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A New Acivicin Prodrug Designed for Tumor-Targeted Delivery

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Abstract—Acivicin is an antitumor agent known to inhibit cell growth. A new prodrug **9b** of acivicin **10** was synthesized, based on a *p*-hydroxybenzylcarbamate self-immolative spacer capable to release acivicin under esterase activity. The prodrug includes a maleimide-containing arm for linkage with thiol-containing macromolecules such as antibodies. This molecule is intended for the conception of bioconjugates to target an inactive acivicin precursor to tumor cells, when linked to a monoclonal antibody (mAb) which recognizes a tumor-specific antigen. Prodrug cleavage by plasmatic esterases will then restore the acivicin's activity toward tumor cells. We report here the synthesis and the in vitro characteristics of the prodrug. As expected, its inhibitory activity against the γ-glutamyl transpeptidase (γ-GT) enzyme and its cytotoxicity towards HL-60 cells were highly reduced compared to the parent drug. The chemical and plasmatic hydrolysis kinetics of the compound was studied by HPLC. The prodrug is stable, being slowly hydrolyzed in pH 7.6 buffer at 37 °C with a half-life of 37 h. It is converted into an active acivicin under the effect of pig liver esterase, and its half-life in human plasma is 3 h. These results indicate this compound may be further used as a prodrug–antibody conjugate, to target acivicin to malignant cells.

Introduction

Acivicin [(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, AT-125, NSC-165301, U42126] **10** (Scheme 1), which was originally isolated as a fermentation product of *Streptomyces sveceus* for its growth inhibition properties[1] is a glutamine antimetabolite[2] and a potent γ-glutamyl transpeptidase (γ-GT) inhibitor.[3] Beside its antiproliferative activity against a variety of tumor cell models[4] and animal models,[5] acivicin induces a dose- and time-dependent inhibitory growth effect on myeloid lineage cells associated with an induction of morphological features characteristic of macrophage maturation.[6,7] These observations suggest acivicin is a peculiar attractive molecule for potential treatment of certain malignancies, including acute myeloid leukemias.

To prevent the severe side effects usually observed in clinical trials with this molecule,[5,8] we have considered a construction enabling its targeting to the tumoral cell surface. Immunoconjugates of drugs already demonstrated a dramatic increase of the therapeutic index compared to the free drug[9,10] and some are currently under clinical investigation.[11,12] Here, we have synthesized a prodrug of acivicin designed to be further linked with thiol-containing proteins such as antibodies (Scheme 1).

Conception of this prodrug is based both on our previous experience in the conception of N-*p*-hydroxybenzylcarbamate prodrugs and on its elegant application for solid support recently reported.[13] The self-immolative spacer is designed to release acivicin under the effect of plasmatic esterases. A maleimide-containing arm previously described[14] enables its linkage to the antibody. Acivicin is expected to be inactive as long as its amine is engaged in the carbamate linkage,[15] and to recover its full activity upon esterase action.

In order to collect preliminary data, we synthesized the prodrug **9b**, bearing a mercaptoacetic acid linked on maleimido group to avoid any reaction with thiol-containing molecules during the in vitro tests. Its synthesis, kinetics, enzymatic, and cell effects are reported here.

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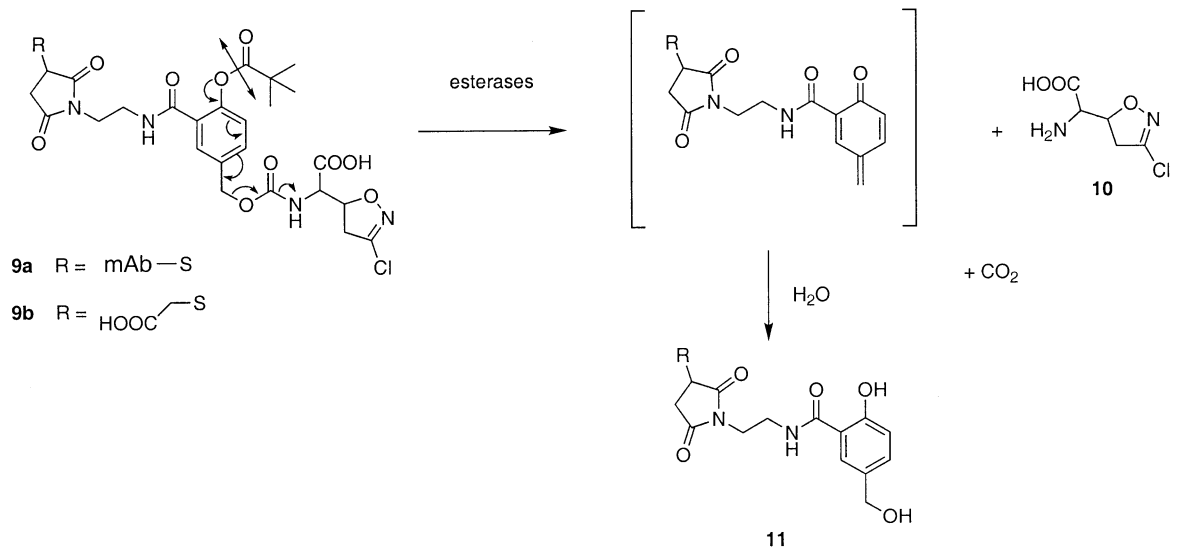
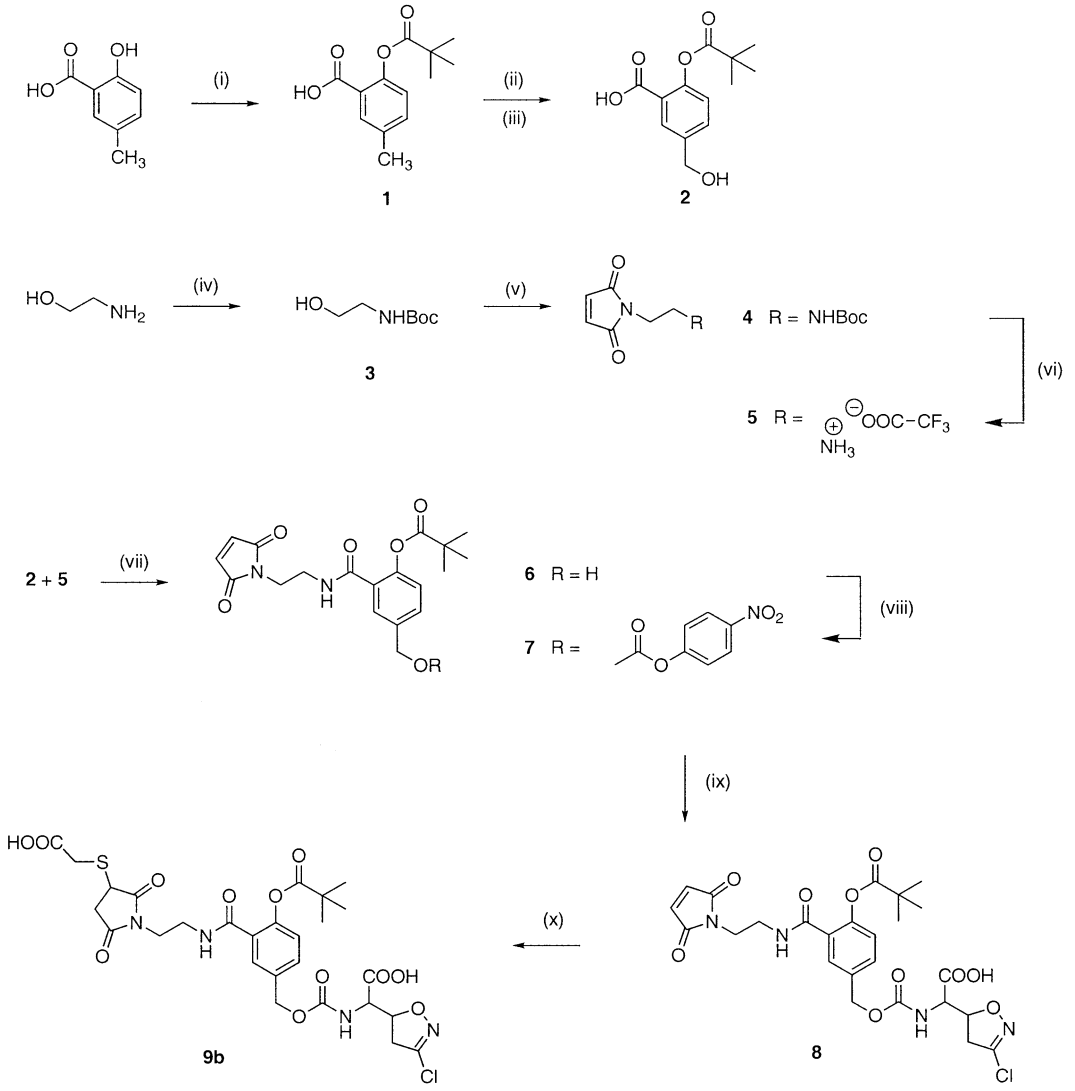


Scheme 1. Self-immolative mechanism of the prodrug leading to the release of acivicin under esterase action.



Scheme 2. Reagents: (i) *tert*-Butylacetate chloride, Et_3N, CH_2Cl_2, rt, 84%; (ii) NBS, AIBN, CCl_4, 80 °C; (iii) $AgNO_3$, H_2O, dioxane, rt, 52% overall yield; (iv) di-*tert*-butoxycarbonylanhydride, NaOH, dioxane, rt, 90%; (v) maleimide, PPH_3O, DEAD, THF, −78 °C, 80%; (vi) TFA, CH_2Cl_2, 0 °C, 78%; (vii) DCC, Et_3N, CH_2Cl_2, rt, 50%; (viii) *p*-nitrophenylchloroformate, pyridine, CH_2Cl_2, rt, 88%; (xi) acivicin, Et_3N, DMF, rt, 95%; (x) mercaptoacetic acid, Et_3N, CH_2Cl_2, 95%.

Results

Chemistry

Synthesis of **9b** started from 5-methyl-2-hydroxybenzoic acid (Scheme 2), which was esterified with pivaloyl chloride to give **1** in 84% yield. Functionalization of the methyl residue into benzylic alcohol was realized in two steps by bromination with *N*-bromosuccinimide and subsequent aqueous silver nitrate hydrolysis which afforded **2** (52% overall yield). Synthesis of the maleimide-containing arm started from ethanolamine, which was *N*-protected with *tert*-butoxycarbonylanhydride (Boc) (80%). The alcohol was substituted by a maleimido group using a Mitsunobu reaction optimized to give **4** in 80% yield. The compound **5** was obtained by Boc deprotection of **4** with trifluoroacetic acid (78%). Subunits **2** and **5** were then coupled using dicyclohexylcarbodiimide to afford **6** (50%), which by treatment with *p*-nitrophenylchloroformate and coupling with acivicin **10** gave **8** in 84% overall yield. Addition of thioacetic acid on the maleimido group afforded **9b** (80%).

HPLC studies

Effect of incubation of prodrug **9b** with pig liver esterase was followed by HPLC analysis. A time-dependent disappearance of the prodrug accompanied by a concomitant release of acivicin was observed (Fig. 1). Quantitation of the prodrug and acivicin was obtained from measurements of the peak areas in relation to those of corresponding standards chromatographed under the same conditions. Another peak appeared proportionally to prodrug degradation, and its UV spectrum was consistent with the structure of **11b** (absorption maximum wavelength at 300 nm). The structure of **11b** was confirmed by the electrospray mass spectrum from samples collected during the HPLC analysis (data not shown).

The stability of the prodrug was investigated in buffer and plasma conditions, and degradation kinetics determined. Half-life of prodrug **9b** was 36 h at 37 °C in protease buffer, but of 3 h when incubated in human plasma.

Enzymatic and cell effects

We first tested whether the carbamate linkage of acivicin was efficient in inactivating acivicin. The prodrug was first incubated for 3 h in the absence or presence of commercial esterase, after which aliquots were assayed on soluble commercial γ-GT activity. The prodrug mainly lost its inhibitory activity towards γ-GT, as shown by the 12-times ratio between the respective IC_{50} of acivicin and prodrug **9b** (Table 1).

As previously described, free acivicin inhibits growth of HL-60 cells. The effects of the prodrug onto HL-60 cell proliferation were measured. A significant shift was observed between the concentrations at which acivicin and the prodrug **9b** are able to inhibit cell proliferation. This result is illustrated by the ratio between their respective IC_{50}, which is 26 (Table 1).

In parallel, the toxicity of acivicin onto HL-60 cells was shown to reach 40% of necrotic cells, at a concentration of 0.5 mM (data not shown). At the same concentration the prodrug was not as much cytotoxic, as only 10% of necrotic cells were count. The prodrug was 27 times less toxic than acivicin, as assessed by their LD_{10} ratio (Table 1).

Second, we tested if the activity of acivicin was restored upon esterase action. Acivicin inhibits surface γ-GT activity of HL-60 cells with an IC_{50} of 25 μM similar to that obtained with soluble γ-GT (data not shown). In the absence of esterase, the prodrug did not markedly affect HL-60 γ-GT (Fig. 2). In contrast addition of esterase to the prodrug leads to the inhibition of γ-GT at a same degree as acivicin (Fig. 2). Of note, esterase by itself did not affect HL-60 activity.

Table 1. Comparison of the effects of acivicin and its prodrug on γ-GT activity, HL-60 cell growth and cytotoxicity

	Acivicin (μM)	Prodrug (μM)	Ratio
γ-GT inhibition (IC_{50})	25	300	12
Cell proliferation (GI_{50})	7	182	26
Cytotoxicity (LC_{10})	7	190	27

IC_{50} stands for the concentration at which commercial γ-GT activity is inhibited by 50%. GI_{50} is the concentration at which HL-60 cell growth is inhibited by 50%. LC_{10} is the concentration at which 10% of HL-60 cells are dead (Trypan Blue positive percentage).

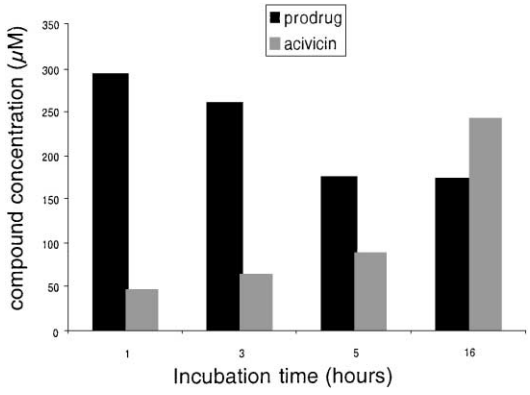


Figure 1. Concomitant prodrug cleavage and acivicin appearance following prodrug incubation with esterase.

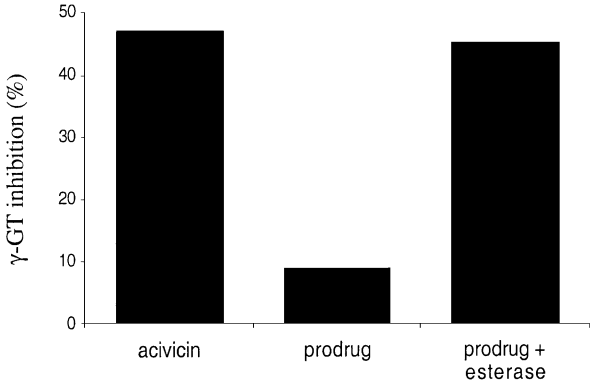


Figure 2. γ-GT inhibition of HL-60 cells by acivicin and its prodrug.

Discussion

As expected, the acivicin linkage to its amino group dramatically reduced its effects on soluble γ-GT activity and HL-60 cell growth and toxicity. Our data (Table 1) are similar to those displayed by the peptidic prodrug of acivicin reported in the past.[15] Parent drug was efficiently released from the prodrug **9b** in presence of esterases. We also showed that the released acivicin recovers its activity, as the cleaved prodrug was found to totally inhibit γ-GT activity. These data ascertain that the synthesized acivicin prodrug is a non-active precursor of the parent drug, capable of releasing acivicin and restoring its inhibitory activity toward γ-GT.

This concept of antibody-targeted prodrug to leukemic cells, to be effective, supposes that the kinetics of antibody binding to target cells is shorter than the prodrug half-life. Previous studies have shown that antibodies directed against blood-circulating antigens like CD33 reach their target within 1 h.[16] If the prodrug half-life in human plasma is a good preview of its in vivo behavior, one may consider that this prodrug, with a plasma half-life of 3 h, is well suited for antibody-targeted drug delivery to hematopoietic cells.

Using thiol-maleimide coupling, the prodrug synthesized here is designed to be easily linked to various proteins, such as antibodies. Its half-life in biological fluids may be increased by modulation of the alkyl ester moiety or by substituting the ester in this position by a carbamate since it has been recently shown in an exhaustive study that phenol carbamates are stable but cleavable in plasma.[17] This prodrug structure could be used with other therapeutic agents, or conjugated to different antibodies, leading to its potential use in various diseases.

Experimental

Reagents

Gamma glutamyl *p*-nitroanilide (γ-Glu-pNA), glycylglycine (Gly-Gly), acivicin, pig kidney γ-GT, pig liver esterase and citrated human plasma were purchased from Sigma Chemical Co (St Louis, MO, USA).

Cell culture

HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, Paisley, Scotland, UK, LPS levels < 0.1 ng/mL), 2 mM L-glutamine, 1 mM sodium pyruvate and 40 μg/mL gentamycin (Flow Laboratories, Rockwell, MD, USA) in a 5% CO_2 humidified atmosphere at 37 °C.

Chemicals

Melting points are uncorrected. IR spectra were recorded using a Perkin–Elmer 1710 spectrophotometer and are expressed in cm^{-1}. ^1H NMR spectra were recorded using a Bruker AM250 (250 MHz), a Bruker AC300 (300 MHz) and a Bruker AM400 (400 MHz) spectrometer. Chemical shifts are expressed in ppm from internal Me_4Si with the notations indicating the multiplicity of the signal. For mass spectra, CI (NH_3) were recorded with a Nermag R10-10C and a Jeol MS 700. Electrospray analyses were performed using an API 2000. TLC was performed on Silica gel $60F_{254}$ (Merck). Silica gel (Merck, particle size 0.040–0.063 nm) was used for flash chromatography. All reactions, except those under aqueous conditions, were performed under an argon atmosphere. Anhydrous reaction solvents were distilled as follows: diethyl ether and tetrahydrofuran from sodium/benzophenone; dichloromethane and carbon tetrachloride from phosphorus pentoxide.

Molecules

2-(2,2-Dimethyl-propionyloxy)-5-methyl-benzoic acid (1). *tert*-Butylacetyl chloride (2.7 mL) was added dropwise to a cooled solution (0 °C) of 5-methylsalicylic acid (3 g, 19.7 mmol) and triethylamine (3 mL) in anhydrous dichloromethane (200 mL). The reaction mixture was stirred at 0 °C for 3 h and then diluted with water and extracted with dichloromethane. The organic layer was separated, washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. Crystallization of the crude solid from EtOAc gave white needles crystals of **1** (3.92 g, 84%), mp 154 °C; ν_{max} ($CHCl_3$) 1698 (C=O), 1752 (C=O), 3502 (OH), δ_H (90 MHz, solvent $CDCl_3$) 1.45 (9H, s, tBu), 2.50 (3H, s, CH_3), 7.1 (1H, d, $J_{cb} = 9$ Hz, H_c), 7.5 (1H, tl, $J_{bc} = 9$ Hz, H_b), 8.1 (1H, s, H_a), 9.6 (1H, sl, COOH); *m/z* (DCI/NH_3) 254 $(M + NH_4)^+$; anal. calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83; found: C, 65.61; H, 6.80.

2-(2,2-Dimethyl-propionyloxy)-5-hydroxymethyl-benzoic acid (2). To a solution of **1** (1 g, 4.24 mmol) and AIBN (0.01 equiv, 7 mg) was added *N*-bromosuccinimide (1.1 equiv, 829 mg). The mixture was then stirred in refluxing CCl_4 (60 mL) under a 120 W lamp for 1 h. After cooling and hydrolysis, the resulting mixture was extracted with dichloromethane, and the organic layer was washed successively with water and brine. Removal of the solvent gave the crude product **2**, which was used without further purification. Silver nitrate (1.35 g) was added to a stirred solution of crude **2** (1.27 g) in dioxane (50 mL) and water (50 mL). After 16 h, the salts were removed by filtration and washed with EtOAc. The organic layer was washed with water. Purification by flash chromatography on silica gel [cyclohexane/ethyl acetate (3:2 v/v)] and subsequent crystallization from ethyl acetate gave white crystals of **3** (52%), mp 142 °C; ν_{max} ($CHCl_3$) 1713 (C=O), 1751 (C=O ester), 3400 (OH), 3669 (OH); δ_H (90 MHz, solvent $CDCl_3$ + DMSO) 1.53 (9H, s, tBu), 4.83 (3H, s, CH_3), 7.2 (1H, d, $J_{cb} = 9$ Hz, H_c), 7.72 (1H, tl, $J_{bc} = 9$ Hz, H_b), 8.2 (1H, s, H_a); *m/z* 270 $(M + NH_4)^+$; anal. calcd for $C_{13}H_{16}O_5$: C, 61.90; H, 6.39; found: C, 61.81; H, 6.42.

(2-Hydroxy-ethyl)-carbamic acid *tert*-butyl ester (3). To a solution of ethanolamine (2 g, 1.98 mmol) and sodium hydroxide (10 mmol) cooled at 0 °C in dioxane was

added ditertbutoxycarbonylanhydride (1.1 equiv, 7.86 g). After stirring for 0.5 h at room temperature, the mixture was extracted with EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$ and the solvent was removed under reduced pressure. Flash chromatography [cyclohexane/ethyl acetate (1:1 v/v)] led to compound **3** isolated as a yellow oil (4.2 g, 80%), ν_{max} ($CHCl_3$) 1170 (C–O), 1707 (C=O ester), 3455 (NH), 3626 (OH); δ_H (90 MHz, solvent $CDCl_3$) 1.50 (9H, s, tBu), 2.10 (1H, s, OH), 3.3 (2H, t, $J=5$ Hz, CH_2–NH), 3.70 (2H, t, $J=5$ Hz, CH_2–OH), 4.95 (1H, sl, NH); m/z 162 $[M+H]^+$, 179 $[M+NH_4]^+$.

[2-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-ethyl]-carbamic acid *tert*-butyl ester (4). A solution of triphenylphosphine (8.6 g, 32.9 mmol) in anhydrous THF (100 mL) was cooled at −78 °C prior successive additions of DEAD (5.2 mL, 32.9 mmol), a solution of **3** (5.3 g, 32.9 mmol) in THF (20 mL), and maleimide (3.19 g, 32.9 mmol). The mixture was stirred for 5 min at −78 °C, and then at room temperature, overnight. After removal of the solvent, diethyl oxide was added. The mixture was then filtered and the filtrate was purified, after concentration, by flash chromatography [cyclohexane/ethyl acetate (3:1 v/v)]. This gave white crystals of **4** (6.3 g, 80%), mp 116 °C; ν_{max} ($CHCl_3$) 1719 (C=O), 3459 (NH); δ_H (90 MHz, solvent $CDCl_3$ + DMSO) 1.45 (9H, s, tBu), 3.3 (2H, m, CH_2–NH), 3.75 (2H, m, CH_2–N), 4.8 (1H, sl, NH), 6.7 (2H, s, CH=CH); m/z (DCI/NH_3) 258 $(M+NH_4)^+$.

2-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-ethylamine trifluoroacetate (5). Trifluoroacetic acid (2.5 mL) was added to a cooled solution (0 °C) of **4** (279 mg) in anhydrous dichloromethane (6 mL). After evaporation of dichloromethane, excess of trifluoroacetic acid was removed by coevaporation with toluene. The residue was dissolved in hot methanol and the trifluoroacetate **5** was precipitated with ethyl dioxide. After filtration, white needles crystals of **5** were collected (247 mg, 78%), mp 127 °C; ν_{max} (KBr) 1676 (C=O), 1714 (C=O); δ_H (90 MHz, solvent CD_3OD) 3.1 (2H, t, CH_2–NH_3^+), 3.8 (2H, t, CH_2–N), 6.8 (2H, s, CH=CH); m/z (DCI/NH_3) 141 $(M+H)^+$, 158 $(M+NH_4)^+$.

2,2-Dimethyl-propionic acid 2-[2-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-ethylcarbamoyl]-4-hydroxymethyl-phenyl ester (6). A solution of the acid **2** (760 mg, 3 mmol), the trifluoroacetate **5** (1 equiv, 760 mg) and triethylamine (1 equiv, 410 μL) in anhydrous dichloromethane (100 mL) was stirred at 0 °C. DCC (1.1 equiv) was added and the mixture was cooled at 0 °C for 0.5 h. The reaction mixture was then stirred at room temperature for 36 h. The urea formed was removed by filtration and the filtrate was evaporated under reduced pressure. The residue obtained was purified by flash chromatography [cyclohexane/ethyl acetate (1:1 v/v)] and crystallization from EtOAc to afford **6** as a white solid (560 mg, 50%), mp 142 °C; ν_{max} ($CHCl_3$) 1715 (C=O), 2928 (CH=CH), 3606 (OH); δ_H (90 MHz, solvent $CDCl_3$) 1.36 (9H, s, tBu), 3.60 (2H, dd, $J_{CH_2NH}=J_{CH_2N}=5.5$ Hz, CH_2NH), 3.77 (2H, t, $J=5.5$ Hz, CH_2N), 4.70 (2H, s, CH_2O), 6.50 (1H, sl, NH), 6.72 (2H, s, CH=CH), 7.02 (1H, d, $J_{cb}=8.4$ Hz, H_c), 7.45 (1H, dd, $J_{bc}=8.4$ Hz, $J_{ba}=1.9$ Hz, H_b), 7.65 (1H, d, $J_{ab}=1.9$ Hz); m/z (DCI/NH_3) 375 $(M+H)^+$, 392 $(M+NH_4)^+$; anal. calcd for $C_{19}H_{22}N_2O_6$: C, 60.95; H, 5.92; N, 7.48; found: C, 61.13; H, 5.85; N, 7.42.

2,2-Dimethyl-propionic acid 2-[2-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-ethylcarbamoyl]-4-(4-nitro-phenoxycarbonyloxymethyl)-phenyl ester (7). To a solution of *p*-nitrophenyle chloroformate (270 mg, 1,36 mmol) and anhydrous pyridine (120 μL, 1,52 mmol) in anhydrous dichloromethane (5 mL) was added a solution of **6** in dichloromethane (5 mL). The reaction mixture was stirred for 3 h at room temperature and extracted with dichloromethane. Purification by chromatography on silica gel gave **7** (190 mg, 88%), ν_{max} ($CHCl_3$) 1669 (C=O amide), 1713 (C=O ester), 1767 (C=O carbonate) 2940 (C=O); δ_H (300 MHz, solvent $CDCl_3$) 1.37 (9H, s, tBu), 3.61 (2H, dd, $J=6$ Hz, CH_2–NH), 3.79 (2H, t, $J=6$ Hz, CH_2–N), 5.29 (2H, s, CH_2–O), 6.54 (1H, t, $J=6$ Hz, NH), 6.71 (2H, s, CH=CH), 7.08 (1H, d, $J_{cb}=8.3$ Hz, H_c), 7.40 (2H, d, $J_{a'b'}=7.6$ Hz), 7.51 (1H, dd, $J_{bc}=8.3$ Hz, $J_{ba}=2$ Hz, H_b), 7.76 (1H, d, $J_{ab}=2$ Hz, H_a), 8.28 (2H, d, $J_{b'a'}=7.6$ Hz, $H_{b'}$)

2,2-Dimethyl-propionic acid 4-[carboxy-(3-chloro-4,5-dihydro-isoxazol-5-yl)-methylcarbamoyloxymethyl]-2-[2-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-ethylcarbamoyl]-phenyl ester (8). To a solution of acivicin (16 mg, 91 mmol) and triethylamine (182 mmol, 25 μL) in anhydrous DMF (5 mL), was added a solution of **7** (49 mg, 91 mmol) in DMF (5 mL). The reaction mixture was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (dichloromethane, methanol 6%, acetic acid 0.5%) affording **8** as a colorless oil (50 mg, 95%). RMN ^1H (300 MHz, $CDCl_3$): δ (ppm) 1.33 (9H, s, tBu), 3.26 (2H, lm, CH_2NH), 3.56 (2H, ls, H_4), 3.72 (2H, ls, CH_2N), 4.53 (1H, ls, H_1), 5.06 (3H, ls, CH_2O, H_3), 6.42 (1H, ls, HN-acivicin), 6.70 (1H, ls, Mal), 6.97 (1H, d, $J_{cb}=8.1$ Hz, H_c), 7.33 (1H, d, $J_{bc}=8.1$ Hz, H_b), 7.62 (1H, s, H_a); RMN ^{13}C (300 MHz, solvent $CDCl_3$): δ (ppm) 26.99 (tBu); 37.29 (CH_2N); 38.94 (CH_2NH); 39.12 (C_4); 65.81 (CH_2O, C_3); 123.10 (C_c); 128.20 (C_a); 130.80 (C_b); 134.30 (Mal); mass spectra (ESI positive) 579.2 $[M+H]^+$, 601.2 $[M+Na]^+$, 617.1 $[M+K]^+$, (ESI negative) 577.1 $[M-H]^+$, 493 $[M-(tBu-C=O)]^+$.

2,2-Dimethyl-propionic acid 4-[carboxy-(3-chloro-4,5-dihydro-isoxazol-5-yl)-methylcarbamoyloxymethyl]-2-[2-(3-carboxymethylsulfanyl-2,5-dioxo-pyrrolidin-1-yl)-ethylcarbamoyl]-phenyl ester (9). To a solution of **8** (16.8 mg, 29 μmol) and triethylamine (35 μmol, 4.9 μL) in anhydrous dichloromethane was added mercaptoacetic acid (35 μmol, 2.5 μL). The mixture was stirred at room temperature for 30 min, and the solvent was evaporated before purification by flash chromatography on silica gel (acetonitrile, water 8%). The final product **9** was isolated after lyophilisation as a white powder (15.5 mg, 80%). δ_H (300 MHz, solvent $CDCl_3$ + CD_3OD) 1.31 (9H, s, tBu), 3.53 (2H, dd, $J_{12}=2$Hz, $J_{1'2}=6.1$ Hz, H_1), 3.72 (2H, dd, $J_{21}=2.0$ Hz, $J_{2'1}=6.1$ Hz, H_2), 5.19 (2H,

s, CH_2O), 6.67 (2H, s, CH=CH), 7.00 (1H, d, $J_{cb}=8.2$ Hz, H_c), 7.41 (1H, dd, $J_{bc}=8.2$ Hz, $J_{ba}=2.1$ Hz, H_b), 7.58 (2H, d, $J_{a'b'}=7.1$ Hz, $H_{a'}$), 7.61 (1H, d, $J_{ab}=2.1$ Hz, H_a), 8.15 (2H, d, $J_{b'a'}=7.1$ Hz, $H_{b'}$), mass spectra (ESI negative) 669.0 $[M-H]^+$, 577.0 $[M-(S-CH_2-COOH)]^+$.

HPLC analysis

The identification of prodrug and cleavage products was performed using HPLC.

Proteins were discarded from the samples either by centrifugal filtration (Ultrafree®-mc Millipore 5000 NMWL), or by precipitation with cold methanol and centrifugation. 50 μL of resulting sample were then injected with an autosampler (Waters 717 plus) onto an analytical column (C18 Waters Symmetry, 4.6×250 mm). The mobile phase consisted of a gradient composed of water (+TFA 0.1%) (during 8 min) and acetonitrile (+TFA 0.1%) (0–80% from 8 to 28 min). Eluted compounds were detected by absorbance at 220 nm (Waters 996 photodiode array detector).

Enzymatic cleavage and plasma stability

The prodrug (300 μM) was incubated at 37 °C with pig liver esterase (50 U/mL) or human plasma (96%).

γ-GT inhibition

To test the prodrug cleavage, the prodrug was incubated for 3 h at 37 °C in protease buffer pH 7.6 containing 100 mM Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 8 mM glucose with pig liver esterase (50 U/mL).

For γ-GT assay, γ-GT transpeptidation activity was assayed according to the colorimetric method of Tate and Meister[18], using γ-Glu-pNA as a substrate of γ-GT hydrolytic activity and Gly-Gly as a glutamate acceptor for the transpeptidation reaction. In a typical experiment, porcine kidney γ-GT (0.2 U/mL) was incubated for 30 min at 37 °C in 0.1 mL protease buffer, in the presence of 1 mg/mL substrate and 20 mM Gly-Gly, and with or without cleaved prodrug/acivicin. The reaction was stopped by adding 0.9 mL of 1 M sodium acetate-acetic acid pH 4. The production of free *p*-nitroaniline was measured by spectrophotometry at 405 nm. The activity is expressed as the percentage of control activity without inhibitor.

Cytotoxicity

HL-60 cells at a density of 2×10^5/mL in 100 μL culture medium containing 10 μM HEPES were grown at 37 °C in the absence or presence of various concentrations of acivicin or prodrug. After 3 days, 10 μL of a trypan blue solution in phosphate-buffered saline (0.4%) was added. The cytotoxicity is expressed as the percentage of cells having incorporated the Trypan Blue.

Cell growth arrest

HL-60 cells at a density of 2×10^5/mL in 100 μL culture medium were grown for 3 days at 37 °C in the absence or presence of various concentrations of acivicin or prodrug. Control cells were exposed to medium only. One μCi (^{3}H-methyl)thymidine (from NEN) was added to each well. After 18 h of culture, cells were harvested onto glass fiber filters using a multiple automated sample harvester and filters were counted for radiation content. Cell proliferation is expressed as the ratio of cpm versus control cells.

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