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Ionic Derivatives of Betulinic Acid Exhibit Strong Antiviral Activity Against Herpes Simplex Virus Type-2 (HSV-2), But Not HIV-1 Reverse Transcriptase

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Abstract

Betulinic acid (**1**) has been modified to ionic derivatives (**2**-**5**) to improve its water solubility and biological activities. The binding properties of these derivatives with respect to human serum albumin (HSA) was examined and found to be similar to current anti-HIV drugs. These compounds did not inhibit HIV reverse transcriptase, however, **1**, **2** and **5** inhibited herpes simplex type 2 (HSV-2) replication at concentrations similar to those reported for acyclovir (IC_{50} ~0.1–10 μM) and with minimal cellular cytotoxicity. IC_{50} values for antiviral activity against HSV-2 186 were 1.6, 0.6, 0.9, 7.2, and 0.9 μM for compounds **1**-**5** respectively.

Abstract

New ionic derivatives of betulinic acid (**1**) such as **2** and **5** show strong inhibition against herpes simplex type 2 (HSV-2) replication with minimal cellular cytotoxicity.

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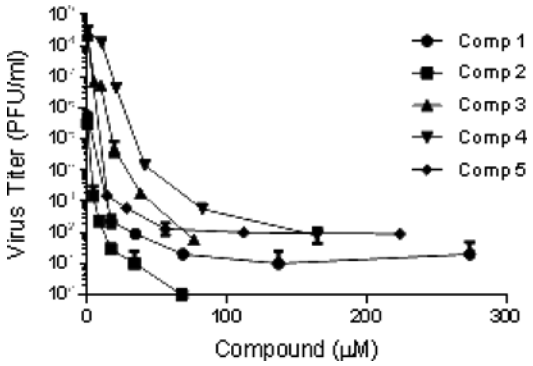
Supplementary data

Supplementary data associated with this article can be found, in the online version, at xxx.



Keywords

Betulinic acid; HIV-1 reverse transcriptase; herpes simplex type 2 (HSV-2); inhibitor

Betulinic acid (**1**), also known as 3β-hydroxy-lup-20(29)-en-28-oic acid, is a natural pentacyclic lupane-type triterpene (Scheme 1) that can be extracted from certain plants including birch trees. This compound and many of its derivatives have a number of medically relevant biological properties such as anticancer, anti-HIV-1 (human immunodeficiency virus type-1), antibacterial, anti-malarial, anti-inflammatory, and anthelmintic activities.[1-6] Betulinic acid and derivatives are of interest because of their anti-HIV-1 activity via several known mechanisms[7] including inhibition of HIV-1 maturation,[8-11] blocking viral infection at a post-binding stage, inhibition of an envelope-dependent step during fusion of the virus to the cell membrane,[12-17] and inhibition of HIV-1 protease.[18, 19] Due to mutation of HIV in response to most chemotherapeutic drugs, there is a constant demand for the development of novel anti-HIV compounds, particularly less expensive and less toxic agents.

The inhibition of betulinic acid and derivatives against other viruses has not been extensively studied. Herpes simplex types 1 (HSV-1) and 2 (HSV-2) are enveloped, double stranded DNA viruses that initially infect mucosal epithelial cells and establish latency in trigeminal and sacral ganglia respectively. HSV-1 is the predominant cause of cold sores whereas HSV-2 primarily causes genital herpes. Genital herpes is a sexually transmitted disease transmitted through contact with genital or oral lesions and secretions. There are more than 700,000 new herpes infections annually in the United States (http://www.cdc.gov/std/Herpes/). The Center for Disease Control estimates that 20.9% and 11.5% of 14–49 year old women and men respectively are infected with HSV-2. Greater than 80% of infected individuals in this age bracket are asymptomatic or have mild symptoms and therefore have never received a HSV-2 positive diagnosis. The fact that virus can be transmitted via shedding from lesion-free skin might explain why infection rates stay high. The development of a HSV-2 vaccine remains a high priority but has been met with only limited success.[20]

Nucleoside analogues make up the majority of currently approved anti-herpesviral drugs (e.g. acyclovir). These drugs inhibit viral replication by targeting new viral DNA replication.[21] Although critical in controlling infections caused by herpes simplex viruses,

nucleoside analogues share a similar mechanism of action. Therefore, treatment options are limited once resistance develops, an important clinical concern for treatment of resistant infections, particularly in immunocompromised individuals. In addition, moderate to severe side effects of some nucleoside analogues make discovery of less toxic drugs desirable. Efforts over the last decade have focused on the identification and development of improved therapies with novel mechanisms of action.

A major obstacle in maximizing the antiviral potency of betulinic acid is its poor solubility in aqueous solutions and, to a lesser extent, in many organic solvents including alcohols, ethers, and esters. The solubility of betulinic acid in water is 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, 4, 24] Anticipating that ionic derivatives of betulinic acid may have improved water solubility, four ionic derivatives (**2**-**5**, Scheme 2) of betulinic acid (**1**, Scheme 1) were prepared previously by our group; their potentials as HIV-1 protease inhibitors and anti-cancer agents were examined.[28, 29] Ionic derivatives had improved water solubilities and thus enhanced biological activity. In this study, the antiviral activities of these derivatives were explored as inhibitors of HIV-1 reverse transcriptase (RT) and herpes simplex virus type-2 (HSV-2) (see experimental procedures in Supplementary data).

Anti-HIV activity was evaluated by infecting Jurkat cells with HIV-1 virus in the presence of ionic derivatives (at a final concentration of 1 μg/mL or 5 μg/mL), and measuring reverse transcriptase (RT) activity. Cell viability was determined by MTT assay while HIV replication was determined by RT assay. DMSO was used as the solvent control, therefore, the overall anti-HIV-1 activity was calculated as the ratio of [MTT activity/(RT activity with compound/RT activity in DMSO)]. A more active HIV-1 inhibitor is expected to have a larger ratio. When comparing with anti-HIV activity in DMSO (Table 1), only compound **2** at 5 μg/mL showed a slightly improved inhibition against HIV-1 RT. The other compounds including betulinic acid (**1**) showed no inhibitory activity of HIV-1 RT, when comparing with results (0.941 at 1 μM and 0.580 at 10 μM) of a known reverse-transcriptase inhibitor azidothymidine (AZT). RT activity was also evaluated using higher concentrations (37.5–150 μg/mL) of **1**-**5** with a Roche colorimetric assay (Figure 1), but no significant inhibition of RT was observed. RT activity was not found to be dose-dependent, which could be due to the interference of slight solution turbidity at high compound concentrations. The overall results are consistent with some earlier studies[9, 12-14] where betulinic acid and derivatives (up to 219 μM [9]) exhibited no inhibition of HIV-1 RT although some pentacyclic triterpenes[25] and triterpenoids[26, 27] were active against HIV-1 RT. Therefore, the anti-HIV properties of betulinic acid and derivatives do not appear to target RT.

Betulin and betulinic derivatives (e.g. betulinic acid and betulonic acid) have been shown to possess anti-viral activity primarily against HSV-1, and in a single study against HSV-2 (Table 1).[28-31] Activity against both acyclovir sensitive and acyclovir resistant HSV strains has been reported. Results from the current study show new betulinic acid derivatives with improved solubility retain significant anti-herpesviral activity against HSV-2 186 (Figure 2).

Compounds **1**-**5** were examined for cytotoxicity in Vero cells (Figure S1 in Supplementary Data). As shown in Table 2, CC_{50}s were greater than 100 μM for compounds **1**, **2**, and **5**. Compounds **3** and **4** had CC_{50}s of approximately 10.5 and 12 μM respectively. Compounds were tested for antiviral activity against HSV-2 186. IC_{50}s were 1.6, 0.6, 0.9, 7.2, and 0.9 μM for compounds **1**-**5** respectively (Table 1). These results compare favorably to IC_{50}s previously reported for acyclovir against multiple HSV-2 isolates, including HSV-2 186 (Table 1).[32, 33] Selectivity indices calculated for compounds **1**-**5** were >62.5, >166.7, 11.6, 1.7, and >111.1 respectively. The IC_{50}s (HSV-2) and SIs of compounds **1**, **2**, and **5** were as good as or better than those previously reported for related compounds (Table 1). The improved solubility of ionic derivatives **2** and **5** likely contributes to improved antiviral activity. The mechanism of action of these new derivatives against HSV-2 strains is currently being investigated.

Lastly, we investigated the binding characteristics of these derivatives with human serum albumin (HSA). HSA is a well-known plasma protein that is responsible for the binding and transport of many endogenous and exogenous substances (e.g. hormones and fatty acids), and drug molecules. Since the binding of drugs to plasma proteins is non-specific, only the unbound form of a drug is considered to interact with its receptor to produce a pharmacological effect.[34] Therefore, a low binding constant or a high dissociation equilibrium constant (K_d) between drug molecules and HSA is desirable for achieving a maximum drug bioavailability. The fluorescence titration of betulinic acid or its derivative with HSA (with excitation at 285 nm [34] or 295 nm [35]) was measured. The titration spectra and calculation plot for compound **5** is provided as an example (Figure S2), showing an increase in fluorescence intensity with an increase in concentration of **5** for up to 0.016 mM. Quenching of fluorescence was observed above this concentration. This could be due to the solubility issue of this compound as the solution appeared to be slightly turbid. The K_d values were further calculated for **1**-**5** and are in the range of $0.16-4.7\times10^{-5}$ M for excitation at 285 nm, and $0.13-3.7\times10^{-5}$ M for excitation at 295 nm (Table 3). The K_d values decrease in the order **2** > **3** >> **1** > **5** > **4**, where ionic derivatives **2** and **3** have much improved K_d values than betulinic acid (**1**). The K_d values of our ionic derivatives are close to those for binding of common anti-HIV drugs to HSA (ranging between 4.4×10^{-5} M and 3.8×10^{-4} M).[36] Subramanyam et al.[34] determined the binding constant of betulinic acid (0.01−0.1 mM) with HSA (0.025 mM) as 1.685×10^{6} M^{-1} (fluorescence excitation at 285 nm), which is equivalent to a K_d value of 0.593×10^{-6} M, approximately one magnitude lower than our K_d value of 0.45×10^{-5} M (Table 3).

In conclusion, betulinic acid (**1**) and ionic derivatives (**2**-**5**) showed no appreciable inhibition against HIV-1 reverse transcriptase, but had significant activity against HSV-2. Compounds **1**, **2**, and **5** had low cytotoxicity with good selectivity indices. The compounds had antiviral activities similar to those reported previously for acyclovir. The binding properties of these compounds with HSA were similar to common anti-HIV drugs with compounds **2** and **3** having relatively higher dissociation equilibrium constants (K_d).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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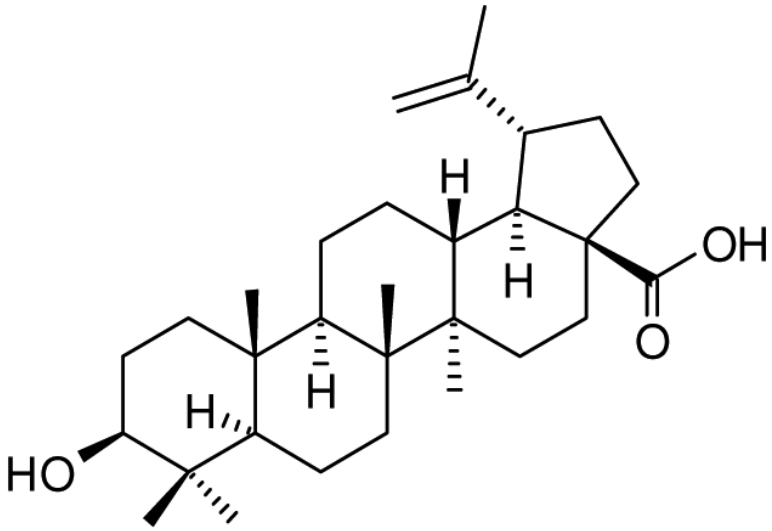
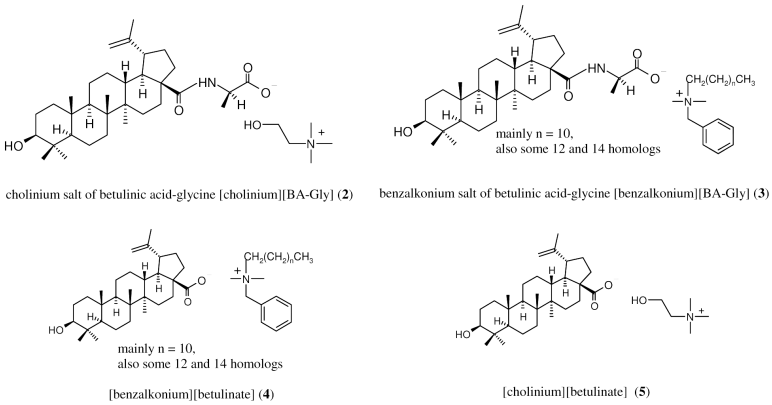


Scheme 1.
Structure of betulinic acid (**1**)



Scheme 2.

Ionic derivatives (**2-5**) of betulinic acid

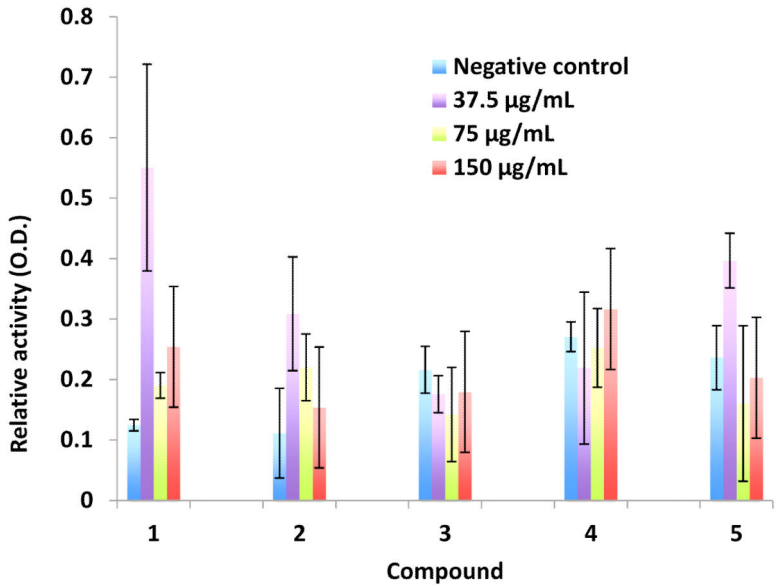


Figure 1.
Effect of betulinic acid (**1**) and derivatives (**2**-**5**) on reverse transcriptase (RT) activity

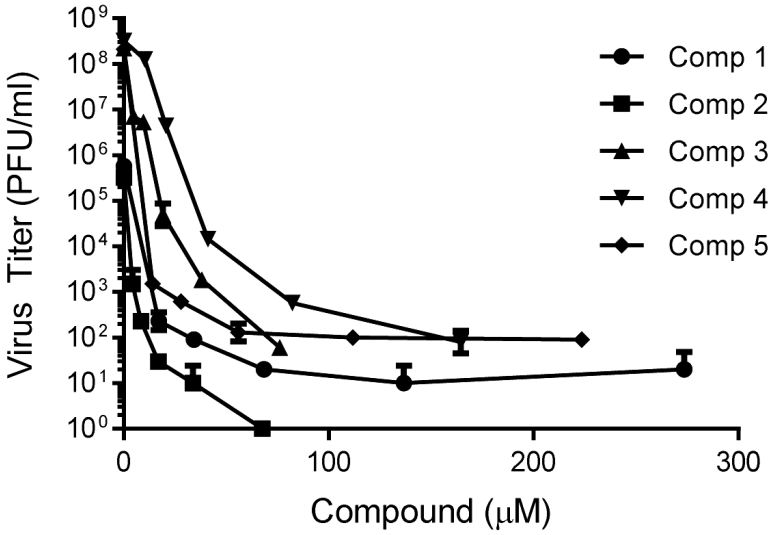


Figure 2.

Anti-herpes viral activity of selected compounds against the HSV-2 strain 186. IC_{50} (μM) values were calculated from viral replication yield reduction assays. The data represent the average viral titer in quadruplicate samples at each indicated drug concentration. Compounds **1** and **2**, and compounds **3**, **4** and **5** were tested in two separate experiments.

Table 1

Anti-HIV activity in the ratio of [MTT activity/(RT activity with compound/RT activity in DMSO)]

Compound	1 μg/mL	5 μg/mL
Negative control	0.464	0.798
Azidothymidine (AZT)	0.941 (1 μM)	0.580 (10 μM)
DMSO	**0.356**	**0.795**
1	0.158	0.649
2	0.319	0.885
4	0.121	0.474
5	0.201	0.576

Table 2

Antiviral herpesviral activity of derivatives of betulinic acid [a]

Compound	Cell Line	Virus	CC_{50} μM	IC_{50} μM	SI	Reference
Acyclovir	Vero	Multiple HSV-2 (including 186)	>10,000	~0.1–10	> 1,000	32, 33
1 [b]	Vero	HSV-2 186	> 100	1.6	> 62.5	*this study*
2	Vero	HSV-2 186	> 100	0.6	> 166.7	*this study*
3	Vero	HSV-2 186	10.5	0.9	11.6	*this study*
4	Vero	HSV-2 186	12	7.2	1.7	*this study*
5	Vero	HSV-2 186	> 100	0.9	> 111.1	*this study*
Betulin (Lup-20(29)-ene-3β,28-diol)	Vero	HSV-1 F	165	0.9	183	29
Betulin (Lup-20(29)-ene-3β,28-diol)	Vero	HSV-2 G	165	9.4	17.6	29
Betulin (Lup-20(29)-ene-3β,28-diol)	RC-37 (Vero)	HSV-1 KOS	49.7	0.7	71	31
3-α-hydroxylup-20(29)-ene-23,28-dioic acid	Vero	HSV-1 15577	246.9	64.4	3.8	30
3-epi-betulinic acid 3-*O*-sulfate	Vero	HSV-1 15578	228.3	45.7	5	30
betulinic acid (**1**)	RC-37 (Vero)	HSV-1 KOS	10.9	0.7	15.6	31
betulonic acid (3-oxolup-20(29)-en-28-oic acid)	Vero	HSV-1 7401H	35.6	5.7	6.2	28

[a] All previously published value were converted from mg/ml to mM for comparison.

[b] Values for compounds 1-5 are averages of triplicate samples.

Table 3

Dissociation equilibrium constant (K_d) of betulinic acid and derivatives to HSA

Compound	K_d/M(285 nm) [a]	K_d/M(295 nm) [a]	*R*
1	0.45×10^{-5}	0.13×10^{-5}	0.93, 0.94
2	4.7×10^{-5}	3.7×10^{-5}	0.95, 0.97
3	1.9×10^{-5}	1.9×10^{-5}	0.95, 0.97
4	0.16×10^{-5}	0.19×10^{-5}	0.94, 0.95
5	0.39×10^{-5}	0.40×10^{-5}	0.96, 0.99

[a] Fluorescence emission spectra were recorded on with excitation at 285 nm or 295 nm.