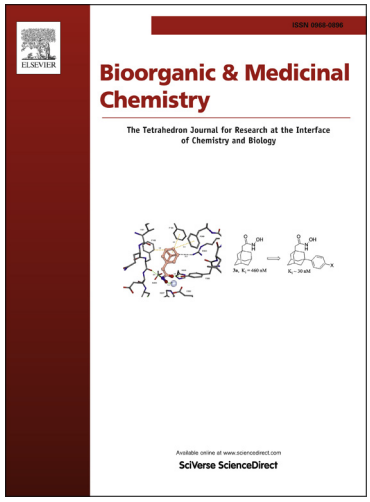

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PII: S0968-0896(13)00885-7
DOI: http://dx.doi.org/10.1016/j.bmc.2013.10.020
Reference: BMC 11171

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 5 September 2013
Revised Date: 30 September 2013
Accepted Date: 17 October 2013

Please cite this article as: Xie, Y., Huang, B., Yu, K., Xu, W., Further Discovery of caffeic acid derivatives as Novel Influenza Neuraminidase inhibitors, *Bioorganic & Medicinal Chemistry* (2013), doi: http://dx.doi.org/10.1016/j.bmc.2013.10.020

Further Discovery of caffeic acid derivatives as Novel Influenza Neuraminidase inhibitors

Yuanchao Xie [a], Bing Huang [b], Kexiang Yu [b], Wenfang Xu [a,*]

[a] Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, 44, West Culture Road, Jinan, Shandong 250012, P. R. China

[b] Institute of poultry science, Shandong Academy of Agricultural Sciences, 1, Jiaoxiao Road, Jinan, Shandong 250023, P. R. China

ABSTRACT

Eight series of compounds, each series containing two to five compounds were prepared by structural modifications of a lead, which was previously discovered as a mild influenza neuraminidase (NA) inhibitor. On the basis of the biological result, a detailed structure-activity relationship (SAR) was derived and discussed. Several caffeic acid derivatives that acted as non-competitive inhibitors were close or superior to the lead and also presented good antiviral activities in cells. Besides, it was interesting to find that modifications of the lead with different strategies could result in selective inhibition against N1 or N2. The preliminary docking analysis indicated that the 150-cavity of the enzymes played an important role in the selective inhibition.

Keywords: Caffeic acid; Influenza; Inhibitor; Neuraminidase.

* Corresponding author: Wenfang Xu

Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University 44, West Culture Road, Jinan 250012, P. R. China

Tel/Fax: +86-531-88382264; E-mail address: wenfxu@gmail.com, yuanchaox623@aliyun.com.

1. Introduction

Influenza viruses that have a high mutation rate pose a great threat to human. In the 20th century, there were three influenza pandemics (H1H1, H2N2 and H3N2), killing tens of millions of people.[1] The first outbreak of the highly pathogenic avian influenza A (H5N1) virus occurred in 1997, and since then this virus has been continuing to evolve and causing global concern. More recently, some novel emerging viral strains, such as the 2009 swine-origin H1N1 virus[2] and the 2013 Chinese H7N9 virus[3] also give rise to seriously public panic.

Flu vaccine is an effective method for preventing influenza virus infection, but it needs reformulating each year to match antigenic variations and is inadequate to cope with an unpredictable pandemic. The influenza neuraminidase (NA) plays an important role in the life cycle of influenza virus and has been discovered as an important target for anti-influenza drugs design.[4] Till now, three NA inhibitors, zanamivir, oseltamivir and peramivir have been approved for the treatment and prophylaxis of influenza. Among them, oseltamivir administered orally is the first line defense drug against influenza. However, in recent years more and more influenza virus strains are resistant to it, such as the seasonal H1N1 viruses[5] and avian H5N1 strains.[6] The other two drugs are mainly administered via inhaled nebulized or intravenous routes, which is very inconvenient for patients. Therefore, it is still necessary to find novel effective drugs with good properties to cope with influenza viruses.

In recent three years, only a few influenza NA inhibitors have been reported and most of them are mainly limited to the derivatives of zanamivir or oseltamivir.[7-12] Because zanamivir and oseltamivir have perfectly occupied the active site of NA, the structural modification of them is hard to discover better compounds. It seems that the development of NA inhibitors has been in a difficult position. However, it is worth noting that many natural products, especially some small plant-derived polyphenols have been found to exert good antiviral effects against influenza virus and show moderate inhibitory activity against NA. These compounds contain various structures, such as flavonoids[13], xanthones[14,15] and diarylheptanoids,[16] which are suitable to be leads for the design of novel influenza NA inhibitors.

In our previous studies, we built a compound library containing a variety of structures based on the reported flavonoid NA inhibitors.[17,18] Among them, we discovered that the caffeic acid derivatives present good anti-influenza virus activity and NA inhibitory activity in vitro, such as compound **1** and **2** (**Fig. 1**). Besides, compound **2** was found to be a non-competitive inhibitor with Ki = 11.5 ± 0.25 μM. In the present study, we took compound **2** as the lead to make further modifications. Eight series of compounds, each series containing two to five compounds were prepared by different synthetic methods. The enzyme inhibition assay indicated that the compounds containing caffeic acid fragment displayed much better activities. Several compounds were closed or superior to the lead and also showed good antiviral activities in cells. Besides, it was interesting to find that modifications of the lead with different strategies could result in

selective inhibition against N1 or N2.

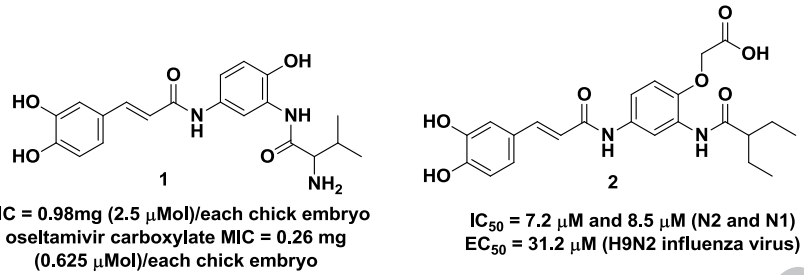


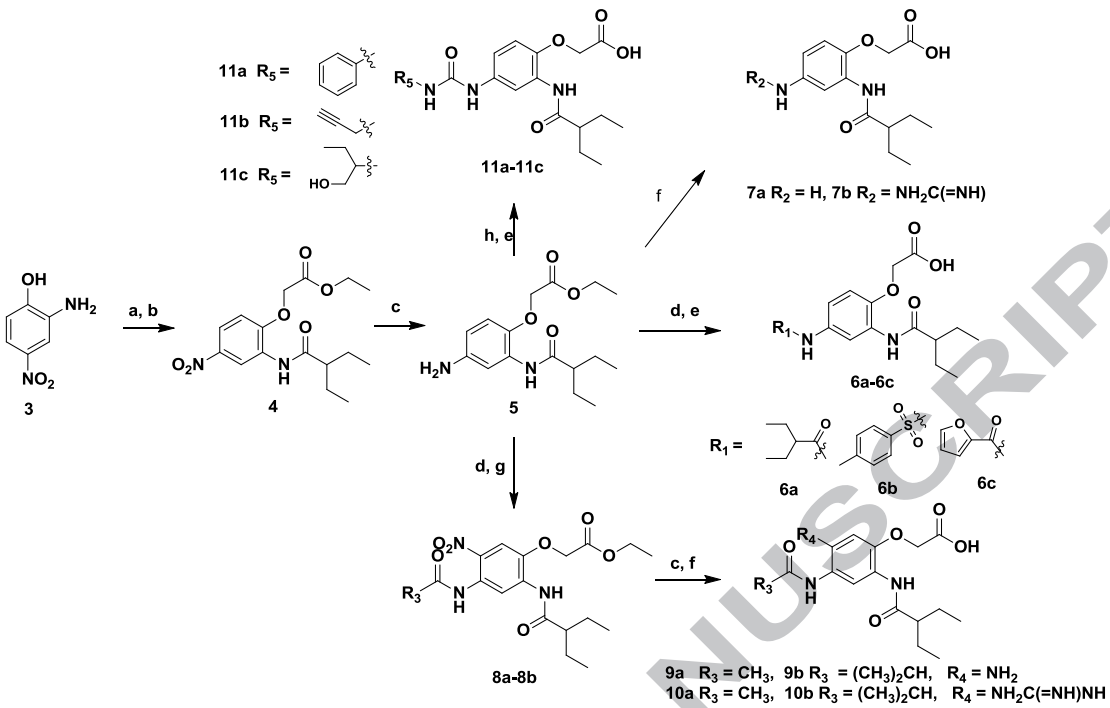
Fig. 1. Two caffeic acid derivatives discovered in our previous studies

2. Chemistry

In order to make sure if the caffeic acid fragment was necessary for the activity and explore the structure-activity relationship (SAR), four series of compounds without the caffeic acid fragment were designed and synthesized in Scheme 1. Compound **5** was a key intermediate that can be synthesized form the starting material, 2-amino-4-nitrophenol (compound **3**) through three serial steps: acylation with 2-ethylbutanoic acid, Williamson ether reaction with ethyl chloroacetate and reduction of the nitro group by hydrogen. The target compounds **6a**-**6c** were prepared by reaction of compound **5** with acyl chloride or sulfuryl chloride and hydrolysis with NaOH.

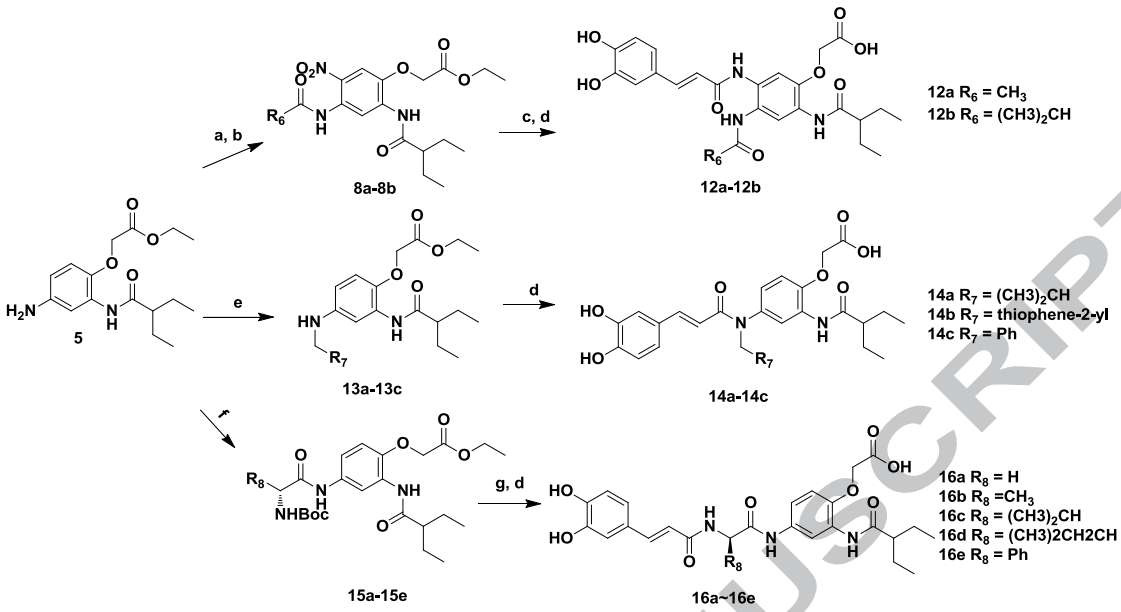
Because the basic group, such as amino group or guanidine group was important for the activity of classical influenza NA inhibitors,[19] compound **7**, **9** and **10** were designed. For synthesizing compound **7a**, the amino group of compound **5** was first protected with Boc, then hydrolyzed to give the Boc-protected acid. Deprotection of the intermediate with TFA afforded the target compound. For synthesizing compound **7b**, compound **5** was first guanylated with *N,N'*-bis(*tert*-butoxycarbonyl)thiourea ($SC(NHBoc)_2$)/$HgCl_2$. The following hydrolysis and Boc deprotection would afford compound **7b**. Compounds **9** and **10** can also be synthesized with the same methods from compounds **8a** and **8b**, which were obtained by two step form compound **5**, acylation with acyl chlorides and nitration with HNO_3/H_2SO_4. The position of the NO_2 on the benzene ring of compounds **8** can be determined by the spectra of ^{1}H-NMR.

To synthesize compounds **11a**-**11c**, compound **5** was first converted the isocyanate and then reacted with different amines. After hydrolysis with NaOH, the urea compounds were obtained.



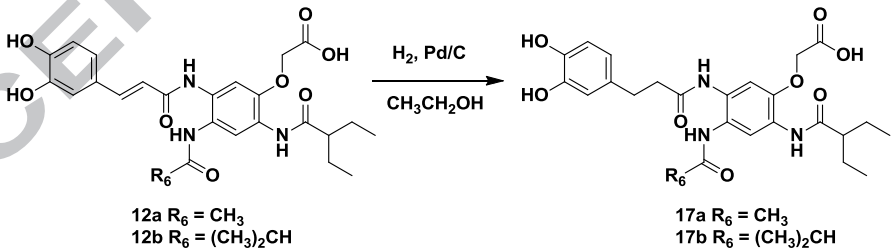
Scheme 1. Reagents and conditions: (a) 2-ethylbutanoyl chloride, $NaHCO_3$, THF, H_2O, rt; (b) ethyl chloroacetate, NaI, K_2CO_3, acetone, reflux; (c) H_2, Pd/C, CH_3CH_2OH, rt; (d) acyl chloride or sulfuryl chloride, $NaHCO_3$, THF,H_2O, rt; (e) NaOH, CH_3OH, H_2O, rt; (f) for 7a: 1. $(Boc)_2O$, Et_3N, THF, rt; 2. NaOH, CH_3OH, H_2O, rt; 3. CH_2Cl_2, CF_3COOH; for 7b: 1. $SC(NHBoc)_2$, $HgCl_2$, Et_3N, DMF; 2. NaOH, CH_3OH, H_2O, rt; 3. CH_2Cl_2, CF_3COOH; (g)HNO_3, H_2SO_4; (h) 1. triphosgene, dioxane, 40 ^oC; 2. THF, Et_3N, R_5NH_2, rt.

In Scheme 2, three series of compounds containing the caffeic acid fragment were designed and synthesized. For improving the yields, the acetylated caffeic acid, (E)-3-(3,4-diacetoxyphenyl)acrylic acid that had a relatively better solubility in common organic solvents was used as the material. This compound was converted into the corresponding acyl chloride by using oxalyl chloride and reacted with the reduced aniline products of compounds **8**. After hydrolysis with NaOH, compounds **12** were obtained. Compounds **13** were prepared by reaction of compound **5** and different aldehydes in the present of $NaBH_3CN$. A little amount of acetic acid could be favorable for this reaction. Condensation of compound **5** with various Boc protected amino acid afforded compounds **15a**-**15f**, which were subsequently treated with 3M HCl/EtOAc to remove the Boc group. Compounds **14a**-**14c** and **16a**-**16f** were prepared with the same methods of synthesizing compounds **12**.



Scheme 2. Reagents and conditions: (a) R_6COCl, $NaHCO_3$, THF, H_2O, rt; (b) H_2SO_4, HNO_3, 0 ^oC; (c) H_2, Pd/C, CH_3CH_2OH, rt; (d) 1. (*E*)-3-(3,4-diacetoxyphenyl)acrylic acid, oxalyl chloride, DMF, anhydrous THF, rt; 2. $NaHCO_3$, THF, H_2O, rt; 3. NaOH, CH_3OH, H_2O, rt; (e) R_7CHO, $NaBH_3CN$, CH_3COOH; (f) Boc-L-amino acid, TBTU, Et_3N,rt; (f) 3M HCl/EtOAc, rt.

In order to explore whether the double bond of the caffeic acid fragment was important for the NA inhibitory activity. In Scheme 3, compounds **17a** and **17b** were prepared by reduction of the double bond of compounds **12a** and **12b**.



Scheme 3. Reagents and conditions: H_2, Pd/C, CH_3CH_2OH, rt.

3. Results and discussion

3.1. In vitro inhibitory activities on NAs

Generally, influenza NAs were divided into two groups: group-1 NAs containing N1, N4, N5, N8 and group-2 NAs containing N2, N3, N6, N7, N9. It had been found that there were some structural differences between the two group NAs in the region adjacent to the enzyme's catalytic center.[20] Therefore, we chose NAs from two kinds of influenza viruses (H9N2 and H5N1) to

determine the inhibitory effects of the designed compounds.

Table 1 listed the in vitro inhibitory effects of compounds **6, 7, 9, 10 and 11** against influenza A NAs. Compared with the lead (compound **2**), the twelve compounds with diverse structures exhibited decreased activities, suggesting that the caffeic acid fragment was important. Among compounds **6a-6a** and **11a-11c**, which had amides and ureas in their structures, Compound **11a** was the best. It seemed that the urea structures with unsaturated groups, especially the aromatic groups favored the activity. Compounds **7a** and **7b** that had basic groups (the amino group and the guanidine group) at C-4 position only presented weak inhibition at the concentration of 100 μM, while compounds **9** and **10** with the basic groups at C-5 position exhibited improved activities. Besides, compounds **9a** and **10a** with the amino group were better than compounds **9b** and **10b** with the guanidine group. According to the pharmacophore model of classical influenza NA inhibitors, the COOH group and basic groups were essential for the activity.[21] However, compounds **9** and **10** did not show potent activities against NA, which was probably because the necessary groups could not bind with the corresponding pockets in the enzymatic active site.

Table 1

In vitro inhibitory effects of compounds **6, 7, 9, 10 and 11** against influenza A NAs

compound	IC_{50} (μM, H9N2 and H5N1)	compound	IC_{50} (μM, H9N2 and H5N1)
6a	> 100	9b	54.3/61.2
6b	78.6/86.7	10a	69.1/75.8
6c	> 100	10b	83.2/NT
7a	> 100	11a	49.7/55.6
7b	> 100	11b	89.8/NT
9a	46.4/58.5	11c	> 100

Table 2 listed the in vitro inhibitory effects of compounds **12, 14** and **16** against influenza A NAs. All the ten compounds containing the caffeic acid fragments showed good NA inhibitory

activities, and several compounds, such as **12a**, **12b**, **16b** and **16d** were superior or closed to the lead. The general order of potency for NA inhibition was compounds 12 > compounds 16 > compounds 14, which indicated that the position of the caffeic acid fragment had a certain effect on the activity. This should be carefully considered in the further studies. Besides, It was noteworthy that compounds **12** and **16** selectively inhibited N2 and compounds **14** selectively inhibited N1. The lead compound also presented slight selective inhibition against N2. Structurally, compounds **14** had branched substituted groups at the amide nitrogen atoms, while compounds **12**, **16** and the lead did not. According to the crystal structures of some group-1 NAs, there were at least two cavities, such as the 150-cavity and 430-cavity adjacent to the active site.[20,22] The branched side chains of compounds **14** may contribute to multiple interactions with N1 and lead to the selective inhibition. This strategy of structural modification could be used to look for highly potent and selective group-1 NA inhibitors. Compound **9a** (Table 1) was a mild NA inhibitor with IC_{50} values of 46.4/58.5 against N2 and N1, respectively, while compound **12a** obtained by condensation of compound **9a** and caffeic acid was about 15-fold more potent against N1. Moreover, modifications on the NH_2 at C-4 position would also affect the activity, since compound **12b** with isobutyryl group at this site was less potent than compound **12a**. Generally, compounds **16a-16e** with different amino acids as linkers connecting the caffeic acid fragment exhibited similar activities to the lead. The side chains of the amino acids had little influence on the activity.

Table 2

In vitro inhibitory effects of compounds **12a**,**12b**, **14a-14c** and **16a-16e** against influenza A NAs

compound	IC_{50} (μM, H9N2 and H5N1)	compound	IC_{50} (μM, H9N2 and H5N1)
12a	3.2/10.7	16a	9.2/17.6
12b	5.8/14.2	16b	7.9/16.1
14a	25.2/11.3	16c	9.4/16.6
14b	24.1/16.4	16d	8.5/14.0

14c	16.0/11.4	16e	9.8/16.2

All compounds were tested in triplicate wells per dilution. Oseltamivir carboxylate was used as the positive control with IC_{50} values = 0.0028 μM (H9N2) and 0.016 μM (H5N1). NT: not tested.

In **Table 3**, it could be found that the activities of compounds **17** were five-fold less potent than those of compounds **12**, which proved that the double bond of caffeic acid fragment contributing to structural constraints played an important role in the activity. Besides, the two compounds also showed obvious selective inhibition against N2 like compounds **12**.

Table 3

In vitro inhibitory effects of compounds **17a** and **17b** against influenza A NAs

compound	IC_{50} (μM, H9N2 and H5N1)
17a	15.8/42.7
17b	14.2/38.2

3.2. Enzyme inhibition kinetics

In the kinetic studies, compounds **12a**, **14c** and **16b** were found to be non-competitive inhibitors with *K*i = 2.70 ± 0.25 μM (N2), 32.5 ± 1.2 μM (N1) and 9.50 ± 0.50 μM (N1), respectively by the means of Lineweaver-Burk and Dixon plots analysis (**Fig. 2**). In Recent years, many natural products, especially polyphenol compounds have also been discovered as non-competitive NA inhibitors. However, the exact binding site of this kind of inhibitors in the enzyme is still unclear.

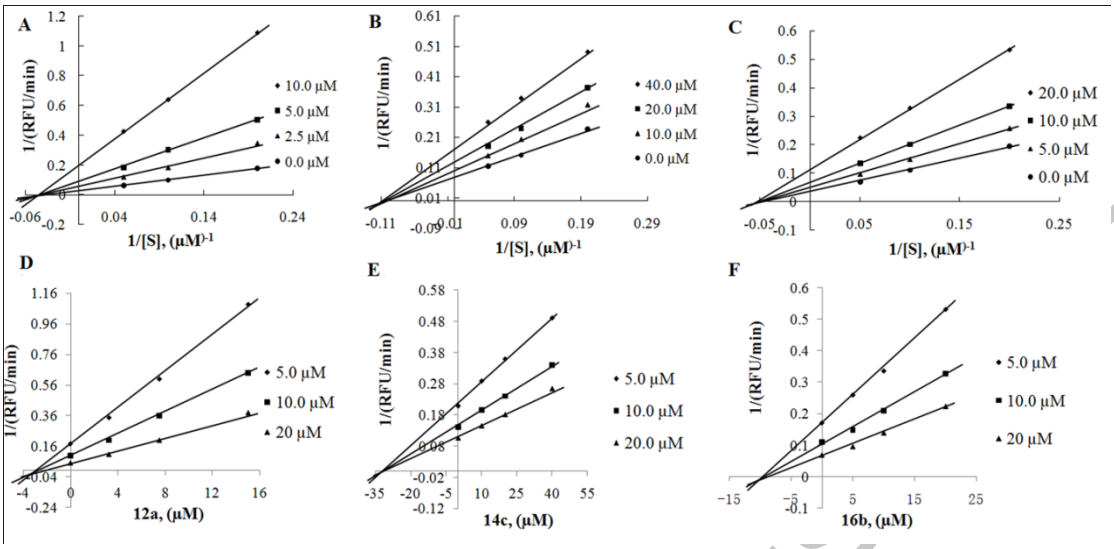


Fig. 2. Graphical determination of the type of inhibition for compound **12a, 14c** and **16c.** (A, B, C): Lineweaver–Burk plot for the inhibition of compound **12a, 14c** and **16c** on NA from influenza virus for the hydrolysis of substrate in the presence of different concentrations. (D, E, F): Dixon plot for inhibition of compound **15d** on NA from influenza virus for the hydrolysis of substrate in the presence of different concentrations of substrate

3.3. Docking analysis

Except for the NA active site and the adjacent several cavities, we did not find other sites that were suitable for the docking analysis in the enzyme surface by using SYBYL-X. Therefore, we made a preliminary binding analysis by docking compound **12a** in the region around the active site of N1 and N2. According to the result, it could be found that there was a relative large cavity, named 150-cavity adjacent to the active site of N1 (**Fig. 3**, A), and the caffeic acid fragment of compound **12a** bound to this pocket. In the N2 enzyme, though the size of the 150-cavity was small and constricted by the closed 150-loop (**Fig. 3**, B), the caffeic acid fragment of compound **12a** could penetrate into the cavity under the closed 150-loop. This kind of interactions would to a great extent increase the binding affinity of compound **12a** with N2 relative to N1. The selective inhibition of this compound against N2 was thus explicable.

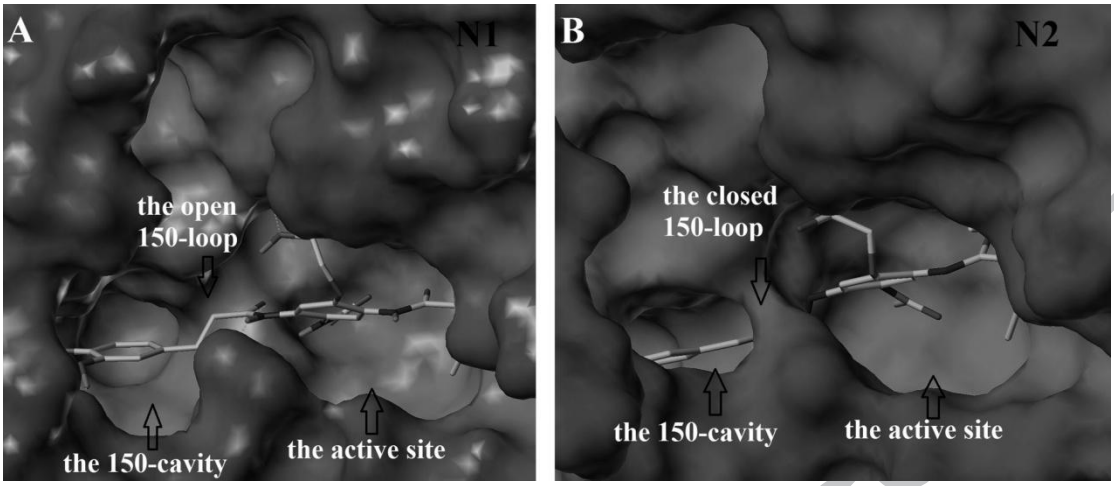


Fig. 3. The docking result of compound 12 with N1 and N2

3.4 In vitro anti-influenza virus activity

Some compounds were further evaluated for their anti-influenza virus activities as well as the cellular toxicity by the CPE reduction assay (**Table 4**).[23] The assay results showed that most of them possessed in vitro anti-influenza virus (H9N2) activity. Among them, compounds **12a**, **12b** and **16d** exhibited good antiviral activities with IC_{50} values of 13.8 μM, 14.5 μM and 13.5 μM, respectively, and were about 2-fold more potent than the lead compound.

Table 4

In vitro anti-influenza virus (A/Chicken/Shandong/LY/08) activities in Chicken embryo fibroblast cells using the CPE reduction assay

Compound	EC_{50} (μM)[a]	CC_{50} (μM)[b]	Compound	EC_{50} (μM)[a]	CC_{50} (μM)[b]
12a	13.8	> 100	16b	25.4	> 100
12b	14.5	> 100	16c	22.9	70.4
14a	56.4	> 100	16d	13.5	89.7
14b	NT	87.6	16e	NT	68.3
14c	NT	78.2	17a	NT	47.2
16a	33.6	> 100	OS[c]	0.24	> 20.0

[a]: EC_{50}: concentration required to achieve 50% protection against virus-induced cytopathic effect

[b]: CC_{50}: concentration of 50% cellular toxicity

[c]: OS: oseltamivir carboxylate, the positive control

NT: not tested.

4. Conclusion

In summary, we designed and synthesized eight series of compounds by structural modifications of compound **2**, a mild influenza NA inhibitor discovered in our previous work. Among them, compounds **12**, **14** and **16** that contained the caffeic acid fragment were much more potent than compounds **6, 7, 9, 10 and 11**. several compounds were closed or superior to the lead, such as compounds **12a**, **12b** and **16c**. Compounds **17** with a saturated bond instead of the double in the caffeic acid fragment exhibited decreased activities. The present study demonstrated that the caffeic acid fragment played an important role in the NA inhibitory activity. Compounds **12a** was the best compound with IC_{50} values of 3.2/10.7 μM and also exhibits good antiviral activity in cells. The docking analysis indicated that the 150-cavity that was different between the two enzymes mainly contributed to the selective inhibition of N2.

H5N1 influenza virus is a great threat to human and many isolated strains of the virus have been resistant to oseltamivir. In this study, we happened to find that compounds **14** could selectively inhibit N1. Though they are not potent, the strategy of structural modifications may be useful to look for more potent and selective NA inhibitors. Caffeic acid itself has a variety of potential pharmacological effects, such as anti-inflammatory, anti-cancer and antiviral activities. In our studies, we have proved that caffeic acid derivatives have good anti-influenza virus activities and NA activities. We hope that they could be new candidates for developing new anti-influenza agents.

5. Experiments

5.1. chemistry

Caffeic acid, amino acid methyl esters, L-ornithine, 2-amino-4-nitro phenol (compound **3**), *N,N'*-bis(*tert*-butoxycarbonyl)thiourea and other compounds not specifically mentioned in the synthetic routes were commercially available. Solvent for anhydrous reaction should be processed before use. ^{1}H-NMR spectra were determined on a Brucker Avance 300 spectrometer or 600 using TMS as an internal standard. The solvents for NMR were DMSO-d_6 (δ 2.5 for 1H), CD_3OD (δ 3.3 for ^{1}H). ESI-MS was determined on API 4000 LC/MS spectrometer (Applied Biosystems, USA) and HRMS analysis was provided by Agilent 6520 Q-TOF LC/MS spectrometer (Agilent, Germany). All reactions were monitored by thin-layer chromatography (TLC) on 25.4×76.2 mm silica gel plates (GF-254). Silica gel used for column chromatography was 200-300 mesh. Melting

points were determined on an electrothermal melting point apparatus and were uncorrected.

5.1.1. The general synthetic procedure for compounds 6a-6c

Compound **5** was prepared according to the reported method from 2-amio-4-nitro phenol, compound **3**.[18] To a mixture of compound **5** (0.46 g, 1.5 mmol) and $NaHCO_3$ (0.50 g, 6 mmol) in 20 mL THF/H_2O (5:1) was added acyl chloride or sulfuryl chloride (1.5 mmol) slowly. The reaction mixture was stirred for 1h at room temperature, then concentrated and extracted with EtOAc. The combined organic extracts were washed with saturated $NaHCO_3$, 1 M aqueous HCl, dried and concentrated.

Without further purification, the obtained crude was dissolved in 15 mL CH_3OH and 5 mL H_2O followed by the addition of NaOH (0.6 g, 15 mmol). The mixture was allowed to be stirred for 15 min at room temperature and then concentrated. The remaining solution was acidated with HCl to a pH of 3 and extracted with EtOAc. The combined extracts were dried over anhydrous $MgSO_4$, and concentrated. Chromatographic purification of the crude product with EtOAc and petroleum afforded the target compounds.

2-(2,4-bis(2-ethylbutanamido)phenoxy)acetic acid (6a)

White solid, yield: 37%. mp = 124-125 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.09 (br, 1H), 9.10 (s, 1H), 8.08 (s, 1H), 8.02 (d, 1H, J = 2.4 Hz), 7.20 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 6.87 (d, 1H, J = 9.0 Hz), 4.66 (s, 2H), 3.29-3.33 (m, 5H), 2.28-2.33 (m, 1H), 1.52-1.59 (m, 2H), 1.41-1.49 (m, 2H), 1.07 (t, 6H, J = 7.2 Hz), 0.87 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{20}H_{31}N_2O_5^+$ [M+H]$^+$ 379.2233; found: 379.2230.

2-(2-(2-ethylbutanamido)-4-(4-methylphenylsulfonamido)phenoxy)acetic acid (6b)

White solid, yield: 42%. mp = 158-160 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.10 (br, 1H), 9.93 (s, 1H), 9.05 (s, 1H), 7.83 (s, 1H), 7.62 (d, 2H, J = 7.8 Hz), 7.32 (d, 2H, J = 7.8 Hz), 6.84 (d, 1H, J = 8.4 Hz), 6.73 (d, 1H, J = 8.4 Hz), 4.65 (s, 2H), 2.33 (s, 3H), 2.28-2.32 (m, 1H), 1.49-1.55 (m, 2H), 1.40-1.45 (m, 2H), 0.84 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{21}H_{27}N_2O_6S^+$ [M+H]$^+$ 435.1590; found: 435.1584.

2-(2-(2-ethylbutanamido)-4-(furan-2-carboxamido)phenoxy)acetic acid (6c)

White solid, yield: 46%. mp = 192-193 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.13 (br, 1H), 10.11 (s, 1H), 9.18 (s, 1H), 8.29 (s, 1H), 7.91 (s, 1H), 7.51 (d, 1H, J = 8.4 Hz), 7.32 (d, 1H, J = 3.0 Hz), 6.97 (d, 1H, J = 8.4 Hz), 6.68 (d, 1H, J = 1.2 Hz), 4.71 (s, 2H), 2.34-2.37 (m, 1H), 1.53-1.60 (m, 2H),

1.44-1.49 (m, 2H), 0.88 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{19}H_{23}N_2O_6^+$ [M+H]$^+$ 375.1556; found: 375.1551.

5.1.2. The synthetic procedure for 2-(4-amino-2-(2-ethylbutanamido)phenoxy)acetic acid trifluoroacetic acid salt (7a)

To a solution of compound **5** (0.31g, 1.0 mmol) and Et_3N (0.29 ml, 2 eq) in 20 ml THF was added $(Boc)_2O$ (0.22g, 1.0 mmol). The reaction mixture was allowed to be stirred for 6h at room temperature, and then concentrated. The obtain residue was hydrolyzed with NaOH by the method above to give Boc-7a. The intermediate was dissolved in CH_2Cl_2 (1.0 mL), followed by the addition of TFA (1.0 mL, 13.0 mmol). The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated and dried under vacuum. The residue was triturated with ether to give a solid product that was collected by filtration. White solid, yield: 47%. mp = 179-181 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.39 (br, 2H), 9.27 (s, 1H), 8.12 (d, 1H, J = 2.4 Hz), 7.04 (d, 1H, J = 9.0 Hz), 6.93 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 4.77 (s, 2H), 2.39-2.47 (m, 1H), 1.39-1.64 (m, 4H), 0.87 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{14}H_{21}N_2O_4^+$ [M+H]$^+$ 281.1501; found: 281.1498.

5.1.3. The synthetic procedure for 2-(2-(2-ethylbutanamido)-4-guanidinophenoxy)acetic acid trifluoroacetic acid salt (7b)

Compound **5** (0.31g, 1.0 mmol), Et_3N (0.29 ml, 2 eq) and $SC(NHBoc)_2$ (0.33 g, 1.2 mmol, 1.2 eq) were dissolved in 15 ml DMF. To the mixture, $HgCl_2$ (0.33 g, 1.2 mmol, 1.2 eq) was slowly added and the reaction mixture was allowed to be stirred at room temperature over night. The suspended reaction solution was diluted with EtOAc and filtered through a pad of Celite. The filtrate was concentrated to give an oil product which was hydrolyzed with NaOH by the method above to give N, N'-diBoc-7b. The following deprotection with TFA afforded compound **7b**. White solid, yield: 38%. mp = 190-192 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.70 (s, 1H), 9.23 (s, 1H), 8.05 (d, 1H, J = 3.0 Hz), 7.41 (br, 4H), 7.04 (d, 1H, J = 8.4 Hz), 6.91 (dd, 1H, J = 8.4 Hz, 3.0 Hz), 4.72 (s, 2H), 2.41-2.43 (m, 1H), 1.53-1.58 (m, 2H), 1.42-1.48 (m, 2H), 0.87 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{15}H_{23}N_4O_4^+$ [M+H]$^+$ 323.1719; found: 323.1714.

5.1.4. The general synthetic procedure for compounds 9a, 9b, 10a and 10b

Compound 5 (0.92 g, 3.0 mmol) was acylated with acetyl chloride or isobutyryl chloride by the reported method. The dried product was dissolved in 5 mL concentrated H_2SO_4 under ice bath. Then, 0.85 mL 65% HNO_3 was slowly added, and the mixture continued to be stirred for less than

10 minutes. The solution was poured into 50 mL water and the suspension was filtrated to give compound 8a and 8b. ethyl 2-(4-acetamido-2-(2-ethylbutanamido)-5-nitrophenoxy)acetate (**8a**): yellow solid, yield: 51%. mp = 132-134 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.17 (s, 1H), 9.43 (s, 1H), 8.45 (s, 1H), 7.61 (s, 1H), 5.00 (s, 2H), 4.19 (q, 2H, J = 7.2 Hz), 2.52-2.57 (m, 1H), 2.03 (s, 3H), 1.53-1.61 (m, 2H), 1.43-1.51 (m, 2H), 1.22 (t, 3H, J = 7.2 Hz), 0.86 (t, 6H, J = 7.2 Hz). ESI-MS m/z: 396.3 $[M+H]^+$.

Compound **8a** or **8b** (2.0 mmol) was dissolved in 20 ml dried CH_3CH_2OH and hydrogenated at room temperature with hydrogen balloon in the presence of 0.15 g of 10% Pd/C for 5h. The mixture was then filtered followed by rotary evaporation of the solvent to give the anilines. According to the synthetic procedure of compounds **7a** and **7b**, compounds **9a**, **9b**, **10a** and **10b** were obtained.

2-(4-acetamido-5-amino-2-(2-ethylbutanamido)phenoxy)acetic acid trifluoroacetic acid salt (9a)

White solid, yield: 35%. mp = 138-140 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.59 (s, 1H), 9.07 (s, 1H), 7.71 (s, 1H), 6.62 (s, 1H), 4.91 (s, 2H), 2.31-2.35 (m, 1H), 2.03 (s, 3H), 1.49-1.56 (m, 2H), 1.40-1.46 (m, 2H), 0.86 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{16}H_{24}N_3O_5^+$ $[M+H]^+$ 338.1716; found: 338.1714.

2-(5-amino-2-(2-ethylbutanamido)-4-isobutyramidophenoxy)acetic acid trifluoroacetic acid salt (9b)

White solid, yield: 37%. mp = 151-152 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.61 (s, 1H), 9.10 (s, 1H), 7.79 (s, 1H), 6.71 (s, 1H), 4.68 (s, 2H), 2.60-2.65 (m, 1H), 2.33-2.37 (m, 1H), 1.50-1.57 (m, 2H), 1.40-1.47 (m, 2H), 1.12 (d, 6H, J = 6.6 Hz), 0.86 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{18}H_{28}N_3O_5^+$ $[M+H]^+$ 366.2029; found: 366.2024.

2-(4-acetamido-2-(2-ethylbutanamido)-5-guanidinophenoxy)acetic acid trifluoroacetic acid salt (10a)

White solid, yield: 30%. mp = 96-98 ºC. ^{1}H NMR (600 MHz, CD_3OD-d_6): δ 8.22 (s, 1H), 7.05 (s, 1H), 4.80 (s, 2H), 2.36-2.47 (m, 1H), 2.17 (s, 2H), 1.50-1.77 (m, 2H), 0.96 (t, 6H, J = 7.5 Hz). HRMS calcd for $C_{17}H_{26}N_5O_5^+$ $[M+H]^+$ 380.1934; found: 380.1929.

2-(2-(2-ethylbutanamido)-5-guanidino-4-isobutyramidophenoxy)acetic acid trifluoroacetic acid salt (10b)

White solid, yield: 37%. mp = 191-193 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.25 (br, 1H), 9.73 (s, 1H), 9.28 (s, 1H), 8.49 (s, 1H), 8.16 (s, 1H), 7.35 (br, 4H), 6.99 (s, 1H), 4.77 (s, 2H), 2.61-2.67 (m, 1H), 2.37-2.41 (m, 1H), 1.52-1.59 (m, 2H), 1.43-1.49 (m, 2H), 1.11 (d, 6H, J = 7.2 Hz), 0.86 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{19}H_{30}N_5O_5^+$ [M+H] $^+$ 408.2247; found: 408.2244.

5.1.5. The general synthetic procedure for compounds 11a-11c

To a solution of compound **5** (0.62 g, 2.0 mmol) and Et_3N (0.84 mL, 6 mmol) in 20 mL dioxane was added triphosgene (0.20 g, 0.67 mmol) in one portion. The whole mixture was stirred at 40 ^{o}C for 15 min, then added into a solution of amine (R_5NH_2, 2.0 mmol) and Et_3N (0.57 mL, 4 mmol) in 15 mL THF. The reaction mixture was stirred for 4h at room temperature, then concentrated and extracted with EtOAc. The combined organic extracts were washed with saturated $NaHCO_3$, 1 M aqueous HCl, dried and concentrated. After hydrolysis and chromatographic purification according to the procedure above, compounds **11a-11c** were obtained.

2-(4-(3-benzylureido)-2-(2-ethylbutanamido)phenoxy)acetic acid (11a)

White solid, yield: 62%. mp = 195-197 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.10 (br, 1H), 9.12 (s, 1H), 8.50 (s, 1H), 7.97 (s, 1H), 7.28-7.34 (m, 4H), 7.20-7.25 (m, 2H), 6.89 (d, 1H, J = 9.0 Hz), 6.44 (t, 1H, J = 6.0 Hz), 4.65 (s, 2H), 4.28 (d, 2H, J = 6.0 Hz), 2.29-2.33 (m, 1H), 1.52-1.59 (m, 2H), 1.42-1.49 (m, 2H), 0.87(t, 6H, J = 7.2 Hz). HRMS calcd for $C_{22}H_{28}N_3O_5^+$ [M+H] $^+$ 414.2029; found: 414.2025.

2.2-(2-(2-ethylbutanamido)-4-(3-(prop-2-yn-1-yl)ureido)phenoxy)acetic acid (11b)

White solid, yield: 67%. mp = 186-187 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.12 (br, 1H), 9.12 (s, 1H), 8.52 (s, 1H), 7.96 (s, 1H), 7.20 (d, 1H, J = 9.0 Hz), 6.90 (d, 1H, J = 9.0 Hz), 6.27 (t, 1H, J = 6.0 Hz), 4.66 (s, 2H), 3.86 (d, 2H, J = 6.0 Hz), 3.09 (s, 1H), 2.29-2.34 (m, 1H), 1.52-1.59 (m, 2H), 1.42-1.49 (m, 2H), 0.87(t, 6H, J = 7.2 Hz). HRMS calcd for $C_{18}H_{24}N_3O_5^+$ [M+H] $^+$ 362.1716; found: 362.1711.

(*R*)-2-(2-(2-ethylbutanamido)-4-(3-(1-hydroxybutan-2-yl)ureido)phenoxy)acetic acid (11c)

White solid, yield: 52%. mp = 117-119 ºC. 1H NMR (600 MHz, DMSO-d_6): δ 13.12 (br, 1H), 9.12 (s, 1H), 8.38 (s, 1H), 7.94 (d, 1H, J = 2.4 Hz), 7.17 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 6.88 (d, 1H, J = 9.0 Hz), 5.82 (d, 1H, J = 7.8 Hz), 4.65 (s, 2H), 3.46-3.52 (m, 1H), 3.42 (dd, 1H, J = 10.8 Hz, 3.6 Hz), 3.32 (dd, 1H, J = 10.8 Hz, 5.4 Hz), 3.86 (d, 2H, J = 6.0 Hz), 2.28-2.34 (m, 1H), 1.50-1.62

(m, 3H), 1.40-1.50 (m, 2H), 1.32-1.38 (m, 1H), 0.80-0.89 (m, 9H). HRMS calcd for $C_{19}H_{30}N_3O_6^+$ $[M+H]^+$ 396.2135; found: 396.2132.

5.1.6. The general synthetic procedure for compounds 12a and 12b

Compounds **8a** and **8b** (2.0 mmol) were reduced with hydrogen in the presence of Pd/C. The acylated caffeic acid, (*E*)-3-(3,4-diacetoxyphenyl)acrylic acid was converted to the acyl chloride [18], and then reacted with the reduction products of compounds **8a** and **8b**. The subsequent hydrolysis and chromatographic isolation afforded compounds **12a** and **12b**.

(*E*)-2-(4-acetamido-5-(3-(3,4-dihydroxyphenyl)acrylamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (12a)

Off-White solid, yield: 47%. mp = 264-266 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.16 (br, 1H), 9.47 (s, 1H), 9.39 (s, 1H), 9.28 (s, 1H), 9.19 (s, 1H), 9.15 (s, 1H), 7.99 (s, 1H), 7.59 (s, 1H), 7.39 (d, 1H, *J* = 15.6 Hz), 7.03 (d, 1H, *J* = 1.8 Hz), 6.91 (dd, 1H, *J* = 8.4 Hz, 1.8 Hz), 6.77 (d, 1H, *J* = 8.4 Hz), 6.64 (d, 1H, *J* = 15.6 Hz), 4.68 (s, 2H), 2.31-2.41 (m, 1H), 2.05 (s, 3H), 1.39-1.61 (m, 4H), 0.87 (d, 6H, *J* = 7.2 Hz). HRMS calcd for $C_{25}H_{30}N_3O_8^+$ $[M+H]^+$ 500.2033; found: 500.2029.

(*E*)-2-(5-(3-(3,4-dihydroxyphenyl)acrylamido)-2-(2-ethylbutanamido)-4-isobutyramidophenoxy)acetic acid (12b)

Off-White solid, yield: 35%. mp = 246-248 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.16 (br, 1H), 9.47 (s, 1H), 9.40 (s, 1H), 9.28 (s, 1H), 9.20 (s, 1H), 9.15 (s, 1H), 8.06 (s, 1H), 7.37-7.43 (m, 3H), 7.02 (d, 1H, *J* = 1.8 Hz), 6.91 (dd, 1H, *J* = 8.1 Hz, 1.8 Hz), 6.77 (d, 1H, *J* = 8.1 Hz), 6.55 (d, 1H, *J* = 15.6 Hz), 4.70 (s, 2H), 2.55-2.65 (m, 1H), 2.32-2.42 (m, 1H), 1.35-1.63 (m, 4H), 1.11 (d, 6H, *J* = 6.9 Hz), 0.87 (d, 6H, *J* = 7.2 Hz). HRMS calcd for $C_{27}H_{34}N_3O_8^+$ $[M+H]^+$ 528.2346; found: 528.2342.

5.1.7. The general synthetic procedure for compounds 14a-14c

To a solution of compound **5** (0.62 g, 2.0 mmol), aldehyde (4.0 mmol, 2eq) and 0.5 ml acetic acid in 30 mL ethanol was added $NaBH_3CN$ (0.50 g, 4eq) slowly. The reaction was stirred at 56 ^{o}C for 6h, and the concentrated. Chromatographic purification gave compounds **13a-13c**. Reaction of compounds **13** and (*E*)-3-(3,4-diacetoxyphenyl)acrylic acid, followed by hydrolysis afforded compounds **14a-14c** based on the methods above.

(*E*)-2-(4-(3-(3,4-dihydroxyphenyl)-N-(2-ethylbutyl)acrylamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (14a)

Light yellow solid, yield: 27%. mp = 108-110 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.10 (br, 1H), 10.02 (br, 1H), 9.39 (br, 1H), 7.99 (d, 1H, J = 2.1 Hz), 7.30 (d, 1H, J = 15.6 Hz), 7.07 (d, 1H, J = 8.4 Hz), 6.92 (dd, 1H, J = 8.4 Hz, 1.8 Hz), 6.65-6.71 (m, 3H), 6.03 (d, 1H, J = 15.6 Hz), 4.59 (s, 2H), 3.66 (d, 2H, J = 6.9 Hz), 2.34-2.42 (m, 1H), 1.31-1.60 (m, 5H), 1.19-1.30 (m, 4H), 0.84 (t, 6H, J = 7.2 Hz), 0.79 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{29}H_{39}N_2O_7^+$ [M+H] $^+$ 527.2757; found: 527.2755.

(*E*)-2-(4-(3-(3,4-dihydroxyphenyl)-N-(thiophen-2-ylmethyl)acrylamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (14b)

Light yellow solid, yield: 35%. mp = 92-94 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.13 (br, 1H), 9.37 (s, 1H), 9.18 (br, 1H), 9.07 (br, 1H), 7.89 (d, 1H, J = 2.1 Hz), 7.42 (d, 1H, J = 15.6 Hz), 7.40 (d, 1H, J = 3.3 Hz), 7.00 (d, 1H, J = 8.7 Hz), 6.86-7.02 (m, 2H), 6.66-6.82 (m, 4H), 6.09 (d, 1H, J = 15.6 Hz), 5.03 (s, 2H), 4.62 (s, 2H), 2.33-2.38 (m, 1H), 1.38-1.59 (m, 4H), 0.84 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{28}H_{31}N_2O_7S^+$ [M+H] $^+$ 539.1852; found: 539.1846.

(*E*)-2-(4-(N-benzyl-3-(3,4-dihydroxyphenyl)acrylamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (14c)

Light yellow solid, yield: 39%. mp = 90-92 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.15 (br, 1H), 9.36 (s, 1H), 9.15 (s, 1H), 9.07 (br, 1H), 7.89 (d, 1H, J = 1.8 Hz), 7.42 (d, 1H, J = 15.6 Hz), 7.20-7.31 (m, 5H), 6.97 (d, 1H, J = 8.7 Hz), 6.82 (dd, 1H, J = 8.7 Hz, 1.8 Hz), 6.66-6.73 (m, 3H), 6.16 (d, 1H, J = 15.6 Hz), 4.93 (s, 2H), 4.76 (s, 2H), 2.31-2.42 (m, 1H), 1.35-1.58 (m, 4H), 0.83 (t, 6H, J = 7.2 Hz). HRMS calcd for $C_{30}H_{33}N_2O_7^+$ [M+H] $^+$ 533.2282; found: 533.2278.

5.1.8. The general synthetic procedure for compounds 16a-16e

Boc-L-amino acid (3.0 mmol) and Et_3N (0.86 ml, 6.0 mmol) were dissolved in 20 mLCH_2Cl_2, and TBTU (1.06 g, 3.3 mmol, 1.1eq) was added in one portion at room temperature. After stirring for 15 min, compound **5** (3.0 mmol) dissolved in 20 mL CH_2Cl_2 was added to the reaction mixture with additional Et_3N (0.43 ml, 3.0 mmol). The reaction was allowed to stir for 4h, then concentrated and extracted with EtOAc. The combined extracts were washed with saturated $NaHCO_3$, 1 M aqueous HCl, dried and concentrated to give intermediates **15a**-**15e**.

Compounds **15** were dissolved in 20 mL 3M EtOAc/HCl and stirred overnight. The solvent was removed under reduced pressure to give the amine products. According to the synthetic method for compounds **12**, compounds **16a**-**16e** were prepared.

(*E*)-2-(4-(2-(3-(3,4-dihydroxyphenyl)acrylamido)acetamido)-2-(2-ethylbutanamido)phenoxy) acetic acid (16a)

White solid, yield: 24%. mp = 123-125 ºC. ^{1}H NMR (600 MHz, DMSO-d_6): δ 13.16 (br, 1H), 9.95 (s, 1H), 9.37 (br, 1H), 9.14-9.15 (br, 2H), 8.30 (t, 1H, J = 6.0 Hz), 8.14 (d, 1H, J = 2.4 Hz), 7.40 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 7.26 (d, 1H, J = 15.6 Hz), 6.97 (d, 1H, J = 1.8 Hz), 6.94 (d, 1H, J = 9.0 Hz), 6.86 (dd, 1H, J = 8.4 Hz, 1.8 Hz), 6.75 (d, 1H, J = 8.4 Hz), 6.46 (d, 1H, J = 15.6 Hz), 4.69 (s, 2H), 3.96 (d, 2H, J = 6.0 Hz) 2.31-2.36 (m, 1H), 1.43-1.59 (m, 4H), 1.11 (d, 6H, J = 6.9 Hz), 0.87 (d, 6H, J = 7.2 Hz). HRMS calcd for $C_{25}H_{30}N_3O_8^+$ [M+H] $^+$ 500.2033; found: 500.2025.

(S,*E*)-2-(4-(2-(3-(3,4-dihydroxyphenyl)acrylamido)propanamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (16b)

White solid, yield: 28%. mp = 134-136 ºC. $[\alpha]_D^{25}$ = +21.4 (c, 1.35, CH_3OH). ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.16 (br, 1H), 9.99 (s, 1H), 9.37 (br, 1H), 9.14-9.15 (br, 2H), 8.26 (d, 1H, J = 7.2 Hz), 8.18 (d, 1H, J = 2.4 Hz), 7.42 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 7.25 (d, 1H, J = 15.6 Hz), 6.93-6.96 (m, 2H), 6.84 (dd, 1H, J = 8.4 Hz, 1.8 Hz), 6.75 (d, 1H, J = 8.4 Hz), 6.47 (d, 1H, J = 15.6 Hz), 4.69 (s, 2H), 4.49-4.54 (m, 1H), 2.26-2.39 (m, 1H), 1.38-1.64 (m, 4H), 1.32 (d, 3H, J = 6.9 Hz), 0.87 (d, 6H, J = 7.2 Hz). HRMS calcd for $C_{26}H_{32}N_3O_8^+$ [M+H] $^+$ 514.2189; found: 514.2184.

(*S*,*E*)-2-(4-(2-(3-(3,4-dihydroxyphenyl)acrylamido)-3-methylbutanamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (16c)

Light yellow solid, yield: 39%. mp = 138-140 ºC. $[\alpha]_D^{25}$ = +60.4 (c, 0.5, CH_3OH). ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.06 (br, 1H), 10.061 (s, 1H), 9.34 (s, 1H), 9.12 (s, 1H), 9.11 (s, 1H), 8.19 (d, 1H, J = 2.4 Hz), 8.11 (d, 1H, J = 8.7 Hz), 7.43 (dd, 1H, J = 9.0 Hz, 2.4 Hz), 7.25 (d, 1H, J = 15.6 Hz), 6.92-6.96 (m, 2H), 6.84 (dd, 1H, J = 8.1 Hz, 1.8 Hz), 6.74 (d, 1H, J = 8.1 Hz), 6.59 (d, 1H, J = 15.6 Hz), 4.69 (s, 2H), 4.36-4.42 (m, 1H), 2.26-2.39 (m, 1H), 1.97-2.10 (m, 1H), 1.38-1.65 (m, 4H), 0.92 (d, 3H, J = 6.6 Hz), 0.87 (d, 6H, J = 7.2 Hz). HRMS calcd for $C_{28}H_{36}N_3O_8^+$ [M+H] $^+$ 542.2502; found: 542.2490.

(*S*,*E*)-2-(4-(2-(3-(3,4-dihydroxyphenyl)acrylamido)-4-methylpentanamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (16d)

Light yellow, yield: 33%. mp = 139-142 ºC. $[\alpha]_D^{25}$ = +36.2 (c, 0.77, CH_3OH). ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.09 (br, 1H), 10.06 (s, 1H), 9.35 (br, 1H), 9.12 (br, 2H), 8.19 (d, 1H, J = 8.1

Hz), 8.17 (d, 1H, *J* = 2.4 Hz), 7.45 (dd, 1H, *J* = 8.7 Hz, 2.4 Hz), 7.25 (d, 1H, *J* = 15.6 Hz), 6.92-6.96 (m, 2H), 6.84 (dd, 1H, *J* = 8.4 Hz, 1.8 Hz), 6.75 (d, 1H, *J* = 8.4 Hz), 6.48 (d, 1H, *J* = 15.6 Hz), 4.79 (s, 2H), 4.53-4.61 (m, 1H), 2.29-2.39 (m, 1H), 1.38-1.67 (m, 7H), 0.85-0.95 (m, 12H). HRMS calcd for $C_{29}H_{38}N_3O_8^+$ [M+H]$^+$ 556.2659; found: 556.2652.

(*S*,*E*)-2-(4-(2-(3-(3,4-dihydroxyphenyl)acrylamido)-3-phenylpropanamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (16e)

Light yellow solid, yield: 28%. mp = 154-156 ºC. $[\alpha]_D^{25}$ = +24.8 (c, 1.15, CH_3OH). ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.12 (s, 1H), 9.54 (br, 1H), 9.35 (br, 1H), 9.15 (br, 1H), 8.35 (d, 1H, *J* = 8.1 Hz), 8.16 (d, 1H, *J* = 2.4 Hz), 7.43 (dd, 1H, *J* = 8.4 Hz, 2.4 Hz), 7.15-7.33 (m, 6H), 6.93-6.96 (m, 2H), 6.82 (dd, 1H, *J* = 8.4 Hz, 1.8 Hz), 6.73 (d, 1H, *J* = 8.4 Hz), 6.42 (d, 1H, *J* = 15.6 Hz), 4.72-4.81 (m, 1H), 4.61 (s, 2H), 3.07 (dd, 1H, *J* = 13.5 Hz, 4.8 Hz), 2.88 (dd, 1H, *J* = 13.5 Hz, 9.6 Hz), 2.29-2.39 (m, 1H), 1.41-1.63 (m, 4H), 0.88 (d, 6H, *J* = 7.2 Hz). HRMS calcd for $C_{32}H_{36}N_3O_8^+$ [M+H]$^+$ 590.2502; found: 590.2501.

5.1.9. The general synthetic procedure for compounds 17a and 17b

Compounds **12a** or **12b** (0.2 mmol) was dissolved in 5 ml dried CH_3CH_2OH and hydrogenated at room temperature with hydrogen balloon in the presence of 50 mg of 10% Pd/C over night. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated, and then dried to give compounds **17a** and **17b**.

2-(4-acetamido-5-(3-(3,4-dihydroxyphenyl)propanamido)-2-(2-ethylbutanamido)phenoxy)acetic acid (17a)

Off-White solid, yield: 89%. mp = 198-200 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.14 (br, 1H), 9.24 (s, 1H), 9.18 (s, 1H), 9.16 (s, 1H), 8.62 (br, 1H), 7.99 (s, 1H) 7.29 (s, 1H), 6.61-6.64 (m, 2H), 6.47 (dd, 1H, *J* = 8.1 Hz, 1.8 Hz), 4.64 (s, 2H), 2.69-2.74 (m, 2H), 2.50-2.56 (m, 2H), 2.29-2.37 (m, 1H), 2.00 (s, 3H), 1.35-1.63 (m, 4H), 0.86 (d, 6H, *J* = 7.2 Hz). HRMS calcd for $C_{25}H_{32}N_3O_8^+$ [M+H]$^+$ 502.2189; found: 502.2181.

2-(5-(3-(3,4-dihydroxyphenyl)propanamido)-2-(2-ethylbutanamido)-4-isobutyramidophenoxy)acetic acid (17b)

Off-White solid, yield: 91%. mp = 218-220 ºC. ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.16 (br, 1H), 9.16-9.20 (m, 3H), 8.62 (br, 1H), 8.05 (s, 1H) 7.17 (s, 1H), 6.60-6.64 (m, 2H), 6.46 (dd, 1H, *J* = 8.1 Hz, 1.8 Hz), 4.65 (s, 2H), 2.71 (t, 2H, *J* = 7.5 Hz), 2.51-2.58 (m, 3H, merged in DMSO),

2.29-2.39 (m, 1H), 1.37-1.63 (m, 4H), 1.09 (d, 6H, *J* = 6.9 Hz), 0.86 (d, 6H, *J* = 7.2 Hz). HRMS calcd for $C_{27}H_{36}N_3O_8^+$ $[M+H]^+$ 530.2502; found: 530.2500.

5.2. Influenza A (H9N2 and H5N1) Neuraminidase Inhibition Assay

The NA inhibition assay was performed according to a standard method[13]. Influenza virus suspensions (H9N2 virus: A/Chicken/Shandong/LY/08 and H5N1 virus: A/duck/China/QJ/01) obtained from the allantoic fluid of embryonated chicken eggs were inactivated and used as the enzyme on the basis that NA was present on the viral surface. The substrate, 2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid sodium salt hydrate (4-MU-NANA) (Sigma, M8639) was cleaved by NA to yield a fluorescent product which can be quantified. Compounds tested were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer (32.5 mM 2-(N-morpholino)-ethanesulfonic acid, 4 mM $CaCl_2$, pH 6.5) if not dissolved in MES directly. In a 96-well plate, 10 μL of the diluted virus supernatant, 70 μL of MES buffer and 10 μL of compounds at different concentration were added successively, and then incubated for 5 min at 37 °C. The reaction was started by the addition of the substrate. After incubation for 30 to 60 min, the reaction was terminated by adding 150 μL 0.2 M glycine-NaOH (pH 10.2) or 0.034 M NaOH in water. Fluorescence was recorded (excitation at 360 nm and emission at 450 nm), and substrate blanks were subtracted from the sample readings. The 50% inhibitory concentration (IC_{50}) was calculated by plotting percent inhibition of neuraminidase activity versus the inhibitor concentration.

5.3. In vitro anti-influenza virus assay and cytotoxicity assay

In vitro anti-*influenza* virus assay and cytotoxicity assay were determined by MTS assay with slight modification[23]. Chicken embryo fibroblast cells were grown in 96-well plates for 24h at 37 ^oC. The compounds dissolved in DMSO were serially diluted by two-fold in DMEM (containing 1% serum). 50 μL of compound at different concentrations was mixed with an equal volume of 1 $TCID_{50}$ (50% tissue culture infective dose) of influenza virus (H9N2 virus: A/Chicken/Shandong/LY/08). After 1 hour incubation at 37 °C, the compound-virus preparation was added to the cells. At 2 days post infection, 20 μL MTS and PMS mixture solution was added per well and the plate was incubated for 3h away from light. The optical density was measured at OD490nm to determine the antiviral activity, expressed as the compound concentration producing 50% inhibition of virus-induced CPE (EC_{50}), as well as the cytotoxicity of the compounds,

expressed as the compound concentration causing 50% cellular toxicity (CC_{50}).

Acknowledgment

This work was supported by the 12th Five Years National Science and Technology Major Project (2011ZX09401-015) and Doctoral Fund of Ministry of Education of China (No 20110131110037). We greatly thank Bing Huang and Kexiang Yu for their work in the biological activity assays.

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Graphical abstract

Compounds containing the caffeic acid fragment exhibited better NA inhibitory activities and selectively inhibited N1 or N2.