Bioorganic & Medicinal Chemistry 16 (2008) 6552–6559

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and antitumor activity evaluations of albumin-binding prodrugs of CC-1065 analog

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ARTICLE INFO

Article history:
Received 17 April 2008
Revised 8 May 2008
Accepted 9 May 2008
Available online 15 May 2008

Keywords:
Prodrug
CC-1065
Albumin
Anticancer

ABSTRACT

An albumin-binding prodrug of the extremely potent CC-1065 analog, (+)-FDI-CBI, has been synthesized. This analog, (+)-FDI-CBIM, formed an albumin conjugate when added to human albumin in vitro. A greater amount (>3-fold) of the prodrug can be administered to animals compared to the free drug. The prodrug had significantly improved antitumor efficacy compared to the free drug in animal models using syngeneic animal tumors and human ovarian xenografted tumor cells. Antitumor drug delivery by in situ formation of drug–albumin conjugate is a promising strategy to improve antitumor efficacy.

1. Introduction

The fungus *Streptomyces zelensis*-derived antibiotic CC-1065 (Fig. 1) is one of the most potent antitumor agents ever found.[1–4] CC-1065 has potent and broad-spectrum antitumor activity in vitro and in vivo, with an IC_{50} value against selected tumor cells in the low pM range.[1–4] CC-1065 binds to double-stranded B-DNA within the minor-groove, where it alkylates the N3 position of the 3′-adenine.[5–7] This mechanism of action differs from those of currently used anticancer agents. Although CC-1065 was not developed as a clinical drug due to its toxic side effect,[8] its extreme potency and unique mechanism of action have attracted intense research interests for the last 20 years. Four CC-1065 and duocarmycin analogs, that is, adozelesin, carzelesin, KW-2189, and bizelesin, have been synthesized and evaluated in clinical trials. Although none of these compounds succeeded in the clinic due to myelotoxicity, measurable antitumor responses were achieved in multiple patients.[9,10]

Trabectedin (also known as Yondelis, ET-743), another DNA minor-groove binding agent, has achieved significant antitumor results in patients with soft tissue sarcoma.[11] In contrast to CC-1065 which alkylates the N3 position of the 3′-adenine, trabectedin alkylates the N2 position of guanines, leading to disruption of the cell cycle and inhibition of cell proliferation. Trabectedin, administered iv once every 3 weeks, was approved in Europe in 2007 as mono-

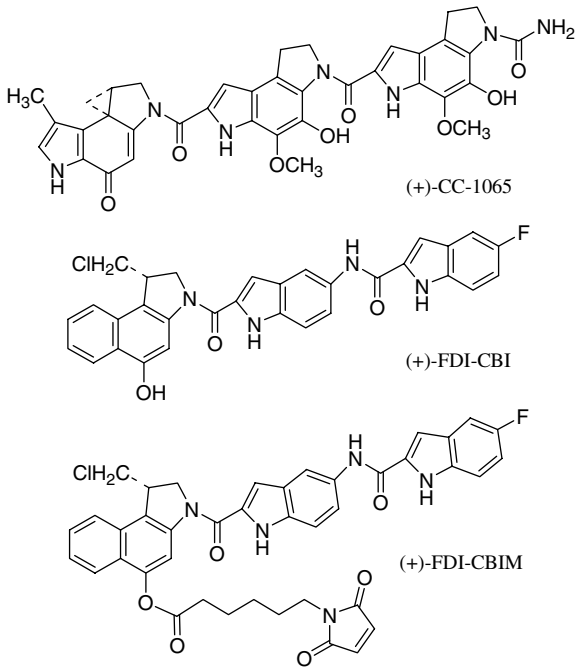

Figure 1. Structures of CC-1065, (+)-FDI-CBI and (+)-FDI-CBIM.

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doi:10.1016/j.bmc.2008.05.025

therapy for use in patients with advanced soft tissue sarcoma after failure of standard therapy with anthracyclines or ifosfamide, or for those patients unsuited to receive these agents. Clinically useful DNA minor-groove binding agents have come of age, and the search for more effective compounds of this class continues.

CC-1065 comprises three indoles linked by amide bonds. The left-hand cyclopropylindole subunit alkylates DNA, and the middle and right-hand subunits enhance the DNA-binding affinity and selectivity. The detrimental delayed toxic effects of CC-1065 were initially thought to be caused by the existence of two methylene moieties in the middle and right-hand subunits of the molecule.[12] In a previous report, we found that CC-1065 analogues that did not have the methylene moieties but possessed a terminal amide moiety also caused animals to die due to delayed toxicity.[12] To address this problem, we replaced the terminal amide moiety with different groups and found that when a terminal amide is replaced with a fluorine, the resulting compound, named (+)-FDI-CBI, did not cause delayed toxicity.[13] In addition, (+)-FDI-CBI had a much improved antitumor efficacy compared to other CC-1065 analogues we had synthesized previously. These findings led to the synthesis of more compounds including prodrugs. Some of the prodrugs were found to be more effective in mouse tumor models.[13]

Human serum albumin (HSA), perhaps the best studied plasma protein, is taken up by endocytosis and degraded in the lysosomes. HSA is known to bind various endogenous metabolites, metal ions, and drugs.[14,15] Binding of drugs to serum albumin affects their metabolism, efficacy, and body distribution.[16] Because HSA is biodegradable, non-toxic, and non-immunogenic, it is widely used as a stabilizing component in pharmaceutical and biologic products including vaccines, recombinant therapies, and coatings for medical devices.

HSA preferentially accumulates in solid tumors.[17] Several factors regarding the metabolism of HSA are noteworthy. Among these are (a) HSA is an important source of energy and nitrogen for tumors; (b) because of the enhanced proliferative rate, tumor cells take up HSA at a greater rate than normal cells; (c) the abnormal vasculature of tumors is highly permeable, which makes them take up large molecules more efficiently than normal cells; (d) the poor lymphatic drainage of tumors cannot readily remove large molecules, leading to an accumulation of HSA molecules in tumors[18–20]; (e) the HS-group of Cys34 of HSA is the most abundant, chemically accessible thiol in human plasma.[21–24] Thus, an HSA-binding prodrug is expected to function as a reservoir of the drug, resulting in improved efficacy for drugs known to have a narrow therapeutic index.[16]

Over the last 10 years, Kratz and coworkers have reported the synthesis and evaluations of conjugates of HSA with numerous anticancer drugs including doxorubicin (Dox).[21] The HSA–doxorubicin conjugate, DOXO-EMCH, is a 6-maleimidocaproyl)hydrazone derivative of Dox, and was found to have improved anticancer efficacy with reduced toxic side effects compared to the parent Dox. In a phase I clinical trial, DOXO-EMCH showed a good safety profile and induced tumor regressions in anthracycline-sensitive tumors, such as breast cancer, small cell lung cancer, and sarcoma.[25]

In our endeavor to find more effective CC-1065 analogs, we have synthesized a conjugate of (+)-FDI-CBI and 6-maleimidocaproic acid. Herein, we report details of the synthesis and biological activities of this novel compound.

2. Results and discussion

2.1. Drug design and chemical synthesis

Conjugation of cytotoxic anticancer agents to albumin has been demonstrated to be a useful prodrug approach to further enhance the efficacy of the parent drug. For this approach to work, several requirements are necessary:

(a) Excessive conjugation is not desirable because albumin plays important biological functions;
(b) The prodrug can be less active than the free drug to limit damage to normal tissues prior to delivery to the intended targets;
(c) The chemical linkage between albumin and the free drug can be cleaved chemically or enzymatically to produce the cytotoxic free drug;
(d) The prodrug must readily react with albumin in the blood stream to form the albumin–drug conjugate.

(+)-FDI-CBI meets all of the above requirements and in addition it is >1000-fold more active than Dox against L1210 leukemia cells in vitro (Table 1). Previous work showed that (+)-FDI-CBI prodrugs were significantly less active than the free drug, for example, the hexanoic acid and *cis*-4,7,10,13,16,19-docosahexenoic acid (DHA) ester prodrugs of the racemic (±)-FDI-CBI were approximately 18- and 45-fold less active than the free drug, respectively (IC_{50} values of 4.6 nM and 13 nM for the hexanoic acid and DHA prodrugs versus 0.29 nM of the free drug).[26] Although we did not directly demonstrate that the ester linkage of the hexanoic acid and DHA prodrugs of (±)-FDI-CBI is cleaved, it is well-known that such ester bonds are readily cleaved by carboxylic esterase, an enzyme activity abundant in human blood. The maleimido moiety has been widely used to link small molecules to proteins, and work by Kratz et al. has shown that the maleimido moiety of 6-maleimidocaproic acid reacts with HSA within minutes in blood to form HSA–drug conjugates.[21,27] For these reasons, we choose to couple (+)-FDI-CBI to 6-maleimidocaproic acid to make a (+)-FDI-CBI and maleimido prodrug [(+)-FDI-CBIM]. Following parenteral administration of this prodrug, we predicted, first a quantitative conjugation reaction with the large excess of HSA in blood, followed by slow carboxylic esterase hydrolysis to release the cytotoxic free drug, leading to tumor cell death (Fig. 2).

(+)-FDI-CBI was synthesized in our laboratory according to a previously reported procedure.[13] Specifically, (+)-FDI-CBI in acetonitrile was treated with 6-maleimidocaproic acid catalyzed by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in the presence of diisopropylethylamine to afford (+)-FDI-CBIM (Scheme 1).

2.2. Cytotoxicity

Compounds were tested in vitro versus L1210 leukemia and SKOV-3 human ovarian cancer cells (Table 1). In agreement with previous results, the ester prodrugs are substantially less potent than their corresponding free drugs.[26] For example, free drug (+)-FDI-CBI has an IC_{50} value of 0.17 nM, while that of (+)-FDI-CBIM is only 25 nM, 147-fold less potent than free drug (+)-FDI-CBI. In the 72-h assay against SKOV-3 human ovarian

Table 1
Antitumor activity against L1210 leukemia in vitro

Drug	IC_{50} (nM)	
	L1210[a]	SKOV-3[b]
(+)-FDI-CBI	0.17 ± 0.06	14 ± 3
(+)-FDI-CBIM	25 ± 8	34 ± 5
Dox	182 ± 93	—

Results were reported as the minimal drug concentration that inhibits uptake of ^{3}H-thymidine by 50%.
[a] Cytotoxicity was measured in a 48-h proliferation assay.
[b] Cytotoxicity was measured in a 72-h proliferation assay.

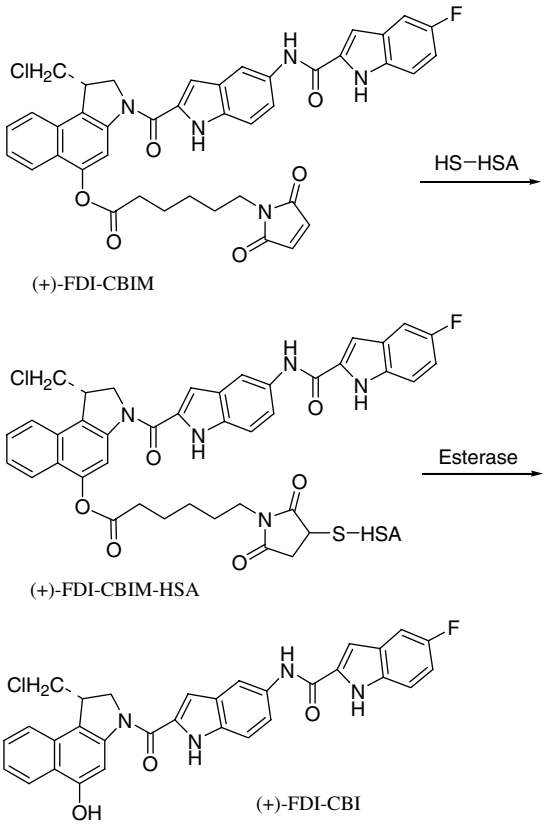
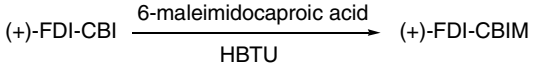


Figure 2. Formation of HSA conjugate and production of (+)-FDI-CBI.



Scheme 1. Synthesis of (+)-FDI-CBIM.

cancer cells, prodrug (+)-FDI-CBIM was approximately 2-fold less active than its corresponding free drug. The reduced cytotoxicity of the prodrug is expected to correlate with decreased damage to normal tissues while the drug is in circulation before reaching its tumor target.

2.3. Formation of albumin–(+)-FDI-CBIM conjugate

(+)-FDI-CBIM is designed to form an albumin conjugate in blood. The sulfhydryl group of cysteine-34 of HSA attacks the double bond of the maleimido moiety through the Michael reaction (Fig. 2). To determine if (+)-FDI-CBIM reacts with albumin to form conjugates, (+)-FDI-CBIM was incubated with human albumin in phosphate buffer. Reaction products were analyzed by HPLC (Fig. 3). Although the retention times of HSA and the conjugate are similar (~60 min), conjugation can be measured at 320 nm where (+)-FDI-CBIM is easily identified while HSA had no absorption. After 30 min of incubation at 37 °C, 35% of (+)-FDI-CBIM had reacted with HSA, after 105 min, 72% of (+)-FDI-CBIM had reacted, and at 180 min <5% free drug was found. Kratz et al. reported that the reaction of albumin-binding Dox and camptothecin prodrugs with human HSA in vitro was very specific with the only product shown on the chromatogram to be the drug–HSA conjugate.[21,27] We found that HSA had a retention time of approximately 60 min when analyzed under the same conditions (with detection wavelength set at 220 nm). These results suggest that (+)-FDI-CBIM readily reacts with HSA.

2.4. Effects on cell cycle arrest in L1210 cells

Cell cycle progression of L1210 leukemia cells exposed to (+)-FDI-CBIM or the free drug (+)-FDI-CBI was investigated by flow cytometry. L1210 leukemia cells were exposed to drug concentrations ranging from 0.0001 to 0.1 μg/mL for 24 h, and the fraction of cells at different phases of the cell cycle were monitored after propidium iodide staining (Fig. 4). Exposure to higher concentrations (i.e., 0.01–0.1 μg/mL for both compounds) decreased the proportion of cells in G1 and increased the fraction in S and G2/M (Fig. 4A and B).

A time course experiment was carried out at a drug concentration set at 0.1 μg/mL with cell cycle analysis at 3, 6, 12, and 24 h (Fig. 4C and D). The effects of drugs on the cell cycle were evident by 3 h, with the proportion of cells in S phase increased compared to control cells (Fig. 4E). By 12 h, the proportion of cells in S phase were significantly increased, and by 24 h, few cells were in G1, and the majority of cells were blocked in S and G2/M phases. This result agrees with what had been reported for adozelesin,[28] another analog of CC-1065. Adozelesin was found to block cells initially in S phase and then subsequently in G2/M. The cell cycle effect of (+)-FDI-CBIM and its free drug (+)-FDI-CBI differs from that of Dox (Fig. 4F), where 12 h exposure to Dox blocked cell proliferation primarily in G2/M phase. This difference between the effects of (+)-FDI-CBIM and Dox on the cell cycle was more apparent when the drugs were compared in the same experiment as illustrated in Figure 3G. With both (+)-FDI-CBIM and (+)-FDI-CBI more cells were blocked cells in S, fewer in G2/M phase, whereas Dox treated cells were blocked primarily in G2/M.

2.5. Antitumor activity in mouse leukemia model

The antitumor activity of (+)-FDI-CBIM was first tested against L1210 leukemia in mice (Table 2). An optimal dose of (+)-FDI-CBIM (0.4 mg/kg) produced an ILS of 133%. By comparison, Dox, one of the most active drugs versus L1210 used as a positive control, demonstrated an ILS of 85%.

2.6. Antitumor activity in mouse colon cancer model

Colon 38 is a well-characterized mouse colon adenocarcinoma, and has been widely used in the primary anticancer activity screening of new agents. (+)-FDI-CBIM was remarkably active against colon 38 in mice (Table 3). At a dose of 0.3 mg/kg, (+)-FDI-CBIM cured 50% of mice (tumor-free on day 60), and at 0.15 mg/kg, it cured 29% of mice. (+)-FDI-CBIM demonstrated tumor growth inhibition (TGI) of 99% at both the 0.3 and 0.15 mg/kg dose levels. In contrast, the free drug, (+)-FDI-CBI, demonstrated a TGI of only 76%, significantly less efficacious. In sharp contrast, at their MTD, both 5-fluorouracil (5-FU) and CPT-11, two of the most effective drugs against colon cancer in the clinic, produced a TGI of 95% and 96%, respectively, without any cures. At the lowest dose of 0.15 mg/kg, (+)-FDI-CBIM killed 10-times more tumor cells than 5-FU which was administered at a very toxic dose of 70 mg/kg. The $\log_{10}$ cell kill (LCK) is 2.6 and 1.1, respectively. (+)-FDI-CBIM was also significantly better than cisplatin, a drug often used for treatment of colon cancer. Importantly, mice could tolerate a greater weight loss with (+)-FDI-CBIM than with 5-FU or CPT-11. For example, with (+)-FDI-CBIM, the mice did not die with a weight loss of −25% of initial body weight. In contrast, mice treated with 5-FU and CPT-11 began to die when weight loss reached −7%.

Why (+)-FDI-CBIM is more effective than the free (+)-FDI-CBI is not clear presently. We hypothesize that (+)-FDI-CBIM forms HSA conjugates and this contention is supported by the in vitro incubation experiments (Fig. 3). The HSA-(+)-FDI-CBI conjugates are expected to selectively accumulate in tumor tissues leading to

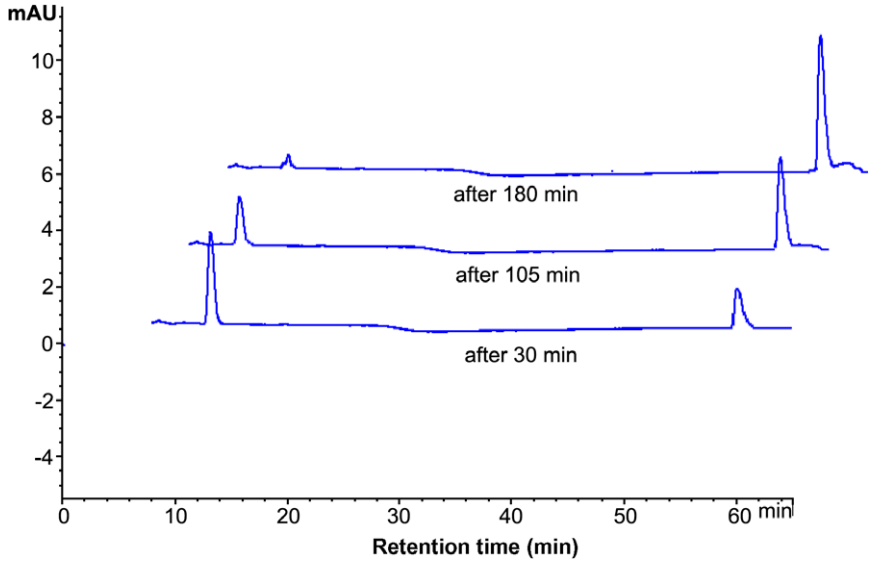


Figure 3. Formation (+)-FDI-CBIM–HSA conjugate.

selective toxicity toward cancer cells. In addition, HSA-(+)-FDI-CBI conjugates may have a longer half-life than the free drug, leading to an increase in bioavailability. Whatever the mechanism, these animal model results support the concept of albumin–drug conjugates as a means to improve drug efficacy.

2.7. Antitumor activity in mouse breast cancer model

JC is an epithelial-like cell line established in 1983 from a spontaneous primary adenocarcinoma along the mouse milk line.[29] JC tumors have a papillary adenocarcinoma morphology in BALB/c mice. (+)-FDI-CBIM was highly active against JC in mice with a TGI of 98% (Table 4 and Fig. 5). (+)-FDI-CBIM was significantly more efficacious than Dox, one of the best drugs against breast cancer. For example, at MTD, while Dox (8 mg/kg) had a TGI of 82%, (+)-FDI-CBIM had a TGI of 98%. At the lowest dose of 0.16 mg/kg, (+)-FDI-CBIM (LCK: 1.5) killed 10-times more tumor cells than Dox at 8 mg/kg (LCK: 0.78).

2.8. Antitumor activity in human ovarian cancer xenografts

Ovarian cancer is currently the most lethal gynecological malignancy in the United States. Although effective therapies exist, the acquisition of multidrug resistance within persisting tumor cell population renders curative therapies elusive for the majority of women with this disease. In clinical trials of bizelesin, one patient with advanced ovarian cancer had a 40% reduction in cancer size lasting for 24 months.[10] Previous work from our laboratory demonstrated that the CC-1065 class of compounds was active against human ovarian cancer in xenograft models (data not shown). For these reasons, (+)-FDI-CBIM was evaluated in a xenograft model of SKOV-3 human epithelial ovarian carcinoma. Since paclitaxel is one of the best chemotherapeutic agents for treatment of ovarian cancer, it was included as a positive control. Dox, another drug used in clinic to treat ovarian cancer was also tested as a positive control. (+)-FDI-CBIM was effective in this xenograft model (Fig. 6 and Table 5), and was more active than both paclitaxel and Dox. (+)-FDI-CBIM showed little toxicity with a weight loss of only 3%.

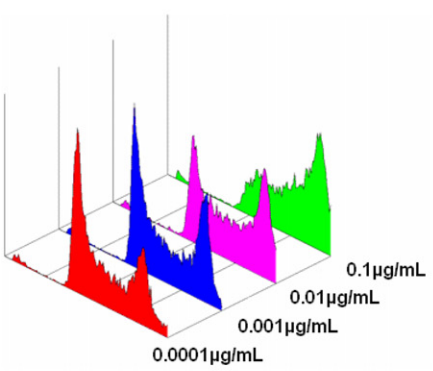
2.9. Preliminary toxicity study

The natural product CC-1065 has delayed toxicity in mice, that is, mice die long after the normal observation period of 15 days for the acute toxicity study.[8] We have previously reported that (+)-FDI-CBI did not cause delayed death.[13,26] To determine if (+)-FDI-CBIM causes delayed death, non-tumor bearing BDF_1 mice were given a dose of 0.5 mg/kg ip of (+)-FDI-CBIM once, and animals observed for 180 days. Although some weight loss was observed, (+)-FDI-CBIM did not cause delayed death.

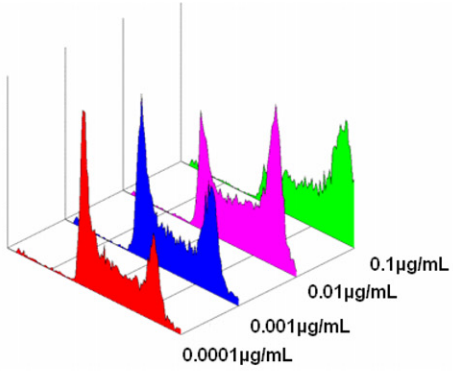
3. Conclusions

To increase therapeutic efficacy and/or reduce toxic side effects of anticancer agents, various drug delivery approaches employing macromolecules have been used. Among these are tumor-specific monoclonal antibody–drug conjugation, liposome encapsulation, pegylation, and so on. These approaches have achieved clinical successes, but their production processes are complex, quality controls are difficult, and thus, the cost is high. The concept of in situ albumin–anticancer drug conjugation to increase therapeutic efficacy and reduce toxic side effects pioneered by Katz and colleagues has several advantages.[21] First of all, this approach takes advantage of the 'enhanced permeability and retention (EPR)' characteristic of tumor tissues.[30–32]. Secondly, albumin itself is a nutrient for tumor cells, leading to selective accumulation in tumor cells; and thirdly, this approach avoids complex and costly production processes.

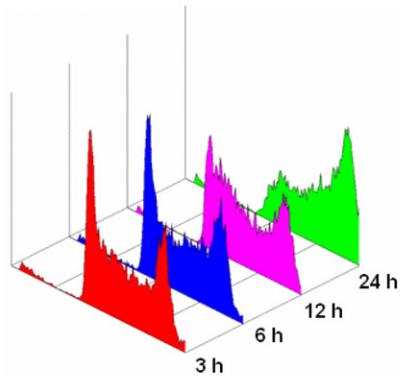
The CC-1065 class of agents is extremely potent, binds to the minor-groove of DNA and causes DNA chain breaks. This mechanism of action differs from other clinically used anticancer drugs. For these reasons, this class of agents has attracted significant interest over the last 30 years. Four drugs of this class, that is, adozelesin, carzelesin, KW-2189 and bizelesin have been evaluated in patients. In the present work, we synthesized (+)-FDI-CBIM, a prodrug of a CC-1065 analog. (+)-FDI-CBIM rapidly reacts with human albumin in vitro. Evaluation of (+)-FDI-CBIM in animal tumor models revealed that antitumor efficacies of the albumin-binding prodrug (+)-FDI-CBIM were significantly improved compared to the free drug (+)-FDI-CBI in several animal tumor models. Furthermore, 3-fold more prodrug can be administered without increasing weight loss, an indication of drug toxicity. This work further validates the concept of anticancer drug delivery by forming drug–albumin conjugates in situ. More work evaluating this promising anticancer agent is in progress, and we will report the results in due course.



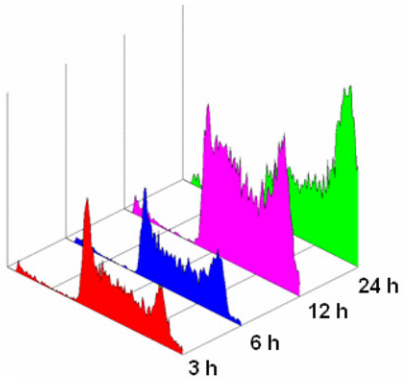
A [(+)-FDI-CBIM]



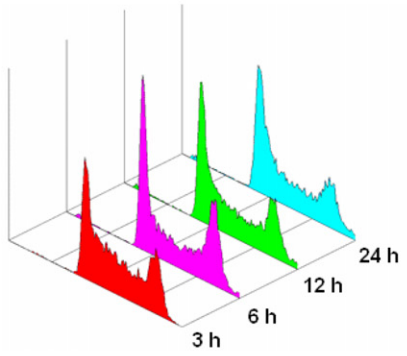
B [(+)-FDI-CBI]



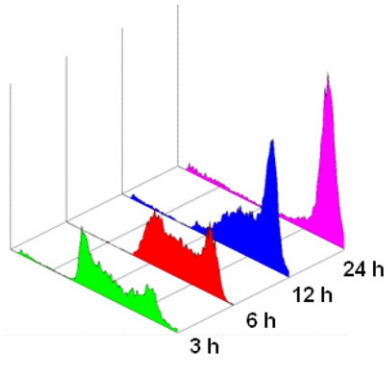
C [(+)-FDI-CBIM, 0.1μg/mL]



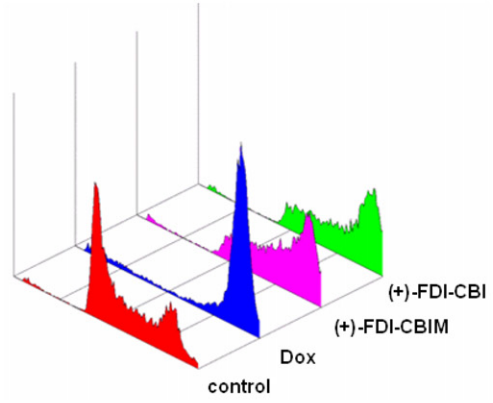
D [(+)-FDI-CBI, 0.1μg/mL]



E (control)



F (Dox, 1μg/mL)



G [Dox, 1μg/mL; (+)-FDI-CBIM, 0.1μg/mL; (+)-FDI-CBI, 0.1μg/mL]

Figure 4. Effects on cell cycle.

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

Drug	Dose (mg/kg)	%ILS
(+)-FDI-CBIM	0.4	133
	0.2	107
	0.1	51
	0.05	40
Doxorubicin	10.0	85

Drugs were administered iv on day 1. The median number of days of survival of the vehicle-treated mice was 7.5.
[a] Female BDF_1 mice (6/group) were injected ip with 10^5 cells on day 0.

4. Experimental

4.1. Chemistry

Melting points were measured using a Mel-Temp II and are uncorrected. ^1H NMR spectra were recorded at ambient temperature on an NT-400 spectrometer. 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. Synthesis of 1-(chloromethyl)-3-[[5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl]-5-(6-hexylmaleimide)oxy-1,2-dihydro-3*H*-benz[*e*]indole [(+)-FDI-CBIM]

(+)-FDI-CBI was synthesized as we previously described.[12,25] To acetonitrile (1.6 mL) were added (+)-FDI-CBI (19 mg, 0.034 mmol), 6-maleimidocaproic acid (22 mg, 0.103 mmol), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 17 mg, 0.045 mmol), and diisopropylethylamine (0.045 mL). The reaction mixture was allowed to proceed overnight. The product was extracted with ethyl acetate, and the organic phase was washed with water. The solution was dried by anhydrous sodium sulfate, and the solution was filtered. Solvent was removed in vacuo, and the product was purified by thin-layer chromatography to afford 10 mg of product in 26% yield as an off

Table 3
Antitumor activity of (+)-FDI-CBIM in mice bearing colon 38[a]

Drug	Dose (mg/kg)	Schedule	%TGI	%Maximum weight loss	Log_{10} cell kill	%Cured mice
(+)-FDI-CBIM	0.3	q4d × 2	99	−25	2.6	50
	0.15	q4d × 3	99	−10	2.3	29
(+)-FDI-CBI	0.05	q4d × 3	76	−7	0.68	0
5-FU[b]	70	q4d × 3	95	−7	1.1	0
CPT-11[c]	100	q4d × 3	96	−7	1.7	0
Cisplatin	2	q4d × 3	42	−8	0.30	0

All drugs were given iv.
[a] Female BDF_1 mice were implanted sc with 10^6 cells on day 0.
[b] 4/6 mice died of drug toxicity.
[c] 1/6 mice died of drug toxicity.

Table 4
Antitumor activity of (+)-FDI-CBIM in mice bearing JC breast cancer[a]

Drug	Dose (mg/kg)	Schedule	%TGI	Log_{10} cell kill (LCK)	%Maximum weight loss
(+)-FDI-CBIM	0.25	q4d × 2	98	2.0	−21
	0.20	q4d × 3	98	1.9	−23
	0.16	q4d × 3	93	1.5	−19
Doxorubicin	8.0	q4d × 3	82	0.78	−25

[a] Female Balb/c mice (8/group) were implanted sc with 10^6 cells on day 0. All drugs were given iv. For all tested drugs, $P < 0.01$ compared with non-drug treated animals.

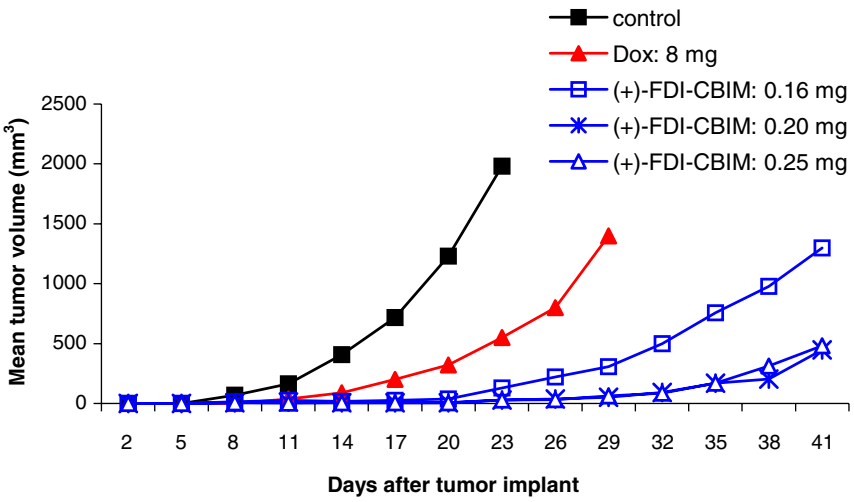


Figure 5. Anticancer activity in mice bearing JC breast cancer.

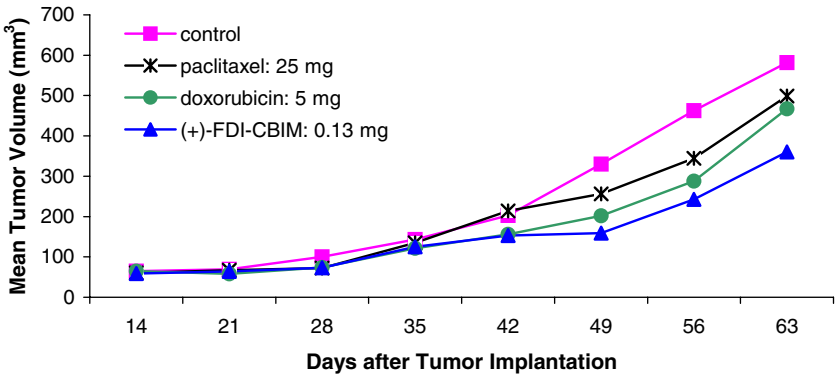


Figure 6. Anticancer activity in SKOV-3 ovarian xenograft.

Table 5
Anticancer activity in SKOV-3 ovarian xenograft[a]

Compound	Dose (mg/kg)	%Weight change[b]	%TGI
(+)-FDI-CBIM	0.13	−3	52
Paclitaxel	25	−1	22
Doxorubicin	5	−4	39

Drugs were given iv on days 15, 24, and 33.
[a] Nude CD1 female mice(7–9/group) were implanted sc with 5×10^6 cells on day 0.
[b] Maximum weight loss. Paclitaxel was given ip. For all tested drugs, $P < 0.01$ compared with non-drug treated animals.

white powder. [α] +16.9° (*c* 0.75, DMSO-d_6); ^{1}H NMR (DMSO-d_6, ppm): 11.84 (s, 1H, NH), 11.76 (s, 1H, NH), 10.23 (s, 1H, NH), 8.24–7.03 (m, 15H), 4.97–4.90 (t, 1H, *J* = 19.85 Hz, NC*H*H), 4.68–4.65 (dd, 1H, *J* = 2.40, 11.19 Hz, NCH*H*), 4.50–4.40 (m, 1H, $ClCH_2CHCH_2$), 4.13–4.09 (dd, 1H, CH*H*Cl), 4.06–4.01 (dd, 1H, *J* = 6.80, 11.59 Hz, CHHCl), 3.45 (t, 2H, *J* = 13.99 Hz, CH_2), 2.83 (t, 2H, *J* = 14.79, CH_2), 1.77–1.71 (m, 2H, CH_2), 1.62–1.56 (m, 2H, CH_2), 1.42–1.36 (m, 2H, CH_2); HRMS calcd for $C_{41}H_{33}ClFN_5O_6Na$ 768.1996, found 768.1995.

4.2. Cytotoxicity

4.2.1. Cell lines

Mouse L1210 leukemia cells were from American Type Culture Collection (ATCC, Manassas, VA), 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 l 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.

4.3. Analysis of albumin-(+)-FDI-CBIM conjugate formation

(+)-FDI-CBIM was dissolved in DMF to make a stock solution at a concentration of 0.5 mg/mL. Human albumin (Guangzhou Beiyi Pharmaceuticals, Guangdong, China; HSA > 80%) was dissolved in a buffer containing 0.15 M sodium chloride, 4 mM sodium phosphate at pH 7.4 to make a 20% solution. (+)-FDI-CBIM solution (0.5 mL) was added to 8 mL of HSA solution at 37 °C, and the reaction mixture was mixed well. At the indicated time, an aliquot (50 μL) of the reaction solution was removed and was analyzed by HPLC. The products were eluted by a gradient of solutions, of which phase A was a solution of acetonitrile:water:diethylamine (66/33.5/0.5) with a flow rate of 1 mL/min and phase B was 100% acetonitrile. The products were detected at a wavelength of 320 nm. At 320 nm, (+)-FDI-CBIM, but not HSA, was detected. Therefore, there were basically only two peaks detected, one of (+)-FDI-CBIM, and the other the (+)-FDI-CBIM–HSA conjugate.

4.4. Effects on cell cycle

The proportion of cells at different phases of the cell cycle was monitored by a flow cytometer. The DNA of cells was stained with propidium iodide (PI). Briefly, exponentially growing cells (2.0×10^5 cells/mL) were incubated in 6-well microtiter plates with medium containing various concentrations of drugs in a final volume of 4.0 mL. After drug treatment, cells were collected and washed with PBS, then fixed and permeabilized in 70% ice-cold ethanol overnight at −20 °C. After washing, fixed cells were incubated with RNase A (200 μg/mL, Sigma) for 1 h at 37 °C and PI in PBS for 30 min. Stained cells were then filtered through 47 μm pore-size nylon meshes to ensure a single cell suspension and immediately subjected to analysis by the flow cytometer (Beckman Coulter, Miami, USA).

4.5. Antitumor activity in mouse tumor models

4.5.1. L1210 leukemia

L1210 leukemia cells (10^5 cells/mouse, 0.1 mL) were injected ip into male BDF_1 mice (6/group) on day 0. Drugs were administered ip on day 1. 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. The median number of days the untreated group of mice (given the vehicle only) died was 6.5. Animals were monitored and weighed daily.

4.5.2. Colon 38 colon cancer

Colon 38 was obtained from the Developmental Therapeutics Program Tumor Depository (Frederick, MD) at NCI, and was maintained by continuous sc passage by implanting 1×10^6 cells in syngeneic female $C_{57}BL/6$ mice. For antitumor efficacy studies, female BDF_1 mice (18–22 g, 8/group) were implanted with 1×10^6 cells sc on day 0. Drugs (0.2 mL/mouse) were given iv on days 1, 5, and 9.

Antitumor activity was expressed as the percentage of tumor growth inhibition (TGI), which was calculated using the formula: $TGI = (1 - T/C) \times 100\%$, where T is the mean treated-tumor weight and C is the mean control-tumor weight. Tumors were measured in two dimensions by caliper, and the recorded measurements are converted to tumor mass using the formula: $[V = (a \times b^2)/2]$, where a is the longer, and b the shorter dimension. TGI was determined when tumor weighed approximately 1–1.5 g. Cured animals were excluded from TGI calculations, and were noted separately. Those animals that were tumor-free on day 60 are considered cured. Log_{10} cell kill $= T - C(\text{days})/3.\ 32 \times \text{Td}$, where $T - C$ represents the time taken for the cells to grow, which is the mean time in days for the tumors of the treated group (T) to reach a predetermined value (1 g for example) and the tumors of the control group (C) to reach the same value, and Td represents the time in days needed for the volume of the tumor in the control group to double.[33]

4.5.3. JC breast cancer

The murine mammary adenocarcinoma JC cell line was obtained from ATCC, and was maintained by continuous sc passage by implanting 1×10^6 cells in female Balb/C (Charles River) mice. For antitumor efficacy studies, female Balb/C mice (8/group) were implanted with 1×10^6 cells sc on day 0. Drugs (0.2 mL/mouse) were given iv on days 1, 5, and 9. Antitumor activity was evaluated using the same method as that described in the colon cancer model.

4.5.4. Ovarian tumor xenografts

SKOV-3 was obtained from the ATCC. Female nude mice (CD1, nu/nu), 19–22 g, 10 mice/group, were implanted sc with SKOV-3 (10^7 cells/mouse), and drugs were administered iv via the tail vein when the tumor grew to approximately 100 mg. Paclitaxel was given ip because of its poor solubility in water. Drugs (0.2 mL/mouse) were given. Antitumor activity was evaluated using the same method as that described in the colon cancer model.

4.5.5. Preliminary toxicity study

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

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (CA82949 to Y.W.) and the Science and Technology Departments of Guangzhou City (2006Z3-E4071 to Y.W.) and Guangdong province (06300573 to Y. W).

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