s, 4H, 2× $CH_2$), 2.05–2.01 (m, 2H, $CH_2$), 1.91–1.84 (m, 2H, $CH_2$), 1.29 (t, 1H, $J$ = 8.0 Hz, $CH_3$), 0.93–0.87 (m, 6H, 2× $CH_3$). Anal. Calcd for ($C_{54}H_{61}N_5O_7 \cdot HCl \cdot 5H_2O$): C, 63.67; H, 7.13; N, 6.87. Found: C, 63.88; H, 6.03; N, 6.71.

**4.1.4. 10-[4-(1-Piperidino)-1-piperidino]carbonyloxycamptothecin.** Carbonate **8** (0.53 g, 1 mmol) in THF (55 mL) was treated with 4-piperidinopiperidine (0.17 g, 1 mmol) and triethylamine (1 mL), and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed in vacuo, and the product was purified by column chromatography, eluting with dichloromethane and methanol (5:1, v/v) to afford PP–HCPT as a yellow solid (0.41 g, 73% yield). The product was dissolved in a mixture of THF and ethyl acetate, and anhydrous HCl in ethyl acetate was added. The precipitate was filtered, and the solid was dried to afford PP–HCPT as a HCl

salt, mp 235 °C (dec). $^1$H NMR (DMSO-$d_6$): 8.66 (s, 1H), 8.19–8.17 (d, 1H, $J$ = 9.2 Hz), 7.92–7.91 (d, 1H, $J$ = 2.8 Hz), 7.70–7.67 (dd, 1H, $J$ = 3.2, 9.6 Hz), 7.35 (s, 1H), 6.54 (s, 1H), 5.44 (s, 2H), 5.30 (s, 2H), 4.29–4.26 (d, $J$ = 13.59 Hz, 1H), 4.12–4.08 (d, $J$ = 13.99 Hz, 1H), 3.06 (t, $J$ = 9.59 Hz, 1H), 2.91 (t, $J$ = 9.59 Hz, 1H), 1.91–1.82 (m, 4H), 1.53–1.42 (m, 8H), 0.89 (t, 3H, $J$ = 7.4 Hz). MS $m/z$ ($M^+$+H): 559.64. Anal. ($C_{31}H_{34}N_4O_6$·HCl·3$H_2O$) C, H, N.

### 4.2. Drugs

For the cytotoxicity study, the drugs were dissolved in DMSO to provide a stock solution of 3 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.

### 4.3. Cytotoxicity

The L1210 mouse leukemia cells were cultured in RPMI 1640 plus 10% FCS with the addition of 100 U/mL penicillin and 100 μg/mL streptomycin. Cytotoxic effects of the drugs were measured by inhibition of DNA synthesis. L1210 leukemia cells in RPMI-1640 plus 10% FCS medium were seeded at $5 \times 10^4$ 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 10 μL of [$^3$H]thymidine (20 μCi/mL). The plate was incubated for another 24 h. The cells were harvested and radioactivity was counted using the Packard Matrix 96 beta counter. The percentage growth inhibition was calculated as follows:

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

This was used to estimate the $IC_{50}$ values.

### 4.4. Antitumor screening in mice

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 8/group for the solid tumor models), and drugs (0.1 mL) were administered ip 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 ip to mice. Antitumor activity was determined by comparing the median survival time of the treated groups ($T$) with that of a control group ($C$), and was expressed as a percentage of ILS [increase of life span, where %ILS = $(T/C-1) \times 100$]. These calculations considered dying animals only. Long-term (30 days) survivors were noted separately. The median number of days the untreated group of mice (given the vehicle only) died was 6.5.

Lewis lung carcinoma was maintained by continuous sc passage by inoculating syngeneic male C57BL/6 mice with $10^6$ tumor cells. For antitumor efficacy studies, mice were implanted sc with $10^6$ cells. The antitumor activity was determined by comparing the tumor weight of the treated groups ($T$) with that of a control group ($C$), and was expressed as a percentage of the tumor growth inhibition (TGI, where %TGI = $1-T/C \times 100$). The TGI was determined when the tumors weighed 1–1.5 g (four weeks). Long-term survivors (60 days) were noted separately.

Colon 38 was maintained by continuous sc passage by implanting $10^6$ cells in syngeneic male $C_{57}$BL/6 mice. For antitumor efficacy studies, mice were implanted with $10^6$ cells sc. The TGI was determined when the tumors weighed 1–1.5 g (4 weeks). Long-term survivors (60 days) were noted separately.

## Acknowledgment

This work was supported in part by a grant from the National Institutes of Health (CA 82949 to Y.W.).

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