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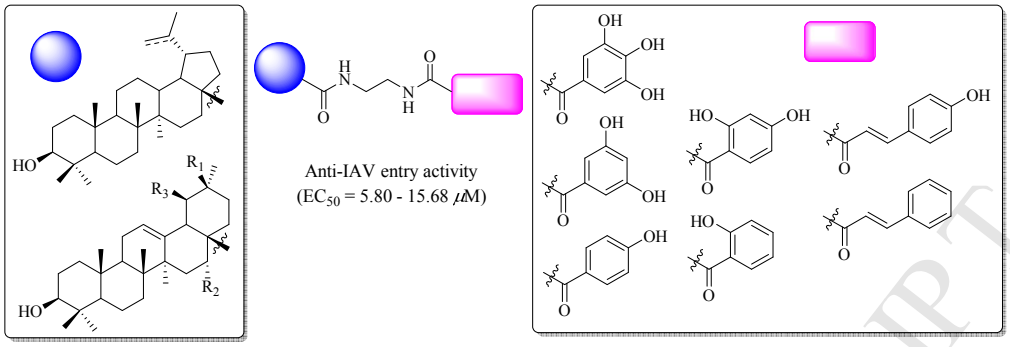
Graphic abstract



Synthesis, structure activity relationship and *in vitro* anti-influenza virus activity of novel polyphenol-pentacyclic triterpene conjugates

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Abstract:

It is urgently necessary to develop more effective anti-influenza agents due to the continuous emergence of drug-resistant strains of influenza virus. Our earlier studies have identified that certain pentacyclic triterpene derivatives are effective inhibitors of influenza virus infection. In the present study, a series of C-28 modified pentacyclic triterpene derivatives via conjugation with a series of polyphenols were synthesized, and their antiviral activities against influenza A/WSN/33 (H1N1) virus in MDCK (Madin-Darby canine kidney) cells were evaluated. Four compounds **23m**, **23o**, **23q** and **23s** displayed robust anti-influenza potency with averaged IC_{50} values at the low-micromole level, surpassing the potency of oseltamivir. In addition, the *in vitro* cytotoxicity activity of the four conjugates against MDCK cells showed no toxicity at 100 μM. Further mechanism studies of compound **23s**, one of the best representative conjugates with IC_{50} value of 5.80 μM and a selective index (SI) value of over 17.2, by hemagglutination inhibition (HI), surface plasmon resonance and molecular modeling indicated that these conjugates bound tightly to the viral envelope hemagglutinin (K_D = 15.6 μM), thus blocking the invasion of influenza viruses into host cells.

Keywords: Influenza virus, pentacyclic triterpene, polyphenol, entry inhibitor, hemagglutinin

1. Introduction

As a single-stranded, helically shaped RNA virus, influenza virus belongs to the *Orthomyxoviridae* family. It can be divided into three classes, A, B and C, according to its internal proteins of nucleoprotein and matrix protein. Influenza A virus, typically encountered more frequently than types B and C, can be further subdivided into different subtypes according to the combinations between the hemagglutinin (HA) (H1~H18) and neuraminidase (NA) (N1~N11), the proteins on the surface of the virus.[1] As a result of genetic variations brought by antigenic shift and sporadic antigenic drift, influenza A viruses possess high pathogenicity and are the main cause of annual epidemics and occasional pandemics of respiratory diseases worldwide.[2] In the 20th century, three influenza pandemics occurred: Spanish influenza in 1918 (~50 million deaths), Asian influenza in 1957 (two million deaths), and Hong Kong influenza in 1968 (one million deaths).[3] The first pandemic of 21st century occurred in April 2009, and a new influenza A virus from H1N1 subtype emerged in Mexico and rapidly spread across the entire globe.[4] Worldwide, these annual epidemics are estimated to result in approximately 3 to 5 million cases of severe illness, and about 290,000 to 650,000 respiratory deaths.[5] Vaccination is currently the most effective strategy to prevent influenza infection. However, influenza viruses can be escaped from the neutralizing activity of previously developed antibodies by antigenic drift and antigenic shift, thus making them ineffective against the infection in the future.[2] Two classes of anti-influenza drugs approved by FDA including M2 ion channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir, zanamivir and peramivir) have several limitations in clinical practice, especially the rapid global spread of drug-resistance,[6, 7] emphasizing the fact that we are constantly required to develop new antiviral agents with different modes of action to currently used anti-influenza drugs. The HA, a spike-shaped glycoprotein which extends from the surface of the virus, plays an important role during the early stage of influenza infection. It can bind to sialylglyconjugates on the surface of the host cells, causing endocytosis of the virus and subsequently membrane fusion of the virus and the host

cells.[8] The crucial role of it in the viral life-cycle makes it an attractive target for the development of therapeutics to inhibit influenza virus infection.[9, 10]

Polyphenols are widely distributed in various plant species, including fruits and vegetables, with one or more hydroxylated aromatic rings in monomer or polymer form. As dietary antioxidants, plant polyphenols are well known for their potentials to contribute to better health.[11] They have been shown potential antioxidant property and can protect cell constituents against oxidative damage, thus limiting the risk of various degenerative diseases associated with oxidative stress.[12] Recently, the antiviral activity and the mode of action of polyphenols, such as CYSTUS052,[13] gallic acid (**1**),[14] epigallocatechin gallate (EGCG, **2**) and its derivatives (**3** and **4**),[15-18] 2,3,4,6-tetragalloylglucose (**5**) and its analog (**6**),[19, 20] 2,3,4-trihydroxybenzoyl dimer (**7**) and its analog (**8**), 2,3,4-trihydroxybenzoyl trimer (**9**),[21] curcumin (**10**) and its derivatives (**11** and **12**),[22, 23] betulinic acid-gallic acid conjugates (**13** and **14**), [24] have attracted increasing attention (Figure 1). CYSTUS052, a well-known polyphenol-rich extract of *Cistus incanus*, can inhibit avain and human influenza A virus replication *in vitro*.[13, 25] Moreover, CYSTUS052-treated patients with influenza A and B virus infection show reduction in symptoms and inflammatory markers in a prospective, randomized, placebo-controlled clinical study.[26] EGCG (**2**), one of the major components of green tea, can bind to a range of virion glycoproteins, thus inhibiting the invasion of HCV, HIV and influenza virus into host cells.[16, 18, 27-30] Therefore, polyphenols have been considered as a useful synthetic precursor to many molecules of potential biological utility.[31, 32]

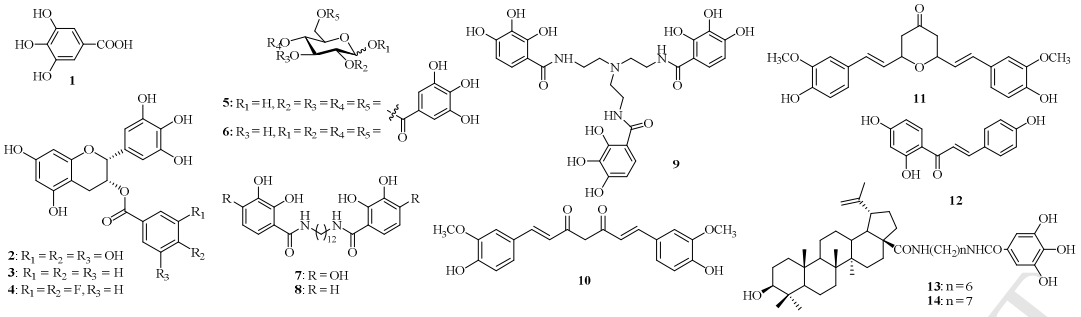


Figure 1. Chemical structures of some polyphenols with antiviral activity.

Pentacyclic triterpenes are described as secondary plant metabolites formed by the regular cyclization of squalene epoxide and widely found in a variety of organisms ranging from bacteria, fungi, plants to mammals.[33] In recent years, the ecological role and biological activities of pentacyclic triterpenes have begun to attract more general interest. They are often the active constituents of many important medicinal plants, such as *Panax ginseng*, *Glycyrrhiza uralensis* and *Radix Bupleuri*, etc. Many compounds of this group show a wide range of biological activities,[34] including hepatoprotective,[35] antiviral,[36] antitumor,[37] anti-inflammatory,[38] antimicrobial,[39] and so on. In our previous study,[40] we have discovered that oleanane-type triterpenoid Q8 (**15**) (Figure 2) shows significant activity against A/WSN/33 (H1N1) virus invasion into MDCK host cells based on the screening of a mini-library of triterpene-conjugates with EC_{50} values of 5.0 μM. Further study has indicated that they bind tightly to the pocket of viral envelope HA protein for sialic acid receptor, thus blocking the attachment of viruses to host cells.

Based on these literature results, and as part of our persistent efforts toward the development of potential antiviral entry inhibitors derived from natural product,[40-44] we described herein the design and synthesis of a total of 40

polyphenol-pentacyclic triterpene derivatives and evaluated their *in vitro* anti-influenza A virus (IAV) activities (Figure 2).

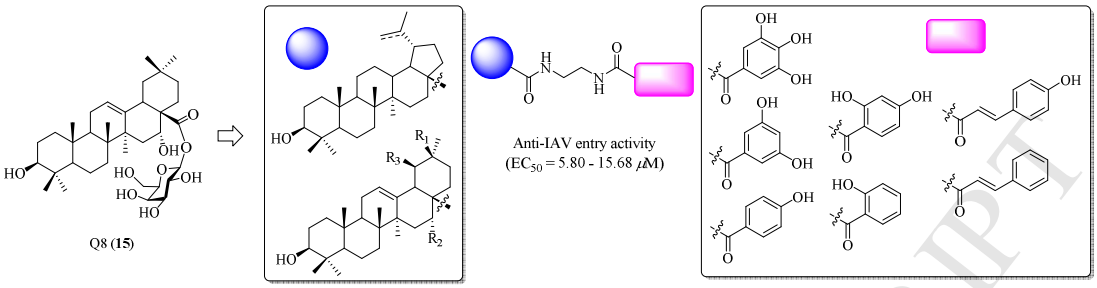


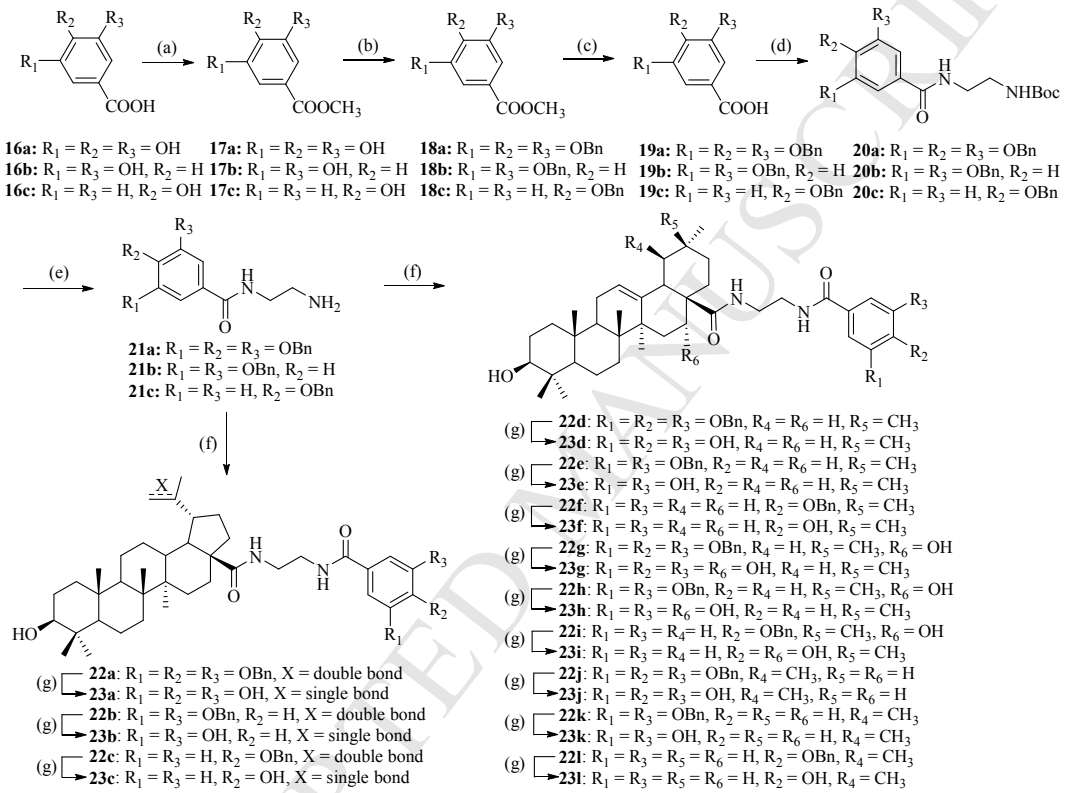
Figure 2. Structures of lead compound Q8 (**15**) and polyphenols-pentacyclic triterpene derivatives.

2. Results and discussion

2.1 Chemistry

Scheme 1 depicts the synthesis of polyphenol-pentacyclic triterpene conjugates **23a-23l,** in which gallic acid (**16a**), 3,5-dihydroxybenzoic acid (**16b**) and 4-hydroxybenzoic acid (**16c**) were attached to four pentacyclic triterpenes, including oleanolic acid (OA), echinocystic acid (EA), ursolic acid (UA) and betulinic acid (BA), at the C28-COOH through a flexible ethylenediamine linker. Firstly, 3,4,5-tribenzyloxybenzoic acid (**19a**) was prepared from commercially available gallic acid (**16a**) in 56% yield via a three-step process described by Ren and colleagues.[45] Compound **19a** was then coupled with *tert*-butyl (2-aminoethyl)carbamate using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*,*N*-(dimethylamino)pyridine (DMAP) as the coupling agents, followed by removal of the Boc group with 3M HCl in EtOAc to provide the key intermediate **21a**, which was used directly in the next step without further purification. Compounds **22a**, **22d**, **22g** and **22j** were synthesized by condensation of **21a** with four pentacyclic

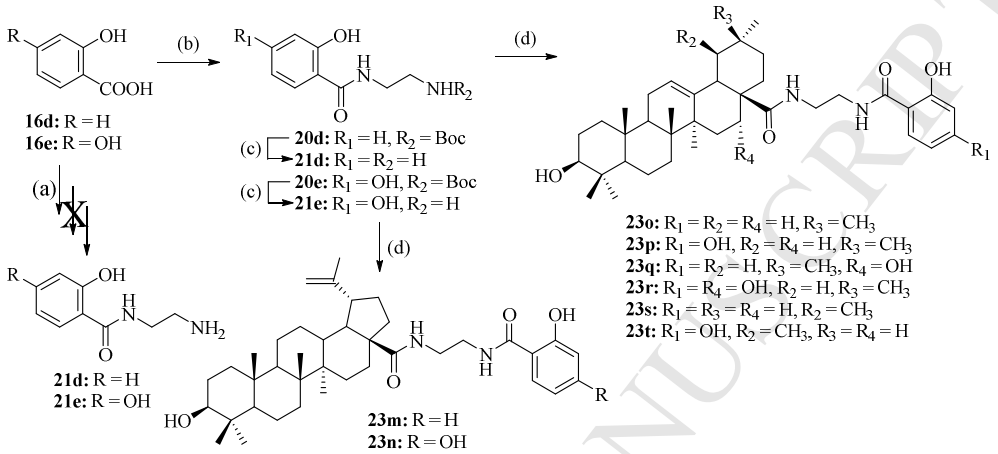
triterpene, followed by debenzylation using H_2/Pd-C to afford polyphenol-pentacyclic triterpene conjugates **23a**, **23d**, **23g** and **23j** with yields ranging from 49 to 74%. Similarly, the conjugation of 3,5-dihydroxybenzoic acid (**16b**) and 4-hydroxybenzoic acid (**16c**) with four pentacyclic triterpenes in a similar manner afforded conjugates **23b-23c**, **23e-23f**, **23h-23i** and **23k-23l**.



Scheme 1. Reagents and conditions: (a) MeOH, H_2SO_4, 120 ^oC, 80-88%; (b) BnBr, K_2CO_3, DMF, 80^oC, 75-82%; (c) NaOH, H_2O-MeOH-dioxane, reflux, 85-96%; (d) *tert*-butyl (2-aminoethyl) carbamate, EDC, DMAP, DCM, rt, 80-88%; (e) 3M HCl/EtOAc, DCM, rt, 90-95%; (f) OA, EA, UA or BA, EDC, DMAP, DCM, rt, 56-80%; (g) Pt/C, H_2, 3 atm, rt, 88-93%.

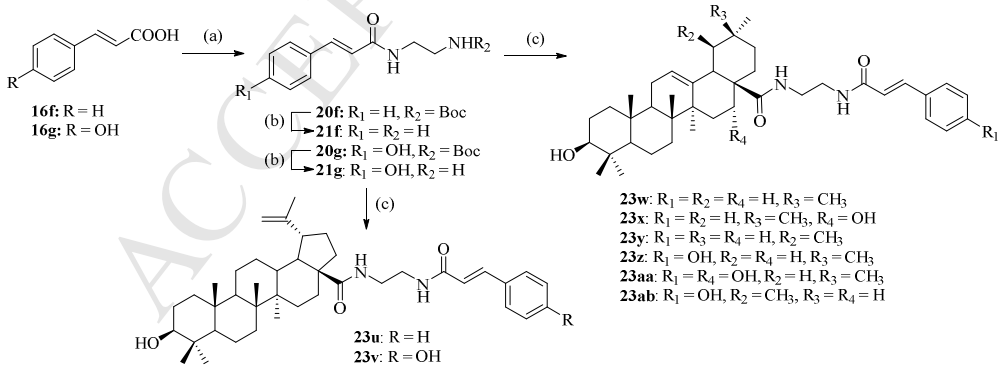
However, the introduction of ethylenediamine at the carboxyl group of **16d** and **16e** in a similar manner as described in Scheme 1 failed to obtain the intermediates **21d** and **21e**. Then a direct and shorter synthetic route involving only one-step

reaction and non-protected polyphenol was attempted (Scheme 2). After removal of the Boc protecting group of **20d** and **20e** by treatment with 3 M HCl in EtOAc, the intermediates were coupled with pentacyclic triterpene to afford **23m-23t** with a three-step yield of 56-72%, calculated from **16d** and **16e**.



Scheme 2. Reagents and conditions: (a) see Scheme 1; (b) *tert*-butyl (2-aminoethyl) carbamate, EDC, DMAP, DCM, rt; (c) 3 M HCl/EtOAc, DCM, rt; (d) BA, OA, EA or UA, EDC, DMAP, DCM, rt.

The cinnamic acid-pentacyclic triterpene conjugates **23u-23ab** were obtained readily by a procedure similar to that discussed for **23m-23t** (Scheme 3).



Scheme 3. Reagents and conditions: (a) *tert*-butyl (2-aminoethyl) carbamate, EDC, DMAP, DCM, rt; (b) 3 M HCl/EtOAc, DCM, rt; (c) BA, OA, EA or UA, EDC, DMAP, DCM, rt.

The ^{1}H NMR, ^{13}C NMR and HRMS spectra of the synthesized compounds **23a-23ab** are shown in supplementary materials.

2.2 Biological assays

2.2.1 Anti-influenza virus activity of polyphenol-pentacyclic triterpene conjugates

The CellTiter-Glo reduction assay and the cytopathic effect (CPE) assay were utilized in parallel to screen the synthesized conjugates as described previously.[40] The CellTiter-Glo screening, an assay monitoring cell proliferation, was utilized to screen and exclude compounds with significant toxicity toward MDCK cells. The CPE screening, an assay measuring the damage to host cells during virus invasion, was utilized to screen and identify compounds displaying a CPE reduction on influenza A/WSN/33 virus. Figure 3A shows that no toxicity was observed when the hydroxyl groups of polyphenol were protected by benzyl groups at the final concentration of 50 μM (compounds **22a-22l**). However, the removal of the benzyl groups of polyphenol (**23a-23aa**) resulted in the significant cytotoxicity at the same concentration, except for compounds **23f**, **23m**, **23o**, **23q**, **23s**, **23u**, **23v**, **23y**, **23z**, **23aa** and **23ab**. indicating that the substituted groups of polyphenol had important effect on the toxicity of polyphenol-pentacyclic triterpene conjugates. Luo et al. have also found that triterpene derivatives, in which 3-(4-hydroxy-3-methoxyphenyl)acrylic acid (ferulic acid) is linked with C3-OH of triterpene, show moderate cytotoxicity against MCF-7 (Michigan Cancer Foundation-7) cells.[46]

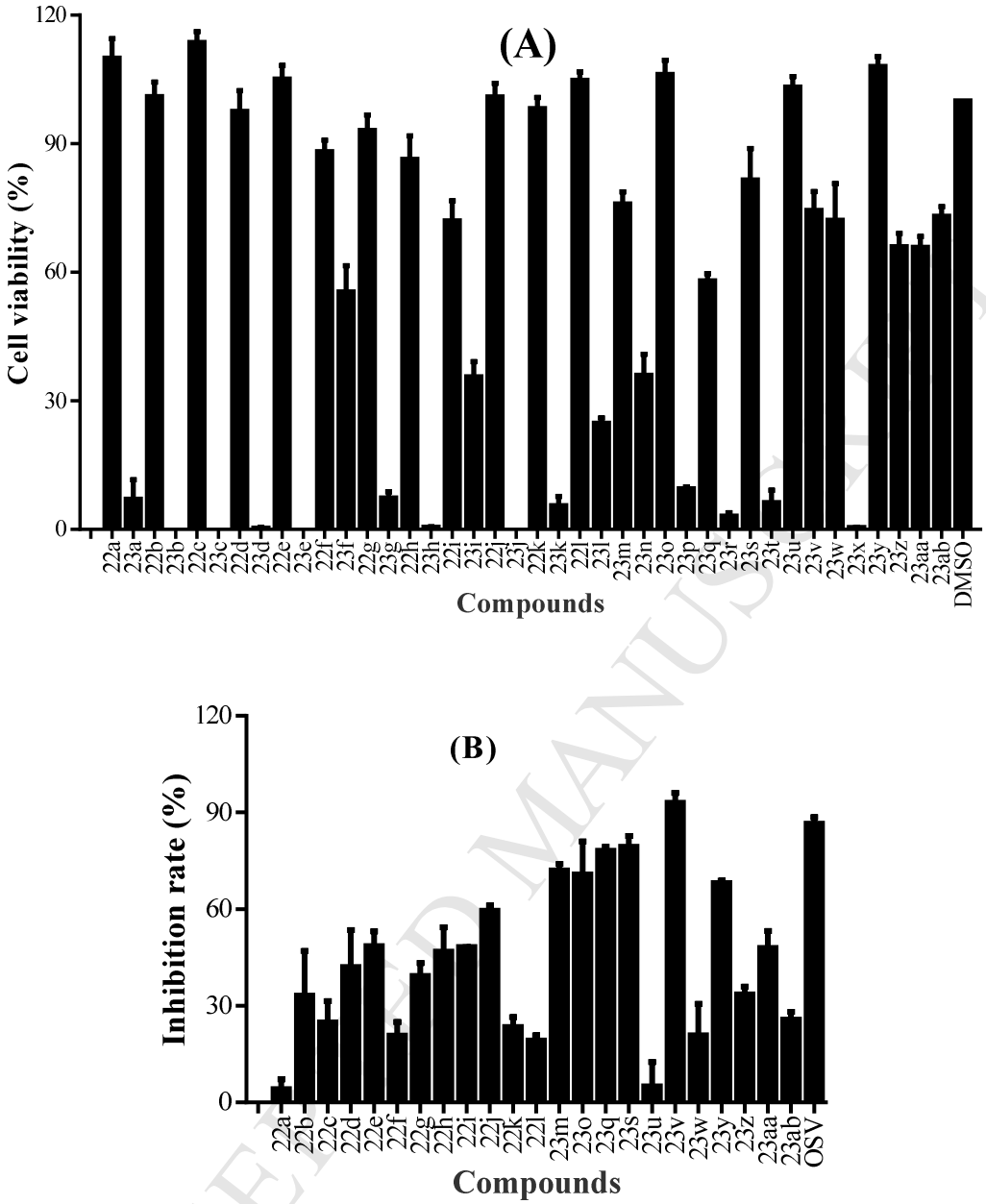


Figure 3. Inhibitory effects of polyphenol-pentacyclic triterpene conjugates against influenza A/WSN/33 (H1N1) virus. (A) The cytotoxic effect-based screening of conjugates **22a-22l** and **23a-23ab** using CellTiter-Glo® assay. DMSO acted as negative control. (B) The CPE-based screening of conjugates **22a-22l** and **23a-23ab**. MDCK was utilized as the host cell to test A/WSN/33 (H1N1) virus infection; 0.5% DMSO (final concentration) was used as negative control, and OSV (oseltamivir) was utilized as positive control. Error bars indicate standard deviations of triplicate experiments.

Except for cytotoxic compounds, the other pentacyclic triterpene-polyphenol conjugates against the influenza A/WSN/33 (H1N1) virus were evaluated by the CPE reduction assay. Compared with DMSO, influenza A/WSN/33 virus causes a severe CPE in MDCK-infected cells (Figure 3B). Compounds **22d**, **22g**, **23a**, **23m**, **23o**, **23q** and **23s** significantly reduced the CPE induced by infection in MDCK cells, indicating that those compounds were able to protect cells from influenza virus-induced CPE. Analysis of the structure-activity relationships revealed that four 2-hydroxybenzoic acid-pentacyclic triterpene conjugates **23m**, **23o**, **23q** and **23s** were active against A/WSN/33 (H1N1) virus, indicating the importance of the 2-hydroxybenzoic acid for the anti-influenza activity. In contrast, the shift of the hydroxyl from position 2 to position 4 of benzoic acid, or introduction of an extra hydroxyl at position 4 all showed no improvement but cytotoxic effect on potency.

The example of the effect of compound **23s** on CPE reduction is shown in Figure 4.

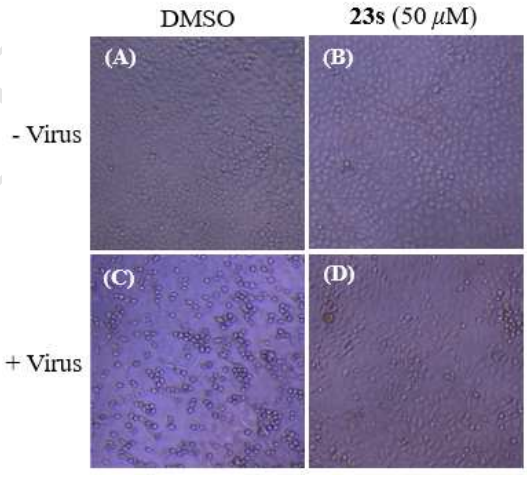


Figure 4. Compound **23s** inhibited the influenza A/WSN/33 (H1N1) virus-induced CPE in MDCK cells. The antiviral efficacy of **23s** was observed in terms of cellular

morphology at 40 h post-infection. MDCK cells were not inflected (A-B) or infected with influenza A/WSN/33 (H1N1) (C-D).

Seven compounds, 22j, **23m**, **23o**, **23q**, **23s**, **23v** and 23y identified with high inhibition rates in the initial screening were selected for the dose response assays. The IC_{50} of OSV used as a positive control was 16.5 μM.[42] Among them, four compounds (**23m**, **23o**, **23q** and **23s**) showed much stronger anti-influenza A/WSN/33 (H1N1) activities with IC_{50} at micromole level (Table 1). Compound **23s** showed the strongest inhibitory activities with IC_{50} value of 5.80 μM in this study. In addition, all the five compounds showed the CC_{50}, which were all higher than 100 μM.

Table 1. *In vitro* anti-influenza virus activity and cytotoxicity of the active compounds

Compounds	IC_{50} (μM)[a]	CC_{50} (μM)[b]
22j	32.87	>100
23m	9.55	>100
23o	7.65	>100
23q	5.84	>100
23s	5.80	>100
23v	15.68	>100
23y	18.34	>100
OSV [c]	16.5	> 200

[a] IC_{50} was measured using influenza A virus (A/WSN/33/H1N1) with MDCK cells by CPE assay. [b] The cytotoxicity (CC_{50}) of all compounds was measured with MDCK

cells by CellTiter-Glo assay. [c] Data taken from the reference.[42]

*2.2.2 Conjugate **23s** inhibits virus-induced hemagglutination*

It is well known that influenza virus HA plays an important role during viral invasion into host cells. HA protein can bind to sialic acid on the surface of red blood cells (RBCs), causing agglutination. To investigate whether the polyphenol-pentacyclic triterpene conjugates blocked the ability of viral particles to bind to cell receptors, a hemagglutination inhibition (HI) assay was performed. Like the capability as anti-HA antibody, conjugate **23s** inhibited the binding of influenza virus A/WSN/33 to RBCs in a concentration-dependent manner, indicating that the compound directly acted on influenza viral particles (Figure 5).

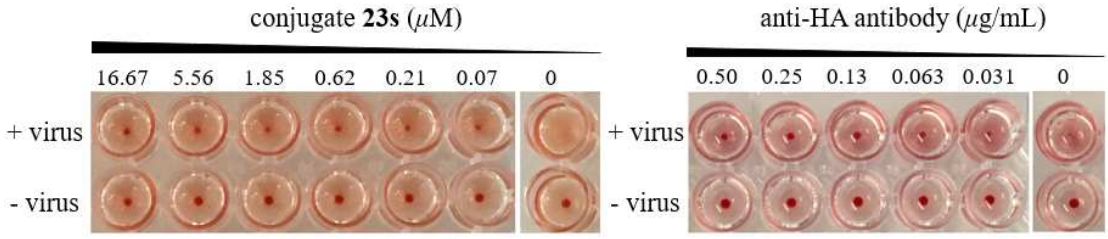


Figure 5. Hemagglutination inhibition assay. (A) Conjugate **23s** was diluted as indicated in 96-well micro titer plates using PBS (Phosphate Buffer Solution) as negative control. (B) The HI activity of anti-HA antibody was tested in a similar manner.

*2.2.3 Conjugate **23o** and **23s** interact with HA*

Surface plasmon resonance (SPR) was used to screen the interaction between a variety of affinity ligands and HA protein.[47] In our study, recombinant influenza HA proteins of influenza A/California/04/2009 (H1N1) (bought from Sino Biological Inc, Beijing, China) were immobilized to a carboxymethylated dextran CM5 chip

surface using an amine coupling kit (GE Healthcare, Buckinghamshire, UK). Then compound **23o** or **23s** at various concentrations was passed over the immobilized HA surface. As shown in Figure 6, the binding curves are fitted well with the Langmuir equation for monovalent binding, which allows the determination of the apparent dissociation constant, K_D. The calculated K_D values for **23o** and **23s** binding with HA protein were 36.0 and 15.6 μM, respectively, indicating that **23s** bound more tightly to HA than **23o**. In addition, we found compound **23o** produced the similar resonance unit signal response to **23s**, but the binding of HA with **23s** was faster than that of **23o**. In contrast, a lower binding affinity was observed between compound UA and HA with a calculated K_D value of 46.5 μM (SI Figure 1), almost one third as potent as that of **23s**, suggesting that 2-hydroxybenzoic acid enhanced the binding of UA with HA protein.

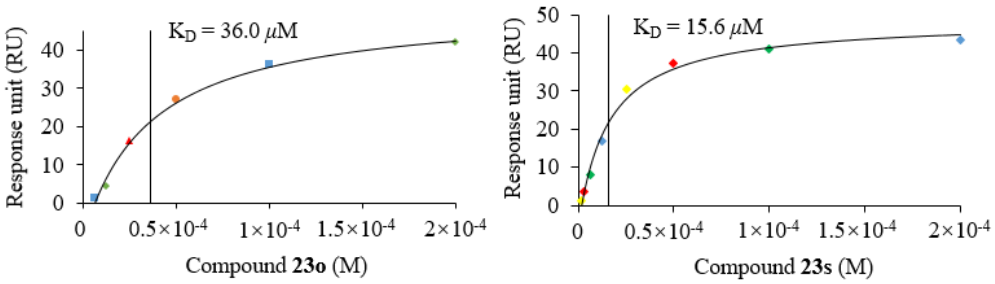


Figure 6. Characterization of the affinity between active compounds (**23o** and **23s**) and HA protein, which were immobilized on a CM5 sensor chip, based on the SPR assay. Their K_D values are labeled on the corresponding curves.

2.2.4 Molecular docking analysis

To further understand the molecular basis of the inhibitory properties of polyphenol-pentacyclic triterpene conjugates, we performed the docking experiments

of the most active compound **23s** and its parent compound UA with influenza HA protein, which was obtained from the RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank, http://www.rcsb.org/pdb/home/home.do, using AutoDock 4.2 program (The scripps research institute, La Jolla, CA, USA).[40]

The docked conformations of HA-**23s** were determined based on the minimum free energy analyses. As shown in Figure 7, the modeled structure of HA complexed with **23s** indicated that C3-OH of UA formed a hydrogen bond with Gln226, while the phenolic hydroxyl of salicylic acid also formed two hydrogen bonds with Lys156 and Gly158, respectively. According to the computer aided docking data, **23s** occupied the binding pocket for sialic acid with an estimated binding energy of -42.43 kcal/mol, while the estimated binding energy between HA and UA, the parent compound of **23s**, is -23.50 kcal/mol as a result of no hydrogen bonds formed between UA and Lys156 and Gly158 (SI Figure 2).

3. Conclusions

In the present study, we reported the synthesis and characterization of a series of polyphenol-pentacyclic triterpene conjugates and tested their anti-influenza A/WSN/33 (H1N1) virus activities in MDCK cells. All the protected polyphenol-pentacyclic triterpene conjugates **22a**-**22l** showed no cytotoxicity to MDCK cells, while their deprotected derivatives showed certain cytotoxicity. The subsequent SAR studies showed that four conjugates **23a**, **23m**, **23o**, **23q** and **23s** exhibited the highest anti-influenza virus activity with IC_{50} at micromole level. In particular, **23s** exhibited a much greater affinity to HA with K_D value of 15.6 μM. The

results indicated that the polyphenol-pentacyclic triterpene might interfere with influenza invasion by its interaction with the sialic acid receptor binding region of HA protein.

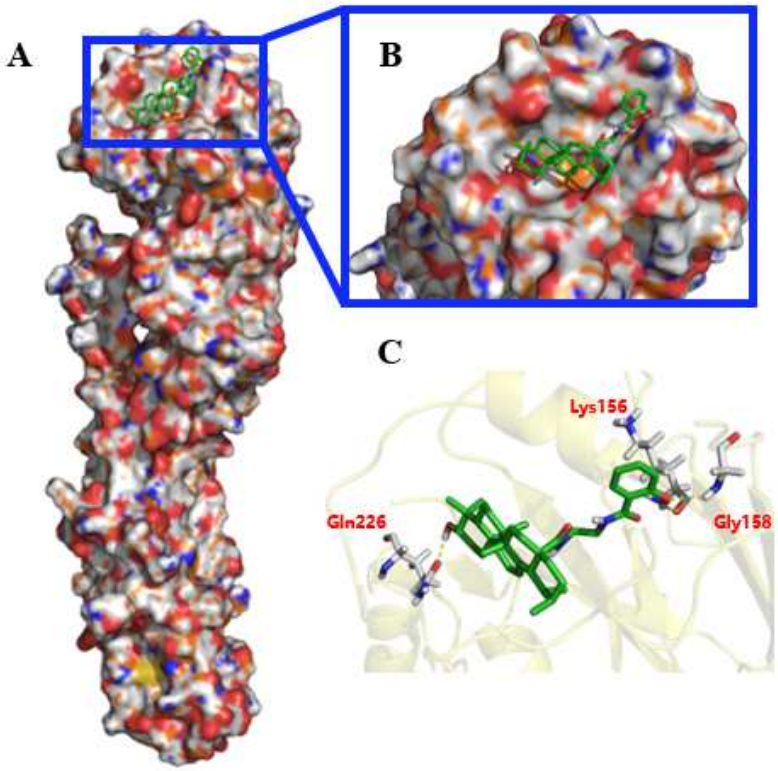


Figure 7. Structural representative of **23s** binding within HA protein (Protein Data Bank: 1RVT) according to blind docking calculation. (A) Overview of HA protein. The inhibitor pocket is highlighted in yellow square. Protein and compound **23s** are shown as orange surface and green sticks, respectively. (B) Closer view of the inhibitor pocket. Compound **23s** is shown as green sticks. (C) Modeled structure of HA complexed with **23s**. Compound **23s** is shown as green sticks. Hydrogen bonds are depicted in dashed lines. Residues in HA close to **23s** are labeled in grey. (For interpretation of the references to color in this figure legend, the red is referred to the web version of this article.)

4. Experimental section

4.1. General information

4.1.1. Materials

MDCK cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL. Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Linz Austria) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2. Influenza A/WSN/33 (H1N1) virus was used in this study.

OSV of 98% purity (Hoffmann-La Roche Ltd., Basel, Switzerland) was used as reference compound in CPE reduction assay. OA and UA were kindly supplied by Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China) and Anboruila Biotechnology Co., Ltd, (Nanjing, China), respectively. BA was supplied by Aladdin-Reagent Co., Ltd. (Shanghai, China). EA was separated from the ethanol/H_2O crude extract of *Gleditsia sinensis Lam*, a traditional Chinese herbal medicine. All the compounds were dissolved in DMSO to 10 mM as stock solutions.

4.1.2. Chemistry

High-resolution mass spectra (HRMS) were obtained with an APEX IV FT_MS (7.0 T) spectrometer (Bruker) in positive ESI mode. NMR spectra were recorded on a Bruker DRX 400 spectrometer at ambient temperature. Chemical shifts of ^{1}H NMR were referenced to the internal standard TMS (δ_H = 0.00) or the solvent signal (δ_H = 3.31 for the central line of MeOD). Chemical shifts of ^{13}C NMR were referenced to the solvent signal (δ_c = 77.00 for the central line of $CDCl_3$, δ_c = 49.00 for the central line of MeOD). Reactions were monitored by thin-layer chromatography (TLC) on a

60 F_{254} plate (pre-coated with silica gel, layer thickness of 0.2 mm; E. Merck, Darmstadt, Germany) and detected by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL), followed by heating.

4.2. General procedures

Compounds **16a-c**, **17a-c**, **18a-c** and **19a-c** were synthesized according to previously published methods.[48, 49] The synthesis of the intermediates **20a-g** and **21a-g** as well as the polyphenol–pentacyclic triterpene conjugates **22d-l**, **23d-l**, **23m-t**, **23w-z**, **23aa** and **23a** were provided in the supporting information. The synthesis of ten polyphenol-BA conjugates, including compounds **22a-22c**, **23a-23c**, **23m**, **23n**, **23u** and **23v**, were obtained as follows.

4.2.1. General procedure for amide bond formation reaction (method A)

To a solution of the carboxyl compound (1.0 equiv.) in DCM, EDC (1.6 equiv.) and DMAP (0.25 equiv.) were added slowly. Then the amino compound (1.2 equiv.) was added to the mixture. The reaction mixture was stirred at room temperature for 1 h and then washed with saturated salt solution (50 mL × 3). The organic layer was collected, dried over Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography over silica gel.

4.2.2. General procedure for reduction reaction (method B)

The starting material (1 equiv.) was dissolved in MeOH (6 mL), and palladium-carbon (0.1 equiv.) was added. The suspension was degassed under vacuum and purged with H_2 for three times, and then it was stirred under H_2 balloon at room

temperature for 24 h. The suspension was filtered through a pad of Celite, and the pad cake was washed with CH_3OH. The combined filtrate was concentrated to dryness. The residue was subjected to flash chromatography to afford the product.

4.2.3. Synthesis of N-(2-(3,4,5-tris(benzyloxy)benzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide ***(22a)***

Compound **22a** was prepared from compounds **21a** (208 mg, 0.43 mmol) and BA (165 mg, 0.36 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 4:1) to yield **22a** as a white solid (288 mg, 78%), m.p. 110-112 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.70 (t, J = 5.5 Hz, 1H), 7.49 – 7.17 (m, 17H), 5.18 (d, J = 11.6 Hz, 2H), 5.15 (d, J = 11.6 Hz, 2H), 5.02 (s, 2H), 4.55 (s, 1H), 4.49 (s, 1H), 3.70 – 3.63 (m, 1H), 3.58 (td, J = 13.0, 2.8 Hz, 1H), 3.45 (dt, J = 13.0, 4.0 Hz, 1H), 3.01 – 3.11 (m, 2H), 2.41 (dt, J = 12.1, 3.2 Hz, 1H), 2.07 (br d, J = 13.5 Hz, 1H), 1.90 – 1.76 (m, 2H), 1.63 – 0.92 (m, other aliphatic ring protons), 1.60, 0.89, 0.83, 0.66 (s, each 3H, 4 × CH_3), 0.62 (s, 6H, 2 × CH_3), 0.57 (d, J = 10.3 Hz, 1H); ^{13}C NMR (100 MHz, MeOD) δ 179.16, 167.26, 152.84, 150.60, 141.31, 137.76, 136.87, 129.27, 128.63, 128.50, 128.27, 128.12, 127.95, 127.76, 109.62, 106.71, 79.06, 75.27, 71.36, 55.99, 55.43, 50.64, 50.21, 47.11, 42.80, 42.62, 40.72, 39.57, 38.93, 38.78, 38.53, 38.16, 37.22, 34.36, 33.81, 31.03, 29.61, 28.05, 27.48, 25.79, 20.95, 19.54, 18.34, 16.19, 16.03, 15.42, 14.79; ESI-HRMS Calcd for $C_{60}H_{77}N_2O_6$ $[M+H]^+$: 921.5776, found 921.5788.

4.2.4. Synthesis of

*N-(2-(3,4,5-trihydroxybenzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23a**)*

Compound **23a** was prepared from compound **22a** (100 mg, 0.11 mmol) according to general procedure B. The residue was purified by column chromatography (DCM/MeOH = 15:1) to yield **23a** as a white solid (62 mg, 88%). Mp: 215-217 ^{o}C. ^{1}H NMR (400 MHz, MeOD): δ 6.88 (s, 2H), 3.54 – 3.40 (m, 3H), , 3.10 (dd, J = 11.3, 4.9 Hz, 1H), 2.40 (dt, J = 12.2, 3.6 Hz, 1H), 2.34 – 2.29 (m, 1H), 2.10 (br d, J = 12.8 Hz, 1H), 1.78 – 0.63 (m, other aliphatic ring protons), 0.93, 0.92 (s, each 3H, 2 × CH_3), 0.85 (d, J = 6.8 Hz, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.75 (d, J = 6.8 Hz, 3H, CH_3), 0.74, 0.73, 0.63 (s, each 3H, 2 × CH_3), 0.64 (d, J = 8.9 Hz, 1H); ^{13}C NMR (100 MHz, MeOD) δ 180.30, 170.65, 146.67, 138.28, 125.55, 107.90, 79.72, 57.49, 56.90, 51.83, 50.93, 45.39, 43.60, 41.87, 41.39, 40.34, 40.09, 39.93, 39.71, 38.95, 38.26, 35.51, 33.96, 31.20, 30.68, 28.63, 28.31, 28.04, 23.96, 23.38, 22.10, 19.38, 16.69, 16.65, 16.11, 14.98, 14.95; ESI-HRMS Calcd for $C_{39}H_{61}N_2O_6$ $[M+H]^+$: 653.4524, found 653.4532.

*4.2.5. Synthesis of N-(2-(3,5-bis(benzyloxy)benzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**22b**)*

Compound **22b** was prepared from compounds **21b** (208 mg, 0.55 mmol) and BA (279 mg, 0.61 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 4:1) to yield **22b** as a white solid (346 mg, 77%), m.p. 123-125 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (br s, 1H), 7.42 – 7.29 (m, 11H), 7.16 (d, J = 2.2 Hz, 2H), 6.71 (t, J = 2.2 Hz, 1H), 6.68 (br s, 1H), 5.06 (d, J = 11.7 Hz, 2H), 5.03 (d, J = 11.7 Hz, 2H), 4.59 (s, 1H), 4.51 (s, 1H),

3.56 – 3.55 (m, 3H), 3.41 – 3.37 (m, 1H), 3.15 – 3.09 (m, 2H), 2.33 (dt, *J* = 9.5, 2.6 Hz, 1H), 2.00 (d, *J* = 13.7 Hz, 1H), 1.89 – 0.80 (m, other aliphatic ring protons), 1.60, 0.92, 0.90, 0.69, 0.66, 0.63 (s, each 3H, 6 × CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.93, 167.59, 160.06, 150.74, 136.56, 136.08, 128.62, 128.13, 127.65, 109.42, 106.12, 105.43, 78.91, 70.26, 55.81, 55.36, 50.58, 50.15, 46.90, 42.47, 42.29, 40.58, 39.46, 38.86, 38.73, 38.43, 37.98, 37.13, 34.24, 33.53, 30.96, 29.55, 28.04, 27.41, 25.69, 20.85, 19.50, 18.25, 16.10, 15.85, 15.45, 14.68; ESI-HRMS Calcd for $C_{53}H_{71}N_2O_5$ $[M+H]^+$: 815.5358, found 815.5357.

*4.2.6. Synthesis of N-(2-(3,5-dihydroxybenzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23b**)*

Compound **23b** was prepared from compound **22b** (100 mg, 0.12 mmol) according to general procedure B. The residue was purified by column chromatography (DCM/MeOH = 15:1) to yield **23b** as a faint yellow solid (66 mg, 85%), m.p. 175-177 ^{o}C. ^{1}H NMR (400 MHz, Pyr-d_5) δ 9.28 (s, 1H), 8.38 (s, 1H), 7.68 (d, *J* = 1.4 Hz, 2H), 7.13 (s, 1H), 3.86 (br s, 3H), 3.72 (br s, 1H), 3.45 (t, *J* = 7.5 Hz, 1H), 2.92 (t, *J* = 9.8 Hz, 1H), 2.76 (t, *J* = 10.4 Hz, 1H), 2.05 (br d, *J* = 13.0 Hz, 1H), 2.01 (dd, *J* = 11.4, 7.1 Hz, 1H), 1.86 – 1.16 (m, other aliphatic ring protons), 1.12, 1.05 (s, each 3H, 2 × CH_3), 1.01 (s, 6H, 2 × CH_3), 0.92 (d, *J* = 6.6 Hz, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.82 – 0.81 (m, 4H); ^{13}C NMR (100 MHz, Pyr-d_5) δ 178.84, 169.18, 160.85, 138.58, 107.35, 107.18, 78.67, 56.96, 56.51, 51.40, 50.72, 44.94, 43.41, 42.17, 42.04, 41.69, 40.65, 40.04, 39.83, 39.59, 38.24, 38.06, 35.40, 34.00, 30.81, 30.51, 29.23, 28.81, 28.12, 24.12, 23.81, 21.83, 19.36, 17.04, 16.99, 16.93, 15.47, 15.29;

ESI-HRMS Calcd for $C_{39}H_{61}N_2O_5$ $[M+H]^+$: 637.4575, found 637.4569.

*4.2.7. Synthesis of N-(2-(4-(benzyloxy)benzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**22c**)*

Compound **22c** was prepared from compounds **21c** (150 mg, 0.56 mmol) and BA (284 mg, 0.62 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 4:1) to yield **22c** as a white solid (315 mg, 80%), m.p. 122-124 ^oC. ^1H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, J = 8.8 Hz, 2H), 7.43 – 7.31 (m, 5H), 7.00 (m, J = 8.8 Hz, 2H), 6.35 (t, J = 5.2 Hz, 1H), 5.09 (s, 2H), 4.72 (s, 1H), 4.59 (s, 1H), 3.59 – 3.56 (m, 3H), 3.49 (dd, J = 10.6, 5.5 Hz, 1H), 3.17 – 3.10 (m, 2H), 2.30 (dt, J = 12.1, 3.5 Hz, 1H), 1.96 (td, J = 13.8, 3.0 Hz, 1H), 1.91 – 0.76 (m, other aliphatic ring protons), 1.66, 0.93, 0.92, 0.72, 0.69, 0.68 (s, each 3H, 6 × CH_3), 0.62 (d, J = 10.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.77, 167.66, 161.54, 150.95, 136.48, 129.09, 128.76, 128.27, 127.56, 126.55, 114.57, 109.46, 79.01, 70.20, 55.86, 55.43, 50.63, 50.26, 46.99, 42.53, 41.96, 40.68, 39.59, 38.93, 38.80, 38.47, 38.02, 37.23, 34.30, 33.59, 31.00, 29.62, 28.08, 27.48, 25.71, 20.95, 19.62, 18.32, 16.16, 16.07, 15.50, 14.71; ESI-HRMS Calcd for $C_{46}H_{65}N_2O_4$ $[M+H]^+$: 709.4939, found 709.4940.

*4.2.8. Synthesis of N-(2-(4-hydroxybenzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23c**)*

Compound **23c** was prepared from compound **22c** (120 mg, 0.19 mmol) according to general procedure B. The residue was purified by column chromatography (DCM/MeOH = 15:1) to yield **23c** as a white solid (92 mg, 88%),

m.p. 159-161 ^{o}C. ^{1}H NMR (400 MHz, Pyr-d_5) δ 9.11 (br s, 1H), 8.43 (br s, 1H), 8.36 (s, 1H), 8.34 (s, 1H), 7.28 (s, 1H), 7.26 (s, 1H), 3.93-3.85 (m, 3H), 3.75 (t, J = 5.6 Hz, 1H), 3.47 (t, J = 7.6 Hz, 1H), 2.95 (t, J = 10.5 Hz, 1H), 2.77 (t, J = 9.7 Hz, 1H), 2.51 – 2.47 (m, 1H), 2.03 (dd, J = 10.8, 7.3 Hz, 1H), 1.87 – 0.82 (m, m, other aliphatic ring protons), 1.24 (s, 3H, CH_3), 1.03 (s, 9H, 3 × CH_3), 0.92 (d, J = 6.3 Hz, 3H, CH_3), 0.85 – 0.82 (m, 6H, 2 × CH_3); ^{13}C NMR (100 MHz, Pyr-d_5) δ 178.80, 168.50, 162.47, 130.51, 126.85, 116.42, 78.61, 56.89, 56.42, 51.28, 50.68, 44.99, 43.35, 42.20, 41.60, 40.74, 39.99, 39.73, 39.50, 38.17, 37.98, 35.31, 33.92, 30.72, 30.45, 29.15, 28.81, 28.06, 24.04, 23.80, 21.76, 19.26, 16.97, 16.89, 16.85, 15.35, 15.21; ESI-HRMS Calcd for $C_{39}H_{61}N_2O_4$ $[M+H]^+$: 621.4626, found 621.4625.

*4.2.9. Synthesis of N-(2-(2-hydroxybenzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23m**)*

Compound **23m** was prepared from compounds **21d** (100 mg, 0.55 mmol) and BA (278 mg, 0.61 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 2:1) to yield **23m** as a white solid (135 mg, 51%), m.p. 142-144 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.54 (s, 1H), 8.17 (s, 1H), 7.56 (d, J = 7.9 Hz, 1H), 7.36 (t, J = 7.6 Hz, 1H), 6.93 (d, J = 8.3 Hz, 1H), 6.86 (t, J = 7.4 Hz, 1H), 6.33 (t, J = 5.6 Hz, 1H), 4.73 (s, 1H), 4.59 (s, 1H), 3.67 – 3.47 (m, 4H), 3.19 – 3.13 (m, 2H), 2.23 – 2.17 (m, 1H), 1.94 (d, J = 14.0 Hz, 1H), 1.86 (q, J = 10.8 Hz, 1H), 1.74 – 0.81 (m, other aliphatic ring protons), 1.66 (s, 3H, 2 × CH_3), 0.93 (s, 6H, 2 × CH_3), 0.71 (s, 3H, CH_3), 0.65 (s, 9H, 3 × CH_3), 0.60 (d, J = 10.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.69, 170.91, 161.75, 150.73,

134.22, 126.42, 118.76, 118.45, 114.18, 109.61, 79.09, 55.97, 55.46, 50.62, 50.34, 47.15, 42.90, 42.58, 40.71, 38.95, 38.87, 38.81, 38.46, 38.30, 37.22, 34.34, 33.64, 31.07, 29.57, 28.10, 27.51, 25.74, 20.87, 19.67, 19.29, 18.31, 16.07, 15.95, 15.48, 14.76; ESI-HRMS Calcd for $C_{39}H_{59}N_2O_4$ $[M+H]^+$: 619.4469, found 619.4449.

*4.2.10. Synthesis of N-(2-(2,4-dihydroxybenzamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23n**)*

Compound **23n** was prepared from compounds **21e** (108 mg, 0.55 mmol) and BA (27 mg, 0.61 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 1:1) to yield **23n** as a white solid (148 mg, 56%), m.p. 186-188 ^oC. ^1H NMR (400 MHz, MeOD) δ 7.53 (d, J = 8.8 Hz, 1H), 6.29 (dd, J = 8.8, 2.2 Hz, 1H), 6.24 (s, 1H), 4.65 (s, 1H), 4.53 (s, 1H), 3.57 – 3.50 (m, 2H), 3.38 (dd, J = 9.6, 2.7 Hz, 1H), 3.22 – 3.19 (m, 1H), 3.06 (dd, J = 10.7, 5.7 Hz, 2H), 2.32 (td, J = 11.6, 3.1 Hz, 1H), 2.05 (d, J = 13.4 Hz, 1H), 1.89 – 0.57 (m, other aliphatic ring protons), 1.63, 0.89, 0.88, 0.71, 0.69, 0.66 (s, 3H each, 6 × CH3); ^{13}C NMR (100 MHz, MeOD) δ 179.79, 171.72, 163.65, 163.56, 151.84, 129.80, 109.91, 108.37, 108.03, 103.87, 79.46, 56.80, 56.56, 51.63, 51.16, 47.96, 43.23, 41.50, 40.31, 39.81, 39.69, 39.05, 38.86, 38.01, 35.03, 33.87, 31.75, 30.35, 28.51, 27.76, 26.62, 21.75, 19.71, 19.46, 19.16, 16.58, 16.42, 15.99, 15.07; ESI-HRMS Calcd for $C_{39}H_{59}N_2O_5$ $[M+H]^+$: 635.4418, found 635.4405.

*4.2.11. Synthesis of N-(2-cinnamamidoethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23u**)*

Compound **23u** was prepared from compounds **21f** (100 mg, 0.53 mmol) and BA (266 mg, 0.58 mmol) according to general procedure A. The residue was purified by

column chromatography (petroleum ether/ethyl acetate = 4:1) to yield **23u** as a white solid (245 mg, 74%). m.p. 164-166 ^{o}C. ^{1}H NMR (400 MHz, MeOD) δ 7.59 (t, J = 4.6, 1H), 7.56 – 7.51 (m, 3H), 7.40 – 7.33 (m, 3H), 6.60 (d, J = 15.8 Hz, 1H), 4.69 (s, 1H), 4.57 (s, 1H), 3.57 – 3.52 (m, 2H), 3.40 – 3.33 (m, 1H), 3.23 – 3.20 (m, 1H), 3.10 – 3.05 (m, 2H), 2.50 (dt, J = 12.8, 2.9 Hz, 1H), 2.11 (d, J = 12.8 Hz, 1H), 1.94 – 1.79 (m, 2H), 1.69 – 0.80 (m, other aliphatic ring protons), 1.66, 0.93, 0.87, 0.86, 0.72, 0.67 (s, 3H each, 6 × CH_3), 0.58 (d, J = 11.4, 1H); ^{13}C NMR (100 MHz, MeOD) δ 179.58, 168.92, 152.17, 141.84, 136.17, 130.84, 129.96, 128.94, 121.94, 110.02, 79.55, 57.00, 56.76, 51.93, 51.35, 48.05, 43.45, 41.90, 40.39, 40.16, 40.03, 39.86, 39.30, 38.85, 38.23, 35.33, 34.12, 31.94, 30.72, 28.65, 28.01, 26.88, 22.06, 19.76, 19.25, 16.83, 16.75, 16.15, 15.15; ESI-HRMS Calcd for $C_{41}H_{61}N_2O_3$ $[M+H]^+$: 629.4677, found 629.4676.

*4.2.12. Synthesis of N-(2-((E)-3-(4-hydroxyphenyl)acrylamido)ethyl)-3β-hydroxy-lup-20(29)-en-28-amide (**23v**)*

Compound **23v** was prepared from compounds **21g** (50 mg, 0.24 mmol) and BA (11 mg, 0.22 mmol) according to general procedure A. The residue was purified by column chromatography (petroleum ether/ethyl acetate = 1:1) to yield **23v** as a white solid (70 mg, 49%), m.p. 204-206 ^{o}C. ^{1}H NMR (400 MHz, MeOD) δ 7.53 (t, J = 4.4 Hz, 1H), 7.46 – 7.40 (m, 3H), 6.80 (d, J = 8.7 Hz, 2H), 6.39 (d, J = 15.8 Hz, 1H), 4.69 (d, J = 2.9 Hz, 1H), 4.56 (s, 1H), 3.63 – 3.54 (m, 2H), 3.19 – 3.05 (m, 3H), 2.48 (td, J = 12.9, 3.5 Hz, 1H), 2.10 (d, J = 13.2 Hz, 1H), 1.90 – 0.57 (m, other aliphatic ring

protons), 1.67, 0.93, 0.88, 0.85, 0.74, 0.68 (s, each 3H, 6 × CH_3); ^{13}C NMR (100 MHz, MeOD) δ 178.34 , 168.32, 159.28, 150.91, 140.70, 129.35, 126.21, 117.02, 115.36, 108.56, 78.27, 55.67, 55.40, 50.55, 49.97, 42.06, 40.52, 38.88, 38.78, 38.61, 38.49, 37.92, 37.51, 36.85, 33.87, 32.73, 30.52, 29.37, 27.17, 26.61, 25.49, 20.63, 18.22, 17.83, 15.35, 15.29, 14.66, 13.60; ESI-HRMS Calcd for $C_{41}H_{61}N_2O_4$ $[M+H]^+$: 645.4623, found 645.4626.

4.3 Biological assays

4.3.1. CPE reduction assay

The assay was performed as previously described with some modifications. MDCK cells were seeded into 96-well plates, incubated overnight and infected with influenza virus (MOI = 0.1). Cells were suspended in DMEM supplemented with 1% FBS, test compound and 2 mg/mL TPCK-treated trypsin, and a final DMSO concentration of 1% was added in each well. After 40 h of incubation, CellTiter-Glo reagent (Promega Corp., Madison, WI, USA) was added, and the plates were read using a plate reader (Tecan Infinite M2000 PRO; Tecan Group Ltd., Mannedorf, Switzerland).

4.3.2. Cytotoxicity test

Cells were grown in 96-well plates containing 1% FBS overnight and then cultured with increasing amounts of the test compounds for 40 h. Cytotoxicity was assessed with the CellTiter-Glo assay as above described.

4.3.3. HI assay

Compound from a 3-fold serial dilution in saline was mixed with an equal

volume of influenza virus (2 HA units) in the V-bottomed 96-well microplates. Subsequently, 50 µL of freshly prepared chicken RBCs (cRBCs) (1% v/v in saline) was added to each well. The mixture was incubated at room temperature for 30 min before observing cRBC aggregation on the plate.

4.3.4. SPR

Interactions between the influenza HA and the compounds were analyzed using the Biacore T200 system (GE Healthcare, Uppsala, Sweden) at 25 ^{o}C. Recombinant influenza HA (Sino Biological Inc., Beijing, China) was immobilized on a sensor chip (CM5) using an amine coupling kit (GE Healthcare, Buckinghamshire, UK). Final HA-immobilized levels were typically ~16,000 RU. Subsequently, compounds were injected as analytes at various concentrations, and PBS-P (10 mM phosphate buffer with 2.7 mM KCl and 137 mM NaCl, 0.05% surfactant P20, pH 4.5) was used as running buffer. For binding studies, analytes were applied at corresponding concentrations in running buffer at a flow rate of 30 mL/min with a contact time of 60 s and a dissociation time of 60 s. Chip platforms were washed with running buffer and 50% DMSO. Data were analyzed with the Biacore evaluation software (T200 version 1.0) by curve fitting using a binding model of 1:1.

4.3.5. Docking simulation

The protein structure, co-crystallized with LSTC (LS-Tetrasaccharide C), was downloaded from PDB (PDB code: 1rvt). Protein Preparation Wizard of the Schrödinger Suite was used to prepare protein ensuring that the downloaded X-ray structure was reliable and qualitatively considerable for further studies by

optimization and minimization protocols. The subsequent step involved removal of water molecules without any contact and addition of hydrogen atoms to the structure. The optimized model structure was further minimized using the OPLS-all atom (AA) force field until the average root-mean-square deviation (RMSD) of the non-hydrogen atoms reached 0.30 Å using. LigPrep was used to prepare 3D structures of the small molecules to obtain the accessible least-energy ionized conformer for docking simulation. Glide implemented in Schrödinger 2013 was used to molecular docking. The binding region was defined by a box centered on the centroid of the crystal ligand LSTC and in similar size with the ligand. The standard precision (SP) mode was used for the docking and scoring. All other parameters were kept default. The best pose was output on the basis of Glide score and the protein-ligand interactions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://

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Highlights

- A total of 40 novel polyphenol-pentacyclic triterpene conjugates were design and synthesized.
- The anti-influenza activities of those conjugates against A/WSN/33 virus were evaluated.
- Five conjugates displayed strong anti-influenza A virus activity with an IC_{50} at micromole level.
- Mechanistic studies indicated that compound **23s** could specifically bind with HA protein, thus disrupting the interaction of HA with sialic acid receptor.