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Design, Synthesis, and Biological Evaluation of a Cephalosporin–Monohydroguaiaretic Acid Prodrug Activated by a Monoclonal Antibody–β-Lactamase Conjugate

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Abstract—A novel cephalosporin derivative of monohydroguaiaretic acid (cephem-M_3N, **7**) was synthesized and found to possess anticancer activity against human leukemia (K562), breast carcinoma (MCF7), human lung cancer (A549), human colon cancer (Colo205) and pancreatic cancer cells (Capan2 and MiaPaCa2). A tumor targeting fusion protein (dsFv3–β-lactamase) was also used in conjunction with cephem-based M_3N **7** and its potency toward K562, MCF7, A549, Colo205, Capan2, and MiaPaCa2 was found to approach that of the free M_3N (**4**). In the presence of dsFv3–β-lactamase, tumor cells were found to be much more susceptible to conjugate **7** than normal human embryonic lung (HEL) cells and normal fibroblasts (Hef522). These notions provide a new approach to the use of nordihydroguaiaretic acid (NDGA) and its derivatives for antitumor therapy.

Introduction

Nordihydroguaiaretic acid (NDGA, **5**) is a versatile molecule which has an antitumor capability probably related with the inhibition of the lipoxygenases (LOX) activity.[1,2] This antioxidant[3] molecule possesses a wide range of pharmacological activities, including the inhibition of the human papillomavirus,[4] herpes simplex,[5] human immunodeficiency virus,[6] and protects against TPA-induced tumor promotion in mouse skin,[7] TPA-caused induction of ornithine decarboxylase[8] and protein kinase C,[9] as well as having hyperglycemic activity.[10,11] NDGA (**5**) inhibits various kinds of injuries, neoplasm,[12] progestron production,[13] StAR protein expression,[13] and fos–jun–DNA complex formation.[14] Disruption of transcription factor action, fos-jun dimerization, has been shown to impair the transcriptional activation and cell transformation regulated by these proteins.[14]

The mechanism of NDGA in cell differentiation and apoptosis is one of the most important topics under investigation in biology.[15] It was reported[16] that 5-LOX and 12-LOX are upregulated in human pancreatic cancer cells and that blockade of these enzymes by NDGA abolishes the cancer cell growth and induces apoptosis.[17] No specific cell phase arrest following treatment with LOX inhibitor **5** was observed, suggesting that inhibition of pancreatic cancer cell proliferation occurred in all phases of the cell cycle.[16] This is consistent with the effects of LOX metabolites in lung cancer cells.[18] On the other hand, apoptosis induced by LOX inhibitors in breast carcinoma cells was found to be

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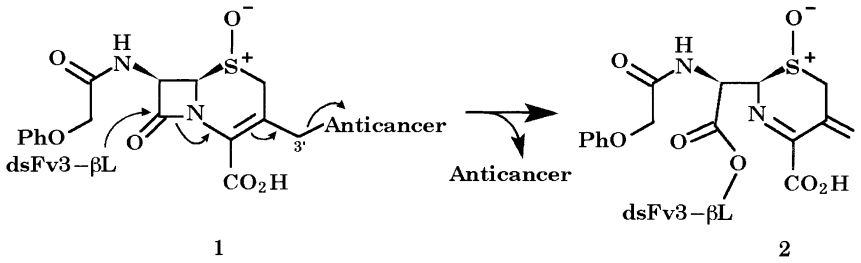

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Scheme 1. Liberation of an anticancer agent from prodrug **1** upon enzyme activation.

accompanied by rapid down-regulation of Bc1-2 protein and dramatic decrease in Bc1-2/Bax ratio, suggesting the involvement of Bc1 proteins in this apoptotic process.[17] Mitochondrial permibility induction and release of cytochrome C by 5-LOX activating protein inhibitors are another critical molecular events for initiating massive apoptosis of human prostate cancer cells.[19,20] Blockade of LOX by NDGA (**5**) also increased intracellular carbonic anhydrase II activity in pancreatic cancer cells, indicating that LOX blockade induced a more differentiated phenotype in the cancer cells.[15,21] As such, perturbation of LOX activity and/or blockade of LOX enzymes by NDGA (**5**) can be associated with induction of apoptosis and cancer cell differentiation. Unfortunately, NDGA (**5**) possesses considerable toxicity in vivo[22] (i.e., long term feeding studies in rats induced lesions in the mesenteric lymph nodes and kidneys). As a result, the compound was removed from the Food and Drug Administration's (FDA's) list. Moreover, despite studies showing that NDGA (**5**) has an anticancer effect in vitro, earlier research by the National Cancer Institute found no such effect in vivo, and even some reports suggest that **5** may stimulate certain malignancies such as renal cell carcinoma.[23]

Chemical modification of the hydroxyl groups[6] in NDGA (**5**) could lead to new derivatives with high potency and low toxicity. Results from biological evaluation of methylated NDGA[6] reveal that permethylated catechol moiety of NDGA (**5**) [i.e., tetra-*O*-methyl NDGA (M_4N) and tri-*O*-methyl NDGA (M_3N, **4**)] possesses good lipophilicity and thus could assist these compounds penetrating the cell membrane.[6] It was claimed that the injection of the water insoluble M_4N into experimentally induced mouse tumors resulted in arrest of tumor growth, followed by the apoptosis and necrosis of the tumor with minimal toxicity to the surrounding normal tissues.[24] Nevertheless, when M_4N[6] was injected into malignant tumors of 25 oral cancer patients, they exposed to toxic side effects; yet it was claimed that in 50% of cases cancerous tumor regressed.[24] To this end, a change in strategy for the selective differentiation of the malignant tumor cells by these versatile molecules, M_4N, M_3N (**4**), and NDGA (**5**), is needed.

The mechanism by which most β-lactamases (βLs) inactivate β-lactam antibiotics is through the acylation of a serine residue at the active site of the enzyme.[25–34] In the case of cephalosporins, a potential leaving group at the C-3′ position is eliminated during the enzymatic reaction,[27,29,34] which most probably proceeds in two steps.[28,33] The leaving group could be an anticancer agent (i.e., **4**). Thus, the enzymatic acylation of the cephalosporin moiety by a serine residue in endogenous proteins releases the anticancer agent.[30,31]

The advent of monoclonal antibodies has revolutionized many aspects of drug development in tumor immunotherapy, where monoclonal antibodies have been used as directed messengers through conjugation, delivery, and release of drugs at the tumor site.[35–42] Most of the cytotoxic agents exert their activities once inside the cell, requiring that the monoclonal antibody carrier facilitate the delivery of the drug to its precise site of activity within the cell. An exciting development in this area of research has involved the conjugation of specific enzymes to monoclonal antibodies, and the enzymatic release of a drug at the tumor site.[42] In principle, one can deliver many drugs to the tumor site with each immunoconjugate molecule and limitations in cancer chemotherapy could perhaps be overcome.

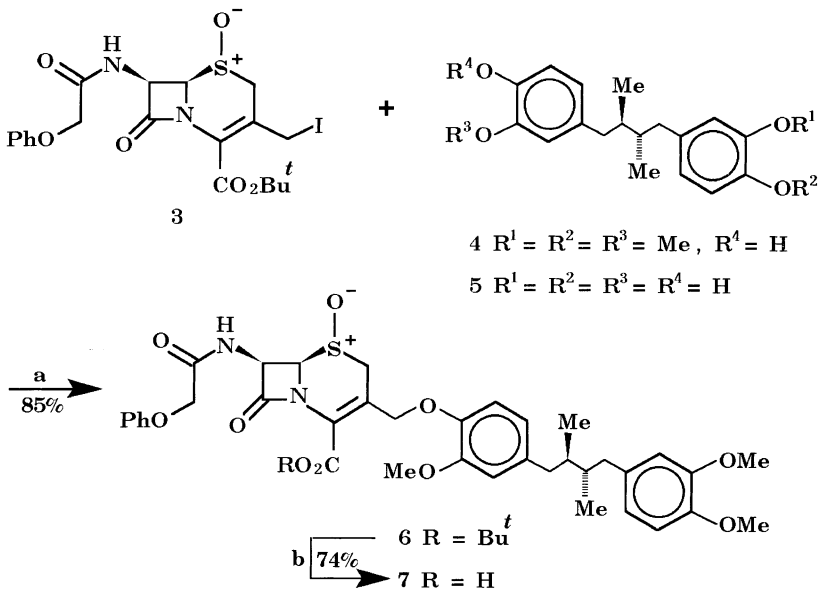
Cephalosporins are highly versatile substrates in the construction of enzyme-activatable prodrugs, including nitrogen mustard drugs,[37,38] a carboplatin analogue,[39] doxorubicin.[40–42] and retinoic acid.[43] βLs are an attractive choice of enzymes for prodrug activation because of their high catalytic efficiency and substrate specificity.[36] Thus, we planned to attach an anticancer agent [e.g., M_3N (**4**)] onto a β-lactam antibiotic at the C-3′ position as shown in Scheme 1.

Herein, we report the chemical synthesis and anticancer property of a new M_3N–cephem conjugate. It includes M_3N attached to a cephalosporin at the C-3′ position (i.e., **7**). Enzyme-activatable prodrug **7** in conjunction with antibody–enzyme fusion protein (dsFv3–βL)[36] exhibited comparable activity to that of M_3N (**4**) against K562, MCF7, A549, Colo205, Capan2, and MiaPaCa2. Its toxicity, however, was found to be much less toward HEL and Hef522 cells.

Results

Synthesis of cephalosporin 3′-M_3N 7 (Scheme 2)

For the preparation of cephem-M_3N conjugate **7**, we condensed 1-oxo-3′-iodocephalosporin **3**[34,44,45] with the sodium salt of M_3N (**4**) to produce the desired intermediate **6** in 85% yield. Conversion of **6** to prodrug **7** (74% yield) was then accomplished by use of CF_3CO_2H-anisole in CH_2Cl_2 at 25 °C.



Scheme 2. Synthesis of prodrug **7**. Reagents: (a) K_2CO_3, DMF, 25 °C, 6.0 h; (b) CF_3CO_2H-anisole, CH_2Cl_2, 25 °C, 2.0 h.

Table 1. Inhibitory concentrations[a] (IC_{50}, μmol) of compounds **4**, **5**, **7**, **7** + βL, and **7** + dsFv3-βL on the growth of malignant cell lines and normal cells in vitro

Compd	K562	MCF7	A549	Colo205	Capan2	MiaPaCa2	HEL	Hef522
4	0.14±0.02	0.08±0.01	0.06±0.00	0.13±0.02	0.28±0.01	0.76±0.05	0.08±0.01	0.05±0.00
5	0.07±0.01	0.11±0.02	0.07±0.01	0.18±0.03	0.31±0.01	0.64±0.03	0.07±0.02	0.07±0.02
7	1.22±0.10	0.98±0.06	0.29±0.02	1.15±0.07	0.92±0.12	1.01±0.08	14.54±1.30	18.12±2.01
7 + βL	0.05±0.01	0.02±0.00	0.02±0.00	0.07±0.01	0.11±0.02	0.27±0.01	0.09±0.02	0.04±0.01
7 + dsFV3-βL	0.04±0.00	0.01±0.00	0.02±0.00	0.08±0.01	0.09±0.01	0.33±0.02	16.35±2.13	21.68±1.01

[a]IThe IC_{50} values were estimated from dose–response curves compiled from at least two independent experiments and represent the compound concentration required to inhibit cell proliferation by 50%.

Solubility and stability of M_3N–cephem conjugate 7 in water

We found that the solubility of M_3N (**4**) and cephalosporin 3′-M_3N **7** in water was, respectively, 0.08 and 1.75 mg/mL. Conjugate **7** was also stable at physiological pH for >20 days. However, at pH = 12, the β-lactam ring in **7** decomposed within 7.0 min. After neutralization of the basic solution, M_3N (**4**) was isolated in 90% yield and characterized by ^{1}H NMR. At pH = 2.5, prodrug **7** underwent hydrolysis to liberate its M_3N (**4**) component after 1.6 h.

Biological activity

Enzymatic hydrolysis study of cephalosporin–M_3N conjugate 7 by ^{1}H NMR. A mixture of DMSO-d_6 and phosphate buffer solution (pD 7.2, 1:1 mL/mL) was used for the ^{1}H NMR study of βL catalyzed hydrolysis.[39] In the presence of sufficient βL from *Escherichia coli* 27C7, the ^{1}H NMR spectrum of **7** changed rapidly to that of the eliminated compound **4** as judged by ^{1}H NMR and HPLC analyses. In a control experiment, in the absence of the βL, **7** was stable to hydrolysis for >20 days at 25 °C.

Kinetic parameters for purified βL and dsFv3-βL with cephalosporin–M_3N conjugate 7. βL and dsFv3-βL were secreted from *E. coli* 27C7 and purified according to an established procedure.[36b] Kinetic parameters for βL and dsFv3-βL with retinoid **7** were determined by HPLC method as described.[36c] The concentrations of prodrug **7** and the resultant M_3N (**4**) were monitored by absorbance at 280 nm. Conjugate **7** was found to be an excellent substrate for βL (k_{cat} 3570 s^{-1}, K_M 91 μM; k_{cat}/K_M 39 s^{-1} μM^{-1}) and dsFv3-βL (k_{cat} 3230 s^{-1}, K_M 80 μM; k_{cat}/K_M 40 s^{-1} μM^{-1}).

Anticancer activity. The anticancer screening experiments for compounds **4**, **5**, **7**, **7** + βL from *E. coli*, and **7** + dsFv3-βL, were carried out in vitro against human leukemia (K562), breast carcinoma (MCF7), human lung cancer (A549), human colon cancer (Colo205) and pancreatic cancer cells (Capan2 and MiaPaCa2).[46] The activity is expressed as the concentration (μmol) required to inhibit tumor cell proliferation by 50% (IC_{50}). Their toxicity toward normal human embryonic cell line (HEL) and normal fibroblasts (Hef522) were also determined. Results are summarized in Table 1.

Discussion

NDGA (**5**) and its derivatives have been shown to possess activity against malignant tumor cells.[16–24] Cephalosporins can react with transpeptidases[25,29] and/or β-lactamases.[36] By applying the dual targeting approach,[47]

we first combined cephalosporin-1-oxide (**3**) with M_3N (**4**) to produce novel anticancer agent **7**. In the presence of a βL from *E. coli* 27C7, oxocephem–M_3N conjugate **7** showed comparable anticancer activity relative to that of **4**. These results indicate that the M_3N (**4**) component can be liberated effectively from **7** at the C-3′ position through a βL induced 1,4-elimination,[34] as shown in Scheme 1. In fact release of M_3N (**4**) from cephalosporin 3′-M_3N **7** by the βL, results in anticancer activity better than that of the M_3N (**4**). This could be attributed to the 25-fold increment in water solubility of **7** compare to **4**.

Prodrugs which are rapidly hydrolyzed by βL, and which exhibit high affinity for the enzyme (high k_{cat}/K_M) are expected to offer a therapeutic advantage in an antibody-directed catalysis delivery system. In the presence of the tumor-targeting fusion protein, dsFv3–βL, prodrug **7** was active (k_{cat}/K_M $40\,s^{-1}\,\mu M^{-1}$) against K562, MCF7, A549, Colo205, Capan2, and MiaPaCa2 and offer a similar activity to that M_3N (**4**); yet its toxicity was not enhanced against HEL and Hef522. This could be due to the capability of monoclonal antibody–βL conjugate (i.e., dsFv3–βL) to bind to cell surface antigenes. Consequently, binding activity of monoclonal antibody may be correlated with the ability of the conjugate to activate prodrugs. This strategy can be utilized in selective tumor directed chemotherapy.

Conclusions

Cephalosporin 3′-M_3N conjugate **7** was prepared through condensation of 1-oxo-3′-iodocephalosporin (**3**) with M_3N (**4**). Prodrug **7** was found to be activated by a βL or the targeting fusion protein, dsFv3–βL, and exhibited remarkable inhibitory activity against human leukemia (K562), breast carcinoma (MCF7), human lung cancer (A549), human colon cancer (Colo205) and pancreatic cancer cells (Capan2 and MiaPaCa2). These results indicate that the M_3N (**4**) component could be effectively released from **7** through a 1,4-elimination process.

Experimental

General methods

For anhydrous reactions, glassware was dried overnight in an oven at 120 °C and cooled in a desiccator over powdered anhydrous calcium sulfate (i.e., $CaSO_4$) or silica gel. Reagents were purchased from Fluka (Switzerland). Solvents, including chloroform, dichloromethane, dimethylformamide, ethyl acetate, hexanes, and pyridine were distilled over CaH_2 under nitrogen. Ethanol was purchased from Merck (Germany) and used as received. Reactions were performed under a nitrogen atmosphere with shielding from light; the apparatus was evacuated and filled with dry nitrogen at least three times.

Melting points were obtained with a Büchi 510 melting point apparatus. Ultraviolet (UV) spectra were recorded on a Cary 118 spectrophotometer and λ_{max} are reported in nm (ε) units. Infrared (IR) spectra were recorded on a Beckman IR-8 spectrophotometer. The wavenumbers reported are referenced to the 1601 cm^{-1} absorption of polystyrene. Proton NMR spectra were obtained on a Varian XL-300 (300 MHz) spectrometer. Chloroform-*d* and D_2O were used as solvent; Me_4Si (δ 0.00 ppm) was used as an internal standard. All NMR chemical shifts are reported as δ values in parts per million (ppm) and coupling constants (*J*) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; br, broad; and m, unresolved multiplet due to the field strength of the instrument. Mass spectra were carried out on a VG 70–250 S mass spectrometer. Microanalysis were performed on a Perkin–Elmer 240-B microanalyzer. Purification on silica gel refers to gravity column chromatography on Merck silica gel 60 (particle size 230–400 mesh). Analytical TLC was performed on precoated plates purchased from Merck (silica gel 60 F_{254}); compounds were visualized by use of UV light.

(±)-1-(3,4-Dimethoxyphenyl)-(2*R*,3*S*)-dimethyl-4-[3-methoxy-4-[(7-phenoxyacetamido)-(1-oxo)-3-cephem-4-*tert*-butoxycarbonyl-3-methoxyphenyl]butane (6). To a solution of **4** (1.69 g, 4.90 mmol) in DMF (40 mL) was added K_2CO_3 (2.10 g, 15.0 mmol). After 15.0 min, to the stirred mixture was added **3** (2.62 g, 4.90 mmol) and further stirred at 25 °C for 6 h. It was then filtered. The filtrate was diluted with EtOAc (280 mL) and H_2O (200 mL). The organic layer was separated and washed with H_2O (3×150 mL). Then it was dried over $MgSO_4$ (s), filtered, and concentrated under reduced pressure. Purification of the residue by use of silica gel column chromatography with $CHCl_3$ as eluant afforded **6** (3.17 g, 4.16 mmol) in 85% yield: mp 128–129 °C; UV λ_{max} (EtOH) 281 nm (ε 7,150); IR (CH_2Cl_2) 3343 (NH), 1790 (β-lactam), 1745 (ester), 1680 (amide), 1150–1031 (ether) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 0.85 (d, $J=6.7$ Hz, 3H, CH_3), 0.86 (d, $J=6.6$ Hz, 3H, CH_3), 1.48 (s, 9H, $(CH_3)_3C$), 1.70–1.84 (m, 2H, 2×CH), 2.27 (dd, $J=13.4$, 1.7 Hz, 1H, CHPh), 2.31 (dd, $J=13.4$, 1.7 Hz, 1H, CHPh), 2.72 (dd, $J=13.4$, 5.5 Hz, 1H, CHPh), 2.75 (dd, $J=13.4$, 5.5 Hz, 1H, CHPh), 3.80 (d, $J=16.5$ Hz, 1H, HCSO), 3.83 (s, 6H, 2×OCH_3), 3.86 (s, 3H, OCH_3), 3.97 (d, $J=16.5$ Hz, 1H, HCSO), 4.29 (br s, 2H, CH_2O), 4.60 (br s, 2H, OCH_2CO), 5.27 (d, $J=4.8$ Hz, 1H, HC(6)), 5.78 (d, $J=4.8$ Hz, 1H, HC(7)), 6.59–7.34 (m, 11H, 3×Ph); MS: 762 (M^+).

(±)-1-(3,4-Dimethoxyphenyl)-(2*R*,3*S*)-dimethyl-4-[3-methoxy-4-[(7-phenoxyacetamido)-(1-oxo)-3-cephem-4-hydroxycarbonyl-3-methoxyphenyl]butane (7). To a solution of **6** (2.82 g, 3.70 mmol) in dry CH_2Cl_2 (100 mL) was added anisole (0.22 g, 2.0 mmol) and CF_3CO_2H (2.85 g, 25.0 mmol). The reaction mixture was stirred at 25 °C under N_2 for 2.0 h. Then, it was concentrated under reduced pressure and the residue was purified by silica gel column chromatography with EtOAc as eluant to give **7** (1.93 g, 2.74 mmol) in 74% yield: mp 141–143 °C; UV λ_{max} (EtOH) 280 nm (ε 6,895); IR (CH_2Cl_2) 3340–3410 (NH, COOH), 1788 (β-lactam), 1704 (C=O), 1678 (amide), 1100–1037 (ether) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 0.84 (d, $J=6.8$ Hz, 3H,

CH_3), 0.85 (d, $J=6.7$ Hz, 3H, CH_3), 1.69–1.79 (m, 2H, 2×CH), 2.31 (dd, $J=13.6$, 1.5 Hz, 1H, CHPh), 2.35 (dd, $J=13.6$, 1.5 Hz, 1H, CHPh), 2.72 (dd, $J=13.6$, 5.7 Hz, 1H, CHPh), 2.75 (dd, $J=13.6$, 5.7 Hz, 1H, CHPh), 3.78 (d, $J=17.0$ Hz, 1H, HCSO), 3.82 (s, 6H, 2×OCH_3), 3.85 (s, 3H, OCH_3), 3.99 (d, $J=17.0$ Hz, 1H, HCSO), 4.31 (br s, 2H, CH_2O), 4.62 (br s, 2H, OCH_2CO), 5.28 (d, $J=5.0$ Hz, 1H, HC(6)), 5.80 (d, $J=5.0$ Hz, 1H, HC(7)), 6.60–7.39 (m, 11H, 3×Ph); MS: 706 (M^+). Anal. calcd for $C_{37}H_{42}N_2O_{10}S$: C, 62.88; H, 5.99; N, 3.96; S, 4.53. Found: C, 62.80; H, 6.01; N, 3.97; S, 4.45.

Determination of solubility of M_3N (4) and M_3N–cephem conjugate 7 in water. A solution of M_3N (**4**) and cephalosporin 3′-M_3N **7** (100 mg) in 1-octanol (15.0 mL) was shaken in a 50-mL volumetric flask with phosphate buffer (0.10 M, 20.0 mL) for 24 h. 1-Octanol was evaporated and the aqueous suspension was agitated for 10 h. This solution was filtered from undissolved solid through a sintered glass funnel (4.0–5.5 mesh ASTM), and the concentration of the solution was determined by UV absorbance.

Enzymatic hydrolysis of cephalosporin–M_3N conjugate 7 in a mixture of DMSO-d_6 and phosphate buffer (1:1 mL/mL, pD 7.2)—(^{1}H NMR study). Prodrug **7** (0.0706 g, 0.0998 mmol) was dissolved in a mixture of DMSO-d_6 (2.0 mL) and 0.10 M deutrated phosphate buffer (2.0 mL, pD 7.2) at 25 °C. The ^{1}H NMR spectrum was taken at this temperature and then 0.58 mL (9.7 units) of βL (from *E. coli* 27C7) buffer solution was added. The ^{1}H NMR spectrum at 25 °C was taken after 5.0 min; the spectrum of **7** changed to that of the eliminated compound **4**. The mixture was extracted with $CDCl_3$ (2×5.0 mL) to remove M_3N (**4**), which was found to be identical with an authentic sample.

Kinetic procedure. Determination of the kinetic parameters for βL and dsFv3-βL (secreted from *E. coli*)[36b] with the substrate **7** was done according to an established procedure.[36c] Briefly, three sets of vials containing 1.5-mL solutions of varying concentrations of substrate **7** at 37 °C in PBS/DMSO (9.5:0.5, pH 7.2) were treated with 0.15 nM of dsFv3–βL. Individual sets of samples were quenched after 60, 90, and 120 s, respectively, by adding 0.50 mL of the reaction solution to 0.50 mL of 40% CH_3CN in 180 mM KH_2PO_4 (pH 4.2). Samples of the quenched reaction mixtures were injected onto a 0.46-cm×15-cm C_{18} reversed-phase HPLC column (50% CH_3CN as eluant) at 1.0 mL/min. Prodrug **7** and product **4** concentrations were monitored by absorbance at 280 nm. Linear rate plots were used to obtain reaction velocities. K_M and k_{cat} were determined from the slope and intercept of Lineweaver–Burk plots.

Anticancer test procedure in vitro. Human leukemia (K562), breast carcinoma (MCF7), human lung cancer (A549), human colon cancer (Colo205), pancreatic cancer cells (Capan2 and MiaPaCa2), normal human embryonic lung (HEL) cells, and normal fibroblasts (Hef522) were cultured in DMEM supplemented with 10% FBS, 2.0 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C and pH 7–7.3.[46] Under this condition, the generation time for K562, MCF7, A549, Colo205, Capan2, MiaPaCa2, HEL and Hef522 cells was about 15, 14, 16, 17, 19, 19, 28, and 30 h, respectively. Compounds **4**, **5**, **7**, **7** + βL (125 nM), and **7** + dsFv3–βL (125 nM), at various concentrations, were added to K562, MCF7, A549, Colo205, Capan2, MiaPaCa2, HEL and Hef522 cells (280 cells/mL) in their exponential phase of growth. The cell numbers of the control cultures, as well as that of the cultures supplemented with the test compounds, were determined after 24, 48, and 72 h of growth. The IC_{50} values were estimated from dose–response curves compiled from two independent experiments and represent the compound concentration (μmol) required to inhibit proliferation of the respective cell lines by 50% after 72 h incubation (Table 1).

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