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Design and Synthesis of a Cephalosporin-Retinoic Acid Prodrug Activated by a Monoclonal Antibody–β-Lactamase Conjugate

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Abstract—Two novel series of all-*trans*-β-retinoic acid derivatives were synthesized and found to possess anticancer activity. The first series, cephalosporin 3′-retinoic esters **6** and **7** were, respectively, obtained by the condensation of all-*trans*-β-retinoic acid (**2**) with cephalosporins **4** and **5**. The second series, 7-(retinamido)cephalosporins **11** and **12**, were synthesized, respectively, by the condensation of **2** with cephalosporins **9** and **10**. These four heretofore undescribed compounds **6**, **7**, **11**, and **12** showed inhibitory activity against murine leukemias (L1210 and P388), sarcoma 180, breast carcinoma (MCF7), and human T-lymphocytes (Molt4/C8 and CEM/0). They also inhibited squamous metaplasia and keratinization in tracheal organ cultures derived from vitamin-A-deficient hamsters. Moreover, cephalosporin 3′-retinoic ester **7** exhibited enhanced activity against keratinization with $ED_{50} = 3.91 \times 10^{-11}$ M in the presence of a β-lactamase from *Staphylococcus aureus* 95. A tumor targeting fusion protein (dsFv3–β-lactamase) was also used in conjunction with cephem-based retinoid **7** and the potency of **7** toward L1210, P388, and MCF7 was found to approach that of the free retinoic acid (**2**). In the presence of dsFv3–β-lactamase, tumor cells were found to be much more susceptible to retinoid **7** than normal human embryonic lung cells. These notions provide a new approach to the use of β-retinoic acid for antitumor therapy.

Introduction

Retinoids are of importance to epithelial tissue for the maintenance of its normal growth.[1] They also prevent cancer in experimental animals,[2] particularly effective for carcinoma of bladder,[3] breast,[4] and skin.[5] Retinoids can induce differentiation of human promyelocytic leukemia cell line HL-60 from mature granulocytes.[6–8] The mechanism of retinoids in cell differentiation and proliferation is one of the most important topics under investigation in biology.[9,10] All-*trans*-β-retinoic acid often binds to specific nuclear receptors in order to regulate special gene expressions.[11–17] Effective doses of retinoids (e.g., all-*trans*-β-retinoic acid) in most prophylactic studies, however, produce undesirable side effects.[18,19]

Chemical modification of the polar carboxylic group,[20–23] the cyclic end group,[24–26] or the polyene chain[6,27] in all-*trans*-β-retinoic acid could lead to new retinoids with high potency and low toxicity. Results from a systematic study on the structure–activity relationship reveal that retinoic acid derivatives of azetidinone exhibit special affinity towards rapidly growing cells and possess anti-leukemic activity in laboratory animals.[28–30]

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.[31–40] In the case of a cephalosporin, a potential leaving group at the C-3′ position is eliminated during the enzymatic reaction,[33,35,40] which most probably proceeds in two steps.[34,39] The leaving group could be an anticancer agent. Thus, the enzymatic acylation of the cephalosporin moiety by a serine residue in endogenous proteins releases the anticancer agent.[36,37]

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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.[41–48] 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.[48] In principle, one can deliver many drugs to the tumor site with each immunoconjugate molecule and limitations in cancer chemotherapy could perhaps be overcome.

We planned to attach all-*trans*-β-retinoic acid (**2**) onto a β-lactam antibiotic at the C-3′ position as shown in Scheme 1. Cephalosporins are highly versatile substrates in the construction of enzyme-activatable prodrugs, including nitrogen mustard drugs,[43,44] a carboplatin analogue,[45] and doxorubicin.[46–48] βLs are an attractive choice of enzymes for prodrug activation because of their high catalytic efficiency and substrate specificity (see Scheme 1).[42]

As a general program aimed at the development of novel and effective retinoids, we have been investigating the prospects of applying the monoclonal antibody–βL conjugates in releasing the retinoic acid in vivo and the obvious potential in tumor directed chemotherapy. Herein, we report the chemical syntheses and anticancer properties of two classes of new retinoic acid–cephem conjugates. They include all-*trans*-β-retinoic acid attached to cephalosporins at the C-3′ position (i.e., **6** and **7**) and at the *N*-7 position (i.e., **11** and **12**). Enzyme-activatable prodrug **7** in conjunction with antibody-enzyme fusion protein (dsFv3–βL)[42] exhibited comparable activity to that of all-*trans*-β-retinoic acid (**2**) against certain malignant tumor cell lines. Its toxicity, however, was found to be much less toward normal human embryonic lung cells (HEL).

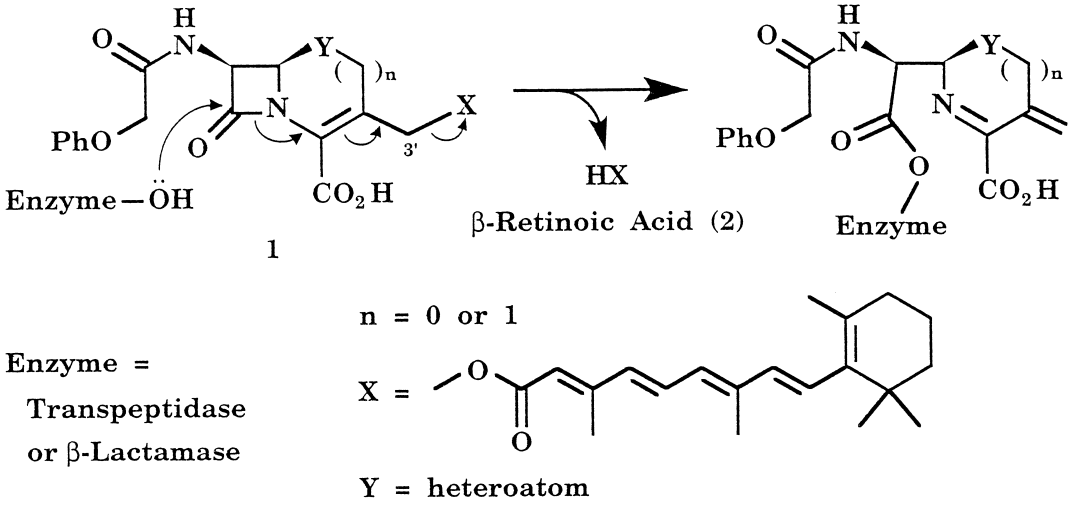
Results

Synthesis of cephalosporin 3′-retinoic esters 6 and 7 (Scheme 2). For the preparation of cephem–retinoic acid conjugate **6**, we silylated (+)-3′-iodocephalosporin **3**[40,49,50] with trimethylsilyl bromide in CH_2Cl_2 (Scheme 2). Without isolation, the resultant silylated ester **4** was treated with the sodium salt of all-*trans*-β-retinoic acid (**2**) in DMF to give the desired ester **6** (48% overall yield) as a single isomer as evidenced by HPLC analysis.

For the synthesis of oxocephem–retinoic acid conjugate **7**, (+)-3′-iodocephalosporin **3** was similarly silylated and then treated, in situ, with 3-chloroperoxybenzoic acid to afford 1-oxocephalosporin **5** (Scheme 2). We then esterified the intermediate **5** with the sodium salt of all-*trans*-β-retinoic acid (**2**) to produce the target ester **7**, as a single isomer, in 40% overall yield.

Synthesis of 7-(retinamido)cephalosporins 11 and 12 (Scheme 3). We considered an alternative way to attach the β-retinoic acid (**2**) onto β-lactams by using their amino group at the *N*-7 position. Accordingly, we silylated (+)-7-aminocephalosporin **8**[40] with trimethylsilyl bromide to give silyl ester intermediate **9** (Scheme 3). In the meantime, all-*trans*-β-retinoic acid (**2**) was treated with ethyl chloroformate in pyridine and CH_2Cl_2 to give the corresponding anhydride. We added this anhydride to the silylated 7-aminocephalosporin **9** to afford a single isomer of retinamido-β-lactam conjugate **11** in 60% overall yield.

To obtain the corresponding *S*-oxide **12**, we treated the silylated intermediate **9** with 3-chloroperoxybenzoic acid in situ to afford sulfoxide **10** (Scheme 3). By the same way described above, all-*trans*-β-retinoic acid (**2**) was converted to the corresponding anhydride, which was added to sulfoxide **10**. In this way, the desired retinamido–β-lactam conjugate **12** was obtained as a single isomer in 42% overall yield.



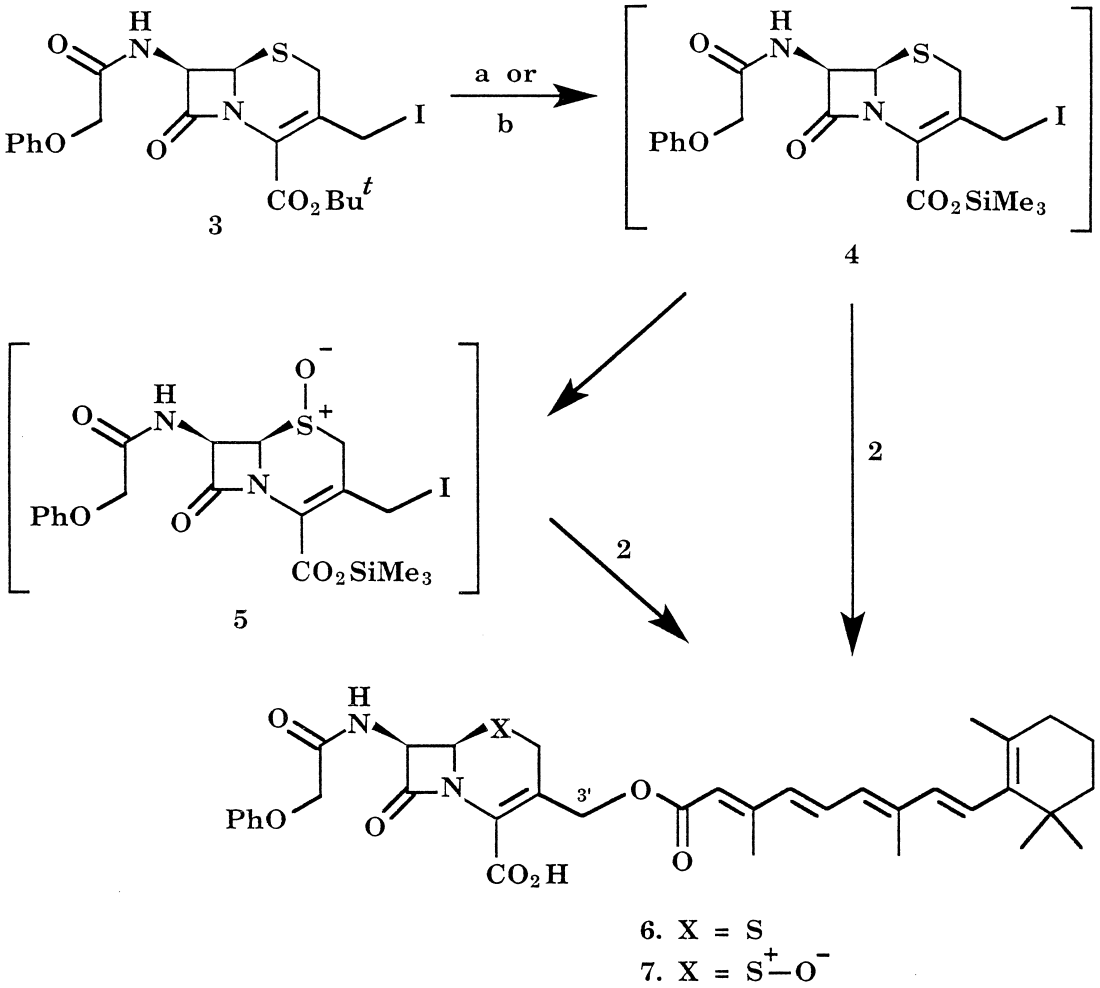
Scheme 1. Liberation of β-retinoic acid (**2**) from β-lactam-containing prodrug **1** upon enzyme activation.

Solubility and stability of retinoic acid–cephem conjugates 6, 7, 11, 12, and retinoic acid (2) in water. We found that the solubility of cephalosporin 3′-retinoic esters **6** and **7** in water was, respectively, 0.16 and 0.32 mg/mL. The compounds were also stable under subdued light at physiological pH for 7 days. The stereochemistry of the side chain remain intact as judged by HPLC and ^{1}H NMR studies. However, at pH = 12, the β-lactam ring in **6** and **7** decomposed within 5 min. After neutralization of the basic solution, all-*trans*-β-retinoic acid **2** was isolated in 87% yield and characterized by ^{1}H NMR. On the other hand, the solubility of 7-(retinamido)cephalosporins **11** and **12** in water was respectively 0.07 and 0.14 mg/mL. They were found to be stable in a phosphate buffer (0.10 M, pH 7.2) in the dark for 25 days. No change in the stereochemistry of the side chain was observed as evidenced by HPLC analysis. However, at pH = 12, decomposition was evident after 35 min, at which time a mixture of unidentified compounds was obtained. It should be noted that the solubility of the reference compound, all-*trans*-β-retinoic acid (**2**), in water, however, was determined to be only 0.008 mg/mL.

Biological activity

Enzymatic hydrolysis study of cephalosporin–retinoic acid conjugate 7 by ^{1}H NMR. Because of the poor solubility of retinoid **7** in neutral phosphate solution, 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.[45] In the presence of sufficient βL from *S. aureus* 95, the ^{1}H NMR spectrum of **7** changed rapidly to that of the eliminated compounds **14** and **2** (Scheme 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 7 days in the dark.

Kinetic parameters for purified βL and dsFv3-βL with cephalosporin–retinoic acid conjugate 7. βL and dsFv3-βL were secreted from *Escherichia coli* 27C7 and purified according to an established procedure.[42b] Kinetic parameters for βL and dsFv3-βL with retinoid **7** were determined by HPLC method as described.[42c] The concentrations of pro-drug **7** and the resultant all-*trans*-β-retinoic acid (**2**) were monitored by absorbance at 352 nm. Retinoid **7** was found to be an excellent substrate



Scheme 2. Synthesis of cephalosporin 3′-retinoic esters **6** and **7**. Reagents: (a) (1) Me_3SiBr, CH_2Cl_2, 25 °C, 10 min; (2) $NaHCO_3$, DMF, 25 °C, 1 h, **3**→**4**→**6** (48%); (b) (1) Me_3SiBr, CH_2Cl_2, 25 °C, 10 min; (2) 3-$ClC_6H_4CO_3H$, 1 h; (3) $NaHCO_3$, DMF, 25 °C, 1 h, **3**→**4**→**5**→**7** (40%).

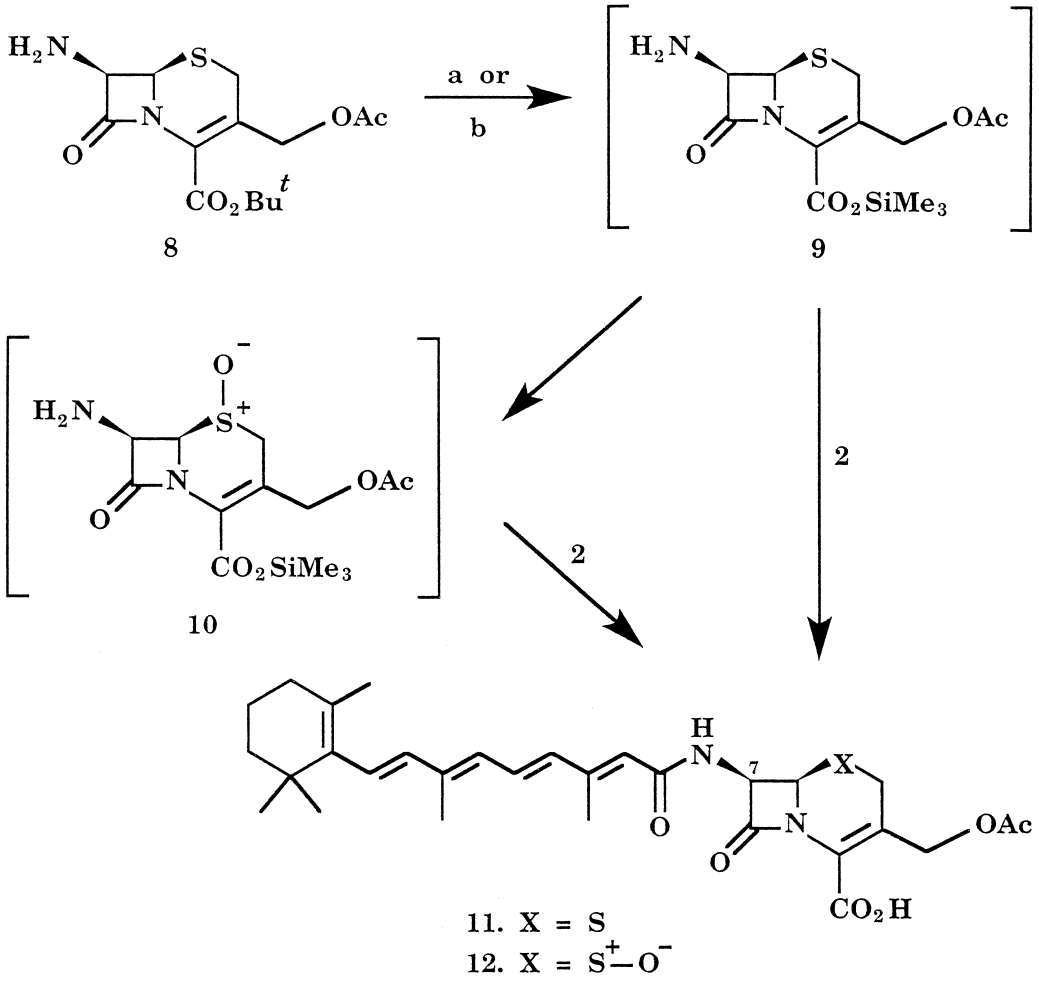
for βL (k_{cat} 2460 s^{-1}, K_M 98 μM; k_{cat}/K_M 25 s^{-1} μM^{-1}) and dsFv3-βL (k_{cat} 1950 s^{-1}, K_M 74 μM; k_{cat}/K_M 26 s^{-1} μM^{-1}).

Inhibition of squamous metaplasia and keratinization. We studied the anti-hyperkeratosis activity of all-*trans*-β-retinoic acid–cephem conjugates **6**, **7**, **11,** and **12**. The experiments were performed according to their ability to inhibit squamous metaplasia and keratinization in organ cultures of trachea derived from vitamin-A-deficient hamsters. β-Retinoic acid **2** with an all *trans* configuration was used as the reference compound.[51]

The percentage of the cultures exhibiting keratinization is illustrated in Figure 1; untreated control exhibited 95% keratinization. We determined mean effective doses (ED_{50}) for the reversal of keratinization of 50% of the explants by probit analysis.[52,53] The ED_{50} values were 1.46×10^{-10}, 3.68×10^{-10}, 2.15×10^{-10}, 4.16×10^{-10}, and 3.46×10^{-10} M for **2**, **6**, **7**, **11**, and **12**, respectively. We also obtained the carcinostatic properties of retinoids in the presence of 0.85 nM of a βL from *S. aureus* 95 in vitro. No significant enhancement of the activity was observed for **2**, **6**, **11**, and **12** against squamous metaplasia and keratinization. In contrast, cephalosporin–retinoic acid conjugate **7** was activated by the βL and exhibited remarkable anti-keratosis activity with $ED_{50} = 3.91\times10^{-11}$ M.

Anticancer activity. The anticancer screening experiments for compounds **6**, **7**, **11**, **12**, **7** + βL from *E. coli*, **7** + dsFv3–βL as well as the reference compounds 9-(β-D-arabinofuranosyl)cytosine (ara-C) and all-*trans*-β–retinoic acid (**2**) were carried out in vitro against murine leukemias (L1210 and P388), sarcoma 180 (S-180), breast carcinoma (MCF7), and human T-lymphocytes (Molt4/C8 and CEM/0).[54] The activity is expressed as the concentration (μM) required to inhibit tumor cell proliferation by 50% (IC_{50}). Their toxicity toward normal human embryonic cell line (HEL) were also determined. Results are summarized in Table 1.

Determination of LD_{50} for all-*trans*-β-retinoic acid (2) and cephem–retinoic acid conjugate 7 in rats. All-*trans*-β-retinoic acid (**2**) and cephem–retinoic acid conjugate **7** were administered subcutaneously (sc). All-*trans*-β-reti-



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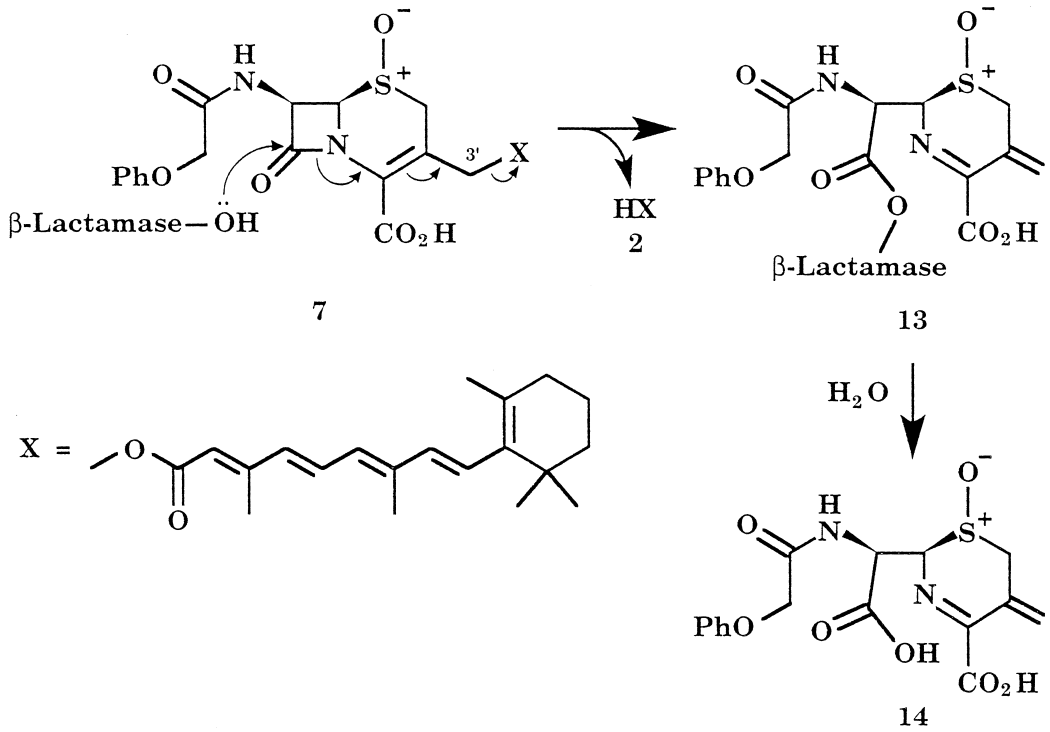
Scheme 3. Synthesis of 7-(retinamdo)cephalosporins **11** and **12**. Reagents: (a) (1) Me_3SiBr, CH_2Cl_2, 25 °C, 10 min; (2) $ClCO_2$ Et, CH_2Cl_2, pyridine, −5 °C, 1.5 h, **8**→**9**→**11** (60%); (b) (1) Me_3SiBr, CH_2Cl_2, 25 °C, 10 min; (2) 3-$ClC_6H_4CO_3$ H, 1 h; (3) $ClCO_2$ Et, CH_2Cl_2, pyridine, −5 °C, 1.5 h, **8**→**9**→**10**→**12** (42%).



Scheme 4. The acyl–enzyme adduct **13** and the corresponding hydrolyzed product **14** resulting from the ring opening of cephalosporin 3′-retinoic ester **7**.

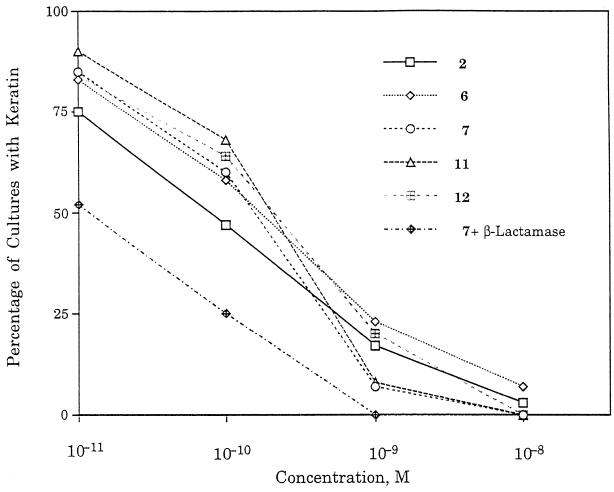


Figure 1. Dose–response relationships of retinoids on the percentage of tracheal organ cultures with keratin.

noic acid (**2**) and the conjugate **7** did not show any toxicity at concentration levels as high as 70 and 105 mg/kg, respectively. All rats were found to be in good conditions after administration for 3 months. Nevertheless, an LD_{50} (sc) value of 95 and 170 mg/kg were obtained, respectively, for all-*trans*-β-retinoic acid (**2**) and oxocephem–retinoic acid conjugate **7**.

Discussion

Retinoids have been used in the prevention and therapy of tumors.[55] Cephalosporins can react with transpeptidases[31,35] and/or β-lactamases[42] (see Scheme 1). By applying the dual targeting approach,[56] we first combined cephalosporins with all-*trans*-β-retinoic acid (**2**) to produce novel anticancer agents **6**, **7**, **11**, and **12**. These newly synthesized retinoids exhibited anti-hyperkeratosis activity comparable with that of all-*trans*-β-reti-

Table 1. Inhibitory concentrations[a] (IC_{50}, μM) of retinoids **6**, **7**, **11**, **12**, **7** + βL, **7** + dsFv3-βL as well as all-*trans*-β-retinoic acid **2** and ara-C on the growth of malignant cell lines and normal human embryonic lung cells in vitro

	Murine leukemias		Sarcoma 180	Breast carcinoma	Human T-lymphocytes		Human lung cell
Compound	L1210	P388	S-180	MCF7	Molt4/C8	CEM/0	HEL
ara-C	0.16±0.02	0.14±0.03	0.38±0.05	0.78±0.13	0.70±0.12	0.77±0.09	1.98×10^{-3}
2	0.06±0.01	0.04±0.00	0.47±0.21	0.02±0.00	0.63±0.05	0.43±0.01	12.16 ± 1.09
6	0.26±0.07	0.70±0.13	1.99±0.15	2.02±0.12	1.77±0.03	1.84±0.10	29.37 ± 1.36
7	0.17±0.02	0.24±0.04	1.18±0.03	0.86±0.02	0.98±0.04	1.36±0.09	24.61 ± 1.02
11	1.01±0.21	2.30±0.08	3.60±0.21	2.16±0.41	1.89±0.18	2.99±0.05	22.13 ± 2.03
12	0.96±0.04	0.87±0.10	1.67±0.06	0.97±0.06	1.21±0.15	2.15±0.00	18.97 ± 0.58
7 + βL	0.04±0.00	0.03±0.01	0.41±0.24	0.01±0.00	0.56±0.07	0.29±0.03	13.13 ± 1.65
7 + dsFv3–βL	0.05±0.02	0.04±0.00	1.27±0.29	0.01±0.00	1.01±0.60	1.35±0.32	26.40±2.03

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

noic acid (**2**) ($ED_{50} = 1.46 \times 10^{-10}$ M). At the second stage, we applied a βL from *S. aureus* 95 to these new compounds individually. Only oxocephem–retinoic acid conjugate **7** showed greater anticancer property ($ED_{50} = 3.91 \times 10^{-11}$ M) than that of **2**. These results indicate that the all-*trans*-β-retinoic acid component can be liberated effectively from **7** at the C-3′ position through a βL induced 1,4-elimination,[40] as shown in Scheme 4. In fact, release of all-*trans*-β-retinoic acid (**2**) from cephalosporin 3′-retinoic ester **7**, by the βL, results in anti-hyperkeratosis activity better than administration of retinoic acid (**2**) alone. This could be attributed to the 40-fold increment in water solubility of **7** compare to **2**.

While βL enhanced only the activity of compound **7**, all compounds (i.e., **6**, **7**, **11**, and **12**) showed retinoid activity. Considering the stability of all-*trans*-β-retinoic acid–cephalosporin linkage, we believe that the retinoic acid receptor β (RARβ) induction[57] by newly synthesized retinoids **6**, **7**, **11**, and **12** may play an important role in mediating growth inhibitory effects of these retinoids in cancer cells. In fact, the induction of RARβ by retinoids is not surprising, because the RARβ gene promoter contains a retinoic acid responsive element (RARE).

Retinoids **6**, **7**, **11**, and **12** were also found to exhibit interesting activity against L1210, P388, S-180, MCF7, Molt4/C8 and CEM/0. In comparison to reference compounds ara-C and all-*trans*-β-retinoic acid (**2**), retinoids **6**, **7**, **11**, and **12** displayed less inhibition on the examined tumor cell lines. They were, however, less toxic against HEL (see Table 1). In contrast, in the presence of free βL from *E. coli*, the potency of pro-all-*trans*-β-retinoic acid **7** approaches that of all-*trans*-β-retinoic acid (**2**). 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 26 s^{-1} μM^{-1}) against L1210, P388, and MCF7 and offer a similar activity to that of all-*trans*-β-retinoic acid (**2**); yet its activity was not enhanced against S-180, Molt4/C8, and CEM/0 (see Table 1). Similarly, toxicity of prodrug **7** toward HEL was found to be similar to that of all-*trans*-β-retinoic acid (**2**) in the presence of free βL, but remained about the same in the presence of dsFv3–βL. 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.

As such, the binding ability of monoclonal antibody to cell surface antigen as well as the capability of its protein conjugate in recognition of prodrugs in vitro may pave the way to further studies involving the effectiveness of monoclonal antibody–β-lactamase conjugates in releasing the retinoic acid in vivo. This strategy can be utilized in selective tumor directed chemotherapy.

Conclusions

For the development of new compounds as anticancer agents, all-*trans*-β-retinoic acid (**2**) was attached to the cephalosporin at either the C-3′ or the *N*-7 position by chemical methods. The first series involves the formation of an ester linkage by condensation of (+)-3′-iodocephalosporin **3** at the C-3′ position with the sodium salt of β-retinoic acid (**2**). In this way, the target cephalosporin 3′-retinoic esters **6** and **7** were synthesized in 40–48% overall yields. The second series involves the formation of an amide linkage by condensation of a silylated 7-aminocephalosporin at the *N*-7 position with an anhydride of all-*trans*-β-retinoic acid (**2**). The desired retinamido-β-lactams **11** and **12** were thus obtained in 42–60% overall yields.

Among these four newly synthesized compounds, oxocephem–retinoic acid conjugate **7** was found to be activated by a βL or the targeting fusion protein, dsFv3–βL, and exhibited remarkable anti-hyperkeratosis ($ED_{50} = 3.91 \times 10^{-11}$ M) as well as inhibitory activity against murine leukemias (L1210 and P388), sarcoma 180 (S-180), breast carcinoma (MCF7), and human T-lymphocytes (Molt4/C8 and CEM/0). These results indicate that the all-*trans*-β-retinoic acid component could be effectively released from **7** through a 1,4-elimination process. Furthermore, the LD_{50} (sc) value of **7** was found to be 170 mg/kg in rats.

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 Chemical Co. Solvents (Switzerland), 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. Reverse phase HPLC (Hewlett-Packard, C_{18} column) was used for identification of retinoids with 20–90% CH_3CN in H_2O as the mobile phase. Peaks were identified as the recovered starting compounds or all-*trans*-β-retinoic acid (**2**) on the basis of mass spectrometry.

3-[(Retinoyloxy)methyl]-7-(phenoxyacetamido)-3-cephem-4-carboxylic acid (6). To a solution of (+)-**3** (0.265 g, 0.499 mmol) in dry CH_2Cl_2 (12 mL) was added trimethylsilyl bromide (0.130 g, 0.824 mmol). The reaction mixture was stirred at 25 °C under N_2 for 10 min. The mixture was then injected into a solution of all-*trans*-β-retinoic acid (**2**, 0.150 g, 0.499 mmol) and $NaHCO_3$ (0.30 g, 3.6 mmol) in DMF (20 mL) and stirred under N_2 for 1.0 h. The solution was diluted with EtOAc (60 mL) and H_2O (80 mL). The organic layer was separated and washed with aqueous HCl solution (1%, 40 mL) and H_2O (50 mL). The organic layer was dried over $MgSO_4$ (s), filtered, and concentrated under reduced pressure. Purification by use of silica gel column chromatography with $CHCl_3$ as eluant afforded single isomer **6** (0.155 g, 0.240 mmol) as a foam in 48% yield: UV λ_{max} (EtOH) 352 nm (ϵ 41,500); IR (CH_2Cl_2) 3410–3300 (NH, COOH), 1782 (β-lactam), 1730 (ester), 1720 (C=O), 1670(amide) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 1.15 (s, 6H, $(CH_3)_2C$), 1.30–2.41 (m, 15H, $3\times CH_3 + 3\times CH_2$), 3.68 (d, $^2J = 19$ Hz, 1H, CHS), 3.82 (d, $^2J = 19$ Hz, 1H, CHS), 4.61 (br s, 2H, OCH_2CO), 4.88 (d, $^2J = 13$ Hz, 1H, CHO), 5.11 (d, $^2J = 13$ Hz, 1H, CHO), 5.18 (d, $^3J = 4.9$ Hz, 1H, HC(6)), 5.78 (d, $^3J = 4.9$ Hz, 1H, HC(7)), 5.61–6.72 (m, 6H, $6\times$ =CH), 6.99–7.40 (br s, 5H, C_6H_5). Anal. calcd for $C_{36}H_{42}N_2O_7S$: C, 66.85; H, 6.55; N, 4.33; S, 4.96. Found: C, 66.79; H, 6.36; N, 4.41; S, 4.99.

3-[(Retinoyloxy)methyl]-7-(phenoxyacetamido)-(1-oxo)-3-cephem-4-carboxylic acid (7). To a solution of (+)-**3** (0.260 g, 0.490 mmol) in dry CH_2Cl_2 (15 mL) was added trimethylsilyl bromide (0.127 g, 0.808 mmol). The reaction mixture was stirred at 25 °C under N_2 for 10 min. Subsequently, 3-$ClC_6H_4CO_3H$ (0.086 g, 0.50 mmol) was added into the reaction mixture, which was stirred for 1 h. Then, this reaction mixture was added to a solution of all-*trans*-β-retinoic acid (**2**, 0.147 g, 0.490 mmol) and $NaHCO_3$ (0.30 g, 3.6 mmol) in DMF (25 mL). After the solution was stirred for 1 h, it was diluted with EtOAc (60 mL) and H_2O (80 mL). The organic layer was separated, washed with aqueous HCl solution (1%, 40 mL) and H_2O (50 mL), dried over $MgSO_4$ (s), filtered, and concentrated under reduced pressure. Purification by use of silica gel column chromatography with $CHCl_3$ as eluant followed by crystallization from Et_2O gave single isomer **7** (0.130 g, 0.196 mmol) as yellow crystals in 40% yield: mp 70–72 °C (dec.); UV λ_{max} (EtOH) 352 nm (ϵ 42,000); IR (CH_2Cl_2) 3420–3300 (NH, COOH), 1789 (β-lactam), 1730 (ester), 1715 (C=O), 1675 (amide) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 1.16 (s, 6H, $(CH_3)_2C$), 1.32–2.43 (m, 15H, $3\times CH_3 + 3\times CH_2$), 3.80 (d, $^2J = 17$ Hz, 1H, HCSO), 3.97 (d, $^2J = 17$ Hz, 1H, HCSO), 4.61 (br s, 2H, OCH_2CO), 4.86 (d, $^2J = 14$ Hz, 1H, CHO), 5.02 (d, $^2J = 14$ Hz, 1H, CHO), 5.29 (d, $^3J = 5.0$ Hz, 1H, HC(6)), 5.79 (d, $^3J = 5.0$ Hz, 1H, HC(7)), 5.60–6.72 (m, 6H, $6\times$ =CH), 7.01–7.42 (br s, 5H, C_6H_5). Anal. calcd for $C_{36}H_{42}N_2O_8S$: C, 65.24; H, 6.39; N, 4.23; S, 4.84. Found: C, 64.98; H, 6.41; N, 4.36; S, 4.97.

3-(Retinamido)cephalosporanic acid (11). To a solution of (+)-**8** (0.144 g, 0.438 mmol) in dry CH_2Cl_2 (12 mL) was added trimethylsilyl bromide (0.260 g, 1.65 mmol). The reaction mixture was stirred at 25 °C under N_2 for 10 min. In a separate flask, all-*trans*-β-retinoic acid (**2**, 0.150 g, 0.499 mmol) in dry CH_2Cl_2 (10 mL) and pyridine (0.201 g, 2.52 mmol) was treated with ethyl chloroformate (0.062 g, 0.571 mmol) under N_2 at −5 °C for 10 min. The resultant solution was transferred by a syringe into the above reaction mixture. After the solution was stirred for 1.5 h, it was washed with H_2O (3×40 mL), dried over Na_2SO_4 (s), filtered, and concentrated under reduced pressure. Crystallization from a mixture of Et_2O hexane (2:1) gave single isomer **11** (0.144 g, 0.263 mmol) as crystals in 60% yield: mp 80–82 °C; UV λ_{max} (EtOH) 355 nm (ϵ 43,000); IR (CH_2Cl_2) 3400–3300 (NH, COOH), 1785 (β-lactam), 1730 (ester), 1710 (C=O), 1668 (amide) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 1.14 (s, 6H, $(CH_3)_2C$), 1.30–2.41 (m, 15H, $3\times CH_3 + 3\times CH_2$), 2.21 (s, 3H, CH_3CO), 3.67 (d, $^2J = 19$ Hz, 1H, CHS), 3.79 (d, $^2J = 19$ Hz, 1H, CHS), 4.81 (d, $^2J = 13$ Hz, 1H, CHO), 5.19 (d, $^2J = 13$ Hz, 1H, CHO), 5.20 (d, $^3J = 5.0$ Hz, 1H, HC(6)), 5.80 (d, $^3J = 5.0$ Hz, 1H, HC(7)), 5.60–6.74 (m, 6H, $6\times$ =CH). Anal. calcd for $C_{30}H_{38}N_2O_6S$: C, 64.96; H, 6.91; N, 5.05; S, 5.78. Found: C, 64.90; H, 6.83; N, 5.16; S, 5.61.

3-(Retinamido)-1-oxocephalosporanic acid (12). To a solution of (+)-**8** (0.140 g, 0.426 mmol) in dry CH_2Cl_2 (15 mL) was added trimethylsilyl bromide (0.253 g, 1.60 mmol). The reaction mixture was stirred at 25 °C under N_2 for 10 min. Then 3-$ClC_6H_4CO_3H$ (0.097 g, 0.56 mmol) was added into the reaction mixture, which was stirred for 1 h. In a separate reaction flask, all-*trans*-β-retinoic acid (**2**, 0.146 g, 0.484 mmol) in dry CH_2Cl_2 (12 mL) and pyridine (0.195 g, 2.44 mmol) was treated with ethyl chloroformate (0.0600 g, 0.554 mmol) under N_2 at −5 °C for 15 min. The resultant solution was transferred by a syringe into the above mixture. After the solution was stirred for 1.5 h, it was washed with H_2O (3×40 mL), dried over Na_2SO_4 (s), filtered, and concentrated under reduced pressure. Crystallization from Et_2O gave **12** (0.102 g, 0.179 mmol) as a single isomer in 42% yield: mp 89–92 °C (dec.); UV λ_{max} (EtOH) 355 nm (ϵ 43,100); IR (CH_2Cl_2) 3420–3310 (NH, COOH), 1791 (β-lactam), 1730 (ester), 1715 (C=O), 1669 (amide) cm^{-1}; ^1H NMR ($CDCl_3/D_2O$) δ 1.15 (s, 6H, $(CH_3)_2C$), 1.35–2.42 (m, 15H, $3\times CH_3 + 3\times CH_2$), 2.22 (s, 3H, CH_3CO), 3.78 (d, $^2J = 16$ Hz, 1H, HCSO), 3.99 (d, $^2J = 16$ Hz, 1H, HCSO), 4.84 (d, $^2J = 13$ Hz, 1H, CHO), 5.08 (d, $^2J = 13$ Hz, 1H, CHO), 5.30 (d, $^3J = 5.0$ Hz, 1H, HC(6)), 5.80 (d, $^3J = 5.0$ Hz,

1H, HC(7)), 5.62–6.73 (m, 6H, 6×=CH). Anal. calcd for $C_{30}H_{38}N_2O_7S$: C, 63.14; H, 6.71; N, 4.91; S, 5.62. Found: C, 63.34; H, 6.60; N, 5.03; S, 5.41.

Determination of solubility of all-*trans*-β-retinoic acid–cephem conjugates 6, 7, 11, 12, and all-*trans*-β-retinoic acid (2) in water

A solution of each compound (80 mg) in 1-octanol (7.0 mL) was shaken in a 30-mL volumetric flask with phosphate buffer (0.10 M, 10.0 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 all-*trans*-β–retinoic acid–cephem conjugate 7 in a mixture of DMSO-d_6 and phosphate buffer (1:1 mL/mL, pD 7.2)—(^{1}H NMR study)

Retinoid **7** (0.0650 g, 0.122 mmol) was dissolved in a mixture of DMSO-d_6 (3.0 mL) and 0.10 M deutrated phosphate buffer (3.0 mL, pD 7.2) at 25 °C. The ^{1}H NMR spectrum was taken at this temperature and then 0.60 mL (9.9 units) of βL (from *S. aureus* 95) buffer solution was added. The ^{1}H NMR spectrum at 25 °C was taken immediately; the spectrum of **7** changed rapidly to that of the eliminated compounds **14** and **2**. The mixture was extracted with $CDCl_3$ (2×3.0 mL) to remove all-*trans*-β-retinoic acid (**2**), which was found to be identical with an authentic sample. The aqueous solution was frozen and lyophilized to afford a residue. Purification by silica gel column chromatography using EtOAc/MeOH 9:1 as eluant gave **14** (0.0420 g, 0.105 mmol) in 86% yield: mp 198–199 °C; ^{1}H NMR (DMSO-d_6/D_2O) δ 3.48 (d, ^2J=13.5 Hz, 1H, CHSO), 3.47 (d, ^2J=13.5 Hz, 1H, CHSO), 4.71 (br s, 2H, OCH_2CO), 4.82 (d, ^3J=4.2 Hz, 1H, HC(6)), 5.36 (d, ^3J=4.2 Hz, 1H, HC(7)), 5.58 (s, 1H, =CH), 5.81 (s, 1H, =CH), 7.38 (br s, 5H, C_6H_5). CIMS: 381 ($M^+ + 1$), 380 (M^+).

Kinetic procedure

Determination of the kinetic parameters for βL and dsFv3–βL (secreted from *E. coli*)[42b] with the substrate **7** was done according to an established procedure.[42c] 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 0.46×15-cm C_{18} reversed-phase HPLC column (50% CH_3CN as eluant) at 1.0 mL/min. Prodrug **7** and product **2** concentrations were monitored by absorbance at 352 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.

Reversal of keratinization in tracheal organ culture by retinoids

After incubation for 24 h, cultures were divided into six groups (10 cultures/group) and treated in the following manner. Group 1: untreated control; group 2: all-*trans*-β-retinoic acid (**2**) with 1.0×10^{-8}, 1.0×10^{-9}, 1.0×10^{-10}, and 1.0×10^{-11} M; group 3: retinoids **6**, **7**, **11**, and **12** with 1.0×10^{-8} M; group 4: retinoids **6**, **7**, **11**, and **12** with 1.0×10^{-9} M; group 5: retinoids **6**, **7**, **11**, and **12** with 1.0×10^{-10} M; and group 6: retinoids **6**, **7**, **11**, and **12** with 1.0×10^{-11} M. In the meantime, retinoid stock solutions were prepared in DMSO and stored at −110 °C. Aliquots of the retinoid solutions were added to the culture medium to give the concentrations of 1.0×10^{-8}–10^{-11} M. Control cultures received equivalent amounts of the solvent.

Under subdued light, fresh retinoids were added to the culture medium every 4 h. After 96 h, all cultures were fixed in 10% buffered formalin and processed for histological examination. Five micron sections were stained in hematoxylin and eosin, and scored with a microscope for the presence of keratin and keratohyaline granules.

A test result was scored as 'inactive' if both keratin and keratohyaline granules were observed. On the other hand, it was scored as 'active' if neither keratin nor keratohyaline granules were observed, or if keratohyaline granules alone were absent.[51] The above experiments were also performed in the presence of 0.85 nM of a βL from *S. aureus*.

Anticancer test procedure in vitro

Murine leukemias (L1210 and P388), sarcoma 180 (S-180), breast carcinoma (MCF7), human T-lymphoblasts (Molt4/C8 and CEM/0), and normal human embryonic lung (HEL) cell lines 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.[54] Under this condition, the generation time for L1210, P388, S-180, MCF7, Molt4/C8, CEM/0, and HEL cells was about 13, 12, 17, 17, 18, 21, and 29 h, respectively. Compounds **6**, **7**, **11**, **12**, **7** + βL (120 nM), **7** + dsFv3-βL (120 nM) as well as the reference compounds ara-C and all-*trans*-β-retinoic acid (**2**), at various concentrations, were added to L1210, P388, S-180, MCF7, Molt4/C8, CEM/0, and HEL cells (250 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 (μM) required to inhibit proliferation of the respective cell lines by 50% after 72 h incubation (Table 1).

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