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Structure-based Design, Synthesis and Anti-influenza A Virus Activities of Substituted Phenyl-coupled Heterocyclic Ethylamide Derivatives

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Abstract A series of new substituted phenyl-coupled heterocyclic ethylamide derivatives was designed and synthesized as anti-influenza agents. *In vitro* anti-influenza A(A/PR/8/34 H1N1 strain) activities of these compounds were investigated and compared to those of the commercial antiviral drugs(Arbidol and Ribavirin) against the influenza. Specifically, among these twelve compounds exhibiting moderate levels of antiviral activity against influenza A, compounds **30**c and **30**d are the most effective ones, and as efficacious as the positive control Ribavirin and much more effective than Ingavirin and Arbidol, indicating that they are prospective candidates for further exploration. These results are also consistent with the docking study results in terms of the design of compounds targeting influenza A *via* viral nucleoprotein.

Keywords Influenza virus A; Nucleoprotein inhibitor; Ingavirin; Nucleozin; Biological evaluation

1 Introduction

Influenza is a highly contagious and acute respiratory and pulmonary disease caused by the influenza virus. Epidemic and pandemic influenza has made a serious impact on worldwide morbidity, mortality and economy[1]. More importantly, the viral genetic shift may eventually cause a breakthrough of interspecies and inter-human transmission barriers and raise the possibility of transmission from animals to human beings or *vice versa*(human beings to animals), which would be disastrous to public health[2—4]. To date, only a few drugs have displayed abilities to treat influenza A including Amantidine, Zanamivir, Arbidol, Ribavirin and Oseltamivir[5—7]. However, the efficacies of these drugs are limited due to the rapid emergence of mutated viral strains and side effects[8,9]. Thereby, it is attractive important and urgent to develop new anti-influenza agents with a new mechanism of action[10—14].

The influenza virus nucleoprotein(NP) encoded by the fifth genome segment is abundantly expressed during the course of infection. Due to its indispensable roles in viral transcription, replication and packaging, the influenza virus NP becomes a novel target for new anti-influenza drug development. Several NP inhibitors have been reported to display promising anti-influenza virus activities[15—21], among which Ingavirin and Nucleozin are considered as novel candidates. Ingavirin(**1**), a small molecule previously known as Ingamine, is the first non-toxic broad spectrum antiviral drug targeting NP directly[20] and has shown anti-influenza activity against influenza viruses A(H3N2 and H5N1) and B in animal models[22]. It is also effective against the pandemic strains of influenza virus A/California/04/2009 and A/California/07/2009[23,24]. Based on the chemical library screening of 50240 compounds, Nucleozin(**2**) was identified because it triggered the aggregation of NPs, inhibited the nuclear accumulation, impeded the replication of influenza A virus *in vitro* with a satisfied effective concentration(EC_{50}) and protected mice challenged with lethal doses of avian influenza A H5N1[16].

According to the structure analysis of Ingavirin(**1**) and Nucleozin(**2**) by means of Schrodinger 2013-1, we designed and synthesized a series of substituted phenyl-coupled heterocyclic ethylamide derivatives(Fig.1). All the derivatives were divided into two fragments: amine fragments including substituted and unsubstituted heterocyclic ethamine fragments that were synthesized and converted to their hydrochloride salts for further use(Scheme 1) and their acid fragments.

All the newly synthesized compounds were measured by the cell toxicity assays and viral inhibition assays. Compounds **30**c and **30**d were chosen to perform the docking study to explain theoretical interaction between the compounds and NP.

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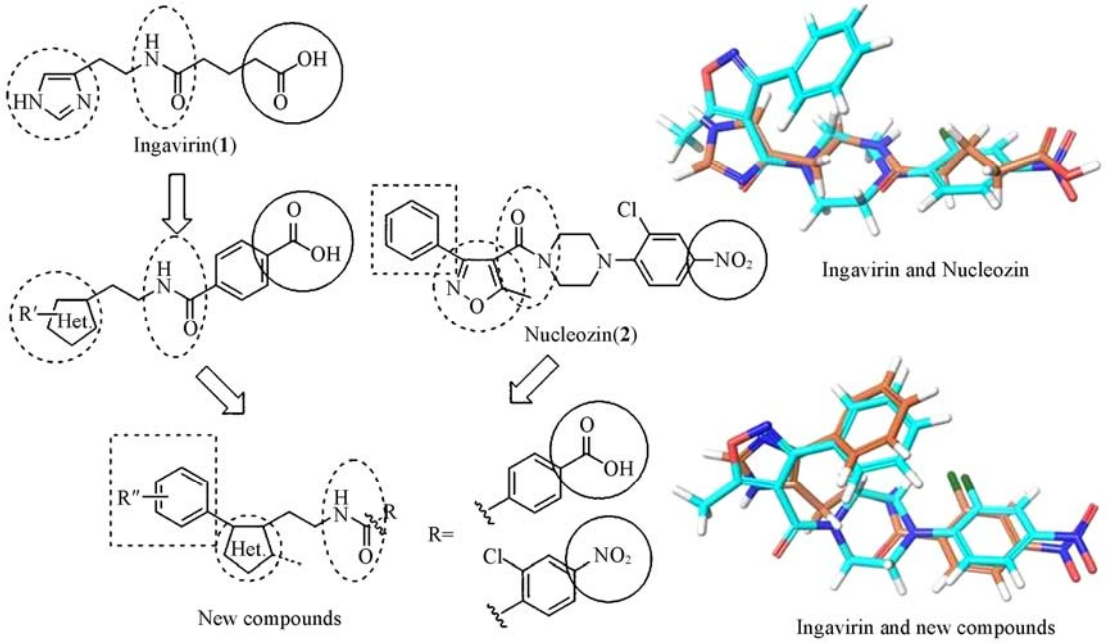
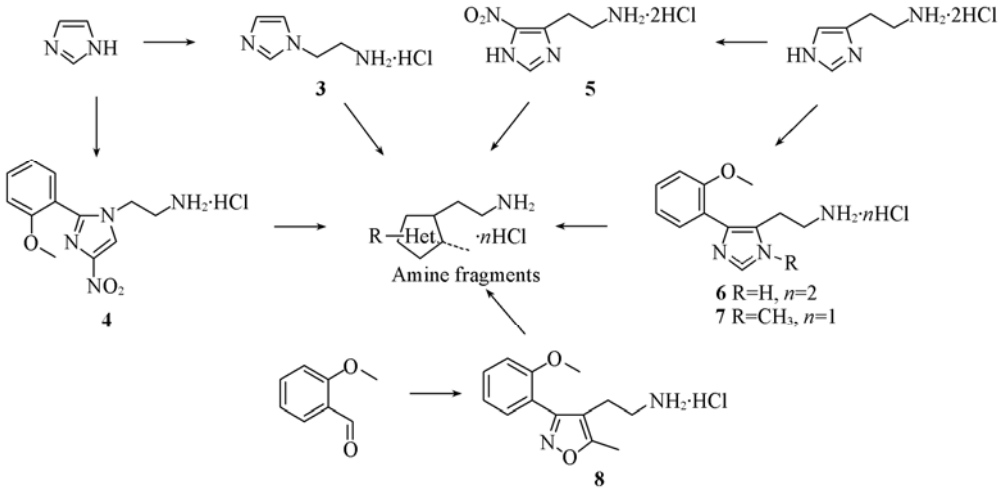


Fig.1 Structure of Ingavirin(1) and Nucleozin(2) as well as the overall strategy for the design of novel anti-influenza viral agents



Scheme 1 General synthesis route of the amine fragments

2 Experimental

2.1 Chemicals and Apparatus

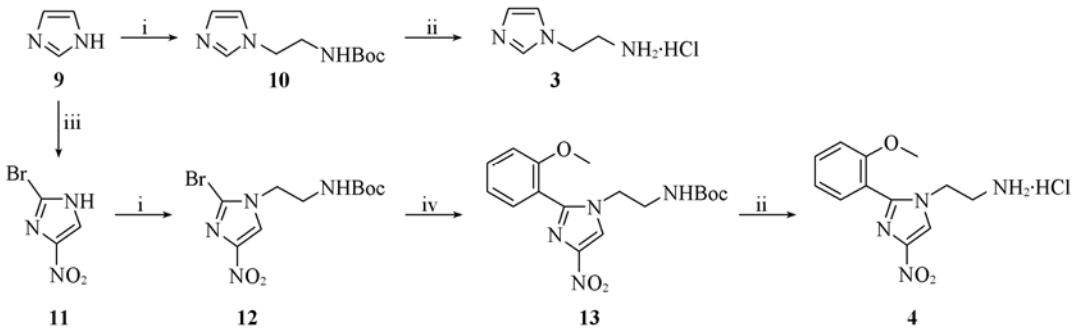
All the reactions were carried out under the protection of argon(Ar) atmosphere. Most chemicals and solvents were analytical grade and used without further purification. Thin layer chromatography(TLC) was performed using precoated silica gel GF254(0.2 mm) while column chromatography was performed using silica gel(100—200 mesh). The melting point was measured on a YRT-3 melting point apparatus(Shantou Keyi Instrument & Equipment Co., Ltd., Shantou, China) without thermometer correction. ^{1}H NMR spectra were collected on a Varian INOVA400(Varian, Palo Alto, USA) using $CDCl_3$, DMSO-d_6 or D_2O as solvent. Chemical shifts were expressed in δ, with tetramethylsilane(TMS) as the internal reference; coupling constants(J) were expressed in Hz. The high-resolution mass(HRMS) spectra were recorded on a Bruker maxis impact Q-TOF instrument(Bruker, Billerica, USA) coupled with a Dionex Ultimate 3000 spectrometer (Dionex, Sunnyvale, USA).

2.2 Synthesis of the Substituted Imidazol-1-yl Ethamine Hydrochloride

The synthetic routes of the substituted imidazol-1-yl ethamine hydrochloride are shown in Scheme 2.

*2.2.1 Synthesis of tert-Butyl 2-(1H-Imidazol-1-yl)ethylcarbamate(**10**)*

To a suspension of imidazole(**9**, 5 g, 73.44 mmol), *N*-boc-2-chloroethylamine(19.79 g, 110.16 mmol) and a catalytic amount of tetrabutylammonium bromide(TBAB, 0.25 g, 5%, mass fraction) in tetrahydrofuran(THF) was added NaOH(8.81 g, 220.32 mmol) in portions. The reaction mixture was refluxed overnight and filtered. The filter cake was washed with dichloromethane(DCM) and the filtrate was evaporated *in vacuum* to obtain brownish oil, which was purified by flash



Scheme 2 Synthesis of the substituted imidazol-1-yl ethamine hydrochloride

Reagents and conditions: (i) *N*-boc-2-chloroethylamine, NaOH, TBAB, THF, r. t. to reflux, overnight, 81% for compound **10** and 72% for compound **12**; (ii) 2 mol/L HCl/MeOH, r. t., overnight, >98%. (iii) (a) conc. HNO_3, conc. H_2SO_4, i.b.(ice bath) to r. t., 1.5 h; (b) fum. HNO_3, AcOH, Ac_2O, i.b. to r. t., 3 h; (c) PhCl, 125 °C, 24 h; (d) 49% HBr, reflux, 5 h, 33% over four steps; (iv) 2-methoxyphenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, 1,4-dioxane, H_2O, reflux, 4 h, 83%.

chromatography on silica gel to get the target compound as a white solid. Yield 80.6%; m. p. 87—89 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.39(s, 9H), 3.35(q, *J*=5.6 Hz, 2H), 4.48(t, *J*=5.6 Hz, 2H), 5.21(brs, 1H), 7.48(d, *J*=3.2 Hz, 1H), 7.57(d, *J*=3.2 Hz, 1H), 8.67(s, 1H).

*2.2.2 Synthesis of 2-(1H-Imidazol-1-yl)ethamine Hydrochloride(**3**)*

A solution of *tert*-butyl 2-(1*H*-imidazol-1-yl)ethylcarbamate(**10**, 5 g, 17.2 mmol) in 2 mol/L HCl/MeOH(75 mL) in an ice bath was stirred at room tempe- rature overnight. The precipitate was collected and washed with MeOH three times and dried under vacuum to obtain a white crystal. Yield 98.4%; m. p. >240 °C; ^{1}H NMR(400 MHz, D_2O), δ: 3.34(t. *J*=6.4 Hz, 2H), 4.47(t, *J*=6.4 Hz, 2H), 7.62(d, *J*=2.4 Hz, 1H), 7.81(d, *J*=2.4 Hz, 1H), 8.55(s, 1H); ESI-HRMS, *m/z*: calcd. for $C_5H_{10}N_3^+$ $[M+H]^+$ 112.0875; found: 112.0864.

*2.2.3 Synthesis of 2-Bromo-4-nitro-1H-imidazole(**11**)*

Compound **11** was prepared following the procedure reported in the literature[25] from the starting material **9** and isolated as a white crystal. Yield 33.4%; m. p. 238—240 °C; ESI-HRMS, *m/z*: calcd. for $C_3H_3BrN_3O_2^+$$[M+H]^+$ 191.9409, 193.9388; found: 191.9404, 193.9389.

*2.2.4 Synthesis of tert-Butyl 2-(2-Bromo-4-nitro-1H-imidazol-1-yl)ethylcarbamate(**12**)*

Compound **12** was synthesized from compound **11** according to the procedure used to prepare compound **10**, and isolated as a yellow solid. Yield 72.4%; m. p. 159—161 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.42(s, 9H), 3.50(q, *J*=6.0 Hz, 2H), 4.20(brs, 2H), 4.88(brs, 1H), 7.79(s, 1H).

*2.2.5 Synthesis of tert-Butyl 2-[2-(2-Methoxyphenyl)-4-nitro-1H-imidazol-1-yl]ethylcarbamate(**13**)*

A suspension of *tert*-butyl 2-(2-bromo-4-nitro-1*H*-imidazol-1-yl)ethylcarbamate(**12**, 2 g, 5.97 mmol), 2-methoxyphenylboronic acid(1 g, 6.57 mmol), terakis(triphenylphosphine)-palladium(0)[$Pd(Ph_3P)_4$, 0.35 g, 0.3 mmol] and Na_2CO_3(3.16 g, 29.85 mmol) in 1,4-dioxane and H_2O(50 mL, 1/1 volume ratio) was repeatedly bubbled with Ar to remove oxygen in the flask. The mixture was refluxed for 4 h until TLC showed no starting materials. The suspension was cooled to room temperature and evaporated to half volume followed by the addition of H_2O and the extraction with ethyl acetate three times. The organic layer was combined, washed with brine three times and dried over Na_2SO_4. After the solvent was evaporated *in vacuum*, brownish oil was obtained, which was further purified by flash column chromatography on silica gel to obtain the product as a yellow solid. Yield 83.2%; m. p. 175—178 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 1.29(s, 9H), 3.13(q, *J*=5.2 Hz, 2H), 3.79(s, 3H), 3.87(t, *J*=5.2 Hz, 2H), 6.89(brs, 1H), 7.10(t, *J*=7.6 Hz, 1H), 7.19(d, *J*=8.0 Hz, 1H), 7.40(d, *J*=6.8 Hz, 1H), 7.55(t, *J*=7.6 Hz, 1H), 8.41(s, 1H).

*2.2.6 Synthesis of 2-[2-(2-Methoxyphenyl)-4-nitro-1H-imidazol-1-yl]ethanamine Hydrochloride(**4**)*

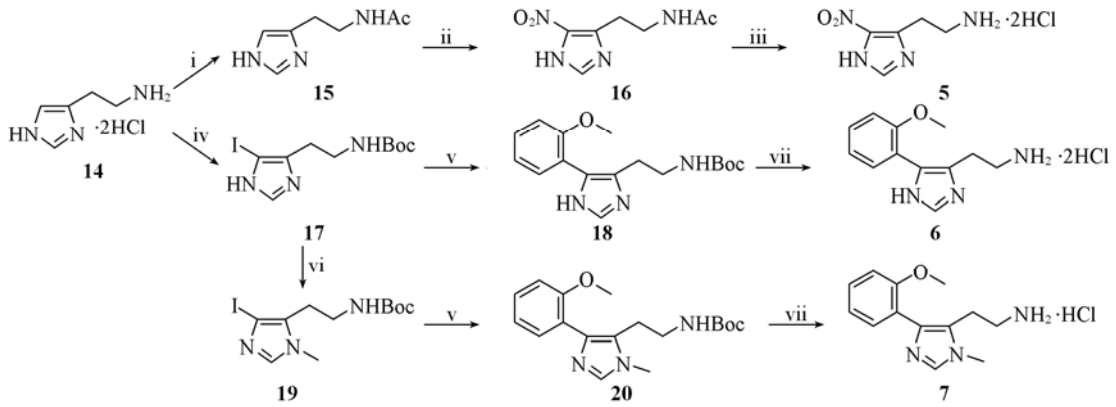
Compound **4** was synthesized from compound **13** according to the procedure used to prepare compound **3** and isolated as a light-yellow solid. Yield 98.5%; m. p. 238—240 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 3.14(t, *J*=6.4 Hz, 2H), 3.82(s, 3H), 4.11(t, *J*=6.4 Hz, 2H), 7.12(t, *J*=7.2 Hz, 1H), 7.23(d, *J*=8.04 Hz, 1H), 7.43(d, *J*=6.4 Hz, 1H), 7.59(t, *J*=7.2 Hz, 1H), 8.07(brs, 3H), 8.67(s, 1H); ESI-HRMS, *m/z*: calcd. for $C_{12}H_{15}N_4O_3^+$$[M+H]^+$ 263.1144; found: 263.1148.

2.3 Synthesis of the Substituted Histamine Hydrochloride

The synthetic routes of the substituted histamine hydrochloride are shown in Scheme 3.

*2.3.1 Synthesis of N-[2-(1H-Imidazol-4-yl)ethyl]-acetamide(**15**)*

NaOH(4.78 g, 119.53 mmol) was added in portions to a suspension of histamine dihydrochloride(**14**, 10 g, 54.33 mmol) in EtOH(200 mL) in an ice bath. The mixture was stirred for 1 h at room temperature and filtered. The filter cake was washed with EtOH and the filtrate was evaporated *in vacuum* to get a colorless oil, which was used without further purification. The oil was dissolved in MeCN(150 mL), and Ac_2O(6.66 g, 65.20 mmol) was added dropwise slowly. The solution was refluxed for 2 h until no starting materials could be detected by TLC. The mixture was cooled to room temperature and poured into water. The suspension was obtained basic with $NaHCO_3$ and extracted with ethyl acetate three times. The organic layer



Scheme 3 Synthesis of the substituted histamine hydrochloride

Reagents and conditions: (i) (a) NaOH, EtOH, 0 °C to r. t., 1 h, 99%; (b) Ac_2O, MeCN, reflux, 2 h, 82%; (ii) fum. HNO_3/conc. H_2SO_4, –10 °C, 75%; (iii) 6 mol/L HCl/MeOH, reflux, overnight, 82%; (iv) (a) NaOH, EtOH, 0 °C to r. t., 1 h, 99%; (b) $(Boc)_2O$, MeCN, 0 °C to r. t., 4 h; (c) K_2CO_3, MeOH, reflux, 3 h; (d) NIS, MeCN, r. t., overnight, 46% over four steps; (v) 2-methoxyphenyl boronic acid, $Pd(PPh_3)_4$, Na_2CO_3, 1,4-dioxane, H_2O, reflux, 4 h, 87% for compound **18** and 81% for compound **20**; (vi) (a) TrtCl, TEA, DCM, i.b. to r. t., overnight; (b) MeI, THF, reflux, 1 h; (c) conc. HCl, MeOH, r. t., overnight; (d) $(Boc)_2O$, MeCN, 0 °C to r. t., 4 h; 71% over four steps; (vii) 2 mol/L HCl, MeOH, 0 °C to r. t., overnight, >98%.

was combined, washed with brine and dried over Na_2SO_4. After the solvent was evaporated *in vacuum*, an off-white solid was obtained and further purified by recrystallization in ethyl acetate to obtain the product as a white crystal. Yield 81.7%; m. p. 137—139 °C; ^{1}H NMR(400 MHz, D_2O), δ: 1.97(s, 3H), 2.82(t, *J*=6.4 Hz, 2H), 3.45(t, *J*=6.4 Hz, 2H), 6.96(s, 1H), 7.73(s, 1H).

*2.3.2 Synthesis of N-[2-(5-nitro-1H-Imidazol-4-yl)ethyl]acetamide(**16**)*

A solution of *N*-[2-(1*H*-imidazol-5-yl)ethyl]acetamide(**15**, 10 g, 65.28 mmol) in conc. H_2SO_4(40 mL) was cooled to –10 °C and a solution of fum. HNO_3(15 mL) in conc. H_2SO_4(15 mL) was added dropwise. The mixture was stirred for 4 h and the solution was poured into ice. The pH value was adjusted to 8 with aqueous ammonia. The precipitate was collected with filtration, washed with water three times and dried *in vacuum* to obtain a white crystal, which was further purified by recrystallization in acetone to get the target product. Yield 74.9%; m. p. 236—238 °C; ^{1}H NMR(400 MHz, DMSO-d_6+ D_2O), δ: 1.74(s, 3H), 3.06(t, *J*=6.4 Hz, 2H), 3.33(t, *J*=6.4 Hz, 2H), 7.58(s, 1H).

*2.3.3 Synthesis of 2-(5-nitro-1H-Imidazol-4-yl)-ethanamine Dihydrochloride(**5**)*

A solution of *N*-[2-(4-nitro-1*H*-imidazol-5-yl)ethyl]-acetamide(**16**, 4 g, 20.18 mmol) and 6 mol/L HCl(20 mL) in MeOH(20 mL) was refluxed for 2 h. And then the solution was cooled to room temperature. When MeOH and most water were evaporated under vacuum, the solution was poured into acetone and stirred for another 30 min. The precipitate was collected, washed with acetone three times and dried *in vacuum* to obtain a white crystal. Yield 82.4%; m. p. >240 °C; ^{1}H NMR(400 MHz, D_2O), δ: 2.94(t, *J*=6.4 Hz, 2H), 3.25(t, *J*=6.4 Hz, 2H), 7.67(s, 1H); ESI-HRMS, *m/z*: calcd. for $C_5H_9N_4O_2^+$ $[M+H]^+$ 157.0726; found: 154.0724.

*2.3.4 Synthesis of tert-Butyl [2-(5-iodo-1H-Imidazol-4-yl)ethyl]carbamate(**17**)*

Compound **17** was prepared following the procedure reported in the literature[26] with the starting material of compound **14**, and isolated as a yellow solid. Yield 46.4%; m. p. 54—56 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.43(s, 9H), 2.81(t, *J*=6.8 Hz, 2H), 3.43(d, *J*=6.0 Hz, 2H), 4.96(brs, 1H), 7.60(s, 1H).

*2.3.5 Synthesis of tert-Butyl 2-[5-(2-Methoxyphenyl)-1H-imidazol-4-yl]ethylcarbamate(**18**)*

Compound **18** was synthesized from compound **17** according to the procedure used to prepare compound **13**, and isolated as a white solid. Yield 87.4%; m. p. 32—35 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.42(s, 9H), 2.90(t, *J*=6.4 Hz, 2H), 3.52(q, *J*=6.0 Hz, 2H), 3.88(s, 3H), 5.40(brs, 1H), 6.99(d, *J*=8.0 Hz, 1H), 7.04(td, *J*=0.8, 7.6 Hz, 1H), 7.31(td, *J*=1.2, 8.4 Hz, 1H), 7.42(d, *J*=6.8 Hz, 1H), 7.64(s, 1H).

*2.3.6 Synthesis of 2-[5-(2-Methoxyphenyl)-1H-imidazol-4-yl]ethanamine Dihydrochloride(**6**)*

Compound **6** was synthesized from compound **18** according to the procedure used to prepare compound **3**, and isolated as a white solid. Yield 98.8%; m. p. 205—207 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ: 2.97(t, *J*=7.6 Hz, 2H), 3.14(brs, 2H), 3.83(s, 3H), 7.22(d, *J*=8.4 Hz, 1H), 7.11(t, *J*=7.2 Hz, 1H), 7.42(dd, *J*=1.6, 7.6 Hz, 1H), 7.52(td, *J*=1.6, 7.6 Hz, 1H), 8.28(brs, 3H), 9.20(s, 1H), 14.78(brs, 2H); ESI-HRMS, *m/z*: calcd. for $C_{12}H_{16}N_3O^+$ $[M+H]^+$ 218.1293; found: 218.1289.

*2.3.7 Synthesis of tert-Butyl 2-(4-iodo-1-Methyl-1H-imidazol-5-yl)ethylcarbamate(**19**)*

Compound **19** was prepared following the procedure reported in the literature[27] with the starting material of compound **17**, and isolated as a yellow solid. Yield 70.8%; m. p. 97—100 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.40(s, 9H), 2.76(t, *J*=6.8 Hz, 2H), 3.24(q, *J*=6.4 Hz, 2H), 3.63(s, 3H), 4.93(brs, 1H), 7.35(s, 1H).

*2.3.8 Synthesis of tert-Butyl 2-[4-(2-Methoxyphenyl)-1-methyl-1H-imidazol-5-yl]ethylcarbamate (**20**)*

Compound **20** was synthesized from compound **19** according to the procedure used to prepare compound **13**, and isolated as a yellow solid. Yield 80.8%; m. p. 35—36 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 1.39(s, 9H), 2.80(t, *J*=6.4 Hz,

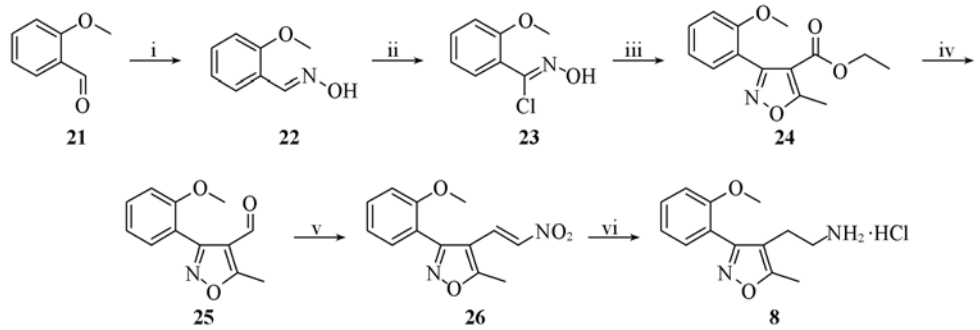
2H), 3.21(q, *J*=6.4 Hz, 2H), 3.66(s, 3H), 3.82(s, 3H), 4.75(brs, 1H), 6.95(d, *J*=8.0 Hz, 1H), 7.00(td, *J*=0.8, 7.6 Hz, 1H), 7.31(td, *J*=1.6, 8.0 Hz, 1H), 7.42(dd, *J*=1.6, 7.6 Hz, 1H), 7.48(s, 1H).

*2.3.9 Synthesis of 2-[4-(2-Methoxyphenyl)-1-methyl-1H-imidazol-5-yl]ethanamine Hydrochloride(**7**)*

Compound **7** was synthesized from compound **20** according to the procedure used to prepare compound **3**, and isolated as a light-yellow solid. Yield 99.1%; m. p. > 240 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ: 2.90(brs, 2H), 3.02(t, *J*=6.8 Hz, 2H), 3.89(s, 3H), 3.82(s, 3H), 7.12(t, *J*=7.2 Hz, 1H), 7.23(d, *J*=8.0 Hz, 1H), 7.39(dd, *J*=1.6, 7.6 Hz, 1H), 7.54(td, *J*=1.6, 8.0 Hz, 1H), 8.19(brs, 3H), 9.18(s, 1H), 14.60(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{13}H_{18}N_3O^+[M+H]^+$ 232.1450; found: 232.1455.

2.4 Synthesis of the Substituted Isoxazol-4-yl Ethamine Hydrochloride

The synthetic routes of the substituted isoxazol-4-yl ethamine hydrochloride are shown in Scheme 4.



Scheme 4 Synthesis of the substituted isoxazol-4-yl ethamine hydrochloride

Reagents and conditions: (i) $NH_2OH{\cdot}HCl$, NaOH, 95% EtOH, H_2O, r. t., 3 h, 87%; (ii) NCS, DMF, r. t., overnight, 78%; (iii) ethyl 3-(pyrrolidin-1-yl)but-2-enoate, TEA, DMF, r. t., overnight, 47%; (iv) (a) $LiAlH_4$, THF, i.b. to r. t., 2 h; (b) PCC, DCM, i.b. to r. t., 6 h, 49% over two steps; (v) CH_3COONH_4, CH_3NO_2, reflux, overnight, 67%; (vi) (a) $LiAlH_4$, THF, reflux, 4 h; (b) 2 mol/L HCl/MeOH, r. t., 1 h, 52% over two steps.

*2.4.1 Synthesis of 3-(2-Methoxyphenyl)-5-methylisoxazole-4-carbaldehyde(**25**)*

Compound **25** was prepared following the procedure reported in the literature[28] from the starting material compound **21**, and isolated as a white solid. Yield for four steps: 15.7%. m. p. 86—87 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 2.76(s, 3H), 3.82(s, 3H), 7.12(d, *J*=8.4 Hz, 1H), 7.39(t, *J*=7.2 Hz, 1H), 7.48—7.58(m, 2H), 9.66(s, 1H).

*2.4.2 Synthesis of 3-(2-Methoxyphenyl)-5-methyl-4-(2-nitrovinyl)isoxazole(**26**)*

To a solution of 3-(2-methoxyphenyl)-5-methylisoxazole-4-carbaldehyde(**25**, 0.8 g, 3.68 mmol) in nitromethane(20 mL) was added ammonium acetate(0.57 g, 7.36 mmol) and the mixture was refluxed overnight. Then, the reaction was monitored by TLC until no starting materials were detected. The suspension was cooled to room temperature and evaporated to dryness *in vacuum*. The residue was dissolved in ethyl acetate and washed with 10% HCl, sat. $NaHCO_3$, and brine three times each. A brown oil was obtained when the organic layer was evaporated under vacuum, which was further purified by flash chromatography on silica gel to get a white solid. Yield 67.4%. m. p. 78—81 °C; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 2.65(s, 3H), 3.80(s, 3H), 6.96(d, *J*=13.6 Hz, 1H), 7.05(d, *J*=8.4 Hz, 1H), 7.10(td, *J*=0.8, 7.6 Hz, 1H), 7.40(dd, *J*=1.6, 7.2 Hz, 1H), 7.54(td, *J*=2.0, 8.4 Hz, 1H), 7.77(d, *J*=13.6 Hz, 1H).

*2.4.3 Synthesis of 2-[3-(2-Methoxyphenyl)-5-methylisoxazol-4-yl]ethanamine Hydrochloride(**8**)*

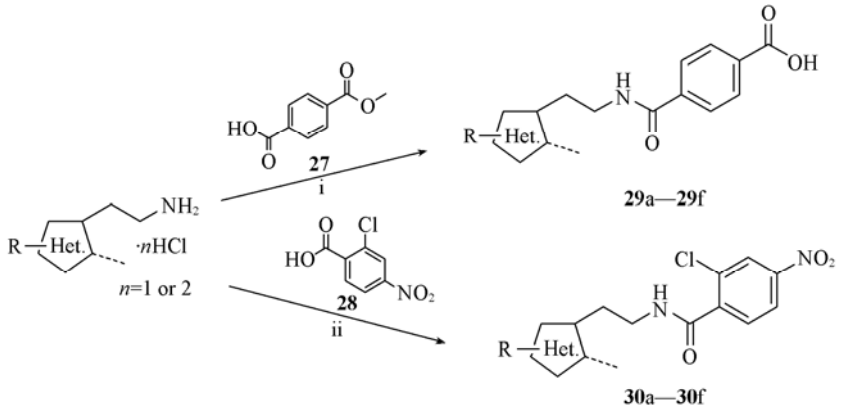
A suspension of lithium aluminium hydride($LiAlH_4$, 0.19 g, 5 mmol) in THF(20 mL) was cooled in an ice bath and a solution of 3-(2-methoxyphenyl)-5-methyl-4-(2-nitrovinyl)-isoxazole(**26**, 0.65 g, 2.5 mmol) in THF(10 mL) was added to it dropwise. The mixture was refluxed for 4 h until the TLC showed no starting materials existing. The suspension was then cooled in an ice bath and water(0.25 mL) was added as well as 10% KOH(0.75 mL). When most THF was evaporated *in vacuum*, the residue was dissolved in ethyl acetate. The organic layer was washed with brine thrice, dried over Na_2SO_4 and evaporated *in vacuum* to get a colorless oil, which was further salified in 2 mol/L HCl/methanol(20 mL) to obtain a white crystal. Yield for two steps: 51.9%; m. p. 180—183 °C; ESI-HRMS, *m/z*: calcd. for $C_{13}H_{17}N_2O_2^+$ $[M+H]^+$ 233.1290; found: 233.1287.

2.5 Synthesis of the Designed Derivatives

The synthetic routes of the substituted phenyl-coulped heterocyclic ethylamide derivatives are shown in Scheme 5.

*2.5.1 General Procedure for the Synthesis of Compounds **29a—29f***

To a suspension of substituted(phenyl coupled) heterocyclic ethylamine hydrochloride in ethanol was added a solution of NaOH(0.084 g, 2.1 mmol) in ethanol dropwise with stirring at room temperature for 1 h. After filtration to remove insoluble substances, the filtrate was evaporated to dryness *in vacuum* and re-dissolved in THF. A suspension of 4-(methoxycarbonyl)benzoic acid(0.43 g, 2.4 mmol) and isobutyl chloroformate(IBCF, 0.33 g, 2.4 mmol) was stirred in an ice bath for 30 min. Then the solution of the corresponding free amine (2 mmol) in THF, and *N*-methylmorpholine(NMM, 0.61 g, 6 mmol) were added to the mixture dropwise successively, and respectively. The mixture was stirred at room temperature overnight, the solution was monitored by TLC until no starting materials were detected, and then evaporated *in vacuum* to



Scheme 5 Synthesis of the substituted phenyl-coulped heterocyclic ethylamide derivatives

Reagents and reactions: (i) (a) NaOH, EtOH, 0 °C to r. t., 1 h; (b) compound **27**, IBCF, NMM, THF, 0 °C to r. t., overnight; (c) $LiOH·2H_2O$, THF/H_2O(2/1, volume ratio), r. t., 3 h, 65%—73% over three steps; (ii) (a) NaOH, EtOH, 0 °C to r. t., 1 h; (b) compound **28**, IBCF, NMM, THF, 0 °C to r. t., overnight, 71%—84% over two steps.

obtain a crude product, which was further purified by flash chromatography on silica gel to get the corresponding methyl ester. The ester was dissolved in THF/H_2O(2/1, volume ratio), treated with $LiOH·2H_2O$(0.13 g, 2.2 mmol) and stirred at room temperature for 3 h until TLC showed no starting materials. The solution was evaporated to dryness *in vacuum* and re-dissolved in H_2O and ethyl acetate. The water layer was adjusted with 10% HCl until the pH was about 2 and the organic layer was separated. The water layer was extracted with ethyl acetate three times. The organic layer was combined and washed with brine three times, dried over Na_2SO_4 and evaporated under vacuum to obtain the final product.

Synthesis of 4-{[2-(1*H*-imidazol-1-yl)ethyl]carbamoyl}-benzoic acid(**29**a): isolated as a white solid; m. p. >240 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 3.65(q, *J*=5.6 Hz, 2H), 4.27(t, *J*=6.0 Hz, 2H), 7.22(s, 1H), 7.44(s, 1H), 7.89(d, *J*=8.0 Hz, 2H), 8.00(d, *J*=8.0 Hz, 2H), 8.29(s, 1H), 8.83(t, *J*=5.2 Hz, 1H), 12.20—14.80(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{13}H_{14}N_3O_3^+[M+H]^+$ 260.1035; found: 260.1047.

Synthesis of 4-{[2-(4-nitro-1*H*-imidazol-5-yl)ethyl]-carbamoyl}benzoic acid(**29**b): isolated as a white solid; m. p. >240 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 3.25(t, *J*=6.4 Hz, 2H), 3.61(q, *J*=6.0 Hz, 2H), 7.69(s, 1H), 7.82(d, *J*=8.0 Hz, 2H), 7.94(d, *J*=8.0 Hz, 2H), 8.80(t, *J*=5.6 Hz, 1H), 12.00—14.60(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{13}H_{13}N_4O_5^+[M+H]^+$ 305.0886; found: 305.0879.

Synthesis of 4-({2-[4-(2-methoxyphenyl)-1*H*-imidazol-5-yl]ethyl}carbamoyl)benzoic acid(**29**c): isolated as a white solid; m. p. 202—204 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 2.76(t, *J*=6.4 Hz, 2H), 3.21(q, *J*=6.4 Hz, 2H), 3.76(s, 3H), 6.96(t, *J*=7.2 Hz, 1H), 7.07(d, *J*=8.0 Hz, 1H), 7.24—7.38(m, 2H), 7.81(s, 1H), 7.87(d, *J*=8.0 Hz, 2H), 7.98(d, *J*=8.0 Hz, 2H), 8.74(t, *J*=6.0 Hz, 1H), 11.60—13.20(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{20}H_{20}N_3O_4^+[M+H]^+$ 366.1454; found: 366.1457.

Synthesis of 4-({2-[4-(2-methoxyphenyl)-1-methyl-1*H*-imidazol-5-yl]ethyl}carbamoyl)benzoic acid(**29**d): isolated as a colorless oil; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 2.90(t, *J*=6.4 Hz, 2H), 3.32(q, *J*=6.4 Hz, 2H), 3.70(s, 3H), 3.87(s, 3H), 6.95(t, *J*=7.2 Hz, 1H), 7.09(d, *J*=8.0 Hz, 1H), 7.27(d, *J*=7.2 Hz, 1H), 7.40(t, *J*=8.0 Hz, 1H), 7.65(s, 1H), 7.78(d, *J*=8.4 Hz, 2H), 7.97(d, *J*=8.4 Hz, 2H), 8.74(t, *J*=6.0 Hz, 1H); ESI-HRMS, *m/z*: calcd. for $C_{21}H_{22}N_3O_4^+[M+H]^+$ 380.1610; found: 380.1621.

Synthesis of 4-({2-[2-(2-methoxyphenyl)-4-nitro-1*H*-imidazol-1-yl]ethyl}carbamoyl)benzoic acid(**29**e): isolated as an amorphous powder; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 2.73(t, *J*=5.2 Hz, 2H), 3.84(s, 3H), 4.07(q, *J*=5.2 Hz, 2H), 7.14(t, *J*=6.8 Hz, 1H), 7.27(d, *J*=8.4 Hz, 1H), 7.38(d, *J*=6.8 Hz, 1H), 7.57(t, *J*=6.0 Hz, 1H), 7.72(d, *J*=8.0 Hz, 2H), 7.96(d, *J*=8.0 Hz, 2H), 8.27(s, 1H), 8.78(t, *J*=5.6 Hz, 1H); ESI-HRMS, *m/z*: calcd. for $C_{20}H_{19}N_4O_6^+[M+H]^+$ 411.1305; found: 411.1314.

Synthesis of 4-({2-[3-(2-methoxyphenyl)-5-methylisoxazol-4-yl]ethyl}carbamoyl)benzoic acid(**29**f): isolated as a colorless oil; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 2.48(s, 3H), 2.77(t, *J*=6.4 Hz, 2H), 3.44(q, *J*=6.4 Hz, 2H), 3.81(s, 3H), 6.11(t, *J*=5.2 Hz, 1H), 6.98—7.09(m, 2H), 7.27(dd, *J*=2.0, 8.4 Hz, 1H), 7.36(td, *J*=2.0, 8.0 Hz, 1H), 7.74(d, *J*=8.4 Hz, 2H), 7.89(d, *J*=8.4 Hz, 2H); ESI-HRMS, *m/z*: calcd. for $C_{21}H_{21}N_2O_5^+[M+H]^+$ 381.1450; found: 381.1447.

*2.5.2 General Procedure for the Synthesis of Compounds **30a—30f***

To a suspension of substituted(phenyl coupled) heterocyclic ethylamine hydrochloride in ethanol was added a solution of NaOH(0.084 g, 2.1 mmol) in ethanol and stirred at room tempe- rature for 1 h. After filtration to remove insoluble substances, the filtrate was evaporated to dryness *in vacuum* and re-dissolved in THF. A suspension of 2-chloro-4-nitrobenzoic acid(0.48 g, 2.4 mmol) and IBCF(0.33 g, 2.4 mmol) was stirred in an ice bath for 30 min and followed by the addition of the corresponding free amine(2 mmol) in THF, and NMM(0.61 g, 6 mmol) dropwise, successively. The mixture was stirred at room temperature overnight, and monitored by TLC until no starting materials were detected, and then evaporated under vacuum to obtain a crude product, which was further purified by flash chromatography on silica gel to get the target compound.

Synthesis of *N*-[2-(1*H*-imidazol-1-yl)ethyl]-2-chloro-4-nitrobenzamide(**30**a): isolated as a white solid; m. p. >240 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 3.74(q, *J*=5.6 Hz, 2H), 4.37(t, *J*=5.6 Hz, 2H), 7.66(s, 1H), 7.70(d, *J*=8.4 Hz, 1H),

7.80(s, 1H), 8.24(dd, *J*=2.0, 8.4 Hz, 1H), 8.34(d, *J*=2.0 Hz, 1H), 8.94(t, *J*=6.0 Hz, 1H), 9.10(s, 1H); ESI-HRMS, *m/z*: calcd. for $C_{12}H_{12}ClN_4O_3^+[M+H]^+$ 295.0598; found: 295.0594.

Synthesis of 2-chloro-4-nitro-*N*-[2-(4-nitro-1*H*-imidazol-5-yl)ethyl]benzamide(**30**b): isolated as a white solid; m. p. 210—212 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 3.23(t, *J*=6.8 Hz, 2H), 3.61(q, *J*=6.8 Hz, 2H), 7.65(d, *J*=8.4 Hz, 1H), 7.75(s, 1H), 8.23(dd, *J*=2.4, 8.4 Hz, 1H), 8.33(d, *J*=2.4 Hz, 1H), 8.84(t, *J*=5.2 Hz, 1H), 13.19(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{12}H_{11}ClN_5O_5^+[M+H]^+$ 340.0449; found: 340.0457.

Synthesis of 2-chloro-*N*-{2-[4-(2-methoxyphenyl)-1*H*-imidazol-5-yl]ethyl}-4-nitrobenzamide(**30**c): isolated as a white solid; m. p. 186—188 °C; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 2.73(t, *J*=5.6 Hz, 2H), 3.50(q, *J*=5.6 Hz, 2H), 3.79(s, 3H), 6.94—7.42(m, 4H), 7.60(s, 1H), 7.66(d, *J*=7.2 Hz, 1H), 8.21(dd, *J*=2.0, 8.4 Hz, 1H), 8.32(d, *J*=2.0 Hz, 1H), 8.74(t, *J*=5.6 Hz, 1H), 12.03(brs, 1H); ESI-HRMS, *m/z*: calcd. for $C_{19}H_{18}ClN_4O_4^+[M+H]^+$ 401.1017; found: 401.1021.

Synthesis of 2-chloro-*N*-{2-[4-(2-methoxyphenyl)-1-methyl-1*H*-imidazol-5-yl]ethyl}-4-nitrobenzamide(**30**d): isolated as a colorless oil; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 3.02(t, *J*=6.4 Hz, 2H), 3.58(q, *J*=6.4 Hz, 2H), 3.70(s, 3H), 3.71(s, 3H), 6.39(t, *J*=5.2 Hz, 1H), 6.88(d, *J*=8.8 Hz, 1H), 6.93(td, *J*=0.8, 7.2 Hz, 1H), 7.27(td, *J*=1.6, 8.8 Hz, 1H), 7.35(dd, *J*=1.6, 7.2 Hz, 1H), 7.38(d, *J*=8.8 Hz, 1H), 7.49(s, 1H), 8.07(dd, *J*=2.0, 8.8 Hz, 1H), 8.21(d, *J*=2.0 Hz, 1H); ESI-HRMS, *m/z*: calcd. for $C_{20}H_{20}ClN_4O_4^+[M+H]^+$ 415.1173; found: 415.1181.

Synthesis of 2-chloro-*N*-{2-[2-(2-methoxyphenyl)-4-nitro-1*H*-imidazol-1-yl]ethyl}-4-nitrobenzamide(**30**e): isolated as an amorphous powder; ^{1}H NMR(400 MHz, DMSO-d_6), δ: 2.73(t, *J*=4.4 Hz, 2H), 3.82(s, 3H), 4.05(q, *J*=4.4 Hz, 2H), 7.11(t, *J*=6.8 Hz, 1H), 7.22(d, *J*=8.4 Hz, 1H), 7.41(d, *J*=6.8 Hz, 1H), 7.50—7.64(m, 2H), 8.23(d, *J*=8.0 Hz, 1H), 8.32(s, 1H), 8.58(s, 1H), 8.80(t, *J*=5.2 Hz, 1H); ESI-HRMS, *m/z*: calcd. for $C_{19}H_{17}ClN_5O_6^+[M+H]^+$ 446.0867; found: 446.0864.

Synthesis of 2-chloro-*N*-{2-[3-(2-methoxyphenyl)-5-methylisoxazol-4-yl]ethyl}-4-nitrobenzamide(**30**f): isolated as a colorless oil; ^{1}H NMR(400 MHz, $CDCl_3$), δ: 2.45(s, 3H), 2.71(t, *J*=6.8 Hz, 2H), 3.44(q, *J*=6.8 Hz, 2H), 3.77(s, 3H), 6.14(t, *J*=5.6 Hz, 1H), 6.94—7.02(m, 2H), 7.30(dd, *J*=2.0, 8.0 Hz, 1H), 7.41(td, *J*=2.0, 8.4 Hz, 1H), 7.60(d, *J*=8.4 Hz, 1H), 8.13(dd, *J*=2.0, 8.4 Hz, 1H), 8.24(d, *J*=2.0 Hz, 1H); ESI-HRMS, *m/z*: calcd. for $C_{20}H_{19}ClN_3O_5^+[M+H]^+$ 416.1013; found: 416.1007.

2.6 Biological Assays

2.6.1 Cells and Viruses

Madin-Darby canine kidney(MDCK) cells were maintained in Dulbecco's modified Eagle medium(DMEM) containing 10% fetal bovine serum in a humidified 5% CO_2 incubator at 37 °C. Influenza A/PR/8/34(H1N1) was propagated in 10-day-old chicken egg embryos at 37 °C and harvested for 48 h after inoculation in pooled allantoic fluid. After a brief centrifugation(3000 r/min at room temperature for 20 min) and a virus titer measurement by hemagglutination test, the virus was aliquot and stored at a –80 °C freezer.

2.6.2 MDCK Cell Based Cell Toxicity Assays

In brief, MDCK cells were added to 96-well plates at a density of 4000 cells per well and cultured for 24 h. The newly synthesized compounds were dissolved in DMSO and diluted to 6 consecutive 3-fold dilutions. Then the compounds were added to the above wells and all the plates were allowed to culture for 72 h. Free DMSO with the same concentration was used as negative control and Nucleozin, Arbidol and Ribavirin were served as positive controls. At the end of the 72-h incubation, the cytopathic effect(CPE) of cells was observed. Cell viability was determined by the alamarBlue® method.

2.6.3 MDCK Cell Based Viral Inhibition Assays

In brief, MDCK cells were added to 96-well plates at a density of 4000 cells per well. After 24 h, the plates were infected by the influenza A(A/PR/8/34 H1N1 strain) virus for 2 h. The newly synthesized compounds were dissolved in DMSO and diluted to 6 consecutive 3-fold dilutions. Then the compounds were added to the above wells and all the plates were allowed to culture for 48 h. Free DMSO with the same concentration was used as negative control while Ingavirin, Arbidol and Ribavirin were utilized as positive control. At the end of the 48 h incubation, the cytopathic effect(CPE) of cells was observed. Cell viability was determined by the 4-methylumbelliferyl-*α*-*N*-acetyl-neuraminate(MUNANA) method.

2.7 Data Processing and Molecular Docking Study

The CC_{50} and EC_{50} values were calculated *via* nonlinear regression using GraphPad Prism 5.

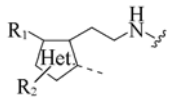
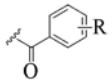
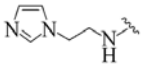
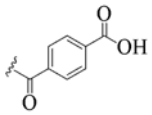
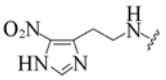
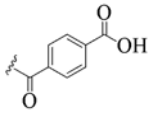
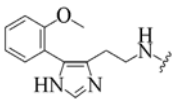
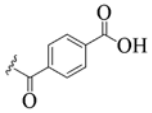
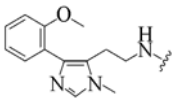
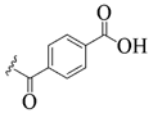
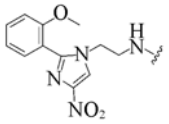
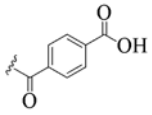
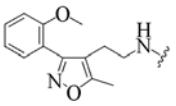
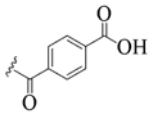
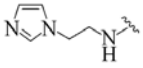
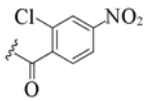
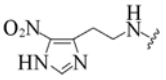
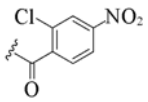
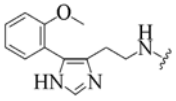
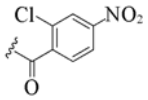
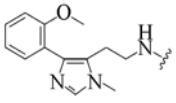
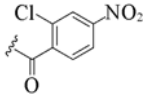
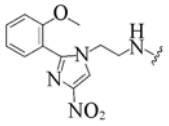
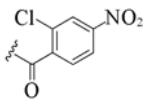
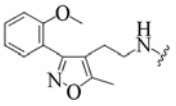
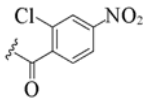
Schrodinger 2013-1 was used to perform the docking simulations. From the RCSB Protein Data Bank, the crystal structure of influenza A virus nucleoprotein(PDB ID: 3RO5) in complex with ligands was retrieved. Afterwards, "Protein Preparation Wizard" in Maestro-8.5(Schrodinger's 2013-1) was used to remove the water molecules and add the polar hydrogens atoms. The protocol was generated based on the ligand in the crystal structure. Other parameter referring the default values was set. Ten poses were generated for each ligand and the lowest energy conformation of each ligand-protein complex was selected for analyzing the interactions between the virus NP and the inhibitor.

3 Results and Discussion

3.1 Anti-influenza A Virus Activity

Although all the active compounds show less activity than the positive control Nucleozin, twelve compounds show antiviral activity against influenza virus A(Table 1). The optimal activities of compounds **30**c and **30**d against influenza A virus can be observed with EC_{50} values of 19.9 and 65.4 μg/mL, and selectivity index(SI) values of 5.18 and 1.74, respectively. They are on the same order of magnitude as that of Ribavirin, indicating that compounds **30**c and **30**d are much more potential than the other positive controls(Ingavirin and Arbidol). These results indicate that the substituted phenyl-coupled

Table 1 Anti-influenza(A/PR/8/34 H1N1 strain) virus activities and cytotoxicities of the derivatives[a]

Compd.			Cytotoxicity	Anti-influenza virus activity	
			CC_{50}[b]/(μg·mL^{-1})	EC_{50}[c]/(μg·mL^{-1})	SI[d]
29a			—[e]	>65	> or x1[f]
29b			—	>125	x1[g]
29c			—	>125	x1
29d			—	>125	x1
29e			—	>41.67	> or x1
29f			—	>125	x1
30a			—	>65	> or x1
30b			—	>125	x1
30c			103	19.9	5.18
30d			113.7	65.4	1.74
30e			—	>41.67	> or x1
30f			—	102.4	>4.88
Ingavirin			—	1191	>8.39
Nucleozin			—	0.03	>694.33
Arbidol			—	6180	>0.81
Ribavirin			62.72	12.35	6.37

a. All the data were the average values from three independent assays; *b*. compound concentration that reduces cell viability by 50% relative to uninfected MDCK cells; *c*. compound concentration that reduces viral replication by 50% relative to infected MDCK cells; *d*. selectivity index: CC_{50}/EC_{50}; *e*. the highest test concentration(500 μg/mL) of the analogues was less than the CC_{50} and the highest test concentrations of some analogues were less than 500 μg/mL; *f*. the highest test concentration that reduces viral replication by 50% relative to infected MDCK cells was lower than the highest test concentration that reduces cell viability by 50% relative to uninfected MDCK cells[29]; *g*. the highest test concentration that reduces viral replication by 50% relative to infected MDCK cells was equal to the highest test concentration that reduces cell viability by 50% relative to uninfected MDCK cells[29].

heterocyclic ethylamide skeleton is a promising structure for the NP inhibitors.

3.2 Cytotoxicity

Cytotoxic activities of the compounds against MDCK cells were also evaluated to monitor the potential cytotoxicity effects. Most of the derivatives show no obvious cellular growth inhibition against MDCK cells at concentrations below 500 μg/mL(the highest test concentration); while Ribavirin displays moderate toxicity against MDCK cells(CC_{50}=62.72 μg/mL). It is worth mentioning that the cytotoxicity of compound **30**f is much lower than the test concentration, though the viral inhibition of it is lower than that of Ribavirin, indicating that compound **30**f is a much lower-cytotoxic compound than Ribavirin and bears a great selectivity index.

3.3 Molecular Docking Study

The X-ray crystal structure of influenza A virus nucleoprotein complex with inhibitors was disclosed. It was demonstrated that six inhibitors bridged two molecules of NP(NP_A and NP_B) protein to form a stable dimer complex[17]. A computational study was performed using Schrodinger 2013-1 to investigate the differences of potential interactions between compounds **30**c, **30**d, and Nucleozin and virus nucleoprotein(PDB ID: 3RO5). The results indicate that compounds **30**c, **30**d and Nucleozin have similar action modes. Although the 2-chlor-4-nitrophenyl fragments of three molecules overlapped well, there is a discrepancy on the other side of the molecule, which might be the reason why there is a disparity in the anti-influenza viral activity(Fig.3). As shown in Fig.4, a strong π-π stacking interaction has been formed between 4-nitro-2-chloro-phenyl moiety and TYR289. The pyrazine ring of Nucleozin also plays a crucial role in making the molecule into a more stable conformation so that Nucleozin could link the two trimers more steadily. This gives a higher inhibitory activity. The diversity of the binding modes has aroused our great interests and encourages us to expansion additional study on this topic despite the fact that all the compounds we reported here have lower activity than Nucleozin.

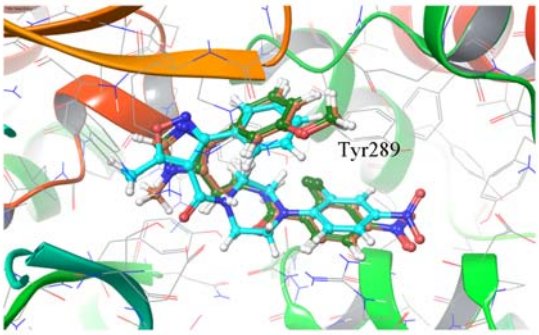


Fig.3 Orientation of the best docked poses of compounds 30c, 30d and Nucleozin in the active site of the viral nucleoprotein

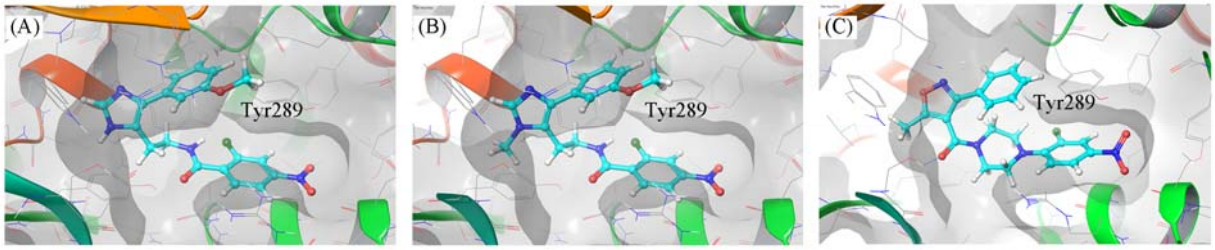


Fig.4 Interaction mode between viral nucleoprotein and compunds 30c(A), 30d(B) and Nucleozin(C)
Hydrogen bonds are highlighted with a dashed line.

4 Conclusions

In summary, we successfully designed and synthesized a series of substituted phenyl-coupled heterocyclic ethylamide derivatives as new anti-influenza A agents based on the chemical structures of Ingavirin and Nucleozin. This new series of low-toxicity anti-influenza viral compounds, most compounds exhibited moderate antiviral activity against the test influenza A virus strain and lower toxicity against MDCK cells than Ribavirin(CC_{50}=62.72 μg/mL). Compounds **30**c, **30**d and **30**f displayed obvious anti-influenza virus activities compared to the positive control Arbidol(EC_{50}=6180 μg/mL) and Ingavirin(EC_{50}=1191 μg/mL).

Importantly, the methyl group of the isoxazole ring was reported to be indispensable for the anti-influenza activity of Nucleozin, and the change in the methyl group would lead to the loss of antiviral activity[30]. However, we found that compound **30**c without the methyl group was 3-fold more potential than compound **30**d. This clearly showed a new approach to targeting NP and offered many more motifs for the future design and development.

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