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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Discovery of a series of novel compounds with moderate anti-avian H5N1 influenza virus activity in chick embryo

Yuanchao Xie · Bing Huang · Kexiang Yu · Fangyuan Shi · Wenfang Xu

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Abstract Enlightened by some flavonoid compounds, which had been found as influenza neuraminidase inhibitors, we designed and synthesized a series of novel compounds containing different amino acid fragments. We also reported a simple synthetic route from oseltamivir to prepare its active form which was used as the positive control. The result of enzyme inhibition assay indicated that all the designed compounds displayed weak inhibitory activity against neuraminidase. However, they showed moderate anti-avian H5N1 influenza virus activity in chick embryo. Besides interfering with the function of neuraminidase, these compounds seemed to inhibit the replication of influenza virus by some other mechanism which deserved deep study.

Keywords Influenza · Neuraminidase · Flavonoid · Amino acid

Introduction

Influenza is a major health problem and can cause serious economic loss. Recently, the worldwide spread of the highly pathogenic avian influenza A (H5N1) virus in birds and the increasing cases of bird-to-human transmission get heightened concern. The viral surface protein, neuraminidase (NA), is an important target for anti-influenza drugs design. Oseltamivir, one kind of NA inhibitors, is currently the first line defense drug against influenza. However, owing to the constant emergence of drug-resistant virus strains, the anti-influenza situation becomes more critical. It is still necessary to find novel effective anti-influenza drugs.

Structure-based drug design has greatly contributed the development of NA inhibitors, leading to three anti-influenza drugs: zanamivir, oseltamivir, and peramivir (Liu *et al.*, 2007). Owing to low bioavailability, zanamivir and peramivir cannot be administered orally, which is not convenient and economical for patient care. In contrast, oseltamivir has high bioavailability, but its wide use has inevitably brought about drug-resistant viral strains (Storms *et al.*, 2012). Therefore, discovering some other effective NA inhibitors with good pharmaceutical properties is the first concern.

According to recent studies, the design of NA inhibitors has been in a difficult position after reaching a peak and it seems hard to discover novel ones which could be better than oseltamivir simply based on the NA active site. Recently, many natural products, the structures of which were very different from those classical NA inhibitors, have been reported with moderate activity against NA. Among them, flavonoids are the largest class (Liu *et al.*, 2008; Upadhyay *et al.*, 2011) (Fig. 1). Unlike the classical ones, most of these compounds acted as noncompetitive NA inhibitors, indicating that they might inhibit the NA activity by binding on some other sites of the enzyme, instead of the catalytic center.

In order to discover some kind of novel NA inhibitors distinct from the classical ones, in this study, we constructed two new skeletons incorporating several structural

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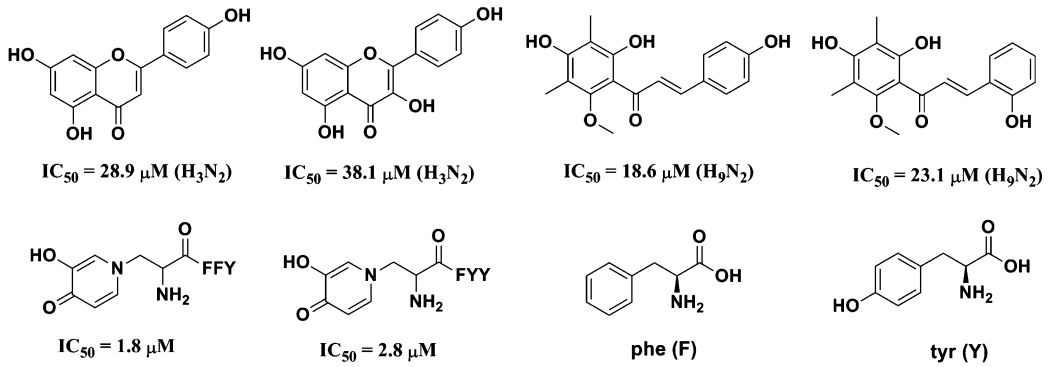
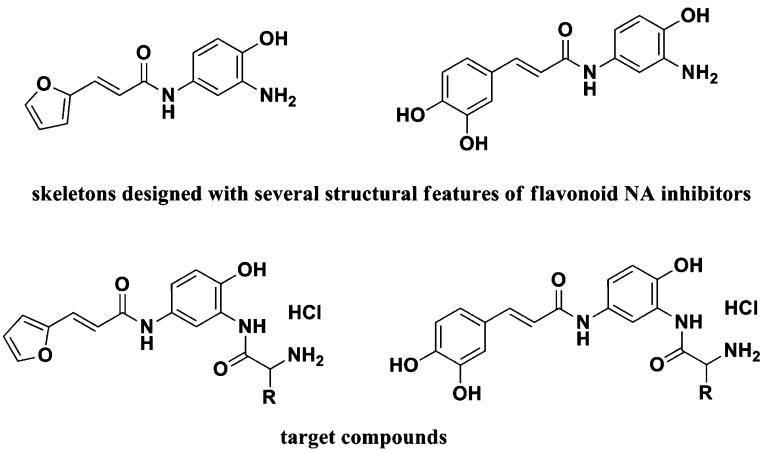


Fig. 1 The flavonoid compounds and mimosine tetrapeptides reported as NA inhibitors

Fig. 2 The two skeletons and target compounds



features which were necessary for NA inhibitory activity of some flavonoid NA inhibitors (Liu *et al.*, 2008). Considering that the enzyme's active site contained some polar residues (von Itzstein, 2007), such as Glu 119, Glu 227, and Glu 276 which preferred to bind with basic groups, and peptides had potential to inhibit NA, we introduced different amino acids into the skeletons to design and synthesize a series of novel compounds (Fig. 2).

Results and discussion

Chemistry

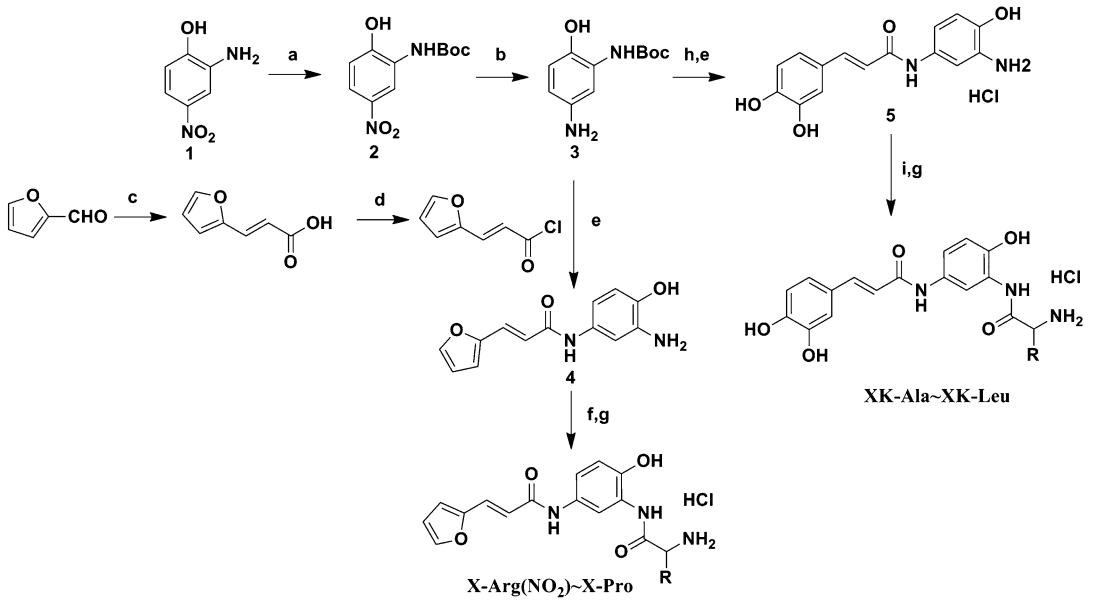
The two skeletons and target compounds were synthesized according to Scheme 1. The amino group of the starting material, 2-amino-4-nitrophenol was first protected with Boc, followed by nitro group reduction with hydrogen to give the important intermediate, compound 3. (*E*)-3-(furan-2-yl)acrylic acid used to construct the skeleton 4 was obtained by the Knoevenagel condensation reaction. Caffeic acid was converted into acyl chloride and reacted with compound 3. After removing the Boc-protecting group, the skeleton 5 was obtained. The target compounds were synthesized by condensation of compound 4 or 5 with Boc-protected amino acids with DCC and deprotection with HCl/EtOAc.

Oseltamivir carboxylate (GS4071) was the active form of oseltamivir and usually used as the positive control for biologic evaluation. This compound was commercially expensive and difficult to obtain by traditional methods. We reported a simple route with high yield for preparing GS4071 hydrochloride from oseltamivir via a three-step sequence: protection of the amino group with Boc, hydrolysis of the carboxylic acid ester mediated by NaOH, and deprotection with HCl/EtOAc (Scheme 2).

Pharmacology

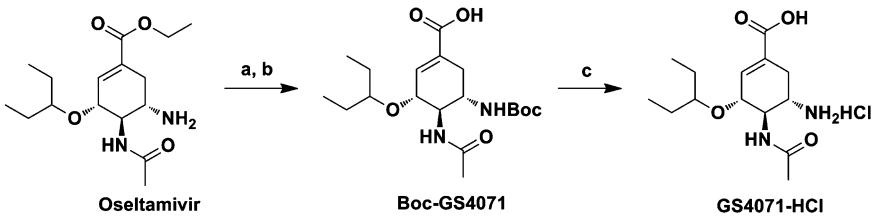
The target compounds were tested for their ability to inhibit NA, Table 1. The enzyme inhibition result showed that they displayed NA inhibitory activity with IC_{50} values from 57.8 to 225 μM, which was not good enough compared with those flavonoid NA inhibitors or mimic peptides.

In general, all the compounds demonstrated a clear structure–activity relationship surrounding the R_1 and R_2 groups. It could be found that the compounds containing a 3,4-dihydroxyphenyl group had better activity relative to



Reagents and Conditions: (a) $(Boc)_2O$, THF+H_2O, 55℃; (b) Pt/C, H_2, CH_3OH, rt; (c) malonic acid, pyridine, reflux; (d) oxalyl chloride, DMF, anhydrous THF, 0℃; (e) $NaHCO_3$, THF+H_2O, rt; (f) Boc-L-amino acid, DCC, rt; (g) HCl/ethyl acetate, rt; (h) caffeic acid, oxalyl chloride, DMF, anhydrous THF, 0℃; (i) Boc-L-amino acid, Et_3N, DCC, rt.

Scheme 1 The synthetic route of target compounds



Reagents and Conditions: (a) $(Boc)_2O$, THF+H_2O, $NaHCO_3$, rt; (b) NaOH, 4 hours, 1M HCl; (c) HCl-ethyl acetate, rt.

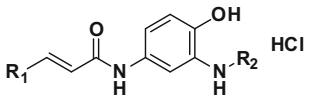
Scheme 2 The synthetic route of GS4071 hydrochloride

the ones with a furyl group at R_1 position, such as compounds **X-Val** and **XK-Val** and compounds **X-leu** and **XK-Leu**. The introduced amino acid fragments at R_2 position also had influence on the activity. From compounds **X-lys**, **X-Arg(NO_2)**, **X-γ-GABA,** and **X-β-Ala**, it was noted that their inhibitory activity was decreased with length increase of the side chains. This trend could also be observed on compound **X-Ala**, **X-thr**, and **X-leu**. However, compound **X-Gly** with the shortest side chain did not present higher inhibitory activity than compound **X-β-Ala**, suggesting that a suitable length was important. The R_2 fragment bearing large aromatic rings or rigid five-membered rings was unfavorable to NA inhibitory activity, which could be easily identified by compounds **X-Trp**, **X-Phe**, **X-Hyp,** and **X-Pro**.

XK-Val exhibited the best inhibitory activity in the series against N2 (H9N2) and N1 (H5N1) NAs. The docking analysis (Fig. 3) performed by means of SYBYL-X 1.1 indicated that there was at least one big cavity adjacent to the enzyme's active center bound by oseltamivir carboxylate. **XK-Val** could occupy both the active site and an adjacent small cavity, but could not penetrate deeply into the enzyme compared with oseltamivir carboxylate. To a certain extent, this may decrease the binding affinity and led to low inhibitory activity. From the docking result, it was also found that the region for the amino acid fragment was very limited and could not be tolerated by large groups, which was consistent with the enzyme inhibition result that the compounds with bulky groups or long side chains at R_2 position were less potent.

The compounds designed in this study exhibited weak NA inhibitory activity and could be further modified. In fact, there were some other cavities close to the active site according to recent studies as well as our docking result

Table 1 The structures and IC_{50} values of the target compounds



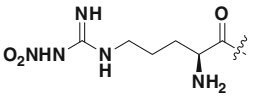
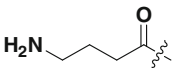
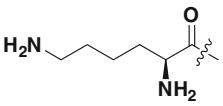
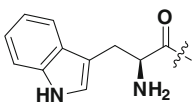
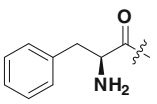
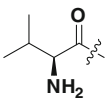
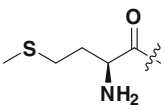
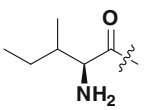
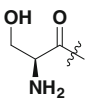
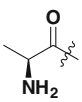
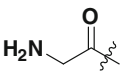
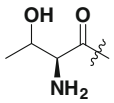
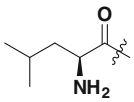
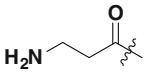
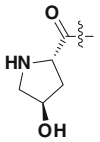
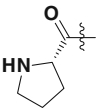
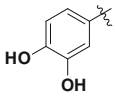
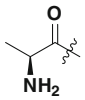
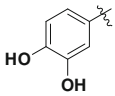
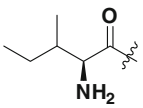
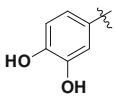
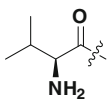
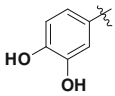
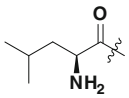
Compounds	R_1	R_2	H9N2 IC_{50} (μM)	H5N1 IC_{50} (μM)
X-Arg(NO2)			193	192
X-γ-GABA			167	132
X-lys			215	225
X-Trp			194	189
X-Phe			143	130
X-Val			165	175
X-Met			144	121
X-Ile			119	103
X-Ser			158	143
X-Ala			91.7	110
X-Gly			175	139

Table 1 continued

Compounds	R_1	R_2	H9N2 IC_{50} (μM)	H5N1 IC_{50} (μM)
X-Thr			138	114
X-Leu			154	135
X-β-Ala			140	113
X-Hyp			200	187
X-Pro			179	148
XK-Ala			118	108
XK-Ile			111	109
XK-Val			85.3	57.8
XK-Leu			105	113
Oseltamivir carboxylate hydrochloride			0.0017	0.012

(Russell *et al.*, 2006). It would probably be better to design NA inhibitors considering all the potential binding sites. Mimosine tetrapeptides were discovered as NA inhibitors with IC_{50} values in the low micromolar range (Upadhyay *et al.*, 2011). Therefore, introducing dipeptides or tripeptides on the skeletons may provide improved activity; besides, finding some new active fragments to construct highly potent NA inhibitors could be feasible by applying the fragment-based drug design strategy.

The chick embryo model had been reported suitably to screen NA inhibitors for their antiviral efficacy against influenza A virus (Sauerbrei *et al.*, 2006). In this study, we further evaluated the preliminary anti-influenza virus activity of the target compounds in chick embryo, Table 2. In previous studies, the efficacy of antiviral compounds was determined on the basis of the survival rate. Differently, we determined the minimal dosage of compounds for keeping chick embryos alive and

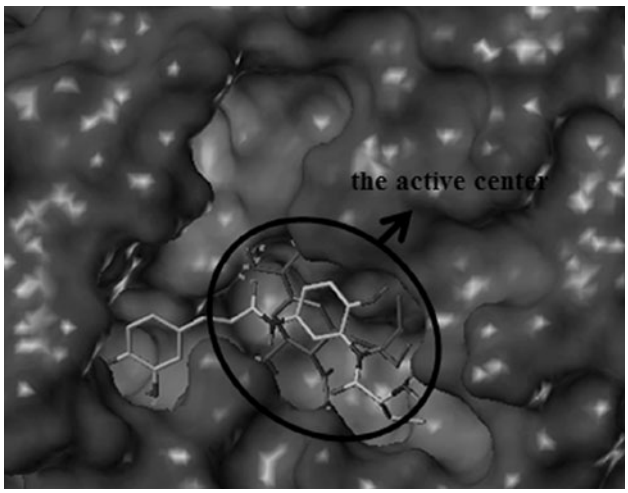


Fig. 3 The docking result of **XK-Val** and oseltamivir carboxylate (the smaller compound) with the active site of NA (PDB ID:2HU0)

Table 2 Antiviral activity of the target compounds in chick embryo

Compound	Effectively inhibition of influenza virus (mg or μmol/each chick embryo)*
X-Arg(NO_2)	2.41 mg (5 μM)
X-γ-GABA	1.83 mg (5 μM)
X-lys	2.04 mg (5 μM)
X-Trp	1.16 mg (2.5 μM)
X-Phe	2.14 mg (5 μM)
X-Val	0.94 mg (2.5 μM)
X-Met	2.06 mg (5 μM)
X-Ile	0.983 mg (2.5 μM)
X-Ser	1.83 mg (5 μM)
X-Ala	1.75 mg (5 μM)
Oseltamivir carboxylate hydrochloride	0.401–0.200 mg (1.25–0.625 μM)
X-Gly	1.68 mg (5 μM)
X-Thr	1.91 mg (5 μM)
X-Leu	1.97 mg (5 μM)
X-β-Ala	1.75 mg (5 μM)
X-Hyp	>1.97 mg (5 μM)
X-Pro	1.89 mg (5 μM)
XK-Ala	1.83 mg (5 μM)
XK-Ile	1.02 mg (2.5 μM)
XK-Val	0.98 mg (2.5 μM)
XK-Leu	1.02 mg (2.5 μM)
Oseltamivir phosphate	0.513–0.257 mg (1.25–0.625 μM)

* The minimal dosage of compounds for keeping chick embryos alive and effectively inhibiting the replication of avian H5N1 influenza virus with a low hemagglutinin (HA) titer (0 log2) in allantoic fluid by hemagglutination assay. The experiment was repeated three times

effectively inhibiting the replication of avian H5N1 influenza virus. From table 2, it could be found that most of the designed compounds had effective activity against avian H5N1 influenza virus and the inhibitory activity was more evident in higher concentration (some data not showed). In general, the four compounds containing 3,4-dihydroxyphenyl group were a little more potent than the other ones. Compared with oseltamivir or its carboxylate, most of the designed compounds were only 4–8-fold less potent, which was not consistent with the result of enzyme inhibition assay. It seemed that these compounds could inhibit the replication of influenza virus mainly by some other mechanism.

Recently, besides the M2 protein, hemagglutinin (HA) and NA, novel targets, such as the nonstructural NS1A protein, the nucleoprotein, and the viral polymerase, have also been identified suitably for the development of new antivirals (Krug and Aramini, 2009). These compounds discovered with weak NA inhibitory activity, but displaying effective anti-H5N1 influenza virus activity in chick embryo deserved deep studies.

Conclusion

In this study, we designed and synthesized a series of novel NA inhibitors. Besides, we also reported a simple synthetic route with high yield from oseltamivir to prepare its active form which was used as the positive control for biologic evaluation.

All the compounds exhibited weak NA inhibitory activity. Among them, compound **XK-Val** was the best with IC_{50} values of 85.3 and 57.8 μM against N2 (H9N2) and N1 (H5N1) NA, respectively. Interestingly, most of the compounds displayed moderate anti-H5N1 influenza virus activity in chick embryo, indicating that they may inhibit the replication of influenza virus by some other mechanism.

NA was still an important target for anti-influenza drugs design. Considering the current ani-influenza situation, it was still necessary to discover novel NA inhibitors. In this study though these compounds designed did not show high inhibitory activity against NA; they really represented a new kind of NA inhibitors and could be further modified.

Experimental

Chemistry

Compound **1**, Boc-protected amino acids, oseltamivir phosphate, and other compounds not specified 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 ^{1}H),

CD_3OD (δ 3.3 for ^1H), and H_2O (δ 4.7 for ^1H). HRMS analysis was provided using 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.

*tert-Butyl 2-hydroxy-5-nitrophenylcarbamate (**2**)*

2-amino-4-nitrophenol (50 mmol, 7.7 g) added in 200 ml THF + H_2O (3:1) was stirred at 75 °C until the material was dissolved. To this solution, *Di-tert*-butyl pyrocarbonate (150 mmol, 32.7 g) was portionwise added in at least 2 h. The reaction mixture was continuously stirred at this temperature for 5 h. After evaporation of the solvent, the obtained residue was extracted with ethyl acetate and the combined extracts were washed with saturated $NaHCO_3$, 1 M aqueous HCl, brine, dried over anhydrous $MgSO_4$, and concentrated. The crude product was recrystallized from ethyl acetate and petroleum ether to give a yellow solid in 50.4 % yield. Mp = 138–140 °C. ^1H NMR (600 MHz, DMSO-d_6): δ 11.56 (s, 1H), 8.64 (s, 1H), 8.21 (s, 1H), 7.86 (dd, 1H, J = 9.0 Hz, J = 3.0 Hz), 6.98 (d, 1H, J = 8.4 Hz), 1.48 (s, 9H). HRMS calcd for $C_{11}H_{13}N_2O_5$ $(M-H)^-$ 253.0824; found: 253.0825.

*tert-Butyl 5-amino-2-hydroxyphenylcarbamate (**3**)*

Compound 2 (2.54 g, 10 mmol) was added in 100 ml dried CH_3OH and hydrogenated at room temperature using hydrogen balloon in the presence of 200 mg of 10 % Pd/C. The reaction was monitored by TLC until the starting material was no longer detected. Evaporation of CH_3OH could afford a light gray solid (1.7 g, yield 74 %). Mp = 162–164 °C. ^1H NMR (600 MHz, DMSO-d_6): δ 8.59 (s, 1H), 7.57 (s, 1H), 7.00 (s, 1H), 6.51 (d, 1H, J = 8.4 Hz), 6.11 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz), 4.47 (s, 2H), 1.45 (s, 9H). HRMS calcd for $C_{11}H_{17}N_2O_3$ $(M+H)^+$ 225.1239; found: 225.1223.

The synthetic procedure of (E)-3-(furan-2-yl)acrylic acid (Jiang et al., 2009)

Furan-2-carbaldehyde (1.92 g, 20 mmol) and malonic acid (5.2 g, 50 mmol) were added in 50 ml pyridine and then heated to reflux at 120 °C. The reaction was monitored by TLC until the starting material was no longer detected. Most of the solvent (pyridine) was removed under vacuum. Then, 20 % NaOH solution 80 ml was added and the residual pyridine was extracted with ethyl acetate. Concentrated HCl was used to adjust the pH to ~ 5 to give the gray solid product. Yield: 67.2 %, mp = 138–139 °C. ^1H NMR (600 MHz, DMSO-d_6): δ 12.42 (br, 1H), 7. 84 (s, 1H), 7.40 (d, 1H, J = 15.6 Hz), 6.93 (d, 1H, J = 2.4 Hz), 6.62–6.63 (m, 1H), 6.15 (d, 1H, J = 15.6 Hz). HRMS calcd for $C_7H_5O_3$ $(M-H)^-$ 137.0239; found: 137.0246.

*(E)-N-(3-amino-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide (**4**)*

To a solution of (*E*)-3-(furan-2-yl)acrylic acid (0.83 g, 6 mmol) and catalytic amount of DMF in 20 ml anhydrous THF was added oxalyl chloride (1.14 ml, 12 mmol) under ice-bath. After the complete conversion of the acid to its acyl chloride, the mixture was concentrated to half its volume by rotary evaporation. The concentrate was added dropwise to a mixture of compound 3 (1.34 g, 6 mmol) and sodium bicarbonate (1.5 g, 18 mmol) in 60 ml THF/H_2O (10:1). After 4 h of stirring at rt, the reaction was complete monitored by TLC. Remove the volatile solvent in vacuum to leave a crude mixture which was dissolved with ethyl acetate and washed with saturated $NaHCO_3$, 1 M aqueous HCl, and brine, and then dried with anhydrous $MgSO_4$. After concentrating to small volume, an off-white solid could be obtained by scratching of the flask with a glass rod.

Without further purification, the solid was added in 20 ml ethyl acetate HCl solution and stirred at rt overnight to give a gray solid. The solid was neutralized with saturated sodium bicarbonate solution and extracted with ethyl acetate. Evaporation of the solvent could afford a gray solid (0.76 g, yield 53.0 %). Mp = 195–196 °C. ^1H NMR (600 MHz, DMSO-d_6): δ 9.73 (s, 1H), 8.80 (s, 1H), 7.79 (s, 1H), 7.29 (d, 1H, J = 15.6 Hz), 7.00 (d, 1H, J = 2.4 Hz), 6.79 (d, 1H, J = 3.0 Hz), 6.72 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz), 6.59–6.62 (m, 2H), 6.55 (d, 1H, J = 8.4 Hz), 4.58 (s, 2H). IR: (KBr) cm^{-1}: 3450, 3368, 3209, 3169, 3061, 1621, 1550, 1453. HRMS calcd for $C_{13}H_{13}N_2O_3$ $(M+H)^+$ 245.0926; found: 245.0930.

*(E)-N-(3-amino-4-hydroxyphenyl)-3-(3,4-dihydroxyphenyl)acrylamide hydrochloride (**5**)*

According to the synthetic route of compound 4, compound 5 prepared from caffeic acid (1.08 g, 6 mmol) and compound 3 (1.34 g, 6 mmol) was obtained as a yellow solid in 48 % yield. Mp = 228–230 °C (decomposed). ^1H NMR (300 MHz, CD_3OD): δ 8.02 (d, 1H, J = 2.4 Hz), 7.53 (d, 1H, J = 15.6 Hz), 7.36 (dd, 1H, J = 8.7 Hz, J = 2.4 Hz), 7.08 (d, 1H, J = 2.1 Hz), 6.99 (d, 1H, J = 8.7 Hz), 6.97 (dd, 1H, J = 8.1 Hz, J = 2.1 Hz), 6.80 (d, 1H, J = 8.1 Hz), 6.56 (d, 1H, J = 15.6 Hz). HRMS calcd for $C_{15}H_{15}N_2O_4$ $(M+H)^+$ 287.1032; found: 287.1024.

The general procedure of compound ***X-Arg(NO$_2$)*** *~* ***X-Pro*** *(Sheehan and Hess, 1955)*

To a solution of compound 4 (0.34 g, 1.5 mmol) and Boc-L-amino acid (1.6 mmol) in 50 ml anhydrous THF, DCC was added (0.46 g, 2.25 mmol). The reaction mixture was stirred at room temperature for 1 h. The insoluble *N*,*N*′-dicyclohexylurea was removed by filtration and the solvent was removed under reduced pressure to give a residue which was subsequently dissolved in ethyl acetate and washed with saturated $NaHCO_3$, 1 M aqueous HCl, and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated. Chromatographic purification of the crude material with EtOAc and petroleum afforded the Boc-protected target compounds which were added to 20 ml HCl/EtOAc and stirred overnight. The precipitate was collected through filtration and washed with EtOAc to give the target compounds in 21–55 % yield.

*(S,E)-2-amino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-5-(3-nitroguanidino)pentanamide hydrochloride (****X-arg(NO$_2$)****)*

Light yellow solid, decomposed ~160 °C. ^{1}H NMR (300 MHz, D_2O): δ 7.64 (d, 1H, J = 2.4 Hz), 7.46 (d, 1H, J = 1.8 Hz), 7.24 (d, 1H, J = 15.6 Hz), 7.07 (dd, 1H, J = 8.7 Hz, J = 2.7 Hz), 6.83 (d, 1H, J = 8.7 Hz), 6.62 (d, 1H, J = 3.6 Hz), 6.43 (dd, 1H, J = 3.6 Hz, J = 1.8 Hz), 6.36 (d, 1H, J = 15.6 Hz), 4.17 (*t*, 1H, J = 6.3 Hz), 3.19–3.22 (m, 2H), 1.93–1.96 (m, 2H), 1.56–1.58 (m, 2H). HRMS calcd for $C_{19}H_{24}N_7O_6$ $(M+H)^+$ 446.1788; found: 446.1809.

*(E)-4-amino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)butanamide hydrochloride (****X-γ-GABA****)*

Light yellow solid, mp = 231–233 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.10 (d, 1H, J = 2.4 Hz), 7.61 (s, 1H), 7.40 (d, 1H, J = 15.6 Hz), 7.26 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz), 6.83 (d, 1H, J = 8.4 Hz), 6.70 (d, 1H, J = 3.6 Hz), 6.60 (d, 1H, J = 15.6 Hz), 6.54 (dd, 1H, J = 3.6 Hz, J = 1.8 Hz), 3.04 (*t*, 2H, J = 7.2 Hz), 2.62 (*t*, 2H, J = 7.2 Hz), 2.02 (m, 2H). IR: (KBr) cm^{-1}: 3324, 3158, 3159, 3051, 1674, 1641, 1532. HRMS calcd for $C_{17}H_{20}N_3O_4$ $[M+H]^+$ 330.1454; found: 330.1469.

*(S,E)-2,6-diamino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)hexanamide hydrochloride (****X-Lys****)*

Yellow solid, decomposed ~189 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.75 (d, 1H, J = 1.8 Hz), 7.59 (s, 1H), 7.39 (d, 1H, J = 15.6 Hz), 7.19 (d, 1H, J = 9.0 Hz), 6.98(d, 1H, J = 9.0 Hz), 6.75 (d, 1H, J = 3.0 Hz), 6.51–6.55 (m, 2H), 4.19 (*t*, 1H, J = 6.6 Hz), 2.99 (*t*, 2H, J = 7.8 Hz), 2.00 (m, 2H), 1.72 (m, 2H), 1.54 (m, 2H). HRMS calcd for $C_{18}H_{22}N_3O_4$ $[M+H]^+$ 373.1876; found: 373.1857.

*(S,E)-N-(3-(2-amino-3-(1H-indol-3-yl)propanamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride (****X-Trp****)*

Yellow solid, decomposed ~210 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.47 (s, 1H),7.42 (d, 1H, J = 8.4 Hz), 7.33 (d, 1H, J = 8.4 Hz), 7.26 (d, 1H, J = 15.6 Hz), 7.22 (s, 1H), 7.03 (*t*, 1H, J = 7.8 Hz), 6.95 (dd, 1H, J = 9.0 Hz, J = 2.4 Hz), 6.90 (*t*, 1H, J = 7.8 Hz), 6.74 (d, 1H, J = 9.0 Hz), 6.66 (s, 1H), 6.63 (d, 1H, J = 3.0 Hz), 6.42–6.44 (m, 1H), 6.37 (d, 1H, J = 15.6 Hz), 4.26–4.29 (m, 1H), 3.38 (dd, 1H, J = 14.4 Hz, J = 6.0 Hz), 3.30 (dd, 1H, J = 14.4 Hz, J = 9.0 Hz). HR-MS calcd for $C_{24}H_{23}N_4O_4$ $[M+H]^+$ 431.1719; found: 431.1702.

*(S,E)-N-(3-(2-amino-3-phenylpropanamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride (****X-Phe****)*

Yellow solid, decomposed ~252 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.21 (d, 1H, J = 2.4 Hz), 7.61 (s, 1H), 7.41 (d, 1H, J = 15.6 Hz), 7.29–7.38 (m, 5H), 7.28 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz), 6.82 (d, 1H, J = 8.4 Hz), 6.70 (d, 1H, J = 3.0 Hz), 6.61 (d, 1H, J = 15.0 Hz), 6.54 (dd, 1H, J = 3.0 Hz, J = 1.2 Hz), 4.41 (*t*, 1H, J = 7.2 Hz), 3.30-3.34 (m, 1H), 3.17 (dd, 1H, J = 14.4 Hz, J = 6.6 Hz). HRMS calcd for $C_{22}H_{22}N_3O_4$ $[M+H]^+$ 392.1610; found: 392.1606.

*(S,E)-2-amino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-3-methylbutanamide hydrochloride (****X-Val****)*

Yellow solid, mp = 199–202 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.21 (d, 1H, J = 1.8 Hz), 7.59 (d, 1H, J = 1.8 Hz), 7.39 (d, 1H, J = 15.6 Hz), 7.28 (dd, 1H, J = 8.4 Hz, J = 2.4 Hz), 6.84 (d, 1H, J = 9.0 Hz), 6.68 (d, 1H, J = 3.0 Hz), 6.59 (d, 1H, J = 15.6 Hz), 6.52 (dd, 1H, J = 3.0 Hz, J = 1.2 Hz), 3.99 (d, 1H, J = 6.0 Hz), 2.26–2.28 (m, 1H), 1.11–1.13 (m, 6H). HRMS calcd for $C_{18}H_{22}N_3O_4$ $[M+H]^+$ 344.1610; found: 344.1593.

*(S,E)-2-amino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-4-(methylthio)butanamide hydrochloride (****X-Met****)*

Yellow solid, mp = 240–242 °C (decomposed). ^{1}H NMR (600 MHz, CD_3OD): δ 8.22 (s, 1H), 7.59 (d, 1H, J = 1.8 Hz),

7.39 (d, 1H, $J = 15.6$ Hz), 7.27 (d, 1H, $J = 8.4$ Hz), 6.84 (d, 1H, $J = 8.4$ Hz), 6.68 (d, 1H, $J = 3.6$ Hz), 6.59 (d, 1H, $J = 15.6$ Hz), 6.52 (dd, 1H, $J = 3.6$ Hz, $J = 1.8$ Hz), 4.26 (*t*, 1H, $J = 6.6$ Hz), 2.63–2.69 (m, 2H), 2.19–2.25 (m, 2H), 2.13 (s, 3H). HRMS calcd for $C_{18}H_{22}N_3O_4S$ $[M+H]^+$ 376.1331; found: 376.1319.

(2S)-2-amino-N-(5-((E)-3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-3-methylpentanamide hydrochloride ***(X-Ile)***

Yellow solid, mp = 256–258 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.23 (d, 1H, $J = 2.4$ Hz), 7.61 (s, 1H), 7.40 (d, 1H, $J = 15.6$ Hz), 7.29 (dd, 1H, $J = 8.4$ Hz, $J = 2.4$ Hz), 6.85 (d, 1H, $J = 8.4$ Hz), 6.70 (d, 1H, $J = 3.6$ Hz), 6.60 (d, 1H, $J = 15.6$ Hz), 6.54 (dd, 1H, $J = 3.6$ Hz, $J = 1.8$ Hz), 4.05 (d, 1H, $J = 6.0$ Hz), 2.01–2.04 (m, 1H), 1.67–1.70 (m, 1H), 1.30–1.33 (m, 1H), 1.11 (d, 3H, $J = 6.6$ Hz), 1.02 (*t*, 3H, $J = 7.2$ Hz). HRMS calcd for $C_{19}H_{24}N_3O_4$ $[M+H]^+$ 358.1767; found: 358.1784.

(S,E)-N-(3-(2-amino-3-hydroxypropanamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride ***(X-Ser)***

Yellow solid, mp = 162–164 °C (decomposed). ^{1}H NMR (600 MHz, D_2O): δ 7.63 (s, 1H), 7.43 (s, 1H), 7.20 (d, 1H, $J = 15.6$ Hz), 7.05 (d, 1H, $J = 8.4$ Hz), 6.80 (d, 1H, $J = 8.4$ Hz), 6.59 (s, 1H), 6.40 (s, 1H), 6.32 (d, 1H, $J = 15.6$ Hz), 4.20 (*t*, 1H, $J = 4.8$ Hz), 3.91–3.97 (m, 2H). IR: (KBr) cm^{-1}: 3400 (br), 3259, 3044, 2885, 1685, 1662, 1620, 1542. ^{13}C NMR (75 MHz, D_2O): δ 166.5, 150.7, 145.6, 145.1, 129.9, 128.6, 123.6, 120.5, 117.6, 117.3, 116.2, 115.1, 112.5, 60.3, 54.9. HRMS calcd for $C_{16}H_{18}N_3O_5$ $[M+H]^+$ 332.1246; found: 332.1236.

(S,E)-N-(3-(2-aminopropanamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride ***(X-Ala)***

Yellow solid, decomposed ~196 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.73 (d, 1H, $J = 1.8$ Hz), 7.57 (s, 1H), 7.35 (d, 1H, $J = 15.6$ Hz), 7.19 (d, 1H, $J = 8.4$ Hz), 6.95 (d, 1H, $J = 8.4$ Hz), 6.73 (s, 1H), 6.54 (s, 1H), 6.47 (d, 1H, $J = 15.6$ Hz), 4.29 (q, 1H, $J = 7.2$ Hz), 1.64 (d, 3H, $J = 7.2$ Hz). IR: (KBr) cm^{-1}: 3453 (br), 3256, 3126, 2918, 2850, 1635, 1557, 1514. ^{13}C NMR (75 MHz, D_2O): δ 169.4, 166.3, 150.7, 145.6, 145.0, 129.9, 128.4, 123.5, 120.2, 117.4, 117.3, 116.2, 115.0, 112.4, 49.5, 16.6. HRMS calcd for $C_{16}H_{18}N_3O_4$ $[M+H]^+$ 316.1297; found: 316.1288.

(E)-N-(3-(2-aminoacetamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride ***(X-Gly)***

Yellow solid, mp = 216–219 °C (decomposed). ^{1}H NMR (600 MHz, D_2O): δ 7.64 (d, 1H, $J = 2.4$ Hz), 7.43 (s, 1H), 7.18 (d, 1H, $J = 15.6$ Hz), 7.03 (dd, 1H, $J = 8.4$ Hz, $J = 2.4$ Hz), 6.79 (d, 1H, $J = 8.4$ Hz), 6.58 (d, 1H, $J = 3.6$ Hz), 6.39 (dd, 1H, $J = 3.6$ Hz, $J = 1.8$ Hz), 6.29 (d, 1H, $J = 15.6$ Hz), 3.88 (s, 2H). IR: (KBr) cm^{-1}: 3474 (br), 3129, 3059, 1673, 1638, 1548, 1509. ^{13}C NMR (75 MHz, D_2O): δ 166.3, 165.7, 150.7, 145.3, 145.1, 129.9, 128.4, 123.7, 119.9, 117.3, 117.1, 116.1, 115.0, 112.4, 40.9. HRMS calcd for $C_{15}H_{16}N_3O_4$ $[M+H]^+$ 302.1141; found: 302.1132.

(2S)-2-amino-N-(5-((E)-3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-3-hydroxybutanamide hydrochloride ***(X-Thr)***

Yellow solid, mp = 199–201 °C (decomposed). ^{1}H NMR (600 MHz, CD_3OD): δ 8.28 (d, 1H, $J = 2.4$ Hz), 7.59 (s, 1H), 7.39 (d, 1H, $J = 15.6$ Hz), 7.26 (dd, 1H, $J = 8.4$ Hz, $J = 2.4$ Hz), 6.83 (d, 1H, $J = 8.4$ Hz), 6.69 (d, 1H, $J = 3.0$ Hz), 6.59 (d, 1H, $J = 15.6$ Hz), 6.52 (dd, 1H, $J = 3.6$ Hz, $J = 1.8$ Hz), 4.11–4.14 (m, 1H), 3.99 (d, 1H, $J = 5.4$ Hz), 1.35 (d, 3H, $J = 6.6$ Hz). HRMS calcd for $C_{17}H_{20}N_3O_5$ $[M+H]^+$ 346.1403; found: 346.1402.

(S,E)-2-amino-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-4-methylpentanamide hydrochloride ***(X-leu)***

Yellow solid, mp = 250–252 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.22 (d, 1H, $J = 1.8$ Hz), 7.59 (d, 1H, $J = 1.8$ Hz), 7.39 (d, 1H, $J = 15.6$ Hz), 7.26 (dd, 1H, $J = 8.4$ Hz, $J = 2.4$ Hz), 6.84 (d, 1H, $J = 8.4$ Hz), 6.69 (d, 1H, $J = 3.0$ Hz), 6.59 (d, 1H, $J = 15.0$ Hz), 6.53 (dd, 1H, $J = 3.0$ Hz, $J = 1.8$ Hz), 4.18 (*t*, 1H, $J = 7.2$ Hz), 1.75–1.84 (m, 3H), 1.02–1.05 (m, 6H). IR: (KBr) cm^{-1}: 3393, 3255, 2958, 2919, 1691, 1664, 1619, 1536. ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.9, 164.9, 151.3, 145.4, 144.5, 130.5, 127.7, 124.6, 118.4, 118.2, 115.6, 115.0, 113.8, 111.9, 52.2, 40.5, 24.2, 21.7, 20.9. HRMS calcd for $C_{19}H_{24}N_3O_4$ $[M+H]^+$ 358.1767; found: 358.1760.

(E)-N-(3-(3-aminopropanamido)-4-hydroxyphenyl)-3-(furan-2-yl)acrylamide hydrochloride ***(X-β-Ala)***

Yellow solid, mp = 219–224 °C (decomposed). 1H NMR (600 MHz, DMSO-d_6): δ 10.09 (s, 1H), 9.63 (s, 1H), 9.54 (s, 1H), 8.13 (d, 1H, $J = 2.4$ Hz), 7.93 (br, 3H), 7.81 (s, 1H), 7.33 (dd, 1H, $J = 8.4$ Hz, J = 2.4 Hz), 7.32 (d, 1H, $J = 15.6$ Hz), 6.83 (d, 1H, $J = 8.4$ Hz), 8.81 (d, 1H,

$J = 3.0$ Hz), 6.63 (d, 1H, $J = 15.6$ Hz), 6.61 (dd, 1H, $J = 3.0$ Hz, $J = 1.8$ Hz), 3.07 (*t*, 2H, $J = 6.6$ Hz), 2.80 (*t*, 2H, $J = 6.6$ Hz). HRMS calcd for $C_{16}H_{18}N_3O_4$ $[M+H]^+$ 316.1297; found: 316.1287.

*(2S,4R)-N-(5-((E)-3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)-4-hydroxypyrrolidine-2-carboxamide hydrochloride (**X-Hyp**)*

Yellow solid, decomposed ~140 °C, mp = 164–166 °C. ^{1}H NMR (300 MHz, CD_3OD): δ 8.27 (d, 1H, $J = 2.4$ Hz), 7.62 (d, 1H, $J = 1.5$ Hz), 7.43 (d, 1H, $J = 15.6$ Hz), 7.29 (dd, 1H, $J = 8.7$ Hz, $J = 2.4$ Hz), 6.87 (d, 1H, $J = 8.7$ Hz), 6.72 (d, 1H, $J = 3.6$ Hz), 6.62 (d, 1H, $J = 15.6$ Hz), 6.56 (dd, 1H, $J = 3.6$ Hz, $J = 1.8$ Hz), 4.78 (dd, 1H, $J = 7.8$ Hz, $J = 10.2$ Hz), 4.65–4.68 (m, 1H), 3.50 (dd, 1H, $J = 12.0$ Hz, $J = 3.6$ Hz), 3.35–3.39 (m, 1H), 2.57–2.66 (m, 1H), 2.13–2.26 (m, 1H). HRMS calcd for $C_{18}H_{20}N_3O_5$ $[M+H]^+$ 358.1403; found: 358.1378.

*(S,E)-N-(5-(3-(furan-2-yl)acrylamido)-2-hydroxyphenyl)pyrrolidine-2-carboxamide hydrochloride (**X-Pro**)*

Yellow solid, mp = 170–174 °C. ^{1}H NMR (300 MHz, D_2O): δ 7.64 (d, 1H, $J = 2.4$ Hz), 7.46 (d, 1H, $J = 1.8$ Hz), 7.22 (d, 1H, $J = 15.6$ Hz), 7.08 (dd, 1H, $J = 8.7$ Hz, $J = 2.4$ Hz), 6.83 (d, 1H, $J = 8.7$ Hz), 6.61 (d, 1H, $J = 3.3$ Hz), 6.43 (dd, 1H, $J = 3.3$ Hz, J = 1.8 Hz), 6.33 (d, 1H, $J = 15.6$ Hz), 4.44-4.50 (m, 1H), 3.28–3.44 (m, 2H), 2.38–2.50 (m, 1H), 1.95–2.16 (m, 3H). HRMS calcd for $C_{18}H_{20}N_3O_4$ $[M+H]^+$ 342.1454; found: 342.1445.

*The general procedure of compound **XK-Ala** ~ **XK-Val***

To a solution of compound 5 (0.34 g, 1.5 mmol), Et_3N (0.21 ml, 1.5 mmol) and Boc-L-amino acid (1.6 mmol) in 50 ml anhydrous THF, DCC was added (0.46 g, 2.25 mmol). The reaction mixture was stirred at room temperature for 1 h. The insoluble *N*,*N*′-dicyclohexylurea was removed by filtration and the solvent was removed under reduced pressure to give a residue, which was subsequently dissolved in ethyl acetate and then washed with saturated $NaHCO_3$, 1 M aqueous HCl, and brine. The organic phase was dried over anhydrous $MgSO_4$ and concentrated. Chromatographic purification of the crude material with EtOAc and petroleum afforded the Boc-protected target compounds which were added to 20 ml HCl/EtOAc and stirred overnight. The precipitate was collected through filtration and washed with EtOAc to give the target compounds in 25–42 % yield.

*(S,E)-N-(3-(2-aminopropanamido)-4-hydroxyphenyl)-3-(3,4-dihydroxyphenyl)acrylamide hydrochloride (**XK-Ala**)*

Yellow solid, mp = 220–224 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.52 (s, 1H), 7.21 (d, 1H, $J = 15.6$ Hz), 6.96 (d, 1H, $J = 8.4$ Hz), 6.87 (s, 1H), 6.82 (d, 1H, $J = 7.8$ Hz), 6.73 (d, 1H, $J = 8.4$ Hz), 6.65 (d, 1H, $J = 8.4$ Hz), 6.23 (d, 1H, $J = 15.6$ Hz), 4.12 (q, 1H, $J = 6.6$ Hz), 1.47 (d, 3H, $J = 6.6$ Hz). IR: (KBr) cm^{-1}: 3418 (br), 3299 (br), 1684, 1645, 1600, 1550, 1509. ^{13}C NMR (75 MHz, D_2O): δ 169.4, 166.6, 146.2, 145.4, 143.9, 141.6, 130.0, 127.2, 123.4, 122.1, 120.1, 117.4, 117.2, 116.1, 115.9, 114.7, 49.5, 16.6. HRMS calcd for $C_{18}H_{20}N_3O_5$ $[M+H]^+$ 358.1403; found: 358.1377.

*(S,E)-2-amino-N-(5-(3-(3,4-dihydroxyphenyl)acrylamido)-2-hydroxyphenyl)-4-methylpentanamide hydrochloride (**XK-Leu**)*

Yellow solid, mp = 233–236 °C. ^{1}H NMR (600 MHz, D_2O): δ 7.51 (s, 1H), 7.22 (d, 1H, $J = 15.6$ Hz), 6.97 (d, 1H, $J = 8.4$ Hz), 6.89 (s, 1H), 6.84 (d, 1H, $J = 7.8$ Hz), 6.76 (d, 1H, $J = 8.4$ Hz), 6.67 (d, 1H, $J = 7.8$ Hz), 6.25 (d, 1H, $J = 15.6$ Hz), 4.05 (*t*, 1H, $J = 7.2$ Hz), 1.60–1.69 (m, 3H), 0.81–0.84 (m, 6H). HRMS calcd for $C_{21}H_{26}N_3O_5$ $[M+H]^+$ 400.1872; found: 400.1859.

*(2S)-2-amino-N-(5-((E)-3-(3,4-dihydroxyphenyl) acrylamido)-2-hydroxyphenyl)-3-methylpentanamide hydrochloride (**XK-Ile**)*

Yellow solid, mp = 208–210 °C. ^{1}H NMR (300 MHz, D_2O): δ 7.55 (d, 1H, $J = 2.4$ Hz), 7.18 (d, 1H, $J = 15.6$ Hz), 6.97 (dd, 1H, $J = 8.7$ Hz, $J = 2.4$ Hz), 6.85 (d, 1H, $J = 1.8$ Hz), 6.84 (dd, 1H, $J = 8.4$ Hz, $J = 1.8$ Hz), 6.75 (d, 1H, $J = 8.7$ Hz), 6.65 (d, 1H, $J = 8.4$ Hz), 6.18 (d, 1H, $J = 15.6$ Hz), 3.96 (d, 1H, $J = 5.4$ Hz), 1.95–1.99 (m, 1H), 1.42–1.56 (m, 1H), 1.14–1.24 (m, 1H), 0.96 (d, 3H, $J = 6.9$ Hz), 0.85 (*t*, 3H, $J = 7.2$ Hz). HRMS calcd for $C_{21}H_{26}N_3O_5$ $[M+H]^+$ 400.1872; found: 400.1864.

*(S,E)-2-amino-N-(5-(3-(3,4-dihydroxyphenyl)acrylamido)-2-hydroxyphenyl)-3-methylbutanamide hydrochloride (**XK-Val**)*

Yellow solid, decomposed ~194 °C, mp = 204 °C. ^{1}H NMR (600 MHz, CD_3OD): δ 8.23 (d, 1H, $J = 2.4$ Hz), 7.48 (d, 1H, $J = 15.6$ Hz), 7.28 (dd, 1H, $J = 8.4$ Hz, $J = 2.4$ Hz), 7.04 (d, 1H, $J = 1.8$ Hz), 6.95 (dd, 1H, $J = 8.4$ Hz, $J = 1.8$ Hz), 6.85 (d, 1H, $J = 8.4$ Hz), 6.78 (d, 1H, $J = 8.4$ Hz), 6.53 (d, 1H, $J = 15.6$ Hz), 3.99 (d, 1H, $J = 6.0$ Hz), 2.27–2.31 (m, 1H), 1.14 (*t*, 6H,

$J = 7.2$ Hz). IR: (KBr) cm^{-1}: 3447 (br), 2968, 1656, 1609, 1508. ^{13}C NMR (75 MHz, D_2O): δ 168.1, 166.5, 146.2, 145.7, 143.9, 141.5, 130.0, 127.1, 123.1, 122.0, 120.3, 117.3, 116.1, 115.9, 114.7, 58.9, 30.1, 17.7, 16.8. HRMS calcd for $C_{20}H_{24}N_3O_5$ $[M+H]^+$ 386.1716; found: 386.1719.

(3R,4R,5S)-4-acetamido-5-((tert-butoxycarbonyl)amino)-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid ***(Boc-GS4071)***

To a mixture of oseltamivir phosphate (2.05 g, 5 mmol) and $NaHCO_3$ (2.1 g, 25 mmol) in 60 ml THF + H_2O (5:1) $(Boc)_2O$ (1.31 g, 6 mmol) was added in one portion. The reaction was allowed to stand for 6 h at rt. The solvent was removed in vacuum followed by extraction of the residues with EtOAc. After concentration, the obtained oil product was dissolved in 30 ml CH_3OH + H_2O (3:1) and NaOH (1.6 g, 40 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. After evaporation of CH_3OH, 15 ml H_2O was added. The undissolved substance was filtered and the clear filtrate was neutralized with 1 M HCl to give a white solid (1.40 g, yield = 72.9 %). Mp = 212–214 °C. ^1H NMR (600 MHz, DMSO-d_6): δ 12.57 (br, 1H), 7.80 (d, 1H, $J = 9.0$ Hz), 6.59–6.62 (m, 2H), 4.04–4.06 (m, 1H), 3.65–3.71 (m, 1H), 3.52–3.58 (m, 1H), 3.35–3.39 (m, 1H), 2.45 (dd, 1H, $J = 17.4$ Hz, $J = 5.4$ Hz), 2.21 (dd, 1H, $J = 17.4$ Hz, $J = 10.8$ Hz), 1.77 (s, 3 H), 1.33–1.48 (m, 13H), 0.83 (*t*, 3H, $J = 7.2$ Hz), 0.76 (*t*, 3H, $J = 7.2$ Hz). HRMS calcd for $C_{19}H_{31}N_2O_6$ $(M-H)^-$ 383.2182; found: 383.2177.

(3R,4R,5S)-4-acetamido-5-amino-3-(pentan-3-yloxy)cyclohex-1-enecarboxylic acid hydrochloride ***(GS4071-HCl)***

Boc-GS4071 (0.38 g, 1 mmol) was added to 20 ml HCl/EtOAc and stirred overnight. The precipitate was collected through filtration and washed with EtOAc to give a white solid (0.26 g, yield = 81.2 %). Mp = 186–188 °C. ^1H NMR (600 MHz, D_2O): δ 6.67 (1H, s), 4.15 (d, 1H, $J = 7.8$ Hz), 3.88 (dd, 1H, $J = 11.4$ Hz, $J = 9.0$ Hz), 3.35-3.44 (m, 2H), 2.76 (dd, 1H, $J = 17.4$ Hz, $J = 5.4$ Hz), 2.32 (dd, 1H, $J = 17.4$ Hz, $J = 10.8$ Hz), 1.90 (s, 3H), 1.35–1.42 (m, 3H), 1.26–1.31 (m, 1H), 0.70 (*t*, 3H, $J = 7.2$ Hz), 0.66 (*t*, 3H, $J = 7.2$ Hz). IR: (KBr) cm^{-1}: 3466 (br), 3040, 2971, 2877, 1703, 1653, 1549. ^{13}C NMR (75 MHz, D_2O): δ 175.2, 168.9, 138.4, 127.5, 84.3, 75.1, 52.5, 49.2, 28.1, 25.4, 25.1, 22.4, 8.51, 8.48. HRMS calcd for $C_{14}H_{25}N_2O_4$ $[M+H]^+$ 285. 1814; found: 285.1810.

Pharmacology

Influenza A (H9N2 and H5N1) NA inhibition assay

The NA inhibition assay was performed according to a standard method (Liu *et al.*, 2008). 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 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 30–60 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 NA activity versus the inhibitor concentration.

Evaluation of anti-influenza virus activity in chick embryo

The test for activity of target compounds against avian H5N1 influenza virus in chick embryo was conducted based on a reported method with a slight modification (Sauerbrei *et al.*, 2006). The target compound dissolved in phosphate buffered saline (PBS) or DMSO was serially diluted by twofold in PBS. Then, 100 µl of compound at different concentrations was mixed with an equal volume of 100 $TCID_{50}$ (50 % tissue culture infective dose) of H5N1 virus (A/duck/China/QJ/01). After 1-h incubation at 37 °C, the compound–virus preparation was injected into 11-day old chick embryos, followed by incubation at 37 °C for 5 days. Define the minimal effective dosage for keeping the eggs alive and inhibiting the viral replication with a low HA titer (0 log2) in allantoic fluid determined by hemagglutination assay. (The eggs of blank control groups were all dead within 36 h).

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References

Jiang D, Wang YY, Xu YN, Dai LY (2009) Doebner condensation in ionic liquids [Bmim] BF4 and [Bpy] BF4 to synthesize [alpha],[beta]-unsaturated carboxylic acid. Chin Chem Lett 20(3):279–282

Krug RM, Aramini JM (2009) Emerging antiviral targets for influenza A virus. Trends Pharmacol Sci 30(6):269–277

Liu Y, Zhang J, Xu W (2007) Recent progress in rational drug design of neuraminidase inhibitors. Curr Med Chem 14(27):2872–2891

Liu AL, Wang HD, Lee SM, Wang YT, Du GH (2008) Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities. Bioorg Med Chem 16(15):7141–7147

Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM, Hay AJ, Gamblin SJ, Skehel JJ (2006) The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 443(7107):45–49

Sauerbrei A, Haertl A, Brandstaedt A, Schmidtke M, Wutzler P (2006) Utilization of the embryonated egg for in vivo evaluation of the anti-influenza virus activity of neuraminidase inhibitors. Med Microbiol Immunol 195(2):65–71

Sheehan JC, Hess GP (1955) A new method of forming peptide bonds. J Am Chem Soc 77(4):1067–1068

Storms AD, Gubareva LV, Su S, Wheeling JT, Okomo-Adhiambo M, Pan CY, Reisdorf E, St George K, Myers R, Wotton JT, Robinson S, Leader B, Thompson M, Shannon M, Klimov A, Fry AM (2012) Oseltamivir-resistant pandemic (H1N1) (2009) virus infections, United States, 2010–11. Emerg Infect Dis 18(2):308–311

Upadhyay A, Chompoo J, Taira N, Fukuta M, Gima S, Tawata S (2011) Solid-phase synthesis of mimosine tetrapeptides and their inhibitory activities on neuraminidase and tyrosinase. J Agric Food Chem 59(24):12858–12863

von Itzstein M (2007) The war against influenza: discovery and development of sialidase inhibitors. Nat Rev Drug Discov 6(12):967–974