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Ionic derivatives of betulinic acid as novel HIV-1 protease inhibitors

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Abstract

Betulinic acid is a natural product possessing abundant and favourable biological activity, including anti-cancer, anti-malarial, anti-inflammatory and anti-HIV properties, while causing minimal toxicity to unaffected cells. The full biological potency of betulinic acid cannot be fully unlocked, however, for a number of reasons, a primary one being its limited solubility in aqueous and biologically pertinent organic media. Aiming to improve the water solubility of betulinic acid without disrupting its structurally related bioactivity, we have prepared different ionic derivatives of betulinic acid. Inhibition bioassays on HIV-1 protease-catalysed peptide hydrolysis indicate significantly improved performance resulting from converting the betulinic acid to organic salt form. Indeed, for one particular cholinium-based derivative, its water solubility is improved more than 100 times and the half maximal inhibitory concentration (IC_{50}) value (22 μg mL^{-1}) was one-third that of wide-type betulinic acid (60 μg mL^{-1}). These encouraging results advise that additional studies of ionic betulinic acid derivatives as a therapeutic solution against HIV-1 infection are warranted.

Keywords

Betulinic acid; anti-HIV; ionic liquid; HIV-1 protease; derivative

Introduction

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) (**1** in Scheme 1) is a pentacyclic lupane-type triterpene. This compound and its derivatives have exhibited a variety of biological properties such as anti-HIV-1 (human immunodefficiency virus type-1), anti-cancer, anti-bacterial, anti-malarial, anti-inflammatory and anthelmintic activities[1–3]. Several mechanisms have been offered as to how betulinic acid and its derivatives are able to act as HIV-1 anti-virals[4]. The first mechanism involves the inhibition of HIV-1 replication and maturation[5–8]. Some major derivatives of betulinic acid investigated for this

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mechanism include dihydrobetulinic acid[5,6], 3-alkylamido-3-deoxy-betulinic acid derivatives[8], and 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid[9,10]. In particular, the last derivative showed a high activity against HIV-induced cytopathic effects in HIV-1-infected MT-4 cells, with the likely mechanism being that this derivative might be involved in the step(s) of virion assembly and/or budding of virions but does not appear to inhibit HIV-1 protease activity[9]; another group[10] also suggested that the last derivative exhibited no inhibition on the HIV-1 protease *in vitro* nor interference with virus assembly or release, but it acts at a late stage of the virus life cycle: it specifically delays the cleavage of Gag between the capsid (CA) and p2, causing the deferred formation of mature viral core and minimised HIV-1 infectivity. By a second mechanism, betulinic acid derivatives block viral infection at a post-binding, envelope-dependent step during the fusion of the virus to the cell membrane, which can be determined from viral binding and syncytium formation[11–16]. These derivatives include ω-undecanoic amides of betulinic acid[11,12], ω-aminoalkanoic acid derivatives[13], betulinic acid derivative RPR103611[14,16] and IC9564 (4*S*-[8-(28-betuliniyl) aminooctanoylamino]-3*R*-hydroxy-6-methylheptanoic acid)[15]. A third mechanism of anti-HIV activity of pentacyclic triterpenes is based upon the inhibitory activity against HIV-1 reverse transcriptase[17]. Since reverse transcriptase is required for early proviral DNA synthesis, inhibition of the reverse transcriptase-catalysed polymerisation of DNA from viral RNA inhibits the viral replication. Reverse transcriptases can be virus-specific and are considered viable chemotherapeutic targets. However, a number of studies have also suggested that betulinic acid and its derivatives may show no inhibition towards HIV-1 reverse transcriptase[6,11,12]. Yet another mechanism is the inhibition of HIV-1 protease by betulinic acid (half maximal inhibitory concentration, IC_{50} = 9 μM[18] or 2.5 μM[19]) and other triterpenes, although some derivatives (e.g. 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid[9] and ω-aminoalkanoic acid derivatives[11,12]) apparently failed to inhibit the activity of HIV-1 protease. The HIV-1 protease consists of two identical monomers which contribute to the construction of only one active site. It is a retroviral aspartyl protease that catalyses the cleavage of newly synthesized polyproteins to produce the mature protein components of an infectious HIV virion; hence inhibition of HIV-1 protease effectively disrupts the virus' ability to replicate and infect additional cells. For this reason, several inhibitors of HIV-1 protease have been developed as chemotherapeutic agents for treating AIDS, including saquinavir, indinavir, tipranavir and darunavir[20,21]. The mechanism of HIV-1 protease inhibition by betulinic acid and derivatives is still not well understood because these compounds may exhibit other inhibition mechanisms as discussed above. Quéré et al.[19] suggested that triterpenes including betulinic acid could inhibit the protease dimerisation, a prerequisite for protease activation; computer modelling showed that several triterpenes could dock into the hydrophobic interface site of the relaxed monomer.

Due to the mutation of HIV in response to most chemotherapeutic drugs, there is a continual demand for the development of novel anti-HIV compounds, particularly agents that are at once inexpensive and minimally toxic. A major impediment to unleashing the anti-HIV potency of betulinic acid lies in its extremely poor solubility in aqueous solutions and, to a lesser extent, in common organic solvents such as alcohols, ethers and esters. The solubility of betulinic acid in water is in fact only about 0.02 μg mL^{-1} at room temperature[22]. Its solubility in common organic solvents at 25°C is also fairly low; e.g. 1% (w/v) in ethanol and 5% (w/v) in DMSO[23]. A limited number of derivatives of betulinic acid were reported to yield improved water solubility and biological activity compared to unmodified betulinic acid[1,3,24]. Building on this early progress using lessons gained from our experience with ionic liquids[23,25], it is anticipated that novel ionic derivatives of betulinic acid may exhibit further improved water solubility and anti-HIV activity. Of particular relevance, betulinic acid coupled with amino acids at the C-28 carboxylic acid position has been reported to exhibit improved water solubility and toxicity against a cultured human melanoma (MEL-2) cell line[26]. In the current study, we initially prepared a glycinyl conjugate of betulinic acid

for subsequent modification, as this form showed the highest water solubility and cytotoxicity against cancerous cells among the amino acid derivatives of betulinic acid studied earlier[26].

Recently, the Rogers group[27–29] introduced a new concept to eliminate the problematic polymorphism of pharmaceutical drugs by converting them into stable liquid forms, so-called “ionic liquid active pharmaceutical ingredients” (IL-API). In its simplest manifestation, the idea is to transform the API into ionic form followed by exchange of the counter ion (metathesis) with a bulky organic counter ion that lends itself towards rendering the salt proper (ion pair) a liquid. Interestingly, the counter ion need not merely serve to frustrate ionic packing (preventing crystallisation) but can furthermore be selected to display desired biological functions complementary to the API. For example, sodium ibuprofen (an anti-inflammatory) can be paired with didecyldimethylammonium bromide (anti-bacterial and anti-inflammatory) to engender the IL-API didecyldimethylammonium ibuprofenate which retains dual biological roles[30]. Motivated by this molecular flexibility, we forecast that betulinic acid derivatives can be designed not only to achieve excellent aqueous solubility and improved anti-HIV activity, but also to show secondary biological function introduced via the counter ion. In a preliminary study, we evaluate a number of ionic betulinic acid derivatives for their ability to inhibit HIV-1 protease activity, while other possible inhibition routes remain a topic of current investigation in our laboratories.

Methods

Materials

The following materials were supplied by Sigma-Aldrich: betulinic acid (90%), glycine methyl ester hydrochloride, *N,N′*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), choline chloride, benzalkonium chloride and Amberlyst® A26 hydroxide form. The chromogenic peptide (Ac-Arg-Lys-Ile-Leu-Phe(4-NO_2)-Leu-Asp-Gly-NH_2, product no. 22089, MW 1047.21) was acquired from AnaSpec (Fremont CA, USA). The HIV-1 protease, recombinant *E. coli* (10,000 U,† product no. 382136), from Calbiochem® was provided by EMD Chemicals, Inc (Gibbstown, NJ, USA).

Preparation of ionic salts of betulinic acid conjugated with glycine

The early steps of the synthesis (synthesis of **4** in Scheme 2) are based upon a modification of a literature protocol[26]. Initially, betulinic acid (**1**, 200 mg, 0.44 mmol), glycine methyl ester (**2**, 100 mg, 0.80 mmol) and triethylamine (81 mg, 0.80 mmol) were dissolved in 50 mL anhydrous tetrahydrofuran (THF) at room temperature. To this solution, DCC (108 mg, 0.52 mmol) and DMAP (30 mg, 0.25 mmol) were added and the reaction mixture stirred at room temperature under nitrogen for 48 h. After completion of the reaction, the precipitate (dicyclohexylurea byproduct) was filtered off and the filtrate evaporated under vacuum to remove THF. The crude product was dissolved in 100 mL of ether/ethyl acetate (2:1, v/v) and extracted with 100 mL of water to remove the excess water-soluble carbodiimide reagent and any remaining dicyclohexylurea. The organic layer was further extracted with 1.0 N HCl (2 × 100 mL) to remove DMAP[31], saturated $NaHCO_3$ solution (1 × 100 mL), and finally with water (1 × 100 mL)[32,33]. The purified organic layer was dried over Na_2SO_4. After filtration, ethyl acetate was evaporated under vacuum to yield the glycine methyl ester conjugate of betulinic acid (**3**) (230 mg, 99% yield).

†One unit (U) is defined as 1 ng of purified protein; one unit will hydrolyse 1.0 pmole of peptide substrate (SQNYPIVQ) per minute at 25°C in pH 4.7 buffer.

The methyl ester (**3**, 200 mg, 0.38 mmol) was dissolved in 100 mL of THF/H_2O (4:1, v/v) solution, followed by the addition of LiOH (45 mg, 1.88 mmol). The reaction mixture was stirred at room temperature for 4 h under N_2. At the end of the reaction, THF was evaporated under vacuum and the product obtained was dissolved in 200 mL of ethyl acetate, followed by washing with water, 0.1 M HCl and water again. The organic layer was then dried with Na_2SO_4, and after filtration the solvent was removed under vacuum to yield glycine conjugate of betulinic acid (**4**) (168 mg, 86% yield).

Choline hydroxide was prepared from choline chloride following an anion-exchange column approach used previously[34,35]. The glycinylated betulinic acid (**4**, 150 mg) was dissolved in 50 mL of THF/H_2O (4:1, v/v), followed by the addition of a five-fold molar excess of choline hydroxide (**5**). The reaction mixture was stirred at room temperature for 24 h. After this time, THF was removed by rotary evaporation. The crude product was extracted into 100 mL of ethyl acetate, and washed with distilled water (2 × 100 mL) to remove the excess choline hydroxide. The organic layer was dried and ethyl acetate removed as above to give the cholinium salt of betulinic acid-glycine ([Choline][BA-Gly], **6**) (130 mg, 72% yield), representing a 61% overall product yield for Scheme 2.

Following the above reaction strategies, we similarly prepared the benzalkonium salt of glycinylated betulinic acid ([Bzk][BA-Gly], **7**; as ionic mixtures, see Scheme 3) (470 mg product from 209 mg of betulinic acid) as well as cholinium betulinate ([Choline][BA], **8**) in 64% yield (120 mg) starting from 153 mg of betulinic acid.

[Choline][BA-Gly] (**6**), m.p. 205°C–210°C. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm) = 0.61 (3H, s), 0.73 (2H, s), 0.83 (4H, s), 0.89 (2H, s), 0.99 (2H, m), 1.02 (5H, m), 1.05–1.06 (3H, m), 1.10 (2H, m), 1.14–1.16 (3H, m), 1.20 (4H, m), 1.24 (4H, m), 1.28 (3H, m), 1.32 (3H, s), 1.66–1.71 (8H, m), 4.23–4.25 (1H, m), 4.52 (1H, m) and 5.53–5.56 (4H, m). ^{13}C NMR (DMSO-d_6), δ (ppm) = 14.9, 16.3, 16.5, 18.5, 19.5, 25.0, 25.9, 27.7, 28.7, 31.0, 33.9, 37.2, 48.1, 77.3, 157.1 and 177.8.

[Bzk][BA-Gly] (**7**), m.p. 175°C–180°C. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm) = 0.62 (3H, s), 0.73 (3H, s), 0.84 (8H, m), 0.88 (3H, s), 1.26 (27H, m), 1.51–1.60 (9H, m), 1.66–1.74 (7H, m), 2.06–2.14 (4H, m), 2.25–2.42 (3H, m), 2.47 (6H, m), 2.91 (7H, m), 3.17–3.23 (6H, m), 4.23 (2H, t), 4.48–4.50 (3H, m), 4.63 (1H, s), 5.70–5.73 (1H, d) and 7.50 (5H, s). ^{13}C NMR (DMSO-d_6), δ (ppm) = 14.5, 14.9, 16.3, 16.5, 18.5, 19.5, 21.0, 22.3, 22.6, 25.0, 25.7, 25.9, 26.4, 27.7, 28.0, 28.6, 29.1, 29.3, 29.6, 30.8, 31.8, 32.7, 33.9, 34.5, 34.7, 37.3, 38.0, 38.8, 39.2, 42.5, 47.2, 48.0, 49.2, 49.7, 50.5, 55.5, 56.1, 61.9, 64.0, 66.8, 68.8, 77.3, 97.7, 109.9, 128.7, 129.5, 130.8, 133.5, 151.3, 157.2, 176.2 and 178.1.

[Choline][BA] (**8**), m.p. 260°C. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm) = 0.61 (3H, s), 0.73 (3H, s), 0.83 (3H, s), 0.87 (3H, s), 1.18 (4H, m), 1.27 (3H, s), 1.37–1.40 (4H, m), 1.59 (3H, s), 1.71–1.77 (2H, m), 2.07–2.11 (1H, d), 2.47 (3H, p), 3.09 (5H, s), 4.48 (1H, m) and 4.61 (1H, m). ^{13}C NMR (DMSO-d_6), δ (ppm) = 14.9, 16.3, 16.5, 18.5, 19.6, 21.1, 25.8, 27.7, 28.6, 29.9, 31.0, 33.2, 34.6, 37.3, 37.6, 37.9, 38.8, 39.0, 42.6, 47.3, 49.4, 50.6, 53.7, 55.5, 55.6, 56.3, 67.7, 77.3, 109.6, 151.8 and 178.2.

Aqueous solubility of betulinic acid analogues

Starting with 50 μL of DMSO solution containing 5 mg mL^{-1} of a particular betulinic acid derivative, distilled water was added in 50 μL increments in order to visually observe a turbid solution. Each time a clear solution resulted, additional water was added in 50 μL steps. The relative water solubility was finally expressed in the dilution steps required to achieve clear solution (i.e. 2x, 3x).

Inhibition of HIV-1 protease activity

To a reaction mixture containing 0.17 mM of the chromogenic peptide substrate Ac-Arg-Lys-Ile-Leu-Phe (4-NO_2)-Leu-Asp-Gly-NH_2 in acetate buffer (0.1 M, pH 4.7, I = 0.2 M)‡ was added 20 μL of a DMSO solution of the desired betulinic acid analogue and 20 μL of HIV-1 protease solution (100 U). The reaction mixture (1.0 mL) was incubated at 37°C, with aliquots periodically withdrawn for high-performance liquid chromatography (HPLC) analysis. A control reaction was carried out under the same conditions, but without the addition of the betulinic acid analogue. The rate of peptide hydrolysis was periodically monitored by a LC-20AT Shimadzu HPLC equipped with a Phenomenex® Kinetex C18 column (100 mm × 4.6 mm, particle size: 2.6 μm). The HPLC column was eluted with a gradient of acetonitrile (15%–25% in 15 min) in 0.2% (v/v) acetic acid at a flow rate of 1.0 mL min^{-1}. The detection wavelength was 280 nm. The initial reaction rate was calculated from the decrease in the peptide substrate peak area in the HPLC chromatograms. All reactions were run in triplicate.

Results and discussion

Preparation of ionic derivatives of betulinic acid

Three ionic derivatives of betulinic acid were successfully prepared, including cholinium and benzalkonium salts of glycinylated betulinic acid (**6** and **7**, respectively) and a cholinium salt of betulinic acid (**8**). Derivatives **6** and **7** were prepared following steps illustrated in Scheme 2, whereas derivative **8** was prepared by simple acid–base neutralisation reaction between betulinic acid and cholinium hydroxide. The purpose of conjugating a water-soluble amino acid (i.e. glycine) at the C-28 carboxylic acid position of betulinic acid is mainly to improve the water solubility, with the possibility to enhance the biological activity as well[26]. The grafting of glycine methyl ester to betulinic acid via amide linkage was based on classical carbodiimide coupling chemistry. This was followed by base-catalysed hydrolysis to generate **4**[26,32,38]. Glycine-conjugated betulinic acid (**4**) was further reacted with cholinium hydroxide or benzalkonium hydroxide to produce the corresponding salts of betulinic acid as shown in step 3 of Scheme 2. There are two motivations for preparing these salts: (i) to further improve the water solubility of betulinic acid by forming more polar ionic compounds and (ii) to introduce secondary biological functionality through the presence of the counter ion. Taking **7** as an example, benzalkonium compounds are known for having anti-bacterial properties[27]. In the case of cholinium compounds such as **6**, many have been shown to be non-toxic, and choline chloride is even an essential micronutrient for human health[39].

Inhibition of HIV-1 protease by ionic derivatives of betulinic acid

To evaluate the effectiveness of our new ionic derivatives of betulinic acid (**6**–**8**) in inhibiting HIV-1 protease activity, we selected the enzymatic hydrolysis of a chromogenic peptide (Ac-Arg-Lys-Ile-Leu-Phe(4-NO_2)-Leu-Asp-Gly-NH_2) for our bioassay, as shown in Scheme 4. This method has evolved into a reliable and mature technique for the following reasons: (i) the peptide substrates are typically designed to be water-soluble, so the hydrolysis reaction can be performed in aqueous buffer with little addition of and interference from organic cosolvents[36,40]; (ii) HIV-1 protease only cleaves the two hydrophobic amino acid residues of substrates occupying the S_1-S_1' sites, thus the reactant and products can be easily detected and quantified[41] and (iii) this reaction can be monitored

‡Although it has been reported that high ionic strength (I) may facilitate the binding between a peptide substrate and HIV-1 protease (see refs. 36,37), we observed that the water-soluble substrate can be “salted-out” by the addition of sufficiently concentrated NaCl; e.g. for I = 0.5 M.

by a number of means, such as UV–vis absorption spectroscopy[36,41], fluorescence spectroscopy[37] and HPLC analysis[41,42].

It has long been known that the addition of NaCl to this assay reaction improves the catalytic activity of protease due to non-selective salting-out of hydrophobic substrates onto the enzyme surface[36,43]. Our experimental data in Figure 1 are in line with this trend: a higher HIV-1 protease activity was observed as the ionic strength increased from 0.05 to 0.50 M. However, we also observed that at I = 0.5 M the substrate became insoluble in the acetate buffer due to the strong salting-out effect, and the use of 5% (v/v) of DMSO was necessary for the complete dissolution of peptide. Since 5% DMSO is toxic for cultured cells, to minimise the deleterious effects of DMSO, we performed the remaining enzymatic assays in a pH 4.7 acetate buffer system at a buffer molarity of 0.1 M (I = 0.20 M), where the peptide remained fully soluble in the aqueous solution.

In the absence of HIV-1 protease, no hydrolysis of the substrate peptide was observed during the assay, confirming that the reaction is predominantly driven by the enzyme. The uninhibited enzyme reaction was initiated by the injection of 100 U of protease into the substrate solution, with the observed initial reaction rate (in units of mM h^{-1} μg^{-1} enzyme) at 37°C reflecting the native (100%) protease activity. Relative to this baseline, the inhibition studies were conducted by adding 20 μL of a DMSO solution of betulinic acid or one of its ionic derivative into the reaction mixture to achieve a final concentration of 10, 25, 50 or 100 μg mL^{-1}. The corresponding reaction rates were compared with that of the betulinic acid-free control reaction, and the degree of inhibition was calculated in terms of the half maximal inhibitory concentration (IC_{50}). As shown in Table 1, pepstatin A, a known natural inhibitor of aspartyl proteases[44], gave an IC_{50} value of 29 μg mL^{-1} (i.e. 44 μM), which is consistent with the published value for acetyl pepstatin[42,45]. In contrast, betulinic acid (**1**) itself is associated with a rather high IC_{50} of 60 μg mL^{-1} (130 μM), presumably due to its poor solubility in aqueous media. In our experience, once the DMSO solution of betulinic acid is injected into the acetate buffer, betulinic acid begins to precipitate, causing the mixture to become cloudy. On the other hand, the ionic derivatives of betulinic acid (**6**–**8**) showed much improved water solubility (Table 1); in particular, [Choline][BA] (**8**) was at least 100 times more soluble in water than the virgin betulinic acid. Linked to this improved water solubility, these ionic derivatives were associated with lower IC_{50} values, especially in the cases of **7** (IC_{50}= 28 μg mL^{-1} or 37 μM) and **8** (IC_{50} = 22 μg mL^{-1} or 40 μM), which gave results better than pepstatin A. These highly encouraging results suggest that suitable ionic derivatives of betulinic acid will display vastly improved aqueous solubility over the natural parent compound with the capability of liberating its latent biological activity in the inhibition of HIV-1 protease. We suspect that this positive outcome will further stimulate the investigation of additional biological functions of these ionic derivatives of betulinic acid, as well as the exploration of ionic forms of other potential drug candidates.

The exact mechanism of HIV-1 protease inhibition by betulinic acid and derivatives is not yet known. The computer modelling has suggested that betulinic acid and other similar triterpenes have the suitable molecular size to be fitted into the hydrophobic interface site of the relaxed monomer, which inhibits the protease dimerisation[19]. In addition to hydrophobic interactions, hydrogen-bonding is also a possible interaction between HIV-1 protease and hydroxyl/carboxyl groups in triterpene scaffold[46]. As the main objective of this study is to prepare more water-soluble derivatives of betulinic acid and screen their inhibitory effect against HIV-1 protease, further studies on other inhibition mechanisms using both biological assays and computer modelling are being carried in our laboratories and will be reported later.

Conclusions

Using straightforward coupling chemistry, we have prepared three new ionic derivatives of betulinic acid which display significantly improved water solubility. These compounds give lower IC_{50} values towards HIV-1 protease than the native betulinic acid, particularly for **7** and **8**, which show IC_{50} values roughly two and three times lower than that of betulinic acid, respectively. These preliminary data suggest the high potential of ionic derivatives of betulinic acid in future treatment of HIV/AIDS. Concurrent studies in our laboratories include the investigation of alternate potential inhibition mechanisms of these new derivatives, such as the inhibition of HIV-1 reverse transcriptase and the blocking of viral entry into the cell, and these results will be reported in due course.

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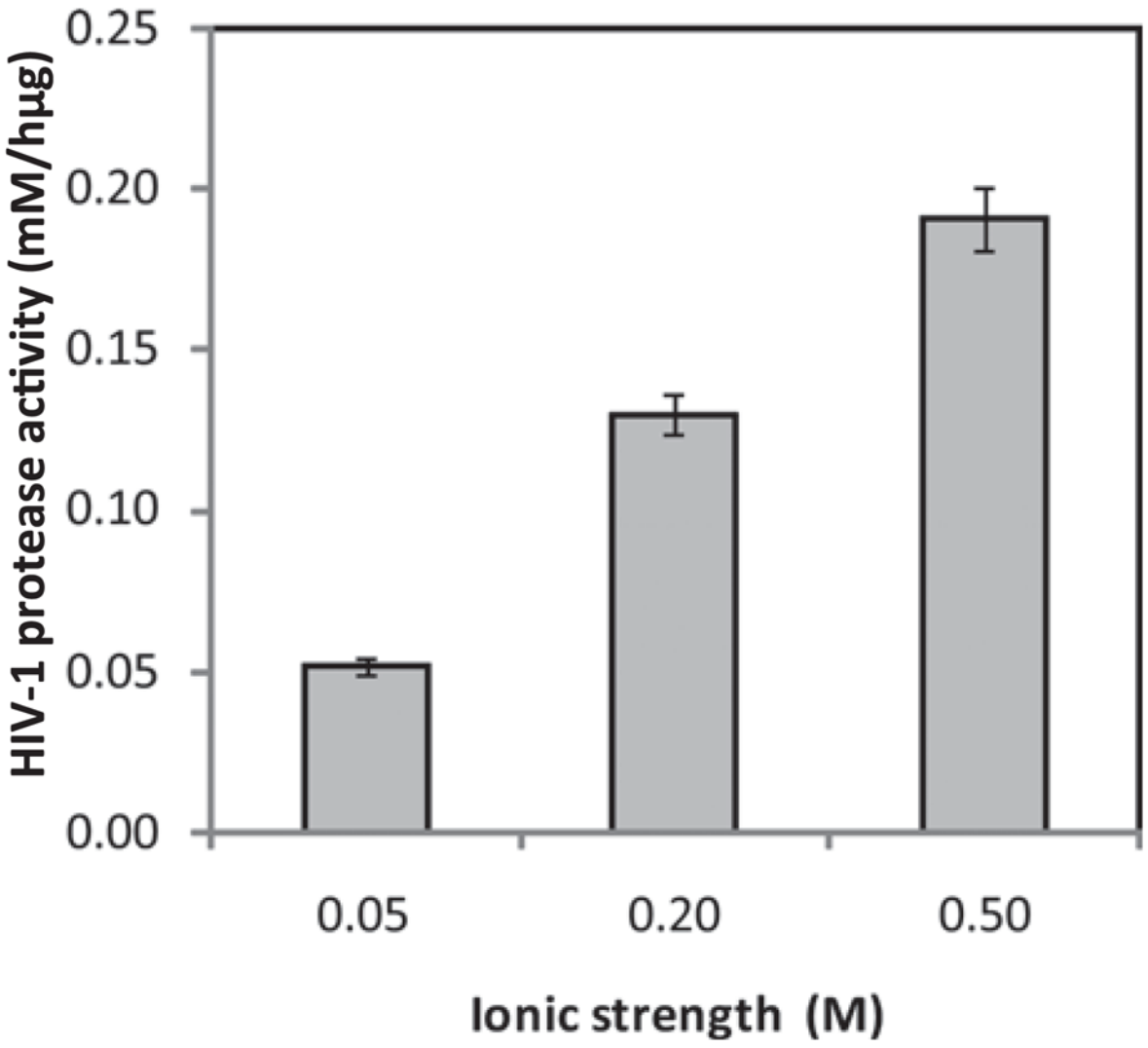
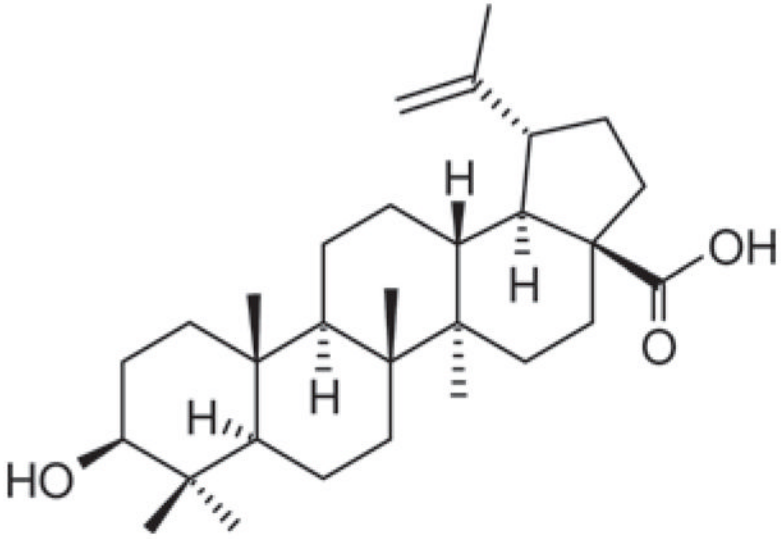
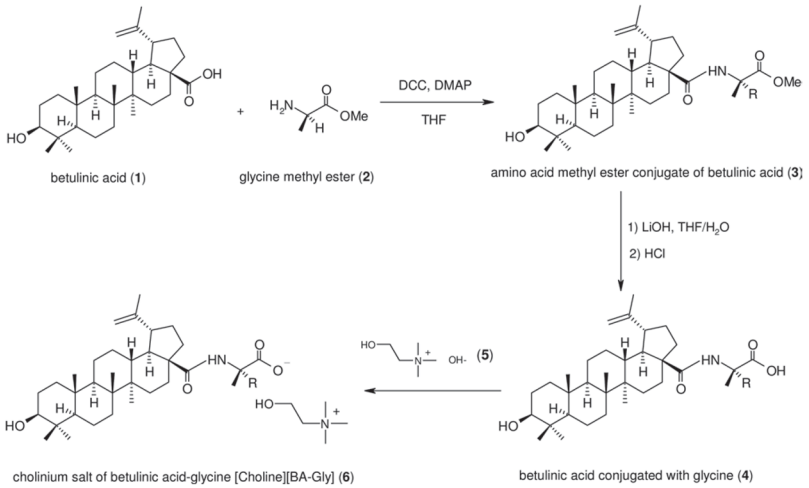


Figure 1.
Effect of buffer ionic strength (I) on HIV-1 protease activity (conditions: 0.17 mM substrate in 1.0 mL of 0.1 M acetate buffer (pH 4.7); 100 U HIV-1 protease; NaCl added to adjust I; assay conducted at 37°C).

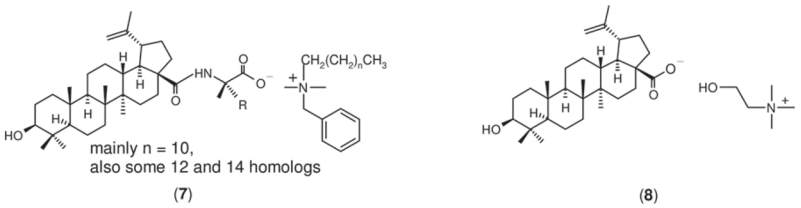


Scheme 1.
Betulinic acid (**1**).



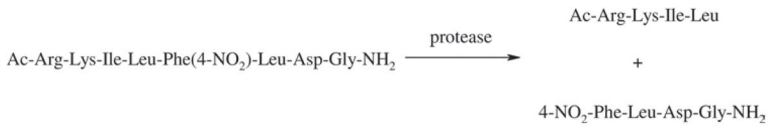
Scheme 2.

General steps for preparing a cholinium salt of betulinic acid conjugated with an amino acid (**6**, [Choline][BA-Gly]).



Scheme 3.

Structures of benzalkonium salt of betulinic acid-glycine (**7**, [Bzk][BA-Gly]), and cholinium salt of betulinic acid (**8**, [Choline][BA]).



Scheme 4.
HIV-1 protease-catalysed cleavage of oligopeptide substrate (see similar reactions in refs. 36,41,42).

Table 1

Inhibition of HIV-1 protease activity (IC_{50}) by betulinic acid and derivatives[a].

		IC_{50}		
HIV-1 protease inhibitor		**μM**	**μg mL^{-1}**	**Water solubility**
	Pepstatin A	29	44	
1	Betulinic acid	60	130	≪ 2x, 0.02 μg mL^{-1} at 25°C (see ref. 22)
6	[Choline][BA-Gly]	54	86	3x
7	[Bzk][BA-Gly]	28	37 *b*	2x
8	[Choline][BA]	22	40	> 100x

Note:

[a] values and water In general, the percentage error of IC_{50} solubility were about 5%;

[b] Estimated using the molar mass of main homologue of benzalkonium salt shown in Scheme 3.