# Inhibition of Human Enterovirus 71 Replication by Pentacyclic Triterpenes and Their Novel Synthetic Derivatives

Chun-hui Zhao,[a,b] Jie Xu,[a] Ying-qiu Zhang,[c,d] Long-xuan Zhao,[b] and Bin Feng*[,e]

[a] *School of Life Sciences, Liaoning Normal University; Dalian 116029, China:* [b] *Liaoning Provincial Key Laboratory of Biotechnology and Drug Discovery; Dalian 116029, China:* [c] *Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences; Beijing 100101, China:* [d] *Institute of Cancer Stem Cell, Dalian Medical University; and* [e] *Department of Biotechnology, Dalian Medical University; Dalian 116044, China.*

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**A large number of bioactive pentacyclic triterpenoids have been shown to have multiple biological activities. This study was conducted to evaluate the inhibitory activities of 6 newly synthesized and novel pentacyclic triterpenoids against enterovirus 71 (EV71). The parent compound, ursolic acid (UA), showed the greatest inhibitory activity against EV71, while oleanolic acid (OA), asiatic acid (AA), and synthetic derivatives of 18-$\beta$-glycyrrhetinic acid (GA) and OA also exhibited inhibitory effects, although to lesser extents. The results suggest these compounds show potential for further optimization as antiviral candidates for treatment of EV71 infections.**

**Key words** enterovirus 71; pentacyclic triterpenoid; inhibitory activity

Human enterovirus 71 (EV71) is a small, non-enveloped virus belonging to the family Picornaviridae. It has been identified as the most frequent causative agent of hand, foot, and mouth disease (HFMD), and EV71 infection is usually accompanied by severe neurological complications which can cause high mortality, such as aseptic meningitis, brainstem encephalitis, neurogenic pulmonary edema, and acute flaccid paralysis.[1)] Children under 5 years old are particularly susceptible to the most severe forms of EV71 infection. EV71 was initially isolated in 1969 from patients in California (U.S.A.) with central nervous system diseases,[2)] and has since been implicated in large worldwide outbreaks of HFMD associated with severe neurological complications, especially in the Asia-Pacific region,[3,4)] including Singapore, Malaysia, Japan, and Taiwan.[5–8)] Since 2008, large outbreaks of EV71 infection in mainland China have resulted in millions of cases and hundreds of deaths in children.[9)] The EV71 capsid comprises four structural proteins: VP1, VP2, VP3, and VP4. Proteins VP1, VP2, and VP3 are located at the surface of the viral capsid and exposed to immune pressures, whereas VP4 is inside the capsid.[10)] In recent years, studies on the molecular biology of EV71 have led to the development of new strategies for treatment and prevention of EV71 infection; however, there is still no effective agent for treating EV71 infections.[11)] Therefore, it is essential to identify novel anti-EV71 agents as candidates for further research and optimization.

To date, *ca.* 20000 triterpenoids have been identified from various parts of medicinal plants.[12)] While numerous triterpenoids have demonstrated antiviral activity,[13)] only the pentacyclic triterpenoid ursolic acid (UA) extracted from *Ocimum basilicum* has been reported to exhibit potent anti-EV activity ($EC_{50}$ value of 0.5 $\mu$g/mL and 50% cytotoxic concentration ($CC_{50}$) value of 100.5 $\mu$g/mL) in a human skin basal cell carcinoma cell line (BCC-1/KMC).[14)] Our knowledge concerning the antiviral activity of natural and synthetic pentacyclic triterpenoids against EV71 is quite limited. The naturally occurring pentacyclic triterpenoids 18-$\beta$-glycyrrhetinic acid (GA), asiatic acid (AA), oleanolic acid (OA), and UA all contain hydroxyl groups and carboxylic groups (Fig. 1). While previous reports have suggested that the activity of these pentacyclic triterpenoids is related to their basic triterpenoid skeletal structure, the attached functional groups offer opportunities for chemical modification and improvement of activity.[15–17)] Optically active D-amino acids are important components in marketed drugs. Among these amino acids, D-phenylglycine is used to synthesize drugs such as ampicillin, aspoxicillin, cefbuperazone, and cefpiramide. D-Phenylglycine, instead of L-phenylglycine, was used as the building block in pharmaceuticals and developmental drugs to enhance enzymatic stability and improve pharmacodynamics and bioavailability.[18,19)] In the current study, functional groups attached to pentacyclic triterpene molecules were modified to generate derivatives while maintaining the pentacyclic triterpene skeletal structure. As a result of these modifications, the carboxylic group was converted to a methyl ester with retention of stereochemistry, which may significantly influence its physical properties or receptor interactions. D-Phenylglycine was introduced into the hydroxyl group at the C-2 position of AA, and at the C-3 positions of GA, OA, and UA. The dihydroxyl groups at C-3 and C-23 of AA were protected with acetonide using 2,2-dimethoxy propane.[15)] The present report describes six novel synthetic pentacyclic triterpenoids and their anti-EV71 activities, as compared to the activities of GA, AA, OA, and UA.

## Results and Discussion

**Synthesis of Pentacyclic Triterpenoid Derivatives** Pentacyclic triterpenoids were used as lead compounds, and structural modifications were made at positions C-3, C-30 of GA, C-2, C-3, C-23, C-28 of AA, and C-3 and C-28 of OA and UA, respectively. Similar procedures were used to synthesize derivatives of GA, UA, and OA, and the synthetic pathways are presented in Charts 1–3. The carboxylic groups (C-30 of GA, and C-28 of OA and UA) were subjected to methyl iodide treatment in the presence of potassium carbonate in dimethyl formamide to create methyl ester moieties with retention of stereochemistry. The hydroxyl group at C-3 of these pentacy-

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* To whom correspondence should be addressed. e-mail: binfeng@dlmedu.edu.cn

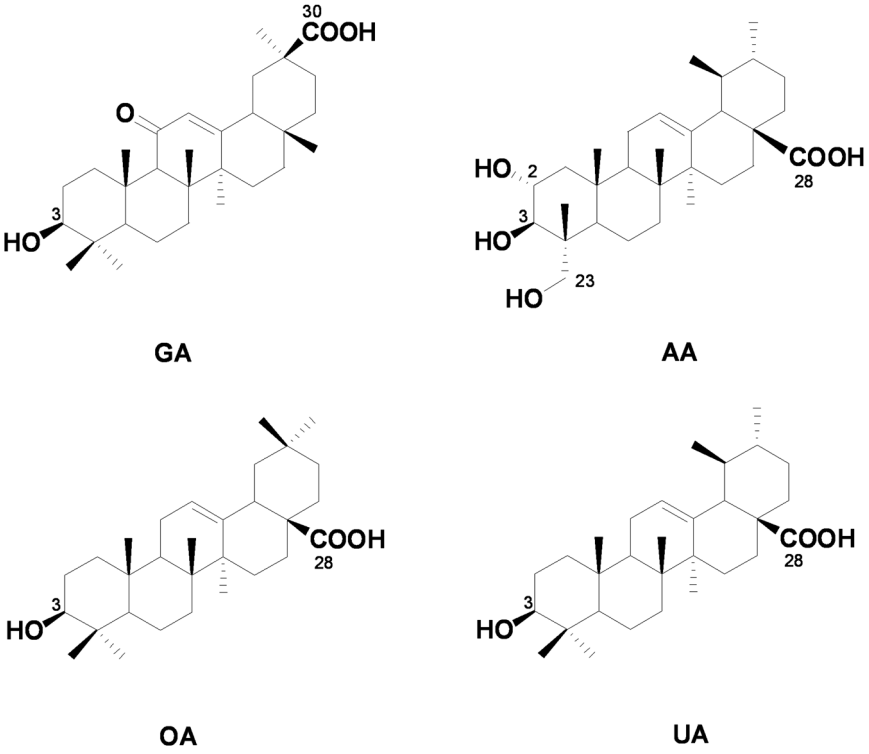
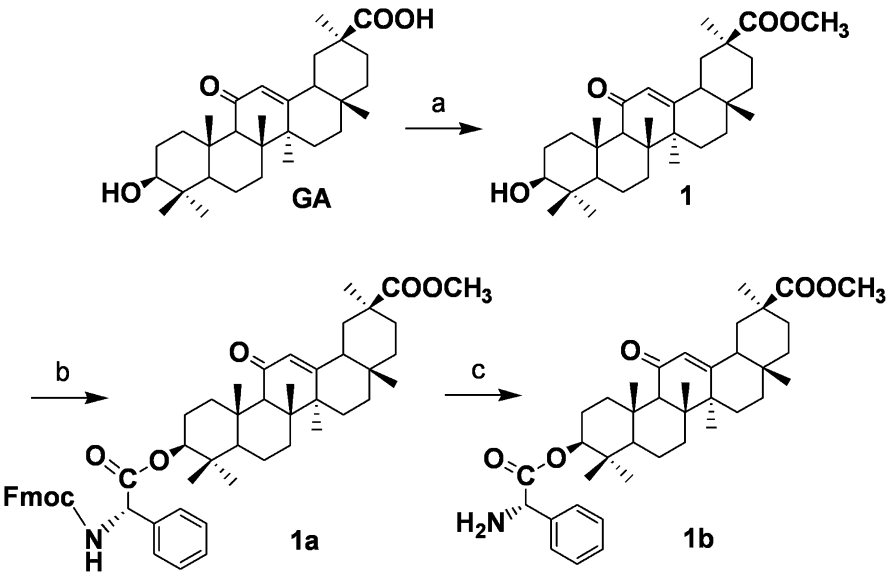



Fig. 1. Chemical Structures of Pentacyclic Triterpenoids

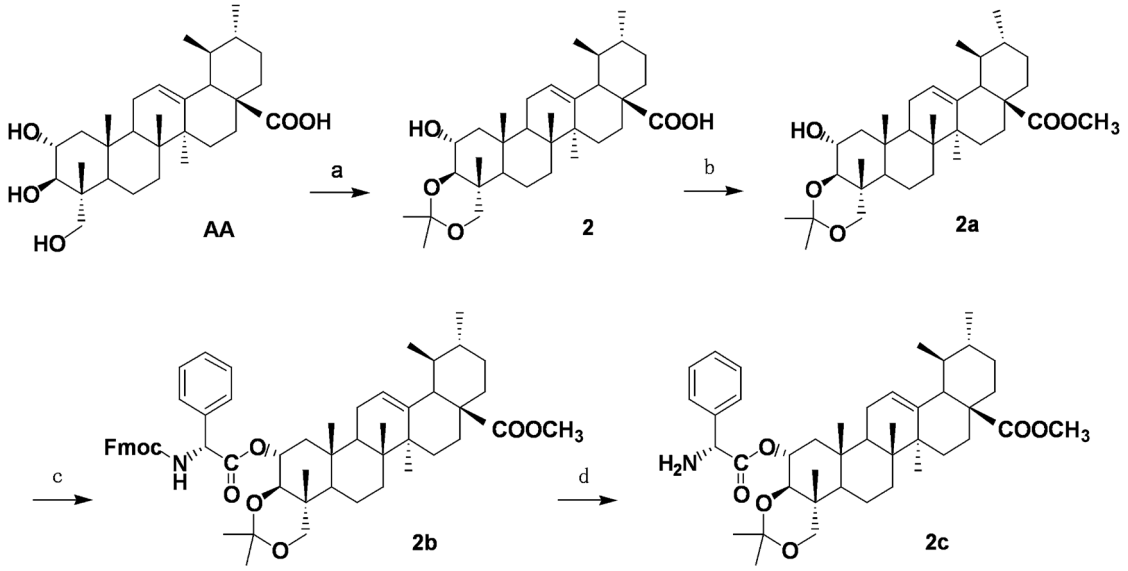



Reagents and conditions: a) $CH_3I$, $K_2CO_3$, DMF; b) Fmoc-D-Phg-OH, EDCI, DMAP, $CH_2Cl_2$; c) $Et_2NH$, $CH_2Cl_2$.

Chart 1. Synthesis of Glycyrrhetinic Acid Derivatives

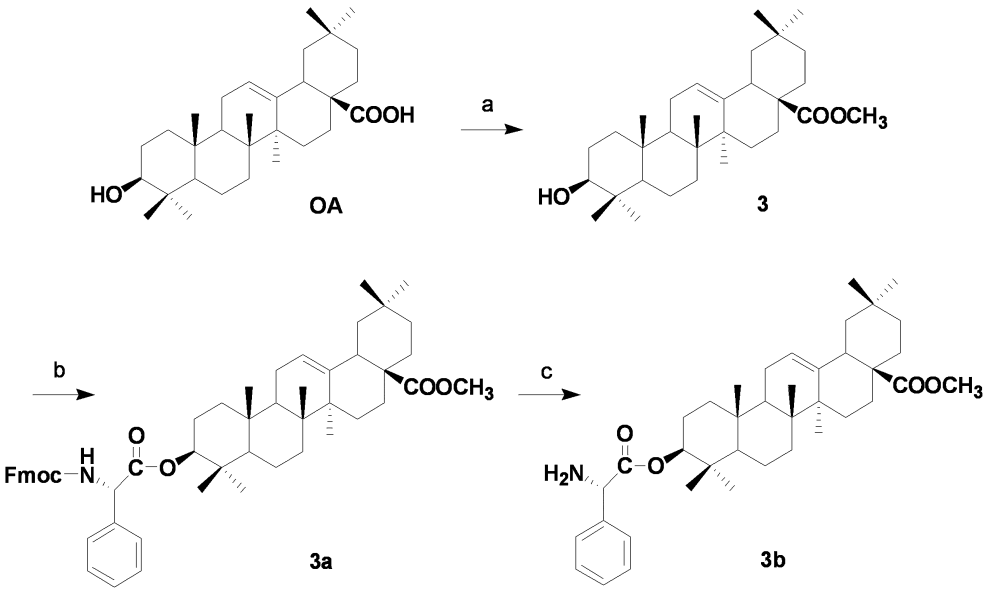
clic triterpenoids was treated with Fmoc-D-phenylglycine in the presence of 4-(dimethylamino)pyridine in dichloromethane to generate an intermediate, which in turn, was treated with diethylamine in dichloromethane to obtain end products with good yield. AA was prepared and its derivatives were synthesized as previously described.[20] As shown in Chart 4, the dihydroxyl groups at C-3 and C-23 of AA were treated with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid in dimethylformamide to generate acetonide. The carboxylic group at C-28 was converted to a methyl ester and the hydroxyl group at C-2 was treated with Fmoc-D-phenylglycine using the same synthetic pathways as used for other pentacyclic triterpenoids. All compounds were dissolved in dimethyl sulfoxide (DMSO) prior to use in biological activity assays.

**Assay of Anti-EV71 Activity** The VP1 gene sequence is specific to EV71 and is used to distinguish EV71 from other enteroviruses and in molecular epidemiologic studies.[21] The VP1 gene can be detected by using real-time polymerase chain reaction (PCR) with primers and probes targeting the VP1 region, and by Western blot analysis with an EV71 VP1 monoclonal antibody.[22,23] To examine the effects GA, AA, OA, UA, and their derivatives on EV71, virus-infected




Reagents and conditions: a) $(CH_3)_2C(OCH_3)_2$, *p*-TsOH, DMF; b) $CH_3I$, $K_2CO_3$, DMF; c) Fmoc-D-Phg-OH, EDCI, DMAP, $CH_2Cl_2$; d) $Et_2NH$, $CH_2Cl_2$.

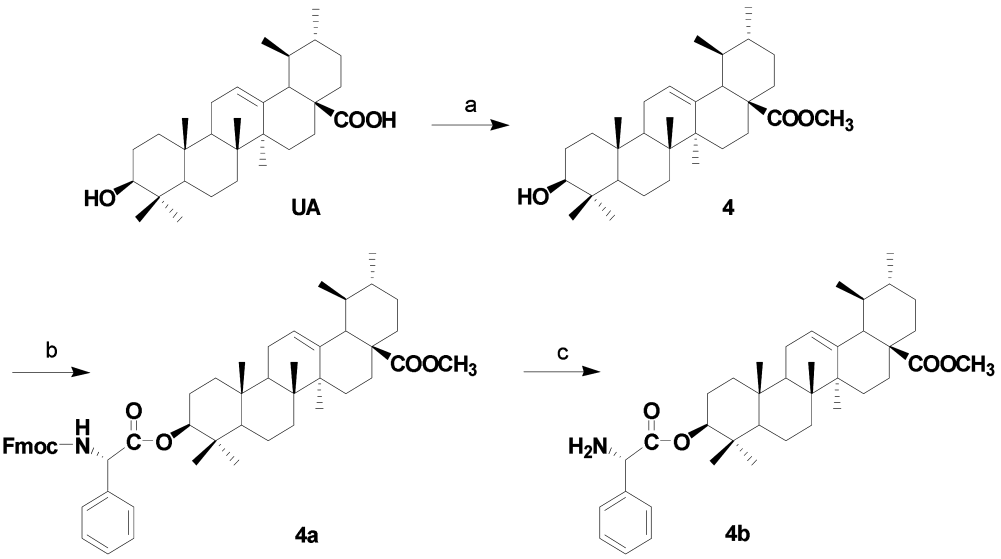
Chart 2. Synthesis of Asiatic Acid Derivatives[19)]




Reagents and conditions: a) $CH_3I$, $K_2CO_3$, DMF; b) Fmoc-D-phg-OH, EDCI, DMAP; c) $Et_2NH$, $CH_2Cl_2$.

Chart 3. Synthesis of Oleanolic Acid Derivatives

human rhabdomyosarcoma (RD) cells were incubated with the selected individual compounds at a concentration of 10 $\mu$g/mL for 48 h, and then harvested. The inhibitory effects of the various compounds on EV71 replication were analyzed by reverse transcription (RT)-PCR and Western blot analysis. RD cells incubated with compound **1b** showed an *ca.* 35% decrease in viral transcripts, while cells incubated with UA showed an *ca.* 45% reduction (Fig. 2A). However, there was no significant decrease of EV71 viral RNA transcripts in the RD cells treated with the other compounds (Fig. 2A). Western blot studies were used to analyze EV71 viral proteins in RD cells treated with various compounds (Fig. 2B). Results showed that RD cells treated with **1b** and UA, had decreased levels of VP1 viral protein (*ca.* 80% and 90% reductions, respectively), which corresponded with results obtained from RT-PCR assays. RD cells treated with AA, OA, **3a**, **3b**, **4a**, or **4b** showed partial suppression of VP protein expression. No significant decrease in VP1 protein levels was observed in RD cells treated with **1a**, **2b**, or **2c**. GA showed slightly inhibition activity (90% of control). These results suggested that modification of GA to form D-phenylglycine at the C-3 position, and modification of OA at the C-2 position may increase antiviral activity, but antiviral activity would not be increased by introduction of D-phenylglycine into the structural skeletons of UA and AA. These results also suggest that the inhibitory effects of various compounds on EV71 infection might be mediated through different mechanisms. Future studies using both of D-phenylglycine and L-phenylglycine to synthesize new compounds would ascertain the structure–activity relationship of the two enantiomers. The cytotoxicity of these




Reagents and conditions: a) $CH_3I$, $K_2CO_3$, DMF; b) Fmoc-D-phg-OH, EDCI, DMAP; c) $Et_2NH$, $CH_2Cl_2$.

Chart 4. Synthesis of Ursolic Acid Derivatives

compounds was evaluated against RD cells *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine ethosulfate (MTS/PMS) assay, and all compounds tested showed a low level of toxicity ($CC_{50}$>50 $\mu$g/mL).

In conclusion, data from the present study collectively suggest that the pentacyclic triterpenoid compounds UA, OA, and AA have inhibitory effects against EV71. Synthetic derivatives of GA and OA showed greater antiviral effects than the natural parent compounds, and lacked significant cytotoxicity. Due to the lack of effective drugs for treatment of EV71 infections, these compounds should be further evaluated as antiviral agents. Future studies will be conducted to better determine the accuracy of $IC_{50}$ values obtained for the individual compounds and the effect of these compounds on key steps involved in EV71 replication; *i.e.*, translation of the internal ribosomal entry site (IRES)-mediated viral polyprotein; the proteolytic activity of viral proteases 2A and/or 3C; and activity of viral 3D-RNA-dependent RNA polymerase (RdRp). Results of such studies will help to elucidate the molecular targets modulated by these compounds.

## Experimental

**Materials and Methods** Melting points (mp, uncorrected) were determined in glass capillaries using an X-5 melting point apparatus (Beijing Tech Instrument Co., Ltd., China). Nuclear magnetic resonance (NMR) experiments ($^1$H-NMR and $^{13}$C-NMR) were conducted using a Bruker Avance 500 MHz spectrometer (Bruker, Karlsruhe, Germany). Infrared radiation (IR) data were analyzed using a Shimadzu IRPrestige-21 FT-IR spectrophotometer (Shimadzu, Kyoto, Japan). Mass spectra were recorded on a Micromass QTof-micro™ spectrometer (Micromass, Manchester, U.K.). Elemental analyses were performed using a Elementar Analysensysteme GmbH (Elementar, Hanau, Germany). All reagents and chemicals were of analytical grade or chemically pure.

**Chemical Synthesis. Synthesis of GA Derivatives** Methyl 3$\beta$-Hydroxyl-11-oxo-olean-12-en-30-oate (**1**): A solution of GA (1 g, 2.125 mmol) and anhydrous potassium carbonate (735 mg, 5.32 mmol) in dry dimethyl formamide (10 mL) was added to methyl iodide (0.26 mL, 4.25 mmol) and stirred at room temperature for 6 h. The reaction mixture was then diluted with ethyl acetate (50 mL), and washed with water (20 mL×3) and saturated sodium chloride solution. The organic layer was dried over anhydrous magnesium sulfate. The solution was filtered, the remaining solvent was removed by evaporation at reduced pressure, and the isolated material was purified by silica gel chromatography using a gradient elution of ethyl acetate–petrol ether (1 : 2, v/v) to afford a white solid (875 mg, 85%), mp 240.5–241.8°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 5.65 (s, 1H, H-12), 3.68 (s, 3H, –$OCH_3$), 3.24–3.21 (m, 1H, H-3), 2.79 (dt, $J_1$=13.2 Hz, $J_2$=3.6 Hz, 1H, H-18), 2.34 (s, 1H, H-9), 1.36, 1.15, 1.14, 1.12, 1.00 (s, each 3H, –$CH_3$), 0.80 (s, 6H, –$CH_3$×2); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 200.22, 176.98, 169.22, 135.37, 128.70, 79.00, 62.08, 55.22, 52.03, 48.67, 45.66, 44.31, 43.46, 41.39, 39.41, 38.03, 37.38, 33.06, 32.13, 31.43, 28.83, 28.63, 28.40, 27.63, 26.79, 26.73, 23.70, 19.00, 17.81, 16.69, 15.89; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3453, 2945, 1723, 1662; electrospray ionization (ESI)-MS: *m*/*z* 485.02 $[M-H]^-$.

Methyl 3$\beta$-*O*-(*N*-Fluorenylmethoxycarbonyl)-phenylglycyl-11-oxo-olean-12-en-30-oate (**1a**): A solution of compound **1** (500 mg, 1.03 mmol), Fmoc-D-phenylglycine (578.7 mg, 1.55 mmol), and *N*,*N*-dimethylaminopyridine (DMAP) (189.3 mg, 1.55 mmol) in methylene chloride (5 mL) was added to 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (197.1 mg, 1.55 mmol) at 0°C and stirred for 3 h at room temperature. The reaction mixture was then diluted with methylene chloride and washed with water and saturated sodium chloride solution (10 mL). The solution was filtered, the remaining solvent was removed by evaporation at reduced pressure, and the isolated material was purified by silica gel chromatography using a gradient elution of ethyl acetate–petroleum ether (1 : 5, v/v) to afford a white solid (347.2 mg, 40.9%), mp 125.4–126.0°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.68 (d, $J$=7.4 Hz, 2H, fluorenyl H-4′, H-5′), 7.51 (d, $J$=4.0 Hz, 2H, fluorenyl H-1′, H-8′), 7.32 (d, $J$=7.0 Hz, 2H, fluorenyl H-3′, H-6′), 7.23 (d, $J$=6.5 Hz, 2H, fluorenyl H-2′, H-7′), 7.34–7.21

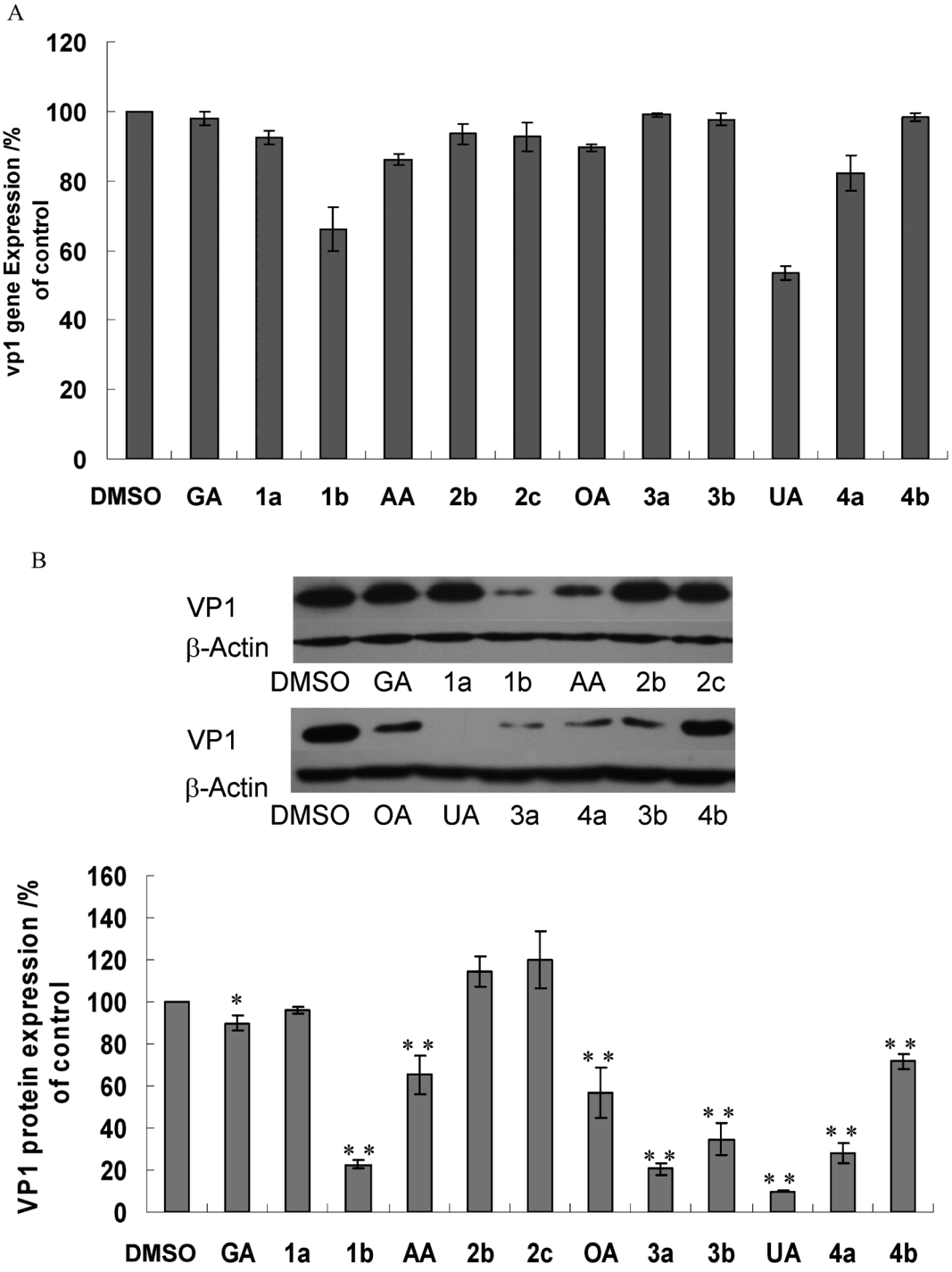



Fig. 2. Inhibition of Enterovirus 71 (EV71) Replication by Various Compounds

(A) Levels of the EV71 RNA transcripts were determined using real-time PCR. Results represent the mean percentage of EV71 RNA transcripts±S.D. from three independent experiments. The ANOVA for comparison of EV71 RNA transcript levels in untreated cells and cells treated with 10 $\mu$g/mL UA or **1b** showed $p<0.05$. (B) Western blot analysis was performed using specific monoclonal antibodies against VP1 structural proteins of EV71. $\beta$-Actin was used as an internal control with anti-$\beta$-actin monoclonal antibodies. Data represent results obtained from three independent experiments. The difference among different treatments was assessed by one-way ANOVA followed by *post-hoc* Tukey's multiple comparison test (*$p<0.05$, **$p<0.001$ *versus* the control group with DMSO).

(m, 5H, Ph-H), 5.96 (d, $J$=7.1 Hz, 1H, Ph-CH), 5.58 (s, 1H, H-12), 5.88 (d, $J$=6.9 Hz, 1H, –NH–), 5.20–5.16 (m, 1H, H-2), 4.52–4.42 (m, 2H, $-CH_2O-$), 4.19 (t, $J$=7.0 Hz, 1H, fluorenyl H-9′), 3.53 (s, 3H, $-OCH_3$), 2.72 (dt, $J_1$=14.0 Hz, $J_2$=3.9 Hz, 1H, H-18), 2.25 (s, 1H, H-9), 1.07, 0.89, 0.85, 0.80,0.79 (s, each 3H, $-CH_3$), 0.71 (s, 6H, $-CH_3\times2$); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 199.92, 176.93, 169.26, 143.91, 128.89, 128.84, 128.54, 128.51, 125.12, 82.70, 77.29, 77.03, 76.78, 67.15, 61.67, 55.05, 51.78, 48.45, 47.21, 45.40, 44.08, 43.24, 41.15, 38.73, 38.25, 38.11, 37.78, 36.92, 32.69, 31.87, 31.18, 29.72, 28.54, 28.34, 26.46, 23.70, 23.37, 22.68, 19.20, 18.70, 17.38, 17.24, 16.65, 16.35, 14.42, 14.12; IR $\nu_{max}$ (KBr, cm$^{-1}$): 3439, 2954, 2890, 1710, 1532, 1438, 1369, 1206, 1156, 796, 710; ESI-MS: $m/z$ 839.83 $[M-H]^-$. *Anal.* Calcd for $C_{54}H_{65}NO_7$: C, 77.20; H, 7.80; N, 1.67. Found: C, 77.13; H, 7.75; N, 1.66.

Methyl 3$\beta$-*O*-Phenylglycyl-11-oxo-olean-12-en-30-oate (**1b**):

A solution of compound **1a** (133 mg, 0.16 mmol) in dry methylene chloride (2 mL) was added to diethylamine (3.32 mL, 32.24 mmol) and stirred at room temperature for 2 h. The solvent was evaporated at reduced pressure, and the residue was purified by silica gel chromatography using a gradient elution of ethyl acetate–petroleum ether (1 : 5, v/v) to afford a white solid (83.2 mg, 85%), mp 226.8–227.1°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.32–7.21 (m, 5H, Ph-H), 5.58 (s, 1H, H-12), 4.53 (s, 2H, $-NH_2$), 4.43 (dd, $J_1$=11.8 Hz, $J_2$=4.8 Hz, 1H, H-3), 4.22 (t, $J$=6.7 Hz, 1H, Ph-CH), 3.61(s, 3H, $-OCH_3$), 2.72 (dt, $J_1$=14.0 Hz, $J_2$=3.9 Hz, 1H, H-18), 2.25 (s, 1H, H-9), 1.27, 1.07, 1.06, 1.03, 0.80, 0.58, 0.38 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 199.98, 176.93, 169.21, 130.90, 128.86, 128.64, 128.53, 127.95, 127.02, 81.72, 65.57, 61.69, 59.09, 54.99, 51.77, 48.44, 45.39, 44.07, 43.22, 41.13, 38.74, 38.23, 37.77, 36.91, 32.69, 31.86, 31.17, 30.61, 28.53, 28.33, 27.47, 26.49, 26.45, 23.68, 23.35, 19.20, 18.69, 17.24, 16.34, 13.73; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3448, 3380, 2967, 2824, 1724, 1653, 1465, 1384, 1256, 1167, 769, 712; ESI-MS: *m/z* 617.44 $[M-H]^-$. *Anal.* Calcd for $C_{39}H_{55}NO_5$: C, 75.81; H, 8.97; N, 2.27. Found: C, 75.66; H, 8.85; N, 2.16.

**Synthesis of Asiatic Acid Derivatives** AA derivatives (compounds **2** and **2a**–**c**, structures shown in Chart 2) were synthesized as previously described.[19] *Anal.* Calcd for $C_{57}H_{71}NO_8$ (**2b**): C, 76.22; H, 7.97; N, 1.56. Found: C, 76.08; H, 7.86; N, 1.43. *Anal.* Calcd for $C_{42}H_{61}NO_6$ (**2c**): C, 74.63; H, 9.10; N, 2.07. Found: C, 74.48; H, 8.98; N, 1.94.

**Synthesis of Oleanolic Acid Derivatives** Methyl 3$\beta$-Hydroxyolean-12-ene-28$\beta$-oate (**3**): A solution of OA (5 g, 11 mmol) and anhydrous potassium carbonate (3.78 g, 27.35 mmol) in dry dimethyl formamide (50 mL) was added to methyl iodide (1.37 mL, 21.89 mmol) and stirred at room temperature for 6 h. The reaction mixture was then diluted with ethyl acetate (300 mL) and washed with water (100 mL×3) and saturated NaCl solution (50 mL). The organic layer was dried over anhydrous magnesium sulfate. The solution was filtered, the remaining solvent was removed by evaporation at reduced pressure, and the isolated material was purified by silica gel chromatography using a gradient elution of ethyl acetate–petrol ether (1 : 5, v/v) to afford a white solid (5 g, 97%), mp 197.0–198.0°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 5.27 (t, $J$=3.6 Hz, 1H, H-12), 3.61 (s, 3H, $-OCH_3$), 3.20 (dd, $J$=10.4, 5.2 Hz, 1H, H-3), 2.85 (dd, $J$=14.0, 4.0 Hz, 1H, H-18), 1.06, 1.00, 0.92, 0.78, 0.73 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 178.25, 143.76, 122.36, 78.96, 77.29, 77.03, 76.78, 55.24, 51.49, 47.63, 46.71, 45.88, 41.63, 41.29, 39.27, 38.74, 38.45, 37.03, 33.86, 33.10, 32.67, 32.37, 30.67, 28.10, 27.70, 27.19, 25.93, 23.63, 23.39, 23.07, 18.33, 16.82, 15.57, 15.29; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3448, 2948, 1728, 1426; ESI-MS: *m/z* 471.28 $[M-H]^-$.

Methyl 3$\beta$-*O*-(*N*-Fluorenonemethyoxycarbonyl)-phenylglycylurs-12-ene-28$\beta$-oate (**3a**): A solution of compound 3 (400 mg, 0.86 mmol) and Fmoc-D-phenylglycine (382 mg, 1.02 mmol) in dry methylene chloride (7 mL) was added to 4-dimethylaminopyridine (124 mg, 1.02 mmol) and stirred. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (197 mg, 1.02 mmol in dry dichloromethane (3 mL)) was added drop-wise to the reaction mixture at 0°C and the mixture was stirred for 5 min at 0°C, followed by additional stirring for 5 h at room temperature. The reaction mixture was then diluted with methylene chloride (50 mL) and washed with water (15 mL×3) and saturated sodium chloride solution (30 mL). The organic layer was dried over anhydrous magnesium sulfate. The residue was filtered, the remaining solvent was removed by evaporation at reduced pressure, and the isolated material was purified by silica gel chromatography with a gradient elution of ethyl acetate–petrol ether (1 : 10, v/v) and the obtained white solid was recrystallized from methanol, providing **3a** (410 mg, 58.1%) as colorless solid; mp 117.7–118.6°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.76 (d, 2H, $J$=7.5 Hz, fluorenyl H-4, H-5), 7.58 (d, 2H, $J$=6.2 Hz, fluorenyl H-1, H-8), 7.30–7.41 (m, 9H, fluorenyl H-2, H-7, H-6, H-3, 5×Ph-H), 5.99 (dd, $J$=6.8 Hz, 1H, CH–NH–CO), 5.32 (dd, 1H, $J$=7.1 Hz, CH–NH–CO), 5.25 (t, 1H, $J$=3.5 Hz, H-12), 4.47–4.58 (m, 1H, H-3), 4.39 (m, 2H, $-CHCH_2OCONH-$), 4.25 (m, 1H, fluorenyl H-9), 3.60 (s, 3H, $OCH_3$), 2.85 (dd, 1H, $J$=14 Hz, H-18), 1.08, 0.95, 0.93, 0.87, 0.75, 0.67, 0.48 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 178.28, 143.86, 128.85, 127.71, 127.21, 127.09, 119.99, 82.99, 55.26, 51.52, 47.21, 45.91, 41.68, 39.31, 38.07, 37.90, 37.77, 36.91, 33.90, 33.12, 32.59, 30.72, 27.71, 25.91, 23.66, 23.43, 23.10, 18.06, 16.85, 16.35, 15.30; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3340, 2922, 1730, 1577, 1472; ESI-MS: *m/z* 825.82 $[M-H]^-$. *Anal.* Calcd for $C_{54}H_{67}NO_6$: C, 78.51; H, 8.17; N, 1.70. Found: C, 78.41; H, 8.03; N, 1.59.

Methyl 3$\beta$-*O*-Phenylglycylurs-12-ene-28-oate (**3b**): A solution of compound **3a** (200 mg, 0.24 mmol) in dry methylene chloride (5 mL) was added to diethylamine (5 mL, 49.5 mmol) and stirred at room temperature for 2 h. The solvent was evaporated at reduced pressure and the residue was purified by silica gel chromatography using a gradient elution of ethyl acetate–petrol ether (1 : 2, v/v) and the obtained white solid was recrystallized from ethyl ether, providing **3b** as colorless solid; (88 mg, 60.0%), mp 195.6–196.9°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.39–7.27 (m, 5H, 5×Ph-H), 5.25 (t, $J$=3.6 Hz, 1H, H-12), 4.60 (d, $J$=13.9 Hz, 1H, $NH_2CHOCO$), 4.52–4.45 (m, 1H, H-3), 3.60 (s, 3H, $-OCH_3$), 2.85 (dd, $J$=13.9, 4.0 Hz, 1H, 4.2 Hz, H-18), 1.12, 0.98, 0.89, 0.87, 0.70, 0.63, 0.44 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 178.29, 143.85, 128.63, 129.90, 126.96, 126.69, 122.28, 81.96, 59.21, 55.27, 51.51, 47.56, 46.76, 41.68, 39.31, 38.10, 37.98, 37.89, 37.76, 36.91, 36.91, 33.90, 30.71, 29.73, 28.12, 27.71, 25.91, 23.66, 23.43, 23.10, 18.08, 16.85, 16.66, 16.37, 15.30; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3444, 2935, 1729, 1530, 1460; ESI-MS: *m/z* 635.39 $[M-H]^-$. *Anal.* Calcd for $C_{39}H_{57}NO_4$: C, 77.58; H, 9.51; N, 2.32. Found: C, 77.46; H, 9.45; N, 2.24.

**Synthesis of Ursolic Acid and Its Derivatives** Methyl 3$\beta$-Hydroxyurs-12-ene-28$\beta$-oate (**4**): Compound **4** was synthesized as a white solid (4.9 g, 95%) using UA (5 g, 11 mmol) as a starting material. The procedure was the same as that used for synthesis of compound **3** from OA, mp 171.0–172.5°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 5.25 (t, $J$=3.6 Hz, 1H, H-12), 3.60 (s, 3H, $-OCH_3$), 3.20 (dd, $J$=10.4, 5.2 Hz, 1H, H-3), 2.22 (d, $J$=11.2 Hz, 1H, H-18), 1.06, 1.00, 0.93, 0.92, 0.85, 0.78, 0.73 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 178.08, 138.17, 125.59, 79.05, 77.29, 77.04, 76.78, 55.25, 52.91, 51.46, 48.11, 47.59, 42.02, 39.51, 39.07, 38.89, 38.77, 38.64, 37.00, 36.66, 33.00, 30.68, 28.16, 28.05, 27.25, 24.25, 23.63, 23.32, 21.20, 18.34, 17.05, 16.93, 15.64, 15.45; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3452, 2923, 1722, 1458; ESI-MS: *m/z* 471.13 $[M-H]^-$.

Methyl 3$\beta$-*O*-(*N*-Fluorenonemethyoxycarbonyl)-phenylglycylurs-12-ene-28$\beta$-oate (**4a**): Compound **4a** was synthesized as a colorless solid (510 mg, 57.7%) from com-

pound **4** (500 mg, 1.07 mmol). The procedure was the same as that used for synthesis of compound **3a** from compound **3**, mp 115.6–116.5°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.76 (d, $J$=7.5 Hz, 2H, fluorenyl H-4, H-5), 7.58 (d, $J$=6.2 Hz, 2H, fluorenyl H-1, H-8), 7.41–7.30 (m, 9H, fluorenyl H-2, H-3, H-6, H-7, 5×Ph-H), 5.99 (d, $J$=6.8 Hz, 1H, –CH–NH–CO), 5.32 (d, $J$=7.05 Hz, 1H, –CH–NH–CO), 5.25 (t, $J$=3.5 Hz, 1H, H-12), 4.57–4.48 (m, 1H, H-3), 4.39–4.38 (m, 2H, –$CHCH_2OCONH$–), 4.22–4.20 (m, 1H, fluorenyl H-9), 3.60 (s, 3H, –$OCH_3$), 2.22 (d, $J$=11.2 Hz, 1H, H-18), 1.08, 0.95, 0.93, 0.87, 0.75, 0.67, 0.48 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 178.04, 143.84, 128.85, 127.71, 127.21, 127.09, 119.99, 83.00, 55.26, 52.91, 41.44, 48.13, 42.03, 39.08, 38.91, 37.89, 37.75, 36.85, 36.67, 32.89, 30.68, 28.04, 24.25, 23.69, 23.60, 23.33, 21.19, 18.05, 17.07, 16.92, 15.44; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3348, 2931, 1722, 1564, 1452; ESI-MS: $m/z$ 826.31 $[M-H]^-$. *Anal.* Calcd for $C_{54}H_{67}NO_6$: C, 78.51; H, 8.17; N, 1.70. Found: C, 78.38; H, 8.05; N, 1.58.

Methyl 3$\beta$-*O*-Phenylglycylurs-12-ene-28$\beta$-oate (**4b**): Compound **4b** was obtained as a colorless solid (78 mg, 53.8%) from **4a** (200 mg, 0.24 mmol). The procedure was the same as that used for synthesis of compound **3b** from compound **3a**, mp 54.0–55.0°C; $^1$H-NMR (500 MHz, $CDCl_3$) $\delta$: 7.40–7.25 (m, 5H, 5×Ph-H), 5.25 (t, $J$=3.5 Hz, 1H, H-12), 4.6 (d, $J$=13.5 Hz, 1H, $H_2NCHOCO$), 4.45–4.52 (m, 1H, H-3), 3.60 (s, 3H, –$OCH_3$), 2.24 (d, $J$=11.2 Hz, 1H, H-18), 1.08, 0.95, 0.93, 0.87, 0.75, 0.67, 0.48 (s, each 3H); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta$: 176.07, 138.20, 128.64, 127.94, 126.94, 126.68, 125.44, 81.98, 59.11, 55.21, 52.88, 51.44, 48.09, 47.44, 41.99, 39.48, 39.04, 38.87, 38.22, 37.85, 36.81, 36.64, 32.85, 30.65, 29.71, 28.00, 27.47, 24.22, 23.65, 23.57, 23.30, 21.19, 18.03, 17.06, 16.89, 16.41, 15.42; IR $\nu_{max}$ (KBr, $cm^{-1}$): 3447, 2937, 1731, 1581, 1436; ESI-MS: $m/z$ 635.51 $[M-H]^-$. *Anal.* Calcd for $C_{39}H_{57}NO_4$: C, 77.58; H, 9.51; N, 2.32. Found: C, 77.43; H, 9.42; N, 2.23.

**Biological Activity. Cells and Viruses** Human rhabdomyosarcoma cells (RD cells, ATCC accession no. CCL-136) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C with 5% $CO_2$. RD cells were used in propagation of enterovirus 71 (BrCr-Ts strain GenBank: AB 204853.1).

**Western Blot Analysis** RD cells were inoculated into 12-well plates containing complete medium at a concentration of $2\times10^5$ cells per well and cultured for 24 h. The pentacyclic triterpenes and their derivatives were added into each well at a final concentration of 0.01 $\mu g\cdot\mu L^{-1}$, respectively. Wells containing cells and control wells received equal amounts of DMSO. Six hours later, the RD cells were infected with 10 $\mu$L of EV71 (tissue culture infective dose ($TCID_{50}$)=$1\times10^9$), followed by incubation at 37°C for 48 h and subsequent extraction of total protein by RIPA buffer (Sigma, St. Louis, MO, U.S.A.). The cells were washed with ice-cold phosphate buffered saline (PBS) solution and lysed in lysis buffer at 0°C for 20 min. Cell lysates were harvested, centrifuged at 12000 rpm for 10 min, and the supernatants were collected. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk in TBST for 1 h at room temperature (RT), washed, and then incubated overnight at 4°C with primary antibody (1 : 2000 dilution) in TBST. Primary antibody specific for EV71 VP1 protein was obtained from Millipore (Billerica, MA, U.S.A.) and $\beta$-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The membrane was then incubated with goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1 : 2000; GE Healthcare, U.S.A.) as a secondary antibody at RT for 1 h. The immuno-reactive bands were detected by an enhanced chemiluminescence detection system (GE Healthcare).

**Real-Time Quantitative PCR** RD cells were treated as for Western blot analysis. Cellular RNA was extracted from cells using TRIzol reagent (Life Technologies, Grand Island, NY, U.S.A.). RNA was reverse-transcribed into cDNA using a reverse transcription kit (Promega, Sunnyvale, CA, U.S.A.) following the manufacturer's instructions. Quantification of mRNA expression was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, U.S.A.). Thermal cycling was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Specific primers for the EV71 vp1 gene were 5′-AGT ATG ATT GAG ACT CGG TG-3′ (forward) and 5′-GCG ACA AAA GTG AAC TCT GC-3′ (reverse). $\beta$-Actin was used to normalize levels of gene expression. Primers for the $\beta$-actin gene were 5′-ACC CAC ACT GTG CCC ATC TAC GA-3′ (forward) and 5′-GCC GTG GTG GTG AAG CTG TAG CC-3′ (reverse). Each reaction was repeated three times. Expression levels of mRNAs were analyzed by $2^{-\Delta\Delta Ct}$.

**Cytotoxicity Analysis** RD cells were seeded into 96-well plates at a concentration of 5000 cells per well and incubated for 24 h at 37°C with 5% $CO_2$. The incubation medium was then replaced with fresh medium and a 2-fold serial dilution of test compound was added to each well. After incubation for 48 h, the culture medium was replaced with 100 $\mu$L DMEM medium containing 20 $\mu$L MTS/PMS (Promega). After incubation at 37°C for 3 h, the absorbance was measured at 490 nm using a microplate reader. Survival rates of treated cells were determined with the following equation: cell viability=($OD_{490}$ of treated cells−$OD_{490}$ of blank)/($OD_{490}$ of control cells−$OD_{490}$ of blank). Data are expressed as means±S.D. of triplicate determinations.

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## References

1) Bek E. J., McMinn P. C., *Future Virol.*, **5**, 453–468 (2010).
2) Schmidt N. J., Lennette E. H., Ho H. H., *J. Infect. Dis.*, **129**, 304–309 (1974).
3) Ooi M. H., Wong S. C., Lewthwaite P., Cardosa M. J., Solomon T., *Lancet Neurol.*, **9**, 1097–1105 (2010).
4) Weng K. F., Chen L. L., Huang P. N., Shih S. R., *Microbes Infect.*, **12**, 505–510 (2010).
5) Chan K. P., Goh K. T., Chong C. Y., Teo E. S., Lau G., Ling A. E., *Emerg. Infect. Dis.*, **9**, 78–85 (2003).
6) Chan L. G., Parashar U. D., Lye M. S., Ong F. G. L., Zaki S. R., Alexander J. P., Ho K. K., Han L. L., Pallansch M. A., Suleiman A. B., Jegathesan M., Anderson L. J., Outbreak Study Group, *Clin. Infect. Dis.*, **31**, 678–683 (2000).

7) Tu P. V., Thao N. T. T., Perera D., Truong K. H., Tien N. T. K., Thuong T. C., How O. M., Cardosa M. J., McMinn P. C., *Emerg. Infect. Dis*., **13**, 1733–1741 (2007).
8) Ho M., Chen E. R., Hsu K. H., Twu S. J., Chen K. T., Tsai S. F., Wang J. R., Shih S. R., *N. Engl. J. Med*., **341**, 929–935 (1999).
9) Yang F., Ren L., Xiong Z., Li J., Xiao Y., Zhao R., He Y., Bu G., Zhou S., Wang J., Qi J., *J. Clin. Microbiol.*, **47**, 2351–2352 (2009).
10) Brown B. A., Pallansch M. A., *Virus Res*., **39**, 195–205 (1995).
11) Wu K. X., Ng M. M. L., Chu J. J. H., *Drug Discov. Today*, **15**, 1041–1051 (2010).
12) Liby K. T., Yore M. M., Sporn M. B., *Nat. Rev. Cancer*, **7**, 357–369 (2007).
13) Rezanka T., Siristova L., Sigler K., *Anti-infective Agents in Medicinal Chemistry*, **8**, 169–192 (2009).
14) Chiang L. C., Ng L. T., Cheng P. W., Chiang W., Lin C. C., *Clin. Exp. Pharmacol. Physiol*., **32**, 811–816 (2005).
15) Zhao L. X., Park H. G., Jew S. S., Lee M. K., Kim Y. C., Thapa P., Karki R., Jahng Y., Jeong B. S., Lee E. S., *Bull. Korean Chem. Soc*., **28**, 970–976 (2007).
16) Shanmugam M. K., Nguyen A. H., Kumar A. P., Tan B. K. H., Sethi G., *Cancer Lett*., **320**, 158–170 (2012).
17) Meng Y. Q., Li Y. Y., Li F. Q., Song Y. L., Wang H. F., Chen H., Cao B., *J. Asian Nat. Prod. Res*., **14**, 844–855 (2012).
18) Ma J. S., *Chim. Oggi*., **21**, 65–68 (2003).
19) Faber K., Biocatalytic applications. *Biotransformations in organic chemistry*, 6th edn. Springer, Germany (2011).
20) Wang L., Xu J., Zhao C. H., Zhao L. X., Feng B., *Chem. Pharm. Bull*., **61**, 1015–1023 (2013).
21) Oberste M. S., Maher K., Kilpatrick D. R., Flemister M. R., Brown B. A., Pallansch M. A., *J. Clin. Microbiol.*, **37**, 1288–1293 (1999).
22) Tan E. L., Tan T. M. C., Tak K. C. V., Poh C. L., *Mol. Ther.*, **15**, 1931–1938 (2007).
23) Zhu Q. C., Wang Y., Liu Y. P., Zhang R. Q., Li X., Su W. H., Long F., Luo X. D., Peng T., *Eur. J. Pharm. Sci*., **44**, 392–398 (2011).