Chemical and Pharmaceutical Bulletin Advance Publication by J-STAGE
DOI:10.1248/cpb.c19-00485

Advance Publication
August 22, 2019

Synthesis, structure activity relationship and anti-influenza A virus evaluation of oleanolic acid-linear amino derivatives

Weijia Li,[a, #] Fan Yang,[a, #] Lingkuan Meng,[a] Jiaqi Sun,[b] Yangqing Su,[a] Liang Shao,[a] Demin Zhou[b] and Fei Yu[a,*]

[a] *Medical School of Kunming University of Science and Technology, Kunming, Yunnan 650500, China*

[b] *State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China*

Address correspondence to:

Dr. Fei Yu

Medical School

Kunming University of Science and Technology

No.727 South Jingming Rd, Chenggong District, Kunming, 650500, China

Tel: +86-18087122003

Email: feiyuz8@kmust.edu.cn

[#] These authors contributed equally to this work.

[*] Correspondence e-mail: feiyuz8@kmust.edu.cn

Summary

Oleanolic acid (OA) was discovered as a mild influenza hemagglutinin (HA) inhibitor in our earlier studies. In the present work, 20 compounds were prepared by structural modifications of OA, and their antiviral activities against influenza A/WSN/33 (H1N1) virus in MDCK cells were evaluated. Based on the biological result, structure–activity relationship (SAR) was discussed. Compound **10** with six-carbon chain and a terminal hydroxyl group showed the strongest anti-influenza activity with an IC_{50} of 2.98 μM, which is an order of magnitude more potent than OA. Hemagglutination inhibition and Surface plasmon resonance (SPR) assay indicated that compound **10** might interfere with influenza invasion by interacting with HA protein.

Keywords

Oleanolic acid; Influenza virus; Entry inhibitor; Cytotoxicity; Synthesis design.

Introduction

Influenza A viruses (IAVs) are the main cause of annual epidemics and occasional pandemics of respiratory diseases worldwide[1-3]. According to estimates by the WHO, IAVs infect 5%-15% of the world's population[4], killing between 250,000 and 500,000 people each year[5]. Based on the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), IAVs are classified into various subtypes. There are 18 variants of HA (H1-H18) and 11 variants of NA (N1-N11) on the IAVs[6], which may form 198 subtypes of IAVs, theoretically[7, 8].

Two classes of anti-influenza drugs, M2 ion-channel inhibitors (amantadine and rimantadine) and NA inhibitors (oseltamivir, zanamivir and peramivir), have been used in clinic for the interruption of specific processes in influenza infections[9, 10]. In 2018, baloxavir was licensed in Japan for the treatment of both adult and pediatric patients infected with influenza virus. It is approved for use in children older than twelve age with acute uncomplicated influenza[11]. However, frequent application of the marketed anti-influenza drugs, in combination with the high mutation rate of the RNA genome of the influenza virus, has led to the rapid emergence of drug-resistant viral strains. Therefore, cost-effective antiviral strategies must be developed to prevent emerging influenza pandemics.

IAVs entry represents a favorable target of drug discovery, because it is the first step of viral infection. Inhibition of this first step should result in the virus propagation effectively blocked[12, 13]. In our previous studies, we found that oleanolic acid (OA) and its derivatives, which belongs to the family of pentacyclic triterpenes, display inhibitory activity against IAVs entry by interrupting the interaction between HA and sialic acid receptor[14].

As a continuation of our project aimed at discovering novel entry-targeting IAVs inhibitors, a total of 20 OA C-28 derivatives were designed, synthesized, and evaluated for their anti-IAVs activities in vitro.

Results and discussion

Chemistry

Certain structural modifications of these triterpenoids, many of which are simple derivatizations of the molecule's functional groups, can have a significant impact on their biological properties. For example, studies have shown increased biological activities of the C-28 amino conjugates of various pentacyclic triterpenic acids as compared to their natural precursors.

As shown in Chart 1, the C-28 of OA was first activated by EDCI and $(CH_3CH_2)_3N$ in the presence of 1-Hydroxybenzotriazole (HOBt), then reacted with amine reagents, giving the corresponding OA derivatives (compounds 1–20) with high yields. The amine reagents were selected with different chain lengths and terminal functional groups to evaluate their influence on the anti-IAVs activity.

Biological evaluation

Anti-IAVs activity of OA derivatives.

All the synthesized OA derivatives were evaluated for their in vitro anti-A/WSN/33 (H1N1) virus in an MDCK cell line at a concentration of 100 μM. The inhibition rates of the compounds were calculated and shown in Figure 1. To exclude the possibility that the observed anti-IAVs virus activity was due to cytotoxicity, a preliminary screening was performed to determine their cytotoxic activity in MDCK cells via the CellTiter-Glo assay and the cytopathic effect (CPE) reduction assay[14]. Oseltamivir (OSV, 100 μM) and 1% DMSO were used as positive and negative controls, respectively.

In Figure 1A, compounds **1-5**, the diamine conjugates of OA, exhibited strong cytotoxicity. This is in agreement with Parra's observation that that triterpene derivatives with a diamine, showed extreme cytotoxicity against cancer-cell lines (B16-F10, HT29 and Hep G2)[15]. On the other hand, no obvious toxicity was observed when the terminal of the side chain is a methylated carboxyl group at the final concentration of 100 μM (compounds **11-15**). However, the removal of the methyl group (compounds **16-20**) resulted in significant cytotoxicity at the same concentration, indicating that the terminal group of the side chain has a significant effect on cytotoxicity. Interestingly, compounds **7** and **8** display much higher cytotoxicity than **6, 9** and **10**, which means the length of the side chain might play a critical role when the terminal is a hydroxyl group (compounds **6-10**). Figure 1B shows that most of the compounds displayed a much higher inhibition rate than OA. However, in light of Figure 1A, this observation might, to a large extent, reflect the cellular toxicity rather than

anti-IAVs potency enhancement. According to the results above, six compounds, **9**, **10**, and **12-15**, with high inhibition rates (>80%) and low cytotoxicity (cell viability >70%) in the initial screening, were selected for the dose response assays. As shown in Table 1, compound **10** displayed the strongest inhibitory activity in this study with an IC_{50} value of 2.98 μM. Compared with DMSO-treated MDCK cells, influenza A/WSN/33 virus causes a severe CPE in MDCK-infected cells. We found that compound **10** (Figure. 2A) could significantly reduce the CPE, which indicating these compounds were able to protect MDCK cells from influenza virus-induced CPE.

Compound 10 inhibits virus-induced hemagglutination.

Hemagglutinin (HA) plays an important role in the early stages of IAVs infection and mediates attachment of IAVs to host cells via the sialic acid receptor[16, 17]. HA can also bind to sialic acid on the surface of red blood cells (RBCs), causing agglutination[18]. To investigate whether compound **10** can block the ability of viral particles to bind to cell membrane receptors, a hemagglutination inhibition (HI) assay was performed. We found that compound **10** inhibited the binding of influenza virus A/WSN/33 to RBCs, suggesting that compound **10** may directly act on the HA protein (Figure. 2B).

In addition, surface plasmon resonance (SPR) assay was used to characterize the a□nity between HA protein and the compounds **10**, OA [19]. We found that compound **10** could bind tightly to the HA immobilized on the sensor chip (CM5) and at concentrations of 0.39−15 μM exerted dose-dependent responses. As shown in Figure 2C, 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 compound **10** and OA were

4 μM and 24.4 μM, respectively, indicating that compound **10** bound more tightly to HA than OA.

Conclusion

In summary, we have designed and synthesized 20 compounds by structural modification of OA, a mild influenza HA inhibitor discovered in our previous work. Among them, compound **10**, which contains a six-carbon chain with a hydroxyl terminal group, was less cytotoxic and much more potent than other compounds with an IC_{50} of 2.98 μM. Hemagglutination inhibition and SPR assays indicated that compound **10** might interfere with influenza invasion by interaction with the HA protein.

Experimental

Chemistry

General procedure A for the synthesis of oleanolic acid derivatives (1-15):

To OA-OBt (0.2 mmol) and Na_2CO_3 (0.6 mmol) stirring in DMF (20 mL) was added the corresponding amine (0.2 mmol) or alcohol (0.4 mmol) or methyl ester (0.6 mmol). The mixture was stirred at room temperature for 12 h. After completion (TLC) the solvent was removed under reduced pressure. The mixture was dissolved in EtOAc and washed with water and brine twice. The organic layer was dried over Na_2SO_4, then filtered and concentrated. The crude product was purified by column chromatography.

General procedure B for the synthesis of oleanolic acid derivatives (16-20):

To compounds **11-15** (0.1 mmol) stirring in methanol (10 mL) was added 1N NaOH (3 mL). The mixture was stirred at rt. After completion (TLC) the reaction mixture was

neutralized with 1N HCl (3 mL). Water was added and the resulting suspension was filtered. Crude product was purified by column chromatography.

OA-OBt This compound was prepared from OA (10 mmol), EDCI (13mmol), $(CH_3CH_2)_3N$ (13mmol) and 1-Hydroxybenzotriazole (HOBt) according to the Lei' s studies[20].

Compound 1 This compound was prepared from OA-OBt (0.2 mmol) and ethylenediamine (2 mmol) according to the general procedure A. The residue was purified by column chromatography (dichloromethane/methanol, 15/1 v/v). Yield: 62 mg, 62%; white solid. Mp 202.5-203.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.76, 0.78, 0.92, 0.94, 0.98, 1.18 (7×CH_3), 0.76-2.00 (m, other aliphatic ring protons), 2.66 (d, J=11.4 Hz, 1H), 2.81 (t, J=6 Hz, 2H), 3.11-3.21 (m, 2H), 3.36-3.43 (m, 2H), 5.40 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 15.00, 15.31, 16.67, 18.07, 23.15, 23.18, 23.27, 25.57, 26.48, 27.07, 27.73, 30.42, 32.26, 32.66 (2C), 33.85, 36.74, 38.35, 38.51, 39.18, 40.44, 40.80, 41.47, 41.69, 46.23, 46.31, 47.35, 55.01, 78.43, 122.79, 144.00, 179.73. ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{32}H_{55}N_2O_2$, 499.4258; found, 499.4257.

Compound 2 This compound was prepared from OA-OBt (0.2 mmol) and 1,3-propanediamine (2 mmol) according to the general procedure A. The residue was purified by column chromatography (dichloromethane/methanol, 15/1 v/v). Yield: 79 mg, 77%; white solid. Mp 187.6-188.4 °C. ^{1}H NMR (400 MHz, CD_3OH) δ: 0.76, 0.78, 0.91, 0.98, 1.17 (7×CH_3), 0.76-2.00 (m, other aliphatic ring protons), 2.56 (d, J=12.4 Hz, 1H), 2.69 (t, J =6.56 Hz, 2H), 3.04-3.10 (m, 1H), 3.17-3.21 (m, 1H), 3.37-3.44 (m, 1H), 5.33 (s, 1H), 5.38 (s, 1H), 7.38 (s, 1H); ^{13}C NMR (100 MHz, CD_3OH) δ: 15.90, 16.32, 17.93, 19.48, 23.98, 24.03, 24.56,

26.49, 27.86, 28.54, 28.76, 31.62, 33.10, 33.56, 33.88, 34.50, 35.12, 37.91, 38.15, 39.81, 39.84, 40.68, 42.55, 42.93, 47.55, 47.67, 56.71, 79.66, 124.03, 145.27, 180.48. ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{33}H_{57}N_2O_2$, 513.4415; found, 513.4413.

Compound 3 This compound was prepared from OA-OBt (0.2 mmol) and 1,4-diaminobutane (2 mmol) according to the general procedure A. The residue was purified by column chromatography (dichloromethane/methanol, 15/1 v/v). Yield: 80 mg, 76%; white solid. Mp 179.3-180.2 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.66, 0.84, 0.86, 0.87, 0.88, 1.07 (7×CH_3), 0.66-2.00 (m, other aliphatic ring protons), 2.77 (d, *J*=13.8 Hz, 1H), 2.89-3.05 (m, 6H), 5.20 (s, 1H), 7.24 (t, *J*=5.4 Hz, 1H), 8.31 (s, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.08, 16.00, 16.81, 18.01, 22.27, 22.92, 23.56, 25.66, 26.59, 26.95 (2C), 28.22, 30.41, 30.54, 32.45, 32.74, 32.95, 33.66, 36.57, 38.08, 38.36, 38.74, 40.45, 41.23, 41.27, 45.15, 46.04, 47.12, 54.83, 76.80, 79.18, 121.34, 144.12, 176.01. ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{34}H_{59}N_2O_2$, 527.4571; found, 527.4572.

Compound 4 This compound was prepared from OA-OBt (0.2 mmol) and 1,5-pentanediamine (2 mmol) according to the general procedure A. The residue was purified by column chromatography (dichloromethane/methanol, 15/1 v/v). Yield: 94 mg, 86%; white solid. Mp 142.3-143.2 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.65, 0.66, 0.83, 0.85, 0.87, 0.88, 1.07 (7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.54-2.56 (m, 2H), 2.77 (d, *J*=9.54 Hz, 1H), 2.87-3.05 (m, 4H), 5.20 (t, *J*=4.92 Hz, 1H), 7.24 (t, *J*=5.64 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.15, 16.10, 16.90, 18.06, 22.30, 22.99, 23.64, 23.92, 25.72, 27.00, 28.30, 28.96, 30.49, 31.21, 32.53, 32.82, 33.02, 33.72, 36.63, 38.13, 38.44, 38.84, 38.96, 40.06, 40.50, 40.80, 41.30, 45.25, 46.11, 47.17, 54.88, 76.89, 121.40, 144.20, 176.18.

ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{35}H_{61}N_2O_2$, 541.4728; found, 541.4728.

Compound 5 This compound was prepared from OA-OBt (0.2 mmol) and 1,6-Hexylenediamime (2 mmol) according to the general procedure A. The residue was purified by column chromatography (dichloromethane/methanol, 15/1 v/v). Yield: 86 mg, 77%; white solid. Mp 138.9-139.5 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.67, 0.85, 0.87, 0.88, 0.89, 1.09 (7×CH_3) 0.67-2.00(m, other aliphatic ring protons), 2.6 (t, *J*=6.8Hz, 2H), 2.79 (d, *J*=10.64 Hz, 1H), 2.91-3.07 (m, 4H), 5.21 (brs, 1H), 5.76 (s, 1H), 7.22 (t, *J*=5.52Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.07, 16.03, 16.85, 18.00, 22.27, 22.93, 23.58, 25.66, 26.02, 26.43, 26.95, 28.23, 29.09, 30.84, 32.47, 32.77, 32.95, 33.66, 38.07, 38.76, 39.78, 40.43 (2C), 40.46, 46.06, 47.11, 54.81, 76.81, 121.32. ESI-HRMS (*m/z*) $[M+H]^+$ calcd for $C_{36}H_{63}N_2O_2$, 555.4884; found, 555.4883.

Compound 6 This compound was prepared from OA-OBt (0.2 mmol) and 2-aminoethanol (0.4 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 1/1 v/v). Yield: 76 mg, 76%; white solid. Mp 232.4-233.8 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.67, 0.84, 0.86, 0.87, 0.89, 1.08 (7×CH_3), 0.67-2.00 (m, other aliphatic ring protons), 2.75(d, *J*=9.28 Hz, 1H), 2.943.02 (m, 2H), 3.12-3.40 (m, 1H), 3.33-3.36 (m, 2H), 4.27 (d, *J*=5.16 Hz, 1H), 4.59 (t, *J*=5.32 Hz, 1H), 5.21 (brs, 1H), 7.15 (t, *J*=5.56 Hz,1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.08, 16.01, 16.72, 17.97, 22.34, 22.91, 23.51, 25.64, 26.93(3C), 28.21, 30.41, 32.36, 32.68, 32.89, 33.61, 36.55, 38.36, 40.54, 41.23, 41.54(2C), 45.24, 46.02, 47.09, 54.78, 59.84, 76.80, 121.48, 144.00, 176.50. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{32}H_{53}NO_3Na$, 522.3918; found, 522.3919.

Compound 7 This compound was prepared from OA-OBt (0.2 mmol) and 3-aminopropanol (0.4 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 1/1 v/v). Yield: 80 mg, 77%; white solid. Mp 210.9-211.3 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.67, 0.84, 0.86, 0.88, 0.89, 1.08 (7×CH_3), 0.67-2.00 (m, other aliphatic ring protons), 2.76 (d, *J*=9.84 Hz, 1H), 2.96-3.04 (m, 2H), 3.07-3.16 (m, 1H), 3.38 (q, *J*=5.44 Hz, 2H), 4.27 (d, *J*=5.12 Hz, 1H), 4.41 (t, *J*=5.24 Hz, 1H), 5.21 (brs, 1H), 7.22 (t, *J*=5.56 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.06, 15.99, 16.79, 17.97, 22.27, 22.90, 23.51, 25.63, 26.93, 28.20, 30.40, 32.25, 32.40, 32.77, 32.89, 33.63, 36.32, 36.55, 38.05, 38.35, 40.06, 40.46(2C), 41.21, 45.17, 46.04, 47.08, 54.78, 58.84, 76.80, 121.43, 144.01, 176.21. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{33}H_{55}NO_3Na$, 536.4074; found, 536.4074.

Compound 8 This compound was prepared from OA-OBt (0.2 mmol) and 4-amino-1-butanol (0.4 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 1/1 v/v). Yield: 86 mg, 81%; white solid. Mp 205.7-206.9 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.66, 0.67, 0.84, 0.86, 0.88, 0.89, 1.08 (7×CH_3), 0.66-2.00 (m, other aliphatic ring protons), 2.78 (d, *J*=9.68 Hz, 1H), 2.90-3.08 (m, 3H), 3.38 (q, *J*=6 Hz, 2H), 4.28 (d, *J*=5.16 Hz, 1H), 4.37 (t, *J*=4.96 Hz, 1H), 5.20 (brs, 1H), 7.21 (t, *J*=4.96 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.08, 16.00, 16.80, 18.00, 22.25, 22.91, 23.56, 25.66, 25.78, 26.95, 28.22, 30.07, 30.42, 32.45, 32.75, 32.94, 33.66, 36.57, 38.07, 38.36, 38.62, 38.74, 40.44, 41.23, 45.14, 45.16, 46.03, 47.11, 54.81, 60.54, 76.82, 121.34, 144.11, 176.00. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{34}H_{57}NO_3Na$, 550.4231; found, 550.4231.

Compound 9 This compound was prepared from OA-OBt (0.2 mmol) and 5-amino-1-pentanol (0.4 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 1/1 v/v). Yield: 74 mg, 68%; white solid. Mp 195.9-200.9 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.69, 0.84, 0.86, 0.88, 0.89, 1.08 (7×CH_3), 0.67-2.00 (m, other aliphatic ring protons), 2.78 (d, *J*=10.2 Hz, 1H), 2.93-3.03 (m, 3H), 3.17 (d, *J*=5.36 Hz, 1H), 3.31-3.38 (m, 3H), 4.28 (d, *J*=3.96 Hz, 1H), 4.33 (t, *J*=5.04 Hz, 1H), 5.20 (s, 1H), 7.19 (t, *J*=5.24 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.07, 16.01, 16.82, 18.01, 22.26, 22.92, 23.10, 23.56, 25.66, 26.95, 28.23, 29.01, 30.42, 32.25, 32.30, 32.47, 32.75, 32.95, 33.67, 36.57, 38.08, 38.37, 40.46, 41.24, 45.16, 46.05, 47.12, 54.83, 60.58, 60.70, 76.70, 76.82, 121.34, 144.12, 176.00. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{35}H_{59}NO_3Na$, 564.4387; found, 564.4385.

Compound 10 This compound was prepared from OA-OBt (0.2 mmol) and 6-amino-1-hexanol (0.4 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 1/1 v/v). Yield: 90 mg, 80%; white solid. Mp 183.8-184.5 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.66, 0.67, 0.84, 0.86, 0.88, 0.89, 1.08 (7×CH_3), 0.66-2.00 (m, other aliphatic ring protons), 2.78 (dd, J=3.96, 13.38 Hz, 1H), 2.92-3.04 (m, 3H), 3.36 (dd, *J*=6.6, 11.76 Hz, 2H), 4.29 (d, *J*=5.22 Hz, 1H), 4.33 (t, *J*=5.16Hz,1H), 5.20 (brs, 1H), 7.20 (t, *J*=5.52 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.07, 16.04, 16.85, 18.00, 22.26, 22.94, 23.59, 25.38, 25.67, 26.62, 26.96, 28.24, 29.20, 30.45, 32.47, 32.63, 32.77, 32.97, 33.67, 36.58, 38.08, 38.39, 38.83, 38.91, 40.06, 40.46, 41.25, 45.19, 46.06, 47.12, 54.83, 60.69, 76.83, 121.33, 144.18, 176.02. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{36}H_{61}NO_3Na$, 578.4544; found, 578.4545.

Compound 11 This compound was prepared from OA-OBt (0.2 mmol) and Glycine methyl ester hydrochloride (0.8 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 3/1 v/v). Yield: 94 mg, 89%; white solid. Mp 137.8-138.6 °C. ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ: 0.64, 0.67, 0.84, 0.87, 0.88, 0.89, 1.08 (7×CH_3), 0.64-2.00 (m, other aliphatic ring protons), 2.76(d, *J*=9.6 Hz, 1H), 2.97-3.00 (m, 1H), 3.59 (s, 3H), 3.64 (dd, *J*=5.52 Hz, 17.04 Hz, 1H), 3.80 (dd, *J*=6Hz, 16.98 Hz, 1H), 4.29 (d, *J*=5.16 Hz, 1H), 5.17(t, *J*=3.36 Hz, 1H), 7.76(t, *J*=5.7Hz, 1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.13, 16.05, 16.60, 18.02, 22.40, 22.94, 23.50, 25.64, 26.84, 26.97, 28.24, 30.42, 32.42, 32.50, 32.90, 33.61, 36.59, 38.09, 38.40, 38.86, 40.35, 40.93, 41.24, 45.19, 46.05, 47.14, 51.51, 54.83, 76.83, 121.42, 143.95, 170.54, 176.94. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{33}H_{53}NO_4Na$, 550.3867; found, 550.3862.

Compound 12 This compound was prepared from OA-OBt (0.2 mmol) and methyl 3-aminopropionate hydrochloride (0.8 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 3/1 v/v). Yield: 90 mg, 83%; white solid. Mp 218.8-219.6 °C. ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ: 0.65, 0.67, 0.84, 0.86, 0.87, 0.89, 1.07 (7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.40-2.43 (m, 2H), 2.74 (dd, *J*=3.96, 13.38 Hz, 1H), 2.97-3.00 (m, 1H), 3.15-3.21 (m, 1H), 3.25-3.21 (m, 1H), 3.57 (s, 3H), 4.28 (d, *J*=4.74 Hz, 1H), 5.19 (t, *J*=3.42 Hz, 1H), 7.35 (t, *J*=5.58 Hz, 1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.10, 16.03, 16.77, 17.99, 22.27, 22.92, 23.52, 25.65, 26.86, 26.96, 28.23, 30.42, 32.40, 32.61, 32.92, 33.57, 33.61, 35.02, 36.57, 38.08, 38.38, 38.89, 40.43, 41.22, 45.22, 46.01, 47.10, 51.26, 54.81, 76.82, 121.48, 143.93, 171.93, 176.47. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{34}H_{55}NO_4Na$, 564.4023; found, 564.4025.

Compound 13 This compound was prepared from OA-OBt (0.2 mmol) and methyl 4-aminobutyrate hydrochloride (0.8 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 3/1 v/v). Yield: 87 mg, 78%; white solid. Mp 190.4-191.7 °C. ^{1}H NMR (400 MHz $(CD_3)_2SO$) δ: 0.54, 0.64, 0.67, 0.84, 0.86, 0.89, 1.08 (7×CH_3), 0.54-1.97 (m, other aliphatic ring protons), 2.27 (t, *J*=7.48 Hz, 2H), 2.78 (d, *J*=10.00 Hz, 1H), 2.97-3.04 (m, 3H), 3.58 (s, 3H), 4.28 (d, *J*=5.08 Hz, 1H), 5.21 (s, 1H), 7.31 (t, *J*=5.72 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.12, 16.08, 16.86, 18.08, 22.27, 22.98, 23.66(2C), 24.47, 25.77, 27.03(2C), 28.30, 30.51, 30.93, 32.53, 32.87, 33.03, 33.71(2C), 36.64, 38.16, 38.20, 38.45, 40.52, 41.30, 45.32, 46.07, 47.19, 51.28, 54.90, 76.90, 121.47, 144.16, 173.22, 176.33. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{35}H_{57}NO_4Na$, 578.4180; found, 578.4183.

Compound 14 This compound was prepared from OA-OBt (0.2 mmol) and methyl 5-aminopentanoate hydrochloride (0.8 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 3/1 v/v). Yield: 77 mg, 67%; white solid. Mp 178.8-179.6 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.65, 0.67, 0.84, 0.86, 0.88, 0.89, 1.08 (7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.27 (t, *J*=7.24 Hz, 2H), 2.76 (d, *J*=9.84 Hz, 1H), 2.93-3.05 (m, 3H), 3.57 (s, 3H), 4.26 (d, *J*=5.16 Hz, 1H), 5.20 (s, 1H), 7.24 (t, *J*=5.52 Hz, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.01, 15.99, 16.79, 17.97, 22.01, 22.22, 22.87, 23.55, 25.64, 26.94, 28.21, 28.50, 30.42, 32.43, 32.77, 32.93, 33.02(2C), 33.63, 36.55, 38.06, 38.31, 38.35, 38.88, 40.44, 41.22, 45.19, 46.02, 47.10, 51.11, 54.79, 76.80, 121.36, 144.08, 173.21, 176.08. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{36}H_{59}NO_4Na$, 592.4336; found, 592.4333.

Compound 15 This compound was prepared from OA-OBt (0.2 mmol) and methyl 6-aminocappoate hydrochloride (0.8 mmol) according to the general procedure A. The residue was purified by column chromatography (petroleum ether/EtOAc, 3/1 v/v). Yield: 95 mg, 81%; white solid. Mp 116.2-117.4 °C. ^{1}H NMR (600 MHz $(CD_3)_2SO$) δ: 0.66, 0.67, 0.84, 0.86, 0.87, 0.89, 1.08 (7×CH_3), 0.66-2.00 (m, other aliphatic ring protons), 2.27 (t, *J*=7.44 Hz, 2H), 2.77 (dd, *J*=4.02, 13.44 Hz, 1H), 2.91-3.04 (m, 3H), 3.57 (s, 3H), 4.27 (d, *J*=5.16 Hz, 1H), 5.20 (t, *J*=3.42 Hz, 2H), 7.21 (t, *J*=5.58 Hz, 2H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.06, 16.03, 16.85, 18.00, 22.26, 22.93, 23.59, 24.23, 25.67, 26.06, 26.96, 28.24, 28.77, 30.45, 32.46, 32.77, 32.97, 33.30, 33.66, 36.58, 38.08, 38.39, 38.60, 38.91, 40.06, 40.46, 41.25, 45.20, 46.05, 47.12, 51.17, 54.82, 76.83, 121.34, 144.17, 173.26, 176.07. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{37}H_{61}NO_4Na$, 606.4493; found, 606.4493.

Compound 16 This compound was prepared from 11 (0.2 mmol) and sodium hydroxide (0.6 mmol) according to the general procedure B. Yield: 84 mg, 82%; white solid. Mp 176.0-177.8 °C. ^{1}H NMR (600 MHz, $(CD_3)_2SO$) δ: 0.64, 0.67, 0.83, 0.87, 0.87, 0.89, 1.08 (7×CH_3), 0.64-2.00 (m, other aliphatic ring protons), 2.76 (dd, *J*=3.84, 13.38 Hz, 1H), 2.97-3.00 (m, 1H), 3.56 (dd, *J*=3.54, 17.28 Hz, 1H), 3.73 (dd, *J*=6.0, 17.28 Hz, 1H), 4.28 (d, *J*=4.44 Hz, 1H), 5.19 (t, *J*=3.36 Hz, 1H), 7.57 (t, *J*=5.58 Hz, 1H), 12.37 (s, 1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.12, 16.05, 16.60, 18.00, 22.45, 22.95, 23.50, 25.65, 26.85, 26.97, 28.24, 30.42, 32.39, 32.48, 32.91, 33.62, 36.58, 38.10, 38.39, 38.88, 40.47, 40.90, 41.24, 45.14, 46.05, 47.15, 54.82, 76.83, 121.48, 143.97, 171.48, 176.68. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{32}H_{51}NO_4Na$, 536.3710; found, 536.3713.

Compound 17 This compound was prepared from 12 (0.2 mmol) and sodium hydroxide

(0.6 mmol) according to the general procedure B. Yield: 90 mg, 85%; white solid. Mp > 250 °C. ^{1}H NMR (600 MHz $(CD_3)_2SO$) δ: 0.65, 0.67, 0.84, 0.86, 0.86, 0.88, 1.07 (7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.33 (t, *J*=7.08 Hz, 2H), 2.72 (dd, *J*=3.78, 13.80 Hz, 1H), 2.97-3.00 (m, 1H), 3.123.15 (m, 1H), 3.23-3.29 (m, 1H), 3.37 (s, 1H), 5.19 (brs, 1H), 7.30 (t, *J*=5.58 Hz, 1H), 12.20 (s,1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) δ: 15.16, 16.09, 16.83, 18.05, 22.33, 22.97, 23.55, 25.69, 26.93, 27.00, 28.28, 30.47, 32.43, 32.66, 32.96, 33.64, 33.82, 35.02, 36.61, 38.13, 38.43, 38.95, 40.56, 41.28, 45.23, 46.05, 47.15, 54.84, 76.87, 121.62, 143.93, 173.21, 176.44. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{33}H_{53}NO_4Na$, 550.3867; found, 550.3865.

Compound 18 This compound was prepared from 13 (0.2 mmol) and sodium hydroxide (0.6 mmol) according to the general procedure B. Yield: 94 mg, 87%; white solid. Mp 228.7-229.5 °C. ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ: 0.65, 0.67, 0.84, 0.86, 0.89, 1.08 (7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.18 (t, *J*=7.4 Hz, 2H), 2.78 (d, *J*=9.68 Hz, 1H), 2.96-3.03 (m, 3H), 3.32 (d, *J*=8.92 Hz, 2H), 4.28 (d, *J*=5.08 Hz, 1H), 5.21 (brs, 1H), 7.29 (t, *J*=5.44 Hz, 1H), 12.0 (s, 1H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ: 15.16, 16.10, 16.92, 18.08, 22.27, 22.99, 23.67, 24.53, 25.76, 27.04, 28.31, 30.52, 31.29, 32.53, 32.87, 33.03, 33.71, 36.65, 38.23, 38.43, 38.46, 40.50, 41.31, 45.29, 45.30, 46.09, 47.19, 54.89, 76.79, 76.90, 121.48, 144.17, 174.36, 176.24. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{34}H_{55}NO_4Na$, 564.4023; found, 564.4024.

Compound 19 This compound was prepared from 14 (0.2 mmol) and sodium hydroxide (0.6 mmol) according to the general procedure B. Yield: 79 mg, 71%; white solid. Mp 139.1-140.9 °C. ^{1}H NMR (600 MHz $(CD_3)_2SO$) δ: 0.65, 0.66, 0.83, 0.86, 0.87, 0.88, 1.07

(7×CH_3), 0.65-2.00 (m, other aliphatic ring protons), 2.17 (t, *J*=7.26 Hz, 2H), 2.78 (dd, *J*=4.08, 13.56 Hz, 1H), 2.94-3.02 (m, 3H), 3.41 (s, 1H), 5.20 (brs, 1H), 7.26 (t, *J*=5.52 Hz, 1H), 11.98 (s, 1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) *δ*: 15.16, 16.09, 16.83, 18.05, 22.33, 22.97, 23.55, 25.69, 26.93, 27.00, 28.28, 30.47, 32.43, 32.66, 32.96(2C), 33.64, 33.82, 35.02, 36.61, 38.13, 38.43, 38.95, 40.06, 40.56, 41.28, 45.23, 46.05, 47.15, 54.84, 76.87, 121.62, 143.93, 173.21, 176.44. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{35}H_{57}NO_4Na$, 578.4180; found, 578.4180.

Compound 20 This compound was prepared from 15 (0.2 mmol) and sodium hydroxide (0.6 mmol) according to the general procedure B. Yield: 82 mg, 72%; white solid. Mp 119.8-120.7 °C. ^{1}H NMR (600 MHz, $(CD_3)_2SO$) *δ*: 0.66, 0.67, 0.84, 0.86, 0.87, 0.88, 1.08 (7×CH_3), 0.66-2.00 (m, other aliphatic ring protons), 2.17 (t, *J*=7.38 Hz, 2H), 2.78 (dd, *J*=3.9, 13.32 Hz, 1H), 2.91-3.04 (m, 3H), 5.20 (brs, 1H), 7.22(t, *J*=5.58 Hz, 2H), 11.98 (s, 1H); ^{13}C NMR (150 MHz, $(CD_3)_2SO$) *δ*: 15.08, 16.05, 16.86, 18.01, 22.27, 22.94, 23.60, 24.30, 25.68, 26.17, 26.97, 28.25, 28.86, 30.46, 32.47, 32.77, 32.98, 33.67(2C), 36.59, 38.08, 38.39, 38.66, 38.91, 40.05, 40.47, 41.26, 45.20, 46.06, 47.13, 54.83, 76.84, 121.35, 144.16, 174.40, 176.06. ESI-HRMS (*m/z*) $[M+Na]^+$ calcd for $C_{36}H_{59}NO_4Na$, 592.4346; found, 592.4344.

Bioassays

Cell activity assay.

MDCK cells were seeded into 96-well plates in DMEM supplemented with 10% FBS cultured overnight at 37 °C in 5% CO_2. Then, cells were suspended in DMEM supplemented with 1% FBS, containing test compound and 2 μg/mL TPCK-treated trypsin, and the cells were further incubated at 37°C in 5% CO_2 for 40 hours. Cell viability was assessed using the CellTiter-Glo assay kit (Promega Corp., Madison, WI, USA) as recommended by the supplier, and the plates were read using a plate reader (Molecular Devices SpectraMax M2). Viability was calculated using the background corrected absorbance as follows: Viability (%) = (CA of experiment well/DA of control well) × 100, where CA, DA represent the reading values of test compound and DMSO, respectively.

Cytopathic Effect (CPE) Reduction Assay.

MDCK cells were seeded into 96-well plates, incubated overnight and infected with influenza virus (MOI = 0.1) suspended in DMEM supplemented with 1% FBS, containing test compound and 2 μg/mL TPCK-treated trypsin, with a final DMSO concentration of 1% in each well. After 40 h of incubation, CellTiter-Glo reagent was added and the plates were read using a plate reader (Molecular Devices SpectraMax M2).

Hemagglutination Inhibition (HI) Assay.

Compound from a 2-fold serial dilution in saline was mixed with an equal volume of influenza virus (The HA titers of A/WSN/33 (H1N1) virus is 1:2) in the V-bottomed 96-well microplate. Subsequently, 50 μL of freshly prepared chicken red blood cells (RBCs) (1% v/v

in saline) were added to each well. The mixture was incubated for 30 min at RT before observing RBCs aggregation on the plate.

Surface plasmon resonance (SPR).

Interactions between the influenza HA and the compounds were analyzed using the Biacore T200 system (GE Healthcare, Uppsala, Sweden) at 25 °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 60s and a dissociation time of 60s. 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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant no. 81560560) and the Scientific Research Foundation of Kunming University of Science and Technology (No. KKSY20169517).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.

References:

1) Bar-On Y., Glasner A., Meningher T., Achdout H., Gur C., Lankry D., Vitenshtein A., Meyers A. F. A., Mandelboim M., Mandelboim O., *Cell. Rep.,* **3**, 1044-1050 (2013).

2) Molinari N-A. M., Ortega-Sanchez I. R., Messonnier M. L., Thompson W. W., Wortley P. M., Weintraub E., Bridges C. B., *Vaccine*, **25***,* 5086-5096 (2007).

3) Chung S-T., Chien P-Y., Huang W-H., Yao C-W., Lee A-R., *Chem. Pharm. Bull.*, **62***,* 415-421 (2014).

4) Morens D. M., Taubenberger J. K., Fauci A. S., *New. Engl. J. Med.*, **361***,* 225-229 (2009).

5) Horimoto T., Kawaoka Y., *Nat. Rev. Microbiol.,* **3***,* 591-600 (2005).

6) van Dongen M. J. P., Kadam R. U., Juraszek J., Lawson E., Brandenburg B., Schmitz F., Schepens W. B. G., Stoops B., van Diepen H. A., Jongeneelen M., Tang C., Vermond J., Eijgen-Obregoso Real A. V., Blokland S., Garg D., Yu W. L., Goutier W., Lanckacker E., Klap J. M., Peeters D. C. G., Wu J., Buyck C., Jonckers T. H. M., Roymans D., Roevens P., Vogels R., Koudstaal W., Friesen R. H. E., Raboisson P., Dhanak D., Goudsmit J., Wilson I. A., *Science*, **363***,* 1-10 (2019).

7) Tong S. X., Zhu X. Y., Li Y., Shi M., Zhang J., Bourgeois M., Yang H., Chen X. Y., Recuenco S., Gomez J., Chen L-M., Johnson A., Tao Y., Dreyfus C., Yu W. L., McBride R., Carney P. J., Gilbert A. T., Chang J., Guo Z., Davis C. T., Paulson J. C., Stevens J., Rupprecht C. E., Holmes E. C., Wilson I. A., Donis R. O., *Plos. Pathog.*, **9***,* 1078-1084 (2013).

8) Tong S. X., Li Y., Rivailler P., Conrardy C., Alvarez Castillo D. A., Chen L-M., Recuenco S., Ellison J. A., Davis C. T., York I. A., Turmelle A. S., Moran D., Rogers S., Shi M., Tao Y., Weil M. R., Tang K., Rowe L. A., Sammons S., Xu X. Y., Frace M., Lindblade K. A., Cox N. J., Anderson L. J., Rupprecht C. E., Donis R. O., *Proc. Natl. Acad. Sci. U.S.A.*, **109***,* 4269-4274 (2012).

9) Bouvier N. M., Palese P., *NIH. Public. Access.*, **26***,* D49-D53 (2008).

10) Amarelle L., Lecuona E., Sznajder J. I., *Arch. Bronconeumol.,* **53***,* 19-26 (2017).

11) Yang J., Huang Y. N., Liu S. W., *Expert. Opin. Investig. Drugs.*, **28***,* 481-4888 (2019).

12) Vanderlinden E., Naesens L., *Med. Res. Rev.*, **34***,* 301-339 (2014).

13) Song G. P., Yang S., Zhang W., Cao Y., Wang P., Ding N., Zhang Z. H., Guo Y., Li Y. X., *J. Med. Chem.*, **52***,* 7368-7371 (2009).

14) Yu M. R., Si L. L., Wang Y. F., Wu Y. M., Yu F., Jiao P. X., Shi Y. Y., Wang H., Xiao S. L., Fu G., Tian K., Wang Y. T., Guo Z. H., Ye X. S., Zhang L. H., Zhou D. M., *J. Med. Chem.*, **57***,* 10058-10071 (2014).

15) Medina-O'Donnell M., Rivas F., Reyes-Zurita F. J., Martinez A., Lupiañez J. A., Parra A., *Eur. J. Med. Chem.*, **118***,* 64-78 (2016).

16) Tian Z. Y., Si L. L., Meng K., Zhou X. S., Zhang Y. M., Zhou D. M., Xiao S. L., *Eur. J. Med. Chem.*, **134***,* 133-139 (2017).

17) Glaser L., Stevens J., Zamarin D., Wilson I. A., García-Sastre A., Tumpey T. M., Basler C. F., Taubenberger J. K., Palese P., *J. Virol.*, **79***,* 11533-11536 (2005).

18) Thontiravong A., Prakairungnamthip D., Chanvatik S., Nonthabenjawan N., Tunterak W., Tangwangvivat R., Oraveerakul K., Amonsin A., *Thai. J. Vet. Med.*, **46***,* 135-142 (2016).

19) Mandenius C. F., Wang R. H., Aldén A., Bergström G., Thébault S., Lustch C., Ohlson S., *NIH. Public. Access.*, **623***,* 66-75 (2008).

20) Xu K., Xu X., Chu F. H., Wang M. N., Wang P. L., Li G. L., Song J. X., Zhang Y. H., Lei H. M., *Res. Chem. Intermed.*, **41***,* 6257-6269 (2015).

Table 1. Anti-IAV activity data of target compounds 9, 10 and 12-15.

Compound	IC_{50} (μM)[a]	CC_{50} (μM)[b]
9	>50	>100
10	2.98	>100
12	5.36	>100
13	39.49	>100
14	>50	>100
15	>50	>100
OSV	1.82	>100
OA	72.27	>100

All data presented are averages of at least three separate experiments. [a]IC_{50}: compound concentration required to achieve 50% inhibition of replication of virus, as determined by the CellTiter-Glo assay. [b]CC_{50}: compound concentration required to cause 50% death of uninfected MDCK cells, as determined by the CellTiter-Glo assay.

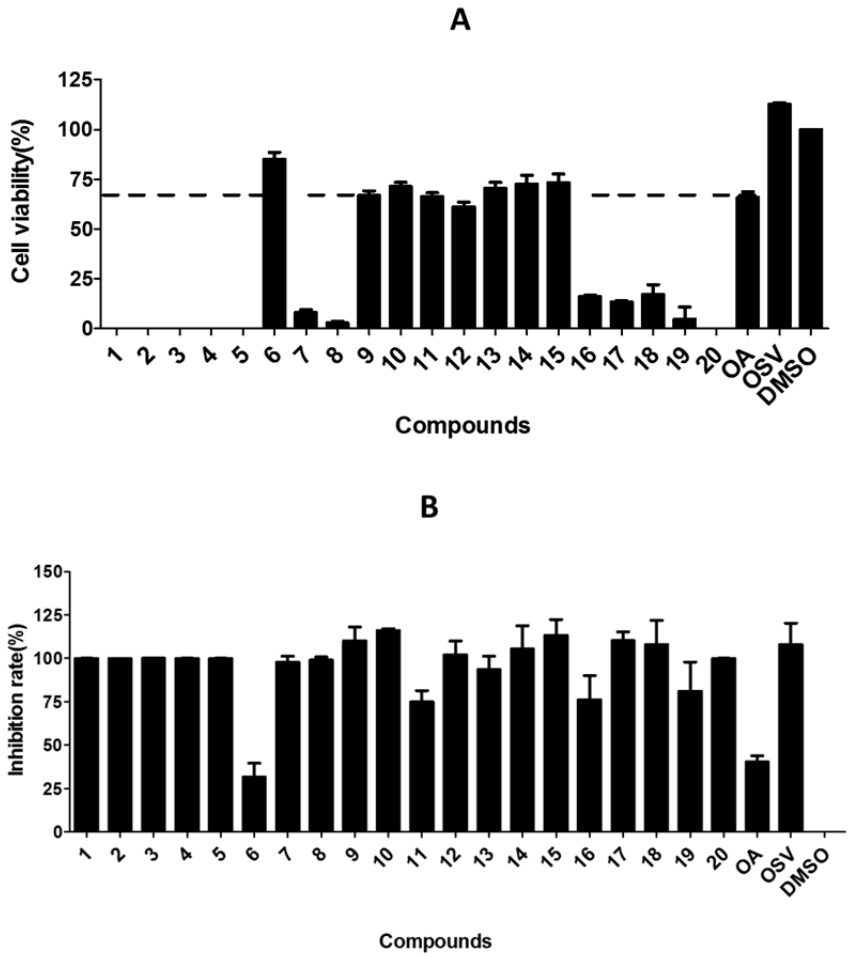

Fig 1. Inhibitory effects of OA derivatives against influenza A/WSN/33 (H1N1) virus. A. Cytotoxicity screening of OA derivatives (final concentration 100 μM) using CellTiter-Glo® assay. B. The CPE-based screening of OA derivatives by using a CellTiter-Glo® assay. MDCK was utilized as the host cell to test A/WSN/33 (H1N1) virus infection; 1% DMSO (final concentration) was used as negative control, and OSV (oseltamivir) was utilized as positive control, respectively. The error bars indicate the standard deviations of triplicate experiments.

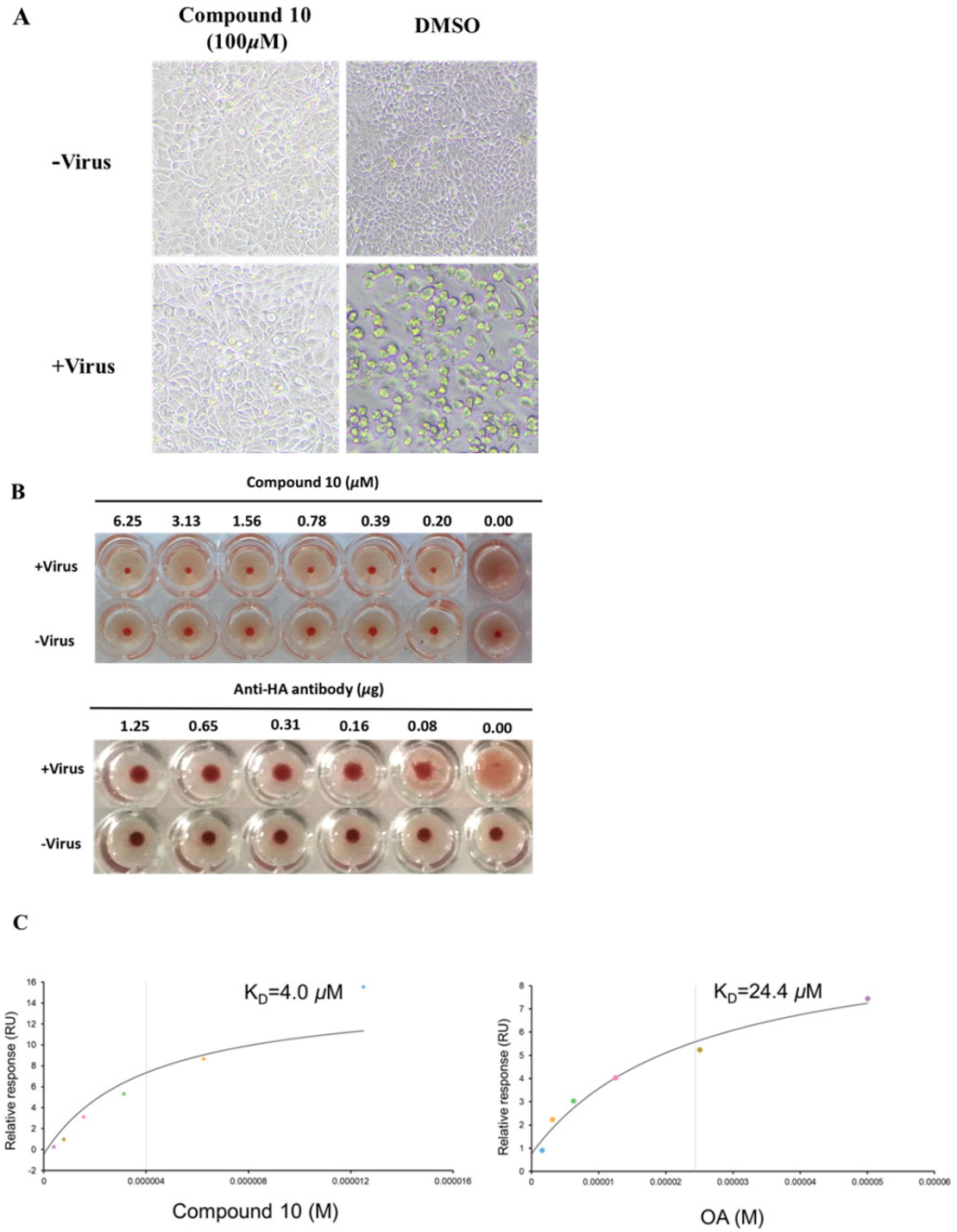

Fig 2. A. Validation of the protection of MDCK cells from influenza A/WSN/33 virus by compound 10. B. Identify HA as the potential target of compound. Comparisons of the behaviors of compound 10 vs anti-HA antibody in inhibition of influenza virus-induced aggregation of chicken erythrocytes. Compound 10 exerted identical capability as anti-HA antibody in inhibition of hemagglutination in a dose dependent manner. C. Characterization of the affinity between compounds (10 and OA) 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.

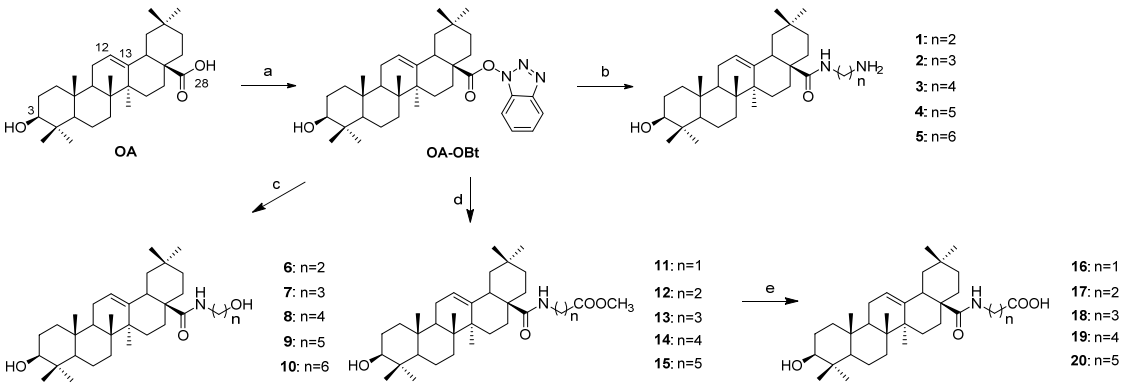

Chart 1. Reagents and conditions: (a) EDCI, HOBt, $(CH_3CH_2)_3N$, CH_2Cl_2, 5-10℃, 12h. (b) Amine compound, Na_2CO_3, DMF, rt, 12h. (c) Alcohol compound, Na_2CO_3, DMF, rt, 12h. (d) Methyl ester compound, Na_2CO_3, DMF, rt, 12h. (e) NaOH, MeOH, rt, 6h.