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Synthesis and antitumor activity of CBI-bearing ester and carbamate prodrugs of CC-1065 analogue

Yuqiang Wang,[a,b,*] Lianfa Li,[b] Zhiming Tian,[b] Wei Jiang[b] and James W. Larrick[b]

[a]*Institute of New Drug Research, Jinan University College of Pharmacy, Guangzhou 510632, China*
[b]*Panorama Research Inc., 2462 Wyandotte Street, Mountain View, CA 94043, USA*

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Abstract—Prodrugs of a CBI-bearing CC-1065 analogue were synthesized. Antitumor activity of the compounds was evaluated against tumor cells in vitro and in mouse tumor models. Compounds **1** and **7**, bearing methylpiperazine and DHA moieties, respectively, showed significant antitumor activity in both the L1210 leukemia and Lewis lung carcinoma mouse tumor models. For the carbamate prodrugs **1**–**4** and **6**, there is a good correlation between the drug's potency both in vitro and in animal tumor models; however, there is no correlation between the prodrug's antitumor activity and the type of bonds linking the free drug. There are no significant differences between the antitumor activities of those that can or cannot be protonated at physiological pH. Compounds **6** and **7**, each bearing a DHA moiety, did not show significantly improved antitumor activity compared to other prodrugs bearing DHA moieties, suggesting that DHA may not be used universally to significantly improve a drug's antitumor efficacy.

1. Introduction

CC-1065 (Fig. 1) was first isolated from *Streptomyces zelensis* in 1978,[1] and was found to be extremely potent against a variety of cancer cells and bacteria both in vitro and in experimental animals.[1–4] In addition to its potency, CC-1065 was found to have a mechanism of action different from other known compounds; that is, it binds to double-stranded B-DNA within the minor groove with the preference of AT-rich sequences, and alkylates the N3 position of the 3′-adenine with its left-hand CPI segment.[5–8] CC-1065 also inhibits gene transcription by interfering with the binding of the TATA box binding protein to its target DNA.[9] Despite its high potency, broad range of antitumor activity, and new mechanism of action, CC-1065 was found to cause delayed death in experimental animals.[10] Thus, it cannot be used as human therapeutics.

Due to its intense potency, novel mechanism of action and broad range of antitumor activity, extensive efforts have been made during the last two decades to find analogues that retain the potency and antitumor activity of the parent compound, but without the delayed toxicity and myelosuppressive effect. One of the approaches to improve a drug's therapeutic efficacy is to make

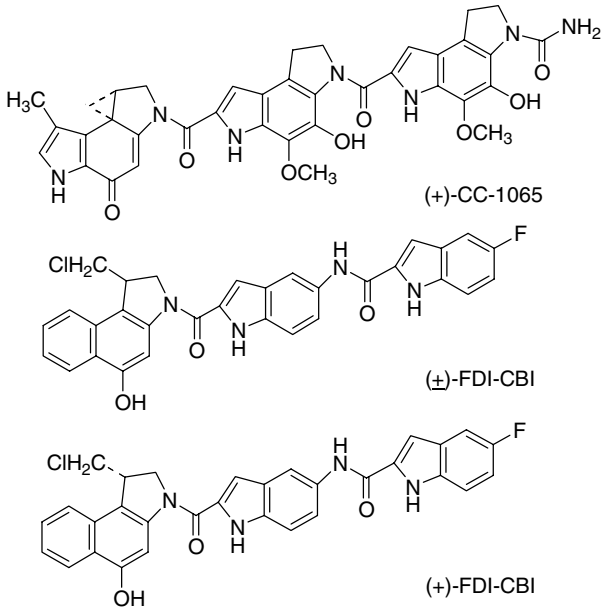

Figure 1. Structures of (+)CC-1065, (±)-FDI-CBI and (+)-FDI-CBI.

Keywords: Anticancer agents; CC-1065; Prodrugs; DHA.
* Corresponding author. Tel.: +1 650 694 4996; fax: +1 650 694 7717; e-mail: yqwangphd@yahoo.com

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prodrugs. For example, carzelesin,[11,12] a carbamate prodrug of CC-1065 analogue, and KW-2189,[13] a carbamate prodrug of duocarmycin, have improved antitumor efficacies and reduced side effects compared to their free drug counterparts. Carzelesin and KW-2189 have been tested in Phase II human clinical trials. Another approach to improve a drug's therapeutic efficacy is to conjugate the drug to a tumor-specific carrier, such as an antibody, polymer or other molecule.

It was reported that tumors avidly take up certain natural fatty acids from blood.[14–17] These fatty acids are presumably used as biochemical precursors and energy sources. Consumption of diets containing n-3 fatty acids, including α-linolenic acid and *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA), has been found to inhibit tumorigenesis,[18,19] growth of rodent tumors[20,21] and human breast cancer xenografts.[22,23] Bradley et al. recently reported the synthesis of DHA-paclitaxel, a 2′-*O*-acyl conjugate of DHA and paclitaxel (Fig. 2).[24] DHA-paclitaxel was shown to have superior therapeutic efficacy to free paclitaxel in experimental tumor models with fewer side effects. The improved efficacy of DHA-paclitaxel over free paclitaxel is attributed mainly to its extensive binding to plasma proteins, which leads to a small volume of distribution (~4 L) and slow systemic clearance (~0.11 L/h).[25] In experimental animals, the concentration of paclitaxel after DHA-paclitaxel injection was maintained at >2 μM for 10 days, while that after paclitaxel administration was only 16 h.[24] Tumors start to grow when the paclitaxel concentration falls below 2 μM.[24] The prolonged half-life may have led to the drug's selective accumulation in tumor cells.[24,25]

Recently, we reported the synthesis and antitumor activity of a DHA and 10-hydroxycamptothecin conjugate (DHA-HCPT) (Fig. 3),[26] in which DHA is chemically linked to 10-hydroxycamptothecin (HCPT), a member of the camptothecin class of compounds which have wide range of antitumor activity both in vitro and in vivo through inhibition of topoisomerase I.[27] It is more potent and less toxic than camptothecin. DHA-HCPT has greatly improved therapeutic efficacies in mouse tumor models including L1210 leukemia, Lewis lung carcinoma, and colon 38 carcinoma, compared to the free HCPT.[26]

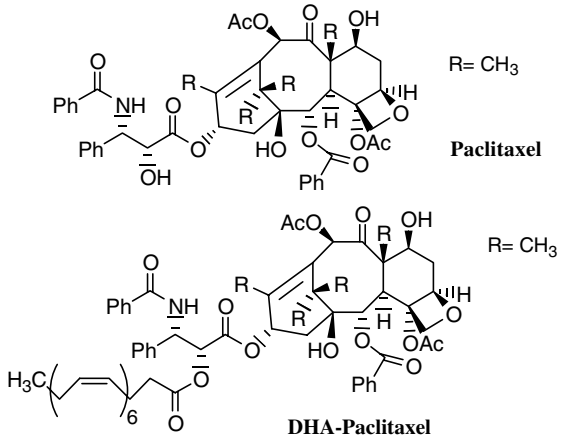


Figure 2. Structures of paclitaxel and DHA-paclitaxel.

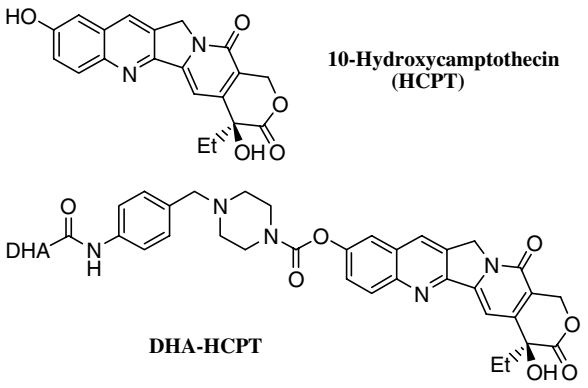


Figure 3. Structures of HCPT and DHA-HCPT.

We previously reported the synthesis and antitumor activity of (±)-FDI-CBI, a CBI-bearing CC-1065 analogue which is intensely potent and has good antitumor activity in experimental animals without the delayed toxicity.[28] CBI is the benzannelated alkylating functional analogue of CC-1065 and was first reported by Boger et al.[29] CBI-indole2 prodrugs have also been reported by the same group.[30] We herein report the synthesis, antitumor activity, and preliminary toxicity study of FDI-CBI carbamate and ester prodrugs including DHA conjugates (Fig. 4).

2. Results and discussion

2.1. Chemistry

The two classes of drugs that are designed are carbamates and esters. Compounds **1**–**4** are simple carbamate prodrugs. Compound **1** incorporated a methylpiperazine used in synthesis of KW-2189 and other duocarmycin prodrugs.[31–34] Compound **2** incorporated a piperidinopiperidine, which has been successfully used in the synthesis of irrinotecan,[35] a clinically used camptothecin analogue with good antitumor efficacy. Compound **3** incorporated a pyridylpiperidine, in which the pyridyl moiety may provide additional water solubility. Compound **4** incorporated a methylhydrazine, which was shown to improve the antitumor efficacy of duocarmycin.[31] Compound **5** is a hexanoic acid ester of (±)-FDI-CBI, which is designed as a control for compounds **6** and **7**, each bearing a DHA moiety. In compound **6**, DHA is first coupled to phenylamine, and then to piperizine. This is to introduce a tertiary amine moiety to compound **6** that can be protonated at physiological pH to increase the compound's water-solubility. Thus, compounds **1**–**4** and **6** should have good water-solubility. In compound **7**, DHA is directly linked to (+)-FDI-CBI. The enantiomeric (+)-FDI-CBI has a higher therapeutic efficacy than the racemic (±)-FDI-CBI. All of these compounds are expected to be cleaved by carboxylate esterase in vivo to release the free drug. It has been

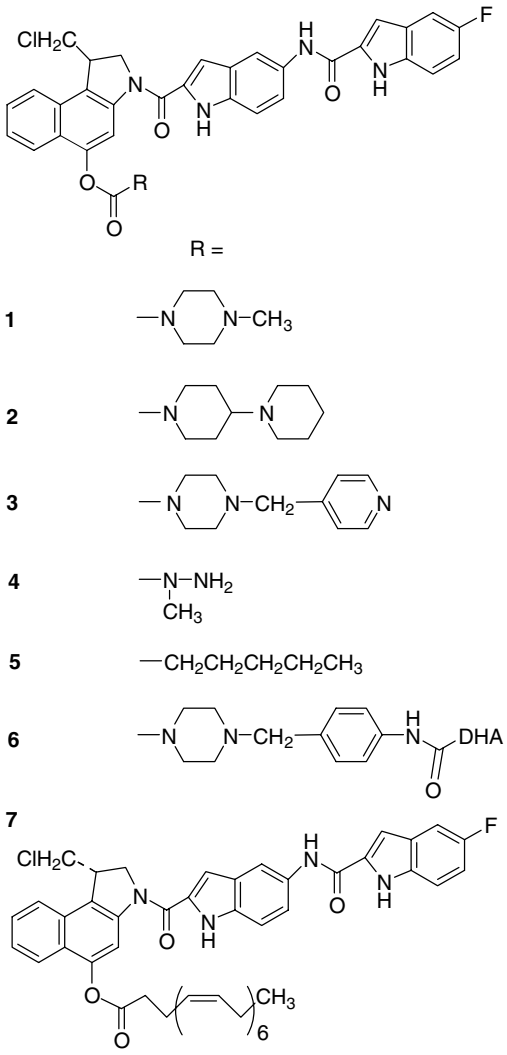
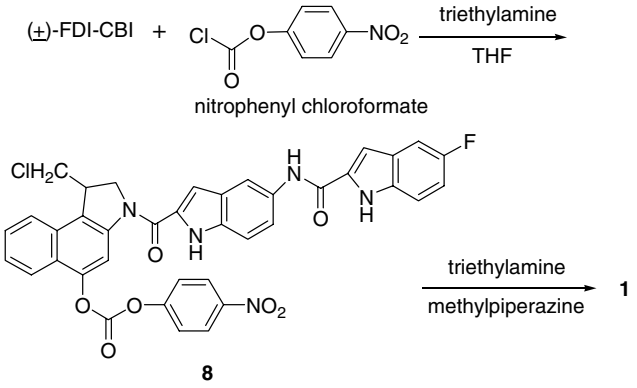


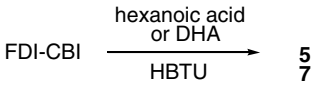
Figure 4. Structures of new CC-1065 analogues.

demonstrated previously that prodrugs of CC-1065[11] and duocarmycin were cleaved by carboxylate esterase,[33,34] releasing the free drug.

The amines for synthesis of carbamates **1**–**4** are commercially available, and the amine for synthesis of **6** was synthesized in our laboratory according to the procedure as reported previously.[26] (±)-FDI-CBI[28] was treated with 4-nitrophenyl chloroformate to afford the key intermediate, carbonate **8**, with a 90% yield. Treatment of the latter with various amines afforded the targeted carbamates **1**–**4** and **6**, with yields ranging from 39% to 67% (Scheme 1). Ester **5** was synthesized by coupling (±)-FDI-CBI with hexanoic acid catalyzed by 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and *N*,*N*-diisopropylethylamine. Ester **7** was synthesized by coupling enantiomeric (+)-FDI-CBI with commercially available DHA (Scheme 2). (+)-FDI-CBI was synthesized by a procedure similar to that as described for the synthesis of (±)-FDI-CBI.[28] (+)-CBI was synthesized according to a procedure reported by Aristoff and Johnson,[36] where resolution of the enantiomers was performed according



Scheme 1. Synthesis of compound **1**.



Scheme 2. Synthesis of compounds **5** and **7**.

to a procedure first reported by Boger and Ishizaki.[37] In both works of Boger and Ishizaki,[37] and Aristoff and Johnson,[36] this procedure produced a diastereomeric purity >99%.

2.2. Cytotoxicity

The new compounds were tested in vitro against L1210 leukemia cells, and the results are shown in Table 1. As expected, all of the carbamates, **1**–**4** and **6**, are substantially less potent than the free drug (±)-FDI-CBI. Compounds **1** and **4** are approximately 20-fold more potent than **2**, **3**, and **6**. This is probably due to the fact that methylpiperazine and methylhydrazine are smaller than piperidinopiperidine, pyridylpiperidine, and the much larger amine used in **6**. The larger amines have a greater steric hindrance to carboxylate esterase than the smaller ones have. These data are in agreement with what have been observed previously.[38] Ester **5** with an IC_{50} value of 4.6 nM is substantially more potent than carbamates **1**–**4** and **6**, although it is still much less potent than the free drug (±)-FDI-CBI with an IC_{50} value of 0.29 nM. The DHA ester **7** with an IC_{50} value of 13 nM is approximately 3-fold less potent than the hexanoic ester **5**, but

Table 1. Cytotoxicity against L1210 leukemia cells

Compound	IC_{50}[a] (nM)
(±)-**1**	12 ± 2.6
(±)-**2**	310 ± 78
(±)-**3**	260 ± 28
(±)-**4**	33 ± 1.4
(±)-**5**	4.6 ± 1.1
(±)-**6**	220 ± 0
(+)-**7**	13 ± 6.8
(±)-FDI-CBI	0.29 ± 0.16
(+)-FDI-CBI	0.17 ± 0.06

[a] IC_{50} values are defined as the minimal drug concentration necessary to inhibit incorporation of [^{3}H]thymidine by 50%, and are the averages of three experiments.

is generally much more potent than the carbamates. The reason that the ester prodrugs are more potent than those of the carbamates is probably because the latter is more resistant than the former to cleavage by carboxylate esterase. The fact that the hexanoic ester **5** is more potent than the DHA ester **7** is because the latter is larger in size than the former; as a result, the latter is more resistant than the former to cleavage by carboxylate esterase.

2.3. Antitumor activity in mouse tumor models

The antitumor activity of selected compounds was first determined in mice bearing L1210 leukemia, and the results are shown in Table 2. Compound **1**, bearing a methylpiperazine, has the highest therapeutic efficacy among all compounds. For example, at an optimal dose of 150 μg/kg, compound **1** produced an ILS of 187% with two long-term survivors. Compound **4** produced an ILS of 185% with no long-term survivors. The cytotoxicity of compounds **1**–**4** is **1** > **4** > **3** > **2**, and their in vivo potencies are **1** > **4** > **2** and **3**. Their therapeutic efficacies are also **1** > **4** > **2** and **3**. Although compound **6** is almost as potent as **2** and **3** in vitro, the former is significantly less potent and efficacious than the latter compounds in vivo. There is a good correlation between the drug's potency both in vitro and in vivo and its therapeutic efficacy among the carbamates. The antitumor efficacy of prodrug **1** (ILS: 187%) with two long-term survivors is much better than that of its corresponding free drug (±)-FDI-CBI (ILS: 160%) with no long-term survivor; however, there were no significant differences between the antitumor efficacies of prodrugs **2** (ILS: 154%), **3** (ILS: 154%), and their corresponding free drug, (±)-FDI-CBI (ILS: 160%).

The antitumor efficacy of DHA ester **7** (ILS: 231% without any long-term survivors) is significantly higher than those of its corresponding free drugs (±)-FDI-CBI (ILS: 160%) and the enantiomeric (+)-FDI-CBI (ILS: 185%) as well as those of carbamates **2**–**4**; however, it is lower than that of carbamate **1** with an ILS of 187% and two long-term survivors. Although DHA ester 7 (ILS: 231%) has a higher therapeutic efficacy than its corresponding free drug (+)-FDI-CBI (ILS: 185%), the difference between their antitumor efficacies is not as great as in cases where DHA was coupled to paclitaxel[24] and 10-hydroxycamptothecin.[26]

The antitumor activity of compounds **7**, (±)-FDI-CBI, and (+)-FDI-CBI was also tested in mice bearing Lewis lung carcinoma, and the results are shown in Table 3. At a dose of 300 μg/kg, compound **7** inhibited tumor growth with a TGI of 78%. (+)-FDI-CBI (TGI: 83%) has a higher therapeutic efficacy than (±)-FDI-CBI (TGI: 60%). There was no significant difference between the efficacies of compound 7 and its corresponding free drug (+)-FDI-CBI, suggesting that DHA has no significant effect on the drug's antitumor activity in this tumor model. All of the compounds are more efficacious than carboplatin in this tumor model. Carboplatin is one of the most effective drugs in the mouse Lewis lung carcinoma tumor model and was used as a positive control in this experiment.

2.4. Preliminary toxicity study

CC-1065 and some of its analogues have delayed toxicity in mice; that is, mice die long after the normal observation period of 15 days for the acute toxicity study.[10] We have reported previously that (±)-FDI-CBI did not cause delayed death.[28] To find out if the prodrugs of FDI-CBI cause delayed death, compounds **1, 7**, and

Table 2. Antitumor activity in mice bearing L1210 leukemia

Compound	Dose (μg/kg)	%weight change[a]	%ILS	30-day survivors
(±)-**1**	67	+2	120	0
	100	0	152	1
	150	−7	187	2
(±)-**2**	100	−8	138	0
	150	−12	154	0
	224	−23	123	0
(±)-**3**	100	−10	108	0
	150	−9	154	0
	224	−25	123	0
(±)-**4**	67	+2	138	0
	100	0	154	0
	150	−3	185	0
(±)-**6**	100	+13	46	0
	200	+9	62	0
	400	+7	88	0
(+)-**7**	134	+2	154	0
	200	0	185	0
	300	−7	231	0
(±)-FDI-CBI	45	+2	137	0
	67	−2	160	0
	100	−7	158	0
(+)-FDI-CBI	22	1	123	0
	34	3	169	0
	50	−10	185	0
CP	125 mg	−5	173	0

CP, cyclophosphamide.
[a] Group bodyweight change between days 0 and 6.

Table 3. Antitumor activity in mice bearing Lewis lung carcinoma[a]

Compound	Dose (μg/kg)	%weight change[b]	%TGI
(+)-**7**	134	0	56
	200	−4	62
	300	−13	78
(±)-FDI-CBI	30	+5	37
	45	−7	45
	67	−19	60
(+)-FDI-CBI	22	+4	42
	34	0	63
	50	−18	83
Carboplatin	20 mg	+3	25

[a] BDF_1 male mice (4–6 week old, 8/group) were used. Each mouse was inoculated with 10^6 cells (0.1 mL), s.c. on the back of the mouse on day 0. Drugs were administered on days 1, 5, and 9, ip. 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 TGI. The tumor weight was measured on day 16, and that of the control group was 1.19 g ± 0.24.
[b] Mean body weight change between days 0 and 12.

Table 4. Delayed death study in mice[a]

Compound	Dose (μg/kg)	No. of mice surviving on day 180
(±)-**1**	100	8/8
(+)-**7**	450	8/8
(+)-FDI-CBI	100	8/8

[a] Drugs were administered on day 1, ip.

(+)-FDI-CBI were administered to non-tumor-bearing BDF_1 mice, and the animals were observed for 180 days. As expected, these drugs did not cause delayed death as seen in Table 4.

3. Conclusions

Carbamate and ester prodrugs of potent CBI-bearing CC-1065 analogues were synthesized. In both the L1210 and the Lewis lung carcinoma mouse tumor models, enantiomer (+)-FDI-CBI has a higher therapeutic efficacy than racemic (±)-FDI-CBI. This observation is in agreement with what we and others have reported in the past.[11,28,39–42,8] A possible explanation for the differences is that the natural enantiomer alkylates DNA more efficiently than the unnatural enantiomer. Compounds **1**, bearing a methylpiperazine, and **7**, bearing a DHA moiety, have the highest therapeutic efficacies in the L1210 leukemia model, and both are more efficacious than the free drug. There is a good correlation between the drug's potency both in vitro and in vivo and its therapeutic efficacy among the carbamate prodrugs. There are no significant differences between the antitumor activities of those that can or cannot be protonated at physiological pH. Although the positive charge has no significant effect on antitumor efficacy, it significantly increased the compound's water-solubility. For example, compounds **1**–**4** dissolved in water easily. In contrast, compounds **5** and **7**, bearing no positive charge, did not readily dissolve in water.

A DHA moiety has been used to make prodrugs such as DHA-paclitaxel and DHA-HCPT that have significantly enhanced antitumor efficacies compared to their corresponding free drugs. However, compounds **6** and **7**, bearing a DHA moiety, did not show significantly enhanced antitumor activity compared to what has been seen in DHA-paclitaxel and DHA-HCPT, suggesting that DHA may not be used universally to greatly improve a drug's antitumor efficacy.

4. Experimental

4.1. Chemistry

Melting points were measured using a Mel-Temp II and are uncorrected. ^{1}H NMR spectra were recorded at ambient temperature on an NT-400 spectrometer. High-resolution mass spectra (FAB) were recorded on a modified MS50 mass spectrometer equipped with a VG 11-250J data system. Atlantic Microlab, Inc., Norcross, Georgia, performed 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 (Merck, 70-230 mesh).

4.1.1. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-hydroxy-1,2-dihydro-3*H*-benz[*e*]indole [(+)-FDI-CBI]. (+)-FDI-CBI was synthesized by a procedure similar to that as described for synthesis of (±)-FDI-CBI.[28] Gray solid, 65% yield. ^{1}H NMR (DMSO-d_6, ppm): 11.83 (s, 1H, NH), 11.74 (s, 1H, NH), 10.44 (s, 1H, OH), 10.22 (s, 1H, NH), 8.23–7.24 (m, 13H, Ar-H), 4.84 (t, 1H, J = 10.0. Hz, NC*H*H), 4.60–4.58 (d, 1H, J = 9.6 Hz, NCH*H*), 4.25–4.23 (m, 1H, $ClCH_2CHCH_2$), 4.06–4.02 (dd, 1H, J = 3.2, 11.2 Hz, C*H*HCl), 3.91–3.87 (dd, 1H, J = 6.8, 10.8 Hz, CH*H*Cl). HRMS (FAB) calcd for $C_{31}H_{22}ClFN_4O_3$ 552.1364. Found 552.1350.

4.1.2. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[(4-nitrophenyl)-carbonyloxy]-1,2-dihydro-3*H*-benz[*e*]indole (8). To (±)-FDI-CBI (53 mg, 96 μmol) in tetrahydrofuran (9 mL) cooled to 0 °C were added 4-nitrophenyl chloroformate (42 mg) and triethylamine (40 μL) subsequently. The reaction mixture were stirred at 0 °C for 20 min. To the reaction mixture was added 4-nitrophenyl chloroformate (42 mg) and triethylamine (40 μL), and the reaction mixture was stirred at 0 °C for another 20 min. Ethyl acetate (100 mL) was added, and the solution was washed with water (3× 50 mL). The solution was dried with sodium sulfate. The solvent was removed in vacuo, and ethyl ether was added. The solid was filtered and washed with ether to afford carbonate **8** as a yellow solid (62 mg, 90% yield). ^{1}H NMR (DMSO-d_6, ppm): 11.85 (s, 1H, NH), 11.78 (s, 1H, NH), 10.24 (s, 1H, OH), 8.41–7.30 (m, 14H, Ar-H), 4.96 (t, 1H, J = 10.2 Hz, NC*H*H), 4.71–4.67 (dd, 1H, J = 2.0, 10.8 Hz, NCH*H*), 4.50–4.46 (m, 1H, $ClCH_2CHCH_2$), 4.15–4.11 (dd, 1H, J = 3.6, 11.6 Hz, C*H*HCl), 4.09–4.05 (dd, 1H, J = 6.4, 11.2 Hz, CH*H*Cl). MS (M+H): 718.09. The product was used for the next reaction without further purification.

4.1.3. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[(4-methylpiperazinyl)carbonyloxy]-1,2-dihydro- 3*H*-benz[*e*]indole (1). To carbonate **8** (10 mg, 18 μmol) in dimethylformamide (0.3 mL) were added methylpiperazine (4 μL) and triethylamine (50 μL). The reaction was allowed to proceed overnight. The solvent was removed in vacuo, and the product was purified by thin-layer chromatography, eluting with ethyl acetate and methanol (1:1, v/v), affording **1** as a grey solid (8 mg, 65% yield). ^{1}H NMR (DMSO-d_6, ppm): 12.00 (s, 1H, NH), 11.75 (s, 1H, NH), 10.33 (s, 1H, NH), 8.25–7.05 (m, 13H, Ar-H), 4.92 (t, 1H, J = 10.2 Hz, NC*H*H), 4.67–4.65 (dd, 1H, J = 1.6, 10.4 Hz, NCH*H*), 4.43–4.41 (m, 1H, $ClCH_2CHCH_2$), 4.12–4.09 (dd, 1H, J = 3.2, 12.0 Hz, C*H*HCl), 4.05–4.01 (dd, 1H, J = 6.8, 11.2 Hz, CH*H*Cl), 3.80 (br s, 2H, CH_2), 3.50 (br s, 2H, CH_2), 2.41 (br s, 2H,

CH_2), 2.27 (s, 3H, NCH_3). HRMS (FAB) calcd for $C_{37}H_{32}ClFN_6O_4$ 678.2158. Found 678.2157.

Compounds **2**–**4** and **6** were synthesized by a similar procedure as that described in detail for synthesis of **1**.

4.1.4. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[[4-(1-piperidino)-1-piperidino]carbonyloxy]-1,2-dihydro-3*H*-benz[*e*]indole (2). Gray solid, 67% yield. ^{1}H NMR (DMSO-d_6, ppm): 11.85 (s, 1H, NH), 11.75 (s, 1H, NH), 10.23 (s, 1H, NH), 8.24–7.06 (m, 13H, Ar-H), 4.92 (t, 1H, J = 10.2 Hz, NC*H*H), 4.67–4.64 (dd, 1H, J = 1.6, 10.8 Hz, NCH*H*), 4.47–4.38 (m, 1H, $ClCH_2CHCH_2$), 4.12–4.00 (m, 3H, CH_2Cl, NC*H*), 3.22–3.16 (m, 1H, NC*H*H), 2.97–2.91 (m, 1H, NCH*H*), 1.90–1.80 (m, 2H, CH_2), 1.62–1.38 (m, 8H, CH_2). HRMS (FAB) calcd for $C_{42}H_{40}ClFN_6O_4$ 746.2748. Found 746.2735.

4.1.5. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[[4-(1-pyridylmethyl)-1-piperidino]carbonyloxy]-1,2-dihydro-3*H*-benz[*e*]-indole (3). Gray solid, 55% yield. ^{1}H NMR (DMSO-d_6, ppm): 11.85 (s, 1H, NH), 11.75 (s, 1H, NH), 10.23 (s, 1H, NH), 8.56–7.06 (m, 17H, Ar-H), 4.92 (t, 1H, J = 10.2 Hz, NC*H*H), 4.68–4.64 (dd, 1H, J = 2.0, 11.2 Hz, NCH*H*), 4.45–4.42 (m, 1H, $ClCH_2CHCH_2$), 4.12–4.09 (dd, 1H, J = 2.8, 10.8 Hz, C*H*HCl), 4.05–4.01 (dd, 1H, J = 6.4, 10.8 Hz, CH*H*Cl), 3.83 (br s, 2H, CH_2), 3.63 (s, 2H, CH_2), 3.53 (br s, 2 H, CH_2), 2.58 (br s, 2H, CH_2). HRMS (FAB) calcd for $C_{42}H_{35}ClFN_7O_4$ 755.2423. Found 755.2420.

4.1.6. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[(1-methylhydrazino)carbonyloxy]-1,2-dihydro-3*H*-benz[*e*]indole (4). Gray solid, 62% yield. ^{1}H NMR (DMSO-d_6, ppm): 11.85 (s, 1H, NH), 11.71 (s, 1H, NH), 10.23 (s, 1H, NH), 8.56–7.06 (m, 13H, Ar-H), 4.94 (t, 1H, J = 10.2 Hz, NC*H*H), 4.69–4.66 (dd, 1H, J = 2.0, 11.2 Hz, NCH*H*), 4.45–4.42 (m, 1H, $ClCH_2CHCH_2$), 4.13–4.109 (dd, 1H, J = 2.8, 10.8 Hz, C*H*HCl), 4.06–4.02 (dd, 1H, J = 6.4, 10.8 Hz, CH*H*Cl), 3.38 (br s, 2H, CH_3). HRMS (FAB) calcd for $C_{33}H_{26}ClFN_6O_4$ 624.1688. Found 624.1669.

4.1.7. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-(hexanoyloxy)-1,2-dihydro-3*H*-benz[*e*]indole (5). To (±)-FDI-CBI (25 mg, 45 μmol) in acetonitrile (2 mL) were added 2-(1-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 22 mg) *N*,*N*-diisopropylethylamine (80 μL), and hexanoic acid (16 mg, 136 μmol). The reaction mixture was stirred at room temperature overnight. Ethyl acetate was added (20 mL), and the solution was washed with water (3× 10 mL). The solution was dried with sodium sulfate, and solvent was removed in vacuo. The product was purified by thin-layer chromatography, eluting with ethyl acetate and hexane (3:2, v/v), affording **5** as a gray solid (17 mg, 58% yield). ^{1}H NMR (DMSO-d_6, ppm): 11.85 (s, 1H, NH), 11.76 (s, 1H, NH), 10.23 (s, 1H, NH), 8.24–7.28 (m, 13H, Ar-H), 4.92 (t, 1H, J = 10.4 Hz, NC*H*H), 4.67–4.64 (dd, 1H, J = 2.0, 11.2 Hz, NCH*H*), 4.45–4.44 (m, 1H, $ClCH_2CHCH_2$), 4.13–4.10 (dd, 1H, J = 3.2, 11.2 Hz, C*H*HCl), 4.06–4.02 (dd, 1H, J = 6.0, 10.8 Hz, CH*H*Cl), 2.84 (t, 3H, J = 7.4 Hz, CH_2), 1.79–1.72 (m, 2H, CH_2), 1.45–1.36 (m, 4H, CH_2CH_2), 0.93 (t, 3H, J = 7.0 Hz, CH_3). HRMS (FAB) calcd for $C_{37}H_{32}ClFN_4O_4$ 650.2096. Found 650.2090.

4.1.8. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-[[[4-(*cis*-4,7,10,13,16,19-docosahexenoyl)amino]benzyl]-1-piperazino]carbonyloxy]-1,2-dihydro-3*H*-benz[*e*]indole (6). Compound **6**. Gray solid, 39% yield. ^{1}H NMR (DMSO-d_6, ppm): 11.84 (s, 1H, NH), 11.74 (s, 1H, NH), 10.23 (s, 1H, NH), 9.89 (s, 1H, NH), 8.24–7.06 (m, 17H, Ar-H), 5.38–5.25 (m, 12H, CH=CH), 4.95 (t, 1H, J = 10.19 Hz, NC*H*H), 4.67–4.65 (d, 1H, J = 10.4 Hz, NCH*H*), 4.45–4.42 (m, 1H, $ClCH_2CHCH_2$), 4.12–4.10 (d, 1H, J = 10.4 Hz, C*H*HCl), 4.06–4.02 (dd, 1H, J = 6.4, 10.8 Hz, CH*H*Cl), 3.75 (br s, 2H, CH_2), 3.47 (br s, 4H, CH_2), 2.84–2.76 (m, 8H, CH_2), 2.38–2.33 (m, 6H, CH_2), 2.02 (m, 2H, CH_2), 0.91 (t, 3H, J = 8.0 Hz, CH_3). HRMS (FAB) calcd for $C_{65}H_{67}ClFN_7O_5$ 1079.4876. Found 1079.4875.

4.1.9. 1-(Chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-(*cis*-4,7,10,13,16,19-docosahexenoyloxy)-1,2-dihydro-3*H*-benz[*e*]indole (7). (+)-FDI-CBI (12 mg, 22 μmol) was dissolved in dimethylformamide (0.5 mL), and *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA, 9 mg) was added, followed by the addition of dicyclohexylcarbodiimide (DCC, 14 mg). The reaction was allowed to proceed overnight at room temperature. Ethyl acetate (20 mL) was added. The solution was washed with water (3× 10 mL) and dried with sodium sulfate. Solvent was removed in vacuo. Purification by thin-layer chromatography, eluting with ethyl acetate, afforded 11 mg of product (59% yield). ^{1}H NMR (DMSO-d_6, ppm): 11.84 (s, 1H, NH), 11.74 (s, 1H, NH), 10.22 (s, 1H, NH), 8.24–7.05 (m, 13H, Ar-H), 4.57–5.24 (m, 12H, CH=CH), 4.92 (t, 1H, J = 10.4 Hz, NC*H*H), 4.68–4.65 (dd, 1H, J = 1.6, 10.8 Hz, NCH*H*), 4.45–4.43 (m, 1H, $ClCH_2CHCH_2$), 4.13–4.00 (dd, 1H, J = 3.2, 11.2 Hz, C*H*HCl), 4.05–4.01 (dd, 1H, J = 6.8, 11.2 Hz, CH*H*Cl), 2.92–2.73 (m, 12H, CH_2), 2.55–2.53 (m, 2H, partially obscured, CH_2), 2.02–1.99 (m, 2H, CH_2), 0.89 (t, 3H, J = 8.0 Hz, CH_3). HRMS (FAB) calcd for $C_{53}H_{52}ClFN_4O_4$ 862.3661. Found 862.3660.

4.2. Cytotoxicity

4.2.1. Cell lines. The mouse L1210 leukemia cells were kindly provided by Dr. Alan Sartorelli of Yale University, and were cultured in RPMI-1640 plus 10% FCS with the addition of 100-U/mL penicillin and 100 μg/mL streptomycin.

4.2.2. Drugs. Drugs were dissolved in DMF to provide a stock solution of 1 mg/mL and were stored at −20 °C. For each experiment, drug solutions were freshly prepared from the stock solution by addition of sterile water to afford concentrations suitable for the experiment.

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 5×10^4 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 with the same medium. The plate was incubated for 24 h at 37 °C followed by addition of 10 μL of [^{3}H]thymidine (20 μCi/mL). The plate was incubated for another 24 h. Cells were harvested and radioactivity was counted using the Packard Matrix 96 beta counter. The percentage growth inhibition was calculated as follows:

% growth inhibition = [(total cpm − experimental cpm)/total cpm] × 100. This was used to estimate the IC_{50} values.

5. Antitumor activity in mouse tumor models

L1210 leukemia cells (10^5cells/mouse, 0.1 mL) were injected ip to male BDF_1 mice (6/group) on day 0. Drugs were administered ip on days 1, 5, and 9. 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) × 100]. These calculations considered dying animals only. Long-term (30 days) survivors were noted separately. Cyclophosphamide was used as a positive control. The median number of days the untreated group of mice (given the vehicle only) died was 6.5. Animals were monitored and weighed daily.

Lewis lung carcinoma (from ATCC, Manassas, Virginia) was maintained by continuous s.c. passage by inoculating syngeneic male C57BL/6 mice with 10^6 tumor cells. For antitumor efficacy studies, male BDF_1 mice, weighing 18–22 g (8/group), were implanted s.c. with 10^6 cells on day 0. Drugs were administered i.p. on days 1, 5, and 9. Carboplatin was used as a positive control. 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 tumor growth inhibition (TGI, where %TGI = 1 − T/C × 100]. Animals were monitored and weighed daily.

6. Preliminary toxicity study

For the preliminary toxicity study, drugs were administered i.p. to male BDF_1 mice on day 0. Animals were monitored daily and were weighed every week. The experiment was terminated on day 180.

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