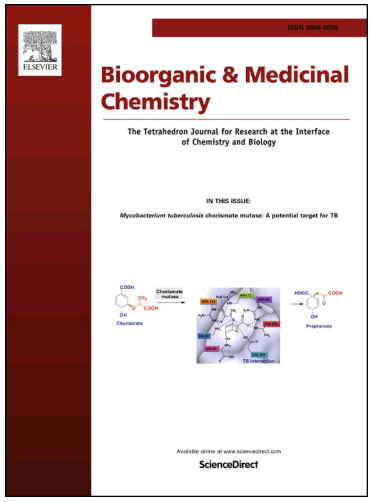
Accepted Manuscript



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Discovery of acylguanidine oseltamivir carboxylate derivatives as potent neuraminidase inhibitors

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Abstract:

In search of novel anti-influenza agents with higher potency, a series of acylguanidine oseltamivir carboxylate analogues were synthesized and evaluated against influenza viruses (H1N1 and H3N2) *in vitro*. The representative compounds with strong inhibitory activities (IC_{50}<40nM) against neureminidase (NA) were further tested against the NA from oseltamivir-resistant strain (H259Y). Among them, compounds **9** and **17** were potent NA inhibitors that exhibited a 5 and 11-fold increase in activity comparing with oseltamivir carboxylate (**2**, OC) against the H259Y mutant, respectively. Furthermore, the effect against influenza virus H259Y mutant (H1N1) replication and cytotoxicity assays indicated that compounds **9** and **17** exhibited a 20 and 6-fold increase than the parent compound **2**, and had no obvious cytotoxicity *in vitro*. Moreover, the molecular docking studies revealed that the docking modes of compounds **9** and **17** were different from that of oseltamivir, and the new hydrogen bonds and hydrophobic interaction were formed in this case. This work provided unique insights in the discovery of potent inhibitors against NAs from wild-type and oseltamivir-resistant strains.

Keywords: Influenza viruses; Neuraminidase inhibitors; Acylguanidine oseltamivir carboxylate; H1N1; H3N2; Oseltamivir-resistant strain (H259Y)

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1. Introduction

Influenza has been a serious threat to human health for centuries. Influenza viruses were found around the world and infected millions of people annually, leading to life threatening respiratory illness. Three pandemic influenza outbreaked during the 20th century, the 1918 Spanish flu, the 1957 Asian flu and the 1968 Hong Kong flu, which represented three different antigenic subtypes of influenza A virus: H1N1, H2N2, and H3N2, respectively.[1-3] In 2009, the swine-origin H1N1 influenza virus spread rapidly throughout the world, bringing people into a renewed panic.[4] Even worse, since the influenza viruses evolve rapidly, H5N1, H7N9 and H10N8 influenza viruses were identified constantly in recent years.[5] Strategies for combating influenza mainly aimed at the M2 ion channel protein and neuraminidase (NA), which were essential for viral replication. However, the M2 ion channel protein inhibitors were no longer recommended for the treatment of influenza infection due to their severe side effects and drug resistance.[6] The NA, a surface glycoprotein of influenza viruses, facilitates the release of viral progeny from infected cells and accelerates viral invasion of the upper airways.[7]

NA is a good therapeutic target because it's active site is relatively well conserved across the influenza A and B viral strains.[7, 8] The NAs are phylogenetically categorized into two groups: group 1 (N1, N4, N5, and N8 subtypes) and group 2 (N2, N3, N6, N7, and N9 subtypes).[9] To date four neuraminidase inhibitors (NAI) have been currently approved for the treatment or prophylaxis of influenza infections in some parts of the world: oseltamivir (**1**),[10] zanamivir (**3**),[11] peramivir (**4**)[12] and laninamivir octanoate (**5**).[13]

Oseltamivir (Tamiflu^{TM}) is the first orally available anti-influenza drug, which is

readily converted to oseltamivir carboxylate (**2**, OC) by endogenous esterases, as the real active form widely distributed in the body. The crystal structure of OC (**2**) bound to NA was investigated by X-ray.[14] The carboxylate was held strongly by Arg292, Arg371 and Arg118. The amino group formed strong hydrogen bond with Glu119 and Asp151. The hydrophobic interaction was formed between the 3-pentyloxy side chain and four amino acid residues (Ile222, Arg224, Ala246 and Glu276). The oxygen atom of the amide interacted with Arg152. However, the oseltamivir-resistant influenza viruses have emerged in recent years.[8,15] Some oseltamivir-resistant mutations have been identified, such as H274Y, I117V, E119A, R292K and so on.[16] The mutations may lead to changes in the interaction effect between functional groups of OC (**2**) and amino acid residues of NA. These emerging problems of oseltamivir increased the demand for the development of novel anti-influenza NA inhibitors.

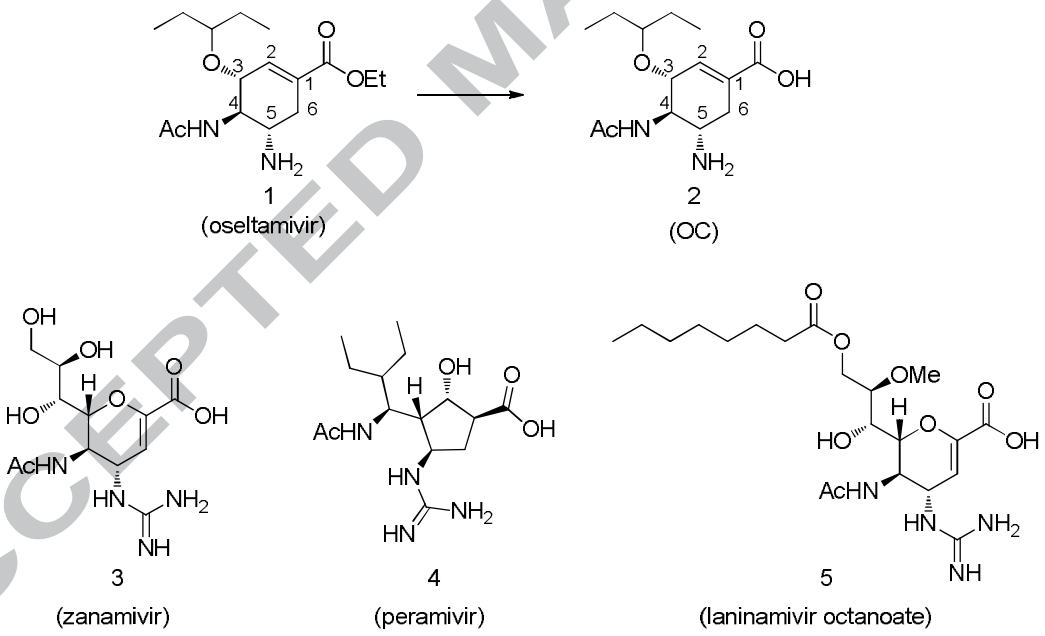


Figure 1. Structures of clinically used NA inhibitors

Fortunately, new characteristics have been found through the analysis of the X-ray crystal structures of NAs. Russell's team found that group-1 and group-2 NAs were structurally distinct, and group-1 NAs contained a so-called "150-cavity" adjacent to

their active sites.[17] In addition, 371-cavity and 430-cavity were also reported by Wong and the Amaro team, respectively.[18-20] These structure characteristics implied additional interactions between NAs and their inhibitors, suggesting the design of new anti-influenza drugs should take advantage of 150-, 371- and 430- cavities. For fully exploiting 150-pocket found in group-1 NA, the groups of Pinto[21], Martin[22] and Xu[23] designed various kinds of oseltamivir analogues bearing different substitutents at the C-5 position of oseltamivir and the results were satisfactory. Wong pointed that tamiphosphor derivatives exhibited potent inhibitory activities against influenza viruses because the 371-cavity could accommodate the alkyl substituents of tamiphosphor monoesters.[20] Lin and co-workers synthesized a series of acylguanidine-modified zanamivir analogs and evaluated their inhibitory activities against the NAs of influenza viruses (H1N1 and H3N2).[24, 25] The results showed that acylguanidine-modified zanamivir analogs bearing hydrophobic groups exhibited inhibitory activity against NAs at the nanomolar scale. Though these compounds didn't show selectivity between H1N1 and H3N2, they correlated well with Amaro's research findings that the 150-cavity may exist not only in group-1 NAs but also in group-2 NAs.[26]

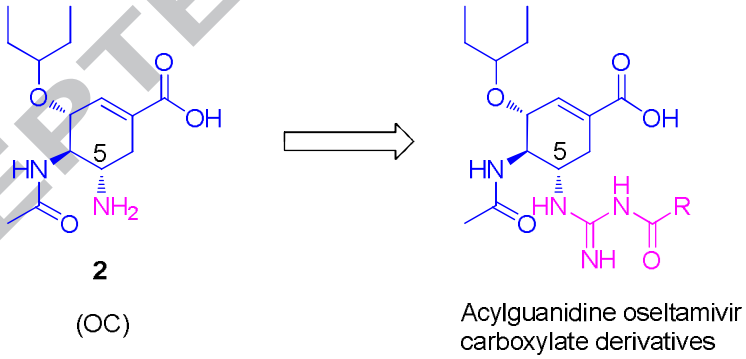


Figure 2. Design of acylguanidine oseltamivir carboxylate derivatives

Enlightened by these newly revealed sites of NA and Lin's work[24, 25], we attempted to find novel anti-influenza agents with higher potency and displayed much more advantages.Therefore, we supposed that more potent NA inhibtors may be obtained by introducing acylguanidine into oseltamivir carboxylate. Making good use of the

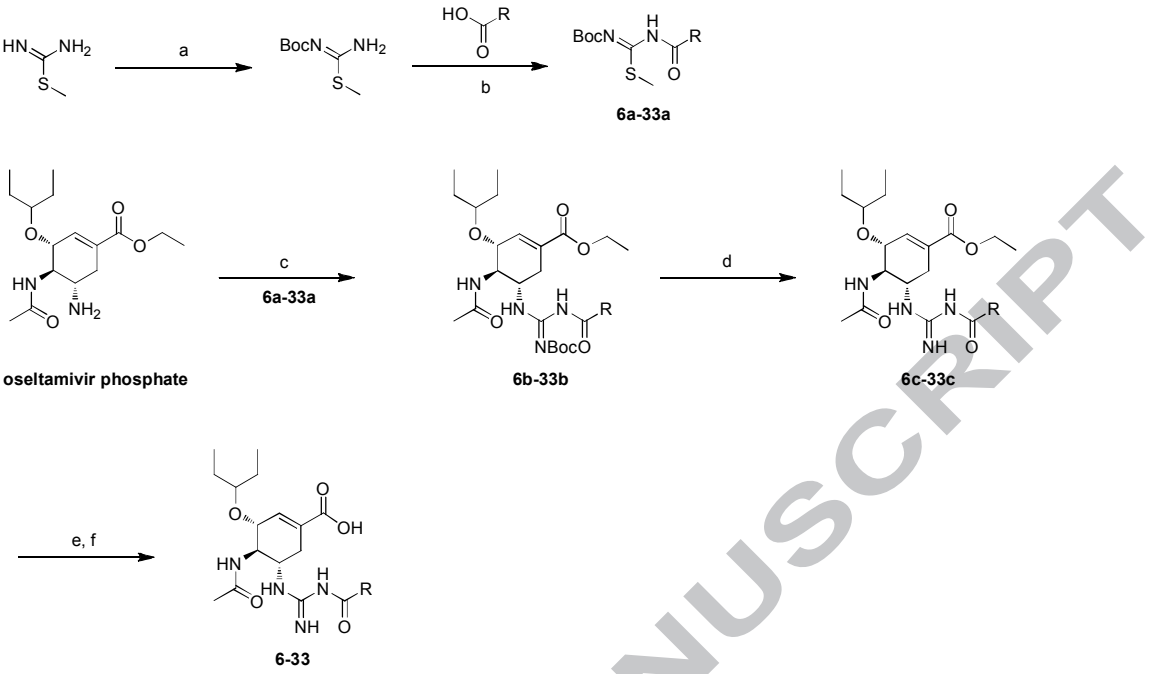
150-cavity, 371-cavity or 430-cavity of the neuraminidase, we modified the C-5 amino group of oseltamivir by substituted acylguanidine analogues for improving interaction with NA (Figure 2) and evaluated the activities of the derivatives against NAs from wild-type and oseltamivir-resistant strains (H259Y). Thus a series of acylguanidine oseltamivir carboxylate derivatives were synthesized and further evaluated for their biological activities.

2. Results and discussion

2.1 Chemistry

The synthetic route of acylguanidine oseltamivir carboxylate derivatives was shown in Scheme 1. Compounds **6a-33a** were prepared according to the previously reported method.[27] Then, commercially available oseltamivir phosphate was used as the starting material to install the acylguanidine to the C-5 position of oseltamivir. The oseltamivir phosphate was subjected to react with **6a**-**33a** in the presence of Mercury(II) chloride ($HgCl_2$), triethylamine (Et_3N) and *N*,*N*-dimethyl formamide (DMF) to give compounds **6b**-**33b**, which were subsequently treated with trifluoroacetic acid (TFA) to afford acylguanidine oseltamivir derivatives **6c**-**33c**. Finally, the hydrolysis of **6c**-**33c**, using the weak base LiOH in H_2O-THF (v/v=1/1) or K_2CO_3 in H_2O-ethanol (v/v=1/4)[24], offered the target acylguanidine oseltamivir carboxylate derivatives in total yield of 40–90% by pre-HPLC.

For the formation of acylguanidine bond, our attempts for the direct coupling reaction of the carboxyl group with the guanidino group failed, even using promoting agents benzotriazol-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) (Scheme 2, Supporting Information). In addition, the strategy that compounds **6b-33b** were directly hydrolysed followed by removal of the Boc protecting group with TFA to afford **6-33**, was not viable because the acylguanidine group protected by Boc was unstable to the alkali (Scheme 3, Supporting Information).



Scheme 1. General route for the synthesis of acylguanidine oseltamivir carboxylate derivatives.

Reagents and conditons: (a) Boc_2O, NaOH, *t*-BuOH, rt; (b) PyBOP, NMM, DMF, rt; (c) $HgCl_2$, Et_3N, DMF, rt; (d) TFA/CH_2Cl_2, rt; (e) LiOH, THF, H_2O, rt; (f) HPLC purify.

2.2 *In vitro* inhibitory activities on NAs

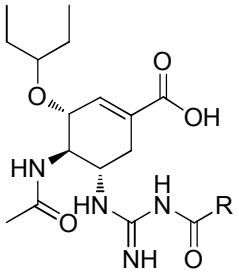
The H1N1 and H3N2 were the representatives of group-1 and group-2 NAs. The inhibitory activities of synthetic compounds **6-33** against the H1N1, H3N2 and H1N1(H259Y) NAs were evaluated, and the results are shown in Table 1. OC (**2**) was used as the positive control with IC_{50} values of 0.76 nM, 0.80 nM and 169.3 nM against H1N1, H3N2 and H1N1(H259Y), respectively. Acylguanidine oseltamivir carboxylate derivatives were found to be less potent than parent compound OC (**2**) against H1N1 and H3N2 NAs and didn't display selectivity for H1N1 and H3N2 NAs. The results were similar to Lin's work[24, 25] and Amaro's research findings[26]. However, to our delight, part of the synthesized compounds showed better inhibitory activities against the H1N1(H259Y) NA than OC (**2**). The further structure activity relationship (SAR) studies showed that the inhibitory activities of these compounds mainly depended on the kinds and positions of the substituents on acylguanidine. Compounds

6-11, with alkyl substituents at the acylguanidine group, had moderate inhibitory activities against all three NAs, and the length of alkyl chain had no obviously impact on the activity. Interestingly, compound **9** bearing unsaturated double bonds at the terminal of the long alkyl chain, was 5-fold (30.5 nM) more potent than OC (169.3 nM) against H1N1(H259Y) NA, and still maintained inhibitory activities against H1N1 and H3N2 NAs with IC_{50} values of 8.1 nM and 5.4 nM respectively. When the steric hindrance of alkyl substituents increased (compounds **10**, **11**), the inhibitory activities gradually decreased, especially against the H1N1 (H259Y) NA. Unfortunately, compounds **19-33**, bearing various aryl substitents, exhibited significantly decreased activities aganist NAs from wild-type strains except compounds **25** and **31-33**, and compounds **31-33** also remained activity against H1N1(H259Y) NA. The SARs of these substituted compounds seemed intricate, mainly depending on the types and positions of the aryl substituents. However, when a methylene unit was introduced between the acylguanidine and aromatic ring (compounds **12-15**), the inhibitory activities against H1N1 and H3N2 NAs were significantly improved, especially compound **14** with a halogen atom (Br) on the benzene ring. At the same time, compound **14** also showed good inhibitory activity against H1N1 (H259Y) with a 3-fold increase relative to OC. Although the potency of inhibition decreased slightly with longer linkers between the acylguanidine and aromatic ring (compounds **16**, **18**), compound **17** with the methoxyl substituents at the C-3 and C-4 positions of the benzene ring, was the most potent NA inhibitor among all these tested compounds. It showed good inhibitory activities against H1N1 (IC_{50}=1.43 nM) and H3N2 (IC_{50}=3.64 nM), and it also exhibited a approximately 11-fold increased activity (IC_{50}=14.47nM) than OC (IC_{50}=169.3 nM) against H1N1(H259Y) NA. Furthermore, the heteroaromatic ring substituented compounds (e.g. pyridine compounds **32, 33**) also displayed good inhibitory activity against H1N1 (H259Y) with a 4-8 folds increase compared with OC.

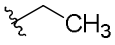
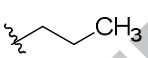
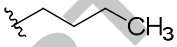
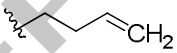
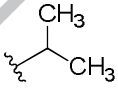
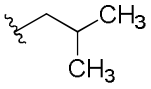
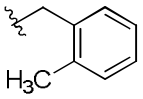
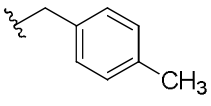
In contrast to the OC (**2**) , most acylguanidine oseltamivir carboxylate derivatives not merely maintained potent inhibitory effects against NAs from wild-type strains but also enhanced activities against NA from the resistant strain (Table 1), especially

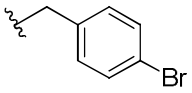
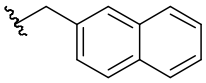
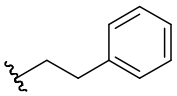
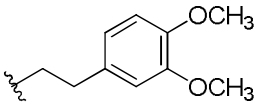
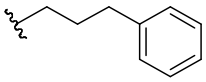
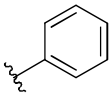
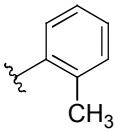
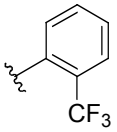
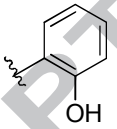
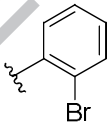
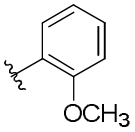
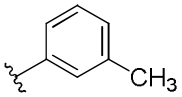
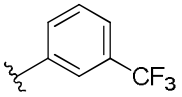
compounds **9** and **17** which exhibited a 5-fold and 11-fold increase, respectively. Furthermore, cell-based assays further indicated that compounds **9** and **17** were safe in vitro (Table 2).

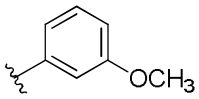
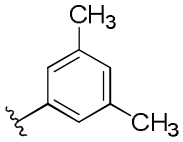
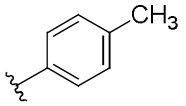
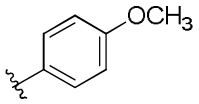
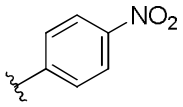
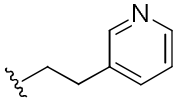
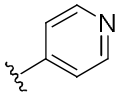
Table 1. Inhibitory activities of novel acylguanidine oseltamivir carboxylate derivatives against NAs



6-33

Compd	R	H1N1[a] IC_{50} (nM)[d]	H3N2[b] IC_{50} (nM)	H1N1-H259Y[c] IC_{50} (nM)
OC(**2**)		0.76±0.06	0.80±0.08	169.3±9.1
6		39.1±1.3	11.4±0.8	74.2±3.4
7		34.8±1.6	11.2±0.8	132.9±7.7
8		37.0±1.8	6.0±0.5	163.3±5.0
9		8.1±0.6	5.4±0.3	30.5±2.0
10		38.2±2.6	13.7±1.1	132.2±5.6
11		88.3±13.8	18.4±1.0	445.2±18.5
12		86.5±7.8	22.8±2.1	225.1±23.6
13		29.4±1.8	9.5±0.7	131.9±10.6

14		14.5±1.8	8.0±0.9	40.6±3.4
15		21.1±3.7	7.2±0.9	66.7±12.2
16		30.1±2.1	9.4±1.7	113.7±18.2
17		1.43±0.21	3.64±0.35	14.47±0.59
18		64.9±5.0	21.4±1.4	221.1±20.1
19		573.8±30.3	334.2±58.0	ND[e]
20		>1000	>1000	ND
21		>1000	911.1±98.7	ND
22		49.8±4.9	184.7±25.1	ND
23		>1000	>1000	ND
24		53.5±4.6	125.2±13.9	ND
25		11.5±1.9	19.5±3.5	>1000
26		216.5±15.5	76.1±4.0	ND

27		175.3±15.2	533.2±47.6	ND
28		155.3±18.1	158.9±10.8	ND
29		>1000	>1000	ND
30		68.6±10.6	214.6±25.1	ND
31		20.1±3.0	14.2±2.0	57.8±4.8
32		10.2±1.8	14.6±2.2	21.1±3.2
33		15.2±2.4	16.1±2.0	36.8±4.6

[a] A/PuertoRico/8/1934(H1N1). [b] A/Hong Kong/498/97(H_3N_2). [c] A/PuertoRico/8/1934 (H259Y, NA resisitant strain). [d] IC_{50}: compound concentration required to inhibit virus production by 50%, as determined by NA activity. [e] ND: not determined.

2.3 Docking analysis

To better understand the effect of acylguanidine group on activity, computer docking studies of the inhibitors with NA were performed. The binding models of compounds **9** and **17** with the H1N1 enzyme were analyzed using Auto Dock Vinaprogam. As shown in Figure 3A, the acylguanidine moiety of compounds **9** and **17** interacts with the space nearby the 150- cavity and 430- cavity by hydrophobic interaction. In addition, to avoid the steric hindrance, the docking conformations of compounds **9** and **17** are different from the docking mode of oseltamivir, as shown in Figure 3B and 3C. Though the structures of compounds **9** and **17** are distorted in active pockets, compound **9** forms five hydrogen bonds with the residues Arg118, Asp151, Ser179, Arg292 and Arg371 and compound **17** also forms five hydrogen

bonds with the residues Arg118, Glu119, Asp151, Arg371 and Tyr406. Furthermore, comparing to compound **9**, pi-pi stacking effect may be formed between the benzene ring of compound **17** and Tyr347. The pi-pi stacking effect may illustrate the slight better inhibitory activity of compound **17** against the H1N1(H259Y) NA. Of course, the methoxyl moiety may also play a role in the action. Overall, although the modification of the guanidino group of oseltamivir results in the loss of interaction with the original active pockets, the new formed H-bonds and hydrophobic interaction, between the acylguanidine moiety and the space nearby the 150-cavity and 430-cavity, compensates for this loss in binding energy.

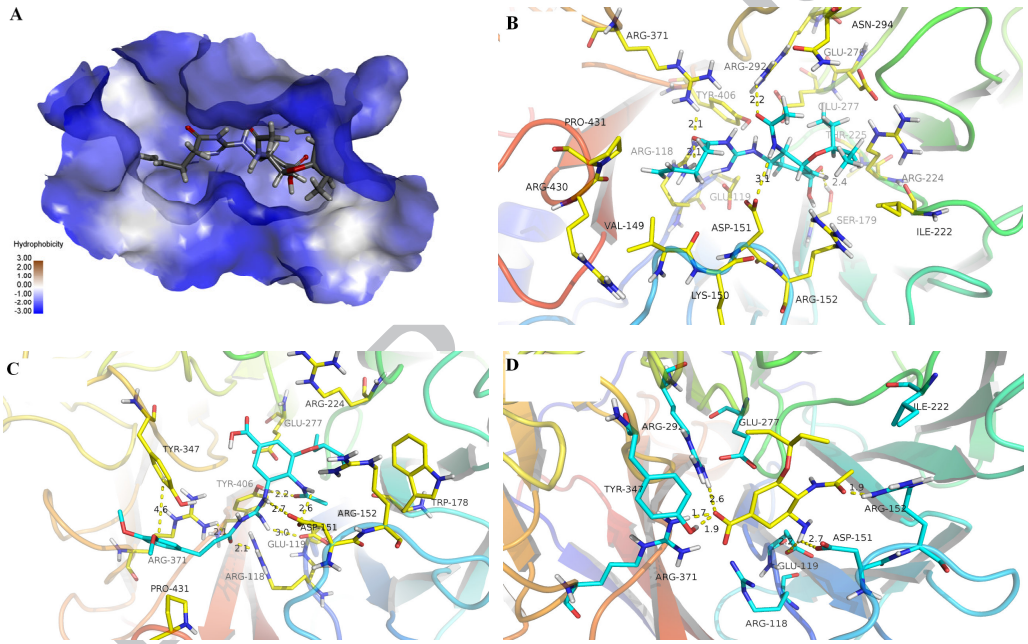


Figure 3. Computational conformations of the compounds in H1N1 neuraminidase. Molecular surface of H1N1 neuraminidase with bound compound **9** (A) and the docking results of compounds **9** (B), **17** (C), and oseltamivir (D) with H1N1 neuraminidase (PDB ID: 2HU4).

2.4 *In vitro* anti-influenza virus activity

Next, we evaluated the anti-influenza activity and cytotoxicity of representative compounds (**6**, **9**, **14**, **15**, **17**, **31**) by cell-based assays (Table 2). The results showed that these compounds could inhibit influenza virus replication *in vitro* and had no

obvious cytotoxicity. The selective values (SI) of these compounds were all higher than OC (**2**), especially compound **9**. These promising results in cells correlated well with their high inhibitory activities against NAs, suggesting these compounds inhibited influenza virus replication by binding to NAs. All the tested compounds showed better inhibitory activities than OC (**2**) in cells infected by H3N2 subtype except compound **31**. In addition, the inhibitory effects of these compounds were significantly improved against the H259Y mutant (H1N1), as comparing with OC (**2**), especially compounds **9** and **17** which exhibited a 20 and 6-fold increase, respectively. Interestingly, compounds **9** and **15** showed better inhibitory activities against the H259Y mutant (H1N1) than compounds **17** using cell-based assays, disagreeing with inhibitory activities against NA. The prominent anti-infulenza activities together with the inhibitory activities against NAs indicate that compounds **9** and **17** are promising for further development of novel anti-influenza drugs.

Table 2. Potency against influenza virus replication and cytotoxicity of compounds by cell-based assays

Compd	H1N1[a] EC_{50} (nM)	H3N2[b] EC_{50} (nM)	H1N1-H259Y[c] EC_{50} (nM)	CC_{50}(nM)	SI[d]
OC(**2**)	8.26	14.77	151.7	> 1000	> 6.6
6	23.88	5.30	37.32	> 1000	> 26.8
9	24.52	9.45	7.18	> 1000	> 139.3
14	12.88	2.58	69.08	> 1000	> 14.5
15	8.45	3.95	11.29	> 1000	> 88.6
17	20.06	5.62	23.02	> 1000	> 43.4
31	159.5	39.17	135.7	> 1000	> 7.4

[a] A/PuertoRico/8/1934(H1N1). [b] A/Hong Kong/498/97(H_3N_2). [c] A/PuertoRico/8/1934 (H259Y, NA resisitant strain). [d] SI (selective index) is determined by the ratio between CC_{50} and EC_{50} (H1N1-H259Y) values.

3. Conclusion

In summary, a series of acylguanidine oseltamivir carboxylate derivatives were synthesized, which showed potent NAs (H1N1 and H3N2) inhibitory effect, especially some compounds exhibited potent inhibition of influenza NAs from the oseltamivir-resistant strain. Among which, compounds **9** and **17** showed the best inhibitory activities against NAs, with IC_{50} values of 30.5 and 14.5 nM, respectively. Though the IC_{50} values of **9** and **17** slightly lower than OC , they exhibited a 5-fold and 11-fold increase in activity against the H259Y mutant. Furthermore, cell-based assays futher illustrated that compounds (**6**, **9**, **14**, **15**, **17**, **31**) were safe and could inhibit influenza virus (H1N1, H3N2 and H1N1- H259Y) replication *in vitro*. Comparing with oseltamivir carboxylate, the inhibitory activities of the compounds **9** and **17** significantly improved with EC_{50} values of 7.18 and 23.02 nM against the H259Y mutant (H1N1), exhibiting a 20 and 6-fold increase, respectively. All the results suggest that compounds **9** and **17** are promising for further development of novel anti-influenza drugs. Moreover, The docking results displayed that the new formed H-bond and the hydrophobic interaction of compounds **9** and **17**, between the acylguanidine moiety and the space nearby the 150-cavity and 430-cavity of NAs, maybe compensate for loss in binding energy, comparing with oseltamivir carboxylate (**2**). We are interested in investigation the active compounds for further development of novel anti-influenza drugs.

4. Experimental sections

4.1 General information

All the reagents were commercially available and used without further purification unless otherwise stated. Reactions and fractions were monitored by thin-layer chromatography on silica gel (GF-254). Flash chromatography was performed on silica gel (300–400 mesh). Some products were prepared by RP-HPLC employing an semi-preparative C_{18} column and a water/acetonitrile gradient moving from 5% to 95% MeCN (0.1% formic acid or trifluoroacetic acid) over 25 min (flow rate 1.0 mL/min). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a BRUKER-ACF-300 or BRUKER-ACF-400 instrument (chemical shifts are expressed as d values relative to

TMS as internal standard). The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad) and dd (doublet of doublets). HRMS analysis was performed using an Agilent 6520 Q-TOF LC/MS spectrometer (Agilent, Germany).

4.2 General procedure for the preparation of **6-33**

Compounds **6a-33a** were prepared according to the previously repored method.[27]

A mixture of oseltamivir phosphate (164 mg, 0.4 mmol), appropriate S-methylisothiourea derivatives (**6a-33a**, 0.4 mmol) and Et_3N (0.166 mL, 1.2 mmol) in anhydrous DMF (4.0 mL) was stirred for 0.5 h and then added $HgCl_2$ (130 mg, 0.48 mmol) at 0°C. The mixture was stirred overnight at room temperature, then diluted with ethyl acetate (40 mL), and filtered through Celite. The filtered solution was washed with water and brine, and the organic layer was dried and concentrated. The residue was purified by column chromatography (PE/EA mixtures) to yield **6b-33b**. To a mixture of **6b-33b** (0.2 mmol) in CH_2Cl_2 (2 mL) at room temperature was added TFA (2 mL), and the reaction mixture was stirred for 6 h at room temperature. After that, the solvent was removed completely under reduced pressure, the oily residue was repeatedly dissolved in CH_2Cl_2 and the solvent was evaporated to remove TFA. A slightly white solid precipitated, after the addition of diethyl ether. Then, the white solid was filtered, washed with cold diethyl ether and dried in vacuo to afford compounds **6c-33c**. To a mixture of **6c-33c** (0.15 mmol) in tetrahydrofuran (4 mL) and water (4 mL) at room temperature was added 1 N LiOH (0.6 mL, 0.6 mmol). The reaction mixture was stirred for 2h, then, neutralized with 1 M aq HCl and concentrated. The residues were purified by preparative HPLC to yield the desired product **6-33** in total yield of 40–90%.

(3R,4R,5S)-4-acetamido-5-(2-propionylguanidino)-3-(pentan-3-yloxy)cyclohex-1-ene -1-enecarboxylic acid (**6**).

White solid, 69%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.00 (1H, d, J = 8.5 Hz), 6.68(1H, s), 4.12-4.10 (1H, m), 4.04-3.94 (1H, m), 3.91-3.85 (1H, m), 3.62-3.59

(1H, m), 2.72 (1H, dd, *J* = 17.4 Hz, 4.7 Hz), 2.42 (2H, q, *J* = 7.3 Hz), 2.34-2.28(1H, m), 1.83 (3H, s), 1.50-1.37 (4H, m), 1.03 (3H, t, *J* = 7.3 Hz), 0.87-0.79 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 176.8, 170.4, 167.9, 153.8, 136.8, 129.5, 81.8, 74.6, 52.7, 49.9, 30.2, 29.6, 26.2, 25.7, 23.1, 9.9, 9.5, 8.7; HR-MS (ESI) *m/z*: calcd for $C_{18}H_{30}N_4O_5$ $[M+H]^+$ 383.2294, $[M+Na]^+$ 405.2114, found 383.2294, 405.2109.

(3R,4R,5S)-4-acetamido-5-(2-butyrylguanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**7**).

White solid, 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.77 (1H, s), 8.98(1H, d, *J* = 7.8 Hz), 8.85 (2H, s), 8.00 (1H, d, *J* = 8.4 Hz), 6.69(1H, s), 4.12-4.05 (1H, m), 4.04-3.95 (1H, m), 3.95-3.85 (1H, m), 3.44-3.36 (1H, m), 2.72(1H, dd, *J* = 17.5 Hz, 4.3 Hz), 2.39 (2H, t, *J* = 7.2 Hz), 2.34-2.29 (1H, m), 1.82 (3H, s), 1.61-1.52 (2H, m), 1.49-1.37 (4H, m), 0.91-0.78 (9H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.6, 170.2, 167.5, 153.4, 137.2, 129.0, 81.8, 74.6, 52.6, 50.0, 38.5, 29.4, 26.2, 25.7, 23.1, 17.8, 13.7, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{19}H_{32}N_4O_5$ $[M+H]^+$ 397.2451, found 397.2478.

(3R,4R,5S)-4-acetamido-5-(2-valerylguanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**8**).

White solid, 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.77 (1H, s), 8.98 (1H, d, *J* = 7.9 Hz), 8.83 (2H, s), 8.00 (1H, d, *J* = 8.4 Hz), 6.69 (1H, s), 4.11-4.06 (1H, m), 4.05-3.95 (1H, m), 3.95-3.85 (1H, m), 3.43-3.35 (1H, m), 2.72 (1H, dd, *J* = 17.4 Hz, 4.4 Hz), 2.40 (2H, t, *J* = 7.2 Hz), 2.36-2.29 (1H, m), 1.82 (3H, s), 1.57-1.49 (2H, m), 1.47-1.39 (2H, m), 1.34-1.24 (4H, m), 0.89-0.78 (9H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.7, 170.3, 167.5, 153.4, 137.2, 129.0, 81.8, 74.6, 52.6, 49.9, 36.4, 29.4, 26.4, 26.1, 25.7, 23.1, 21.9, 14.0, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{20}H_{34}N_4O_5$ $[M+H]^+$ 411.2607, found 411.2624.

(3R,4R,5S)-4-acetamido-5-(2-(4-ene-valeryl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**9**).

White solid, 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.77 (1H, s), 8.98 (1H, d, J = 7.7 Hz), 8.84 (2H, s), 8.01 (1H, d, J = 8.4 Hz), 6.69(1H, s), 5.86-5.76 (1H, m), 5.08-4.99 (2H, m), 4.15-4.06 (1H, m), 4.06-3.94 (1H, m), 3.94-3.85 (1H, m), 3.42-3.38 (1H, m), 2.71 (1H, dd, J = 17.5 Hz, 4.4 Hz), 2.54-2.51 (2H, m), 2.36-2.28(3H, m), 1.82 (3H, s), 1.49-1.37 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 174.9, 170.3, 167.5, 153.4, 137.2, 137.0, 129.0, 116.2, 81.8, 74.6, 52.6, 50.0, 35.8, 29.5, 28.2, 26.2, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{20}H_{32}N_4O_5$ $[M+H]^+$ 409.2451, found 409.2458.

(3R,4R,5S)-4-acetamido-5-(2-(2-methylacetyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**10**).

White solid, 56%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.78 (1H, s), 8.96 (1H, d, J = 8.2 Hz), 8.84 (2H, s), 8.00 (1H, d, J = 8.5 Hz), 6.70 (1H, s), 4.13-4.05 (1H, m), 4.05-3.95 (1H, m), 3.95-3.87 (1H, m), 3.42-3.39 (1H, m), 2.72 (1H, dd, J = 17.5 Hz, 4.6 Hz), 2.65-2.58 (1H, m), 2.37-2.30 (1H, m), 1.82 (3H, s), 1.48-1.39 (4H, m), 1.10 (6H, d, J = 6.8 Hz), 0.86-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 179.3, 170.3, 167.5, 153.7, 137.1, 129.0, 81.8, 74.5, 52.5, 49.9, 35.8, 29.4, 26.1, 25.7, 23.1, 18.9, 18.8, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{19}H_{32}N_4O_5$ $[M+H]^+$ 397.2451, found 397.2478.

(3R,4R,5S)-4-acetamido-5-(2-(3-methylbutyryl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**11**).

White solid, 54%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.77 (1H, s), 9.00 (1H, d, J = 7.6 Hz), 8.83 (2H, s), 7.99 (1H, d, J = 8.4 Hz), 6.69 (1H, s), 4.12-4.05 (1H, m), 4.05-3.96 (1H, m), 3.96-3.88 (1H, m), 3.42-3.37 (1H, m), 2.72 (1H, dd, J = 17.4 Hz, 4.3 Hz), 2.37-2.32 (1H, m), 2.28 (2H, d, J = 7.0 Hz), 2.07-1.97 (1H, m), 1.82 (3H, s), 1.49-1.38 (4H, m), 0.92 (6H, d, J = 6.7 Hz), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.0, 170.3, 167.5, 153.4, 137.1, 129.1, 81.8, 74.6, 52.7, 49.9, 45.6, 29.4, 26.2, 25.7, 25.3, 23.1, 22.4, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{20}H_{34}N_4O_5$ $[M+H]^+$ 411.2607, found 411.2612.

(3R,4R,5S)-4-acetamido-5-(2-(2-(2-methylphenyl)acetyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**12**).

White solid, 69%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.77 (1H, s), 9.00 (1H, s), 8.89 (2H, s), 7.99 (1H, d, J = 8.5 Hz), 7.22-7.13 (4H, m), 6.68 (1H, s), 4.12-4.05 (1H, m), 4.05-3.94 (1H, m), 3.92-3.88 (1H, m), 3.80 (2H, s), 3.39-3.37 (1H, m), 2.72 (1H, dd, J = 17.4 Hz, 4.6 Hz), 2.36-2.30 (1H, m), 2.24 (3H, s), 1.81 (3H, s), 1.45-1.36 (4H, m), 0.85-0.77 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 173.4, 170.3, 167.5, 153.5, 137.4, 137.2, 132.6, 130.8, 130.5, 129.0, 127.9, 126.4, 81.8, 74.5, 52.7, 50.1, 41.2, 29.4, 26.1, 25.7, 23.1, 19.6, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{24}H_{34}N_4O_5$ $[M+H]^+$ 459.2607, found 459.2610.

(3R,4R,5S)-4-acetamido-5-(2-(2-(4-methylphenyl)acetyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**13**).

White solid, 56%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.76 (1H, s), 8.95 (1H, d, J = 8.1 Hz), 8.85 (2H, s), 7.98 (1H, d, J = 8.4 Hz), 7.18-7.13 (4H, m), 6.68(1H, s), 4.12-4.05 (1H, m), 4.05-3.94 (1H, m), 3.92-3.88 (1H, m), 3.71 (2H, s), 3.39-3.37 (1H, m), 2.71 (1H, dd, J = 17.4 Hz, 4.5 Hz), 2.35-2.30 (1H, m), 2.28 (3H, s), 1.81 (3H, s), 1.46-1.36 (4H, m), 0.86-0.76 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 173.7, 170.3, 167.5, 153.5, 137.2, 136.8, 130.8, 129.8, 129.5, 129.0, 81.8, 74.6, 52.6, 50.0, 42.9, 29.4, 26.1, 25.7, 23.1, 21.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{24}H_{34}N_4O_5$ $[M+H]^+$ 459.2607, found 459.2650.

(3R,4R,5S)-4-acetamido-5-(2-(2-(4-Br-phenyl)acetyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**14**).

White solid, 67%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.76 (1H, s), 8.93 (1H, s), 8.85 (2H, s), 7.99 (1H, d, J = 8.5 Hz), 7.54 (2H, d, J = 8.3 Hz), 7.25 (2H, d, J = 8.3 Hz), 6.68(1H, s), 4.12-4.05 (1H, m), 4.04-3.94 (1H, m), 3.92-3.88 (1H, m), 3.77 (2H, s), 3.39-3.37 (1H, m), 2.71 (1H, dd, J = 17.5 Hz, 4.7 Hz), 2.35-2.29 (1H, m), 1.80 (3H, s), 1.46-1.35 (4H, m), 0.85-0.76 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm):

173.1, 170.3, 167.5, 153.6, 137.2, 133.3, 132.3, 131.7, 129.0, 120.9, 81.8, 74.5, 52.6, 50.1, 42.5, 29.4, 26.1, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{31}BrN_4O_5$ $[M+H]^+$ 523.1556 found 523.1564, 525.1546.

(3R,4R,5S)-4-acetamido-5-(2-(2-naphthylacetyl)guanidino)-3-(pentan-3-yloxy)cycloh ex-1-ene-1-enecarboxylic acid (**15**).

White solid, 63%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.76 (1H, s), 8.94 (1H, d, J = 7.6 Hz), 8.86 (2H, s), 7.98 (1H, d, J = 8.5 Hz), 7.92-7.86 (3H, m), 7.82 (1H, s), 7.55-7.49 (2H, m), 7.44 (1H, d, J = 8.3 Hz), 6.68 (1H, s), 4.11-4.06 (1H, m), 4.02-3.97 (1H, m), 3.95 (2H, s), 3.92-3.86 (1H, m), 3.39-3.36 (1H, m), 2.72 (1H, dd, J = 17.4 Hz, 4.6 Hz), 2.36-2.30 (1H, m), 1.80 (3H, s), 1.45-1.35 (4H, m), 0.84-0.75 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 173.5, 170.3, 167.5, 153.4, 137.2, 133.4, 132.5, 131.5, 129.0, 128.6, 128.4, 128.1, 128.0, 127.9, 126.8, 126.5, 81.8, 74.6, 52.6, 50.1, 43.5, 29.4, 26.1, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{27}H_{34}N_4O_5$ $[M+H]^+$ 495.2607, found 495.2618.

(3R,4R,5S)-4-acetamido-5-(2-(3-phenylpropionyl)guanidino)-3-(pentan-3-yloxy)cycl ohex-1-ene-1-enecarboxylic acid (**16**).

White solid, 45%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.78 (1H, s), 9.04 (1H, d, J = 7.0 Hz), 8.78 (2H, s), 8.00 (1H, d, J = 8.4 Hz), 7.31-7.18 (5H, m), 6.69 (1H, s), 4.13-4.07 (1H, m), 4.03-3.95 (1H, m), 3.93-3.86 (1H, m), 3.42-3.36 (1H, m), 2.87 (2H, t, J = 7.3 Hz), 2.76-2.68 (3H, m), 2.35-2.29 (1H, m), 1.81 (3H, s), 1.47-1.38 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 174.9, 170.3, 167.5, 153.4, 140.6, 137.2, 129.0, 128.9, 128.7, 126.7, 81.9, 74.6, 52.6, 50.0, 38.2, 29.9, 29.5, 26.2, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{24}H_{34}N_4O_5$ $[M+H]^+$ 459.2607, found 459.2610.

(3R,4R,5S)-4-acetamido-5-(2-(3-(3,4-bis(methoxyl)phenyl)propionyl)guanidino)-3-(p entan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**17**).

White solid, 43%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.74 (1H, s), 9.00 (1H,

d, J = 7.9 Hz), 8.88 (2H, s), 8.00 (1H, d, J = 8.4 Hz), 6.85 (1H, d, J = 8.2 Hz), 6.84 (1H, s), 6.72 (1H, d, J = 8.2 Hz), 6.69 (1H, s), 4.12-4.08 (1H, m), 4.03-3.95 (1H, m), 3.93-3.87 (1H, m), 3.73 (3H, s), 3.71 (3H, s), 3.42-3.36 (1H, m), 2.81 (2H, t, J = 6.9 Hz), 2.73-2.68 (3H, m), 2.35-2.29 (1H, m), 1.81 (3H, s), 1.49-1.36 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.0, 170.3, 167.5, 153.4, 149.1, 147.7, 137.2, 132.9, 129.0, 120.5, 112.6, 112.3, 81.9, 74.6, 56.0, 55.8, 52.6, 50.0, 38.6, 29.7, 29.5, 26.2, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{26}H_{38}N_4O_7$ $[M+H]^+$ 519.2819, found 519.2830.

(3R,4R,5S)-4-acetamido-5-(2-(3-phenylbutyryl)guanidino)-3-(pentan-3-yloxy)cycloh ex-1-ene-1-enecarboxylic acid (**18**).

White solid, 50%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.76 (1H, s), 8.92 (1H, d, J = 7.4 Hz), 8.78 (2H, s), 8.01 (1H, d, J = 8.4 Hz), 7.29 (2H, t, J = 7.5 Hz), 7.21-7.17 (3H, m), 6.69 (1H, s), 4.11-4.07 (1H, m), 4.03-3.95 (1H, m), 3.93-3.88 (1H, m), 3.41-3.38 (1H, m), 2.71 (1H, dd, J = 17.6 Hz, 4.2Hz), 2.60 (2H, t, J = 7.3 Hz), 2.42 (2H, t, J = 7.3 Hz), 2.36-2.29 (1H, m), 1.91-1.85 (2H, m), 1.82 (3H, s), 1.49-1.39 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.4, 170.3, 167.5, 153.4, 140.7, 137.1, 129.0, 128.8, 128.7, 126.4, 81.9, 74.6, 52.5, 50.0, 36.1, 34.6, 29.4, 26.2, 26.0, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{25}H_{36}N_4O_5$ $[M+H]^+$ 473.2764, found 473.2769.

(3R,4R,5S)-4-acetamido-5-(2-benzoylguanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1 -enecarboxylic acid (**19**).

White solid, 63%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (1H, br), 9.45 (1H , d, J = 8.6 Hz), 9.25 (1H, s), 8.13 (2H, d, J = 7.7 Hz), 7.74 (1H, t, J = 7.7 Hz), 7.61 (2H, t, J = 7.7 Hz), 6.72 (1H, s), 4.24-4.22 (1H, m), 4.17-4.08 (1H, m), 4.02-3.90 (1H, m), 3.45-3.37 (1H, m), 2.79 (1H, dd, J = 17.3 Hz, 4.5 Hz), 2.44-2.37 (1H, m), 1.81 (3H, s), 1.52-1.37 (4H, m), 0.88-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.2, 167.9, 167.5, 154.4, 131.5, 129.4, 128.9, 129.2, 128.8, 127.6, 81.8, 74.7, 52.8, 50.4, 29.7, 26.1, 25.7, 23.2, 9.9, 9.4; HR-MS (ESI) *m/z*: calcd for $C_{22}H_{30}N_4O_5$ $[M+H]^+$

431.2294, found 431.2317.

(3R,4R,5S)-4-acetamido-5-(2-(2-methylbenzoyl)guanidino)-3-(pentan-3-yloxy)cycloh ex-1-ene-1-enecarboxylic acid (**20**).

White solid, 64%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.71 (1H, br), 9.22 (1H, d, J = 8.7 Hz), 9.10 (1H, s), 8.29 (1H, d, J = 7.7 Hz), 7.69 (1H, t, J = 7.7 Hz), 7.52 (1H, t, J = 7.7 Hz), 7.36 (1H, t, J = 7.7 Hz), 6.70 (1H, s), 4.26-4.24 (1H, m), 4.19-4.09 (1H, m), 4.01-3.95 (1H, m), 3.46-3.37 (1H, m), 2.78 (1H, dd, J =17.4 Hz, 4.8 Hz), 2.45 (3H, s), 2.42-2.37 (1H, m), 1.83 (3H, s), 1.52-1.40 (4H, m), 0.88-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.2, 169.1, 167.5, 154.1, 137.8, 137.4, 132.8, 132.5, 131.9, 128.9, 128.6, 126.4, 81.9, 74.6, 52.8, 50.5, 29.6, 26.2, 25.7, 23.2, 20.2, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_5$ $[M+H]^+$ 445.2451, found 445.2530.

(3R,4R,5S)-4-acetamido-5-(2-(2-trifluoromethylbenzoyl)guanidino)-3-(pentan-3-ylox y)cyclohex-1-ene-1-enecarboxylic acid (**21**).

White solid, 68%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.22 (1H, d, J = 8.4 Hz), 8.24 (1H, d, J = 8.7 Hz), 7.93 (1H, d, J = 7.4 Hz), 7.87-7.81 (2H, m), 6.71 (1H, s), 4.26-4.22 (1H, m), 4.18-4.08 (1H, m), 4.02-3.95 (1H, m), 3.43-3.36 (1H, m), 2.76 (1H, dd, J =17.4 Hz, 4.3 Hz), 2.44-2.38 (1H, m), 1.85 (3H, s), 1.53-1.35 (4H, m), 0.88-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.2, 168.3, 167.5, 153.5, 138.3, 137.4, 133.3, 132.5, 129.4, 128.9, 127.4, 126.7, 82.0, 74.5, 52.7, 50.6, 29.5, 26.2, 25.7, 23.1, 15.72, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_9F_3N_4O_5$ $[M+H]^+$ 499.2168, found 499.2200.

(3R,4R,5S)-4-acetamido-5-(2-(2-hydroxylbenzoyl)guanidino)-3-(pentan-3-yloxy)cycl ohex-1-ene-1-enecarboxylic acid (**22**).

White solid, 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.51 (1H, s), 7.97 (1H, d, J = 7.5 Hz), 7.89 (1H, d, J = 7.5 Hz), 7.70 (1H, t, J = 7.5 Hz), 7.35 (1H, t, J = 7.5 Hz), 6.66 (1H, s), 4.21-4.18 (1H, m), 4.14-4.05 (1H, m), 3.95-3.88 (1H, m), 3.46-3.39

(1H, m), 2.70-2.64 (1H, m), 2.42-2.35(1H, m), 1.69 (3H, s), 1.49-1.36 (4H, m), 0.87-0.75 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.1, 167.6, 166.1, 158.6, 153.9, 138.1, 134.7, 129.2, 127.3, 125.7, 117.5, 116.2, 81.5, 75.2, 53.9, 50.4, 29.9, 26.2, 25.7, 23.1, 9.9, 9.4; HR-MS (ESI) *m/z*: calcd for $C_{22}H_{30}N_4O_5$ $[M+H]^+$ 447.2244, found 447.2254.

(3R,4R,5S)-4-acetamido-5-(2-(2-Br-benzoyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**23**).

White solid, 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.15 (1H, s, J = 8.7 Hz), 7.97 (1H, d, J = 7.6 Hz), 7.89 (1H, d, J = 7.6 Hz), 7.70 (1H, t, J = 7.6 Hz), 7.35 (1H, t, J = 7.5 Hz), 6.74 (1H, s), 4.41-4.38 (1H, m), 4.26-4.22 (1H, m), 4.15-4.09 (1H, m), 3.38-3.32 (1H, m), 2.80-2.74 (1H, m), 2.34-2.28(1H, m), 1.84 (3H, s), 1.50-1.35 (4H, m), 0.84-0.74 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 175.7, 172.6, 172.2, 158.5, 142.8, 139.4, 138.6, 138.1, 137.9, 134.2, 132.6, 124.4, 79.0, 65.7, 58.3, 55.3, 34.6, 30.9, 30.5, 27.9, 14.4, 14.1; HR-MS (ESI) *m/z*: calcd for $C_{22}H_{29}BrN_4O_5$ $[M+H]^+$ 509.1400, found 509.1423.

(3R,4R,5S)-4-acetamido-5-(2-(2-methoxylbenzoyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**24**).

White solid, 78%. ^{1}H NMR (MeOD, 400 MHz) δ (ppm): 7.99 (1H, d, J = 7.8 Hz), 7.64 (1H, t, J = 7.8 Hz), 7.24 (1H, d, J = 7.8 Hz), 7.15 (1H, d, J = 7.8 Hz), 6.80 (1H, s), 4.24-4.18 (1H, m), 4.16-4.04 (1H, m), 4.06 (3H, s), 3.89-3.81 (1H, m), 3.49-3.41 (1H, m), 2.74 (1H, dd, J =17.4 Hz, 4.8 Hz), 2.96-2.88 (1H, m), 1.97 (3H, s), 1.58-1.50 (4H, m), 0.97-0.89 (6H, m); ^{13}C NMR (100 MHz, MeOD) δ (ppm): 172.6, 166.7, 158.5, 154.6, 138.6, 138.3, 135.4, 133.7, 132.1, 130.1, 121.8, 112.9, 82.9, 75.9, 56.4, 55.4, 54.1, 32.1, 26.7, 26.3, 14.1, 9.5, 9.3; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_6$ $[M+H]^+$ 461.2400, found 461.2418.

(3R,4R,5S)-4-acetamido-5-(2-(3-methylbenzoyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**25**).

White solid, 85%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.93 (1H, s), 7.73 (1H, d, J = 7.3 Hz), 7.42 (1H, t, J = 7.3 Hz), 7.38 (1H, d, J = 7.9 Hz), 6.72 (1H, s), 4.12-4.08 (1H, m), 4.06-4.01 (1H, m), 4.00-3.93 (1H, m), 3.43-3.38 (1H, m), 2.80 (1H, dd, J = 17.9 Hz, 4.9 Hz), 2.36 (3H, s), 2.30-2.25(1H, m), 1.81 (3H, s), 1.55-1.44 (4H, m), 1.30 (3H, t, J = 7.1Hz), 0.87-0.77 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.0, 168.4, 166.1, 154.0, 138.3, 138.1, 133.8, 132.9, 129.5, 129.1, 128.8, 125.7, 81.8, 61.1, 52.5, 54.9, 48.1, 30.8, 26.1, 25.8, 21.3, 14.5, 9.9, 9.6; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_5$ $[M+H]^+$ 445.2451, found 445.2459.

(3R,4R,5S)-4-acetamido-5-(2-(3-trifluoromethylbenzoyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**26**).

White solid, 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.21 (1H, s), 8.4 (1H, s), 8.18 (1H, d, J = 8.7 Hz), 8.09 (1H, d, J = 8.7 Hz), 7.86 (1H, t, J = 8.7 Hz), 6.72 (1H, s), 4.23-4.20 (1H, m), 4.14-4.10 (1H, m), 4.00-3.94 (1H, m), 3.48-3.38 (1H, m), 2.79 (1H, dd, J = 17.2 Hz, 4.3 Hz), 2.43-2.37 (1H, m), 1.85 (3H, s), 1.50-1.33 (4H, m), 0.88-0.79 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.2, 167.3, 166.3, 154.0, 137.6, 137.4, 132.9, 130.8, 128.9, 125.5, 125.3, 122.8, 81.8, 74.5, 52.7, 50.4, 29.7, 26.2, 25.7, 23.1, 15.6, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_9F_3N_4O_5$ $[M+H]^+$ 499.2168, found 499.2201.

(3R,4R,5S)-4-acetamido-5-(2-(3-methoxylbenzoyl)guanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**27**).

White solid, 75%. ^{1}H NMR ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.61 (1H, s), 8.78 (1H, s), 8.09 (1H, s), 7.54 (1H, s), 7.52 (1H, d, J = 7.9 Hz), 7.41 (1H, t, J = 7.9 Hz), 7.17 (1H, d, J = 7.9 Hz), 6.69 (1H, s), 4.14-4.10 (1H, m), 4.10-3.90 (1H, m), 3.82 (3H, s), 3.45-3.40 (1H, m), 3.40-3.36 (1H, m), 2.80 (1H, dd, J = 17.4 Hz, 4.8 Hz), 2.25-2.19 (1H, m), 1.82 (3H, s), 1.49-1.39 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.0, 167.7, 159.6, 153.9, 137.6, 134.3, 130.1, 129.4, 120.9, 119.5, 113.2, 112.9, 81.6, 75.0, 55.8, 52.6, 48.4, 30.6, 26.1, 25.8, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_6$ $[M+H]^+$ 461.2400, found 461.2426.

(3R,4R,5S)-4-acetamido-5-(2-(3,5-bis(methyl)benzoyl)guanidino)-3-(pentan-3-yloxy) cyclohex-1-ene-1-enecarboxylic acid (**28**).

White solid, 84%. ^{1}H NMR (400 MHz, MeOD) δ (ppm): 7.83 (1H, s), 7.57 (1H, s), 7.36 (1H, s), 6.68 (1H, s), 4.35-4.31 (1H, m), 4.21-4.14 (1H, m), 3.99-3.94 (1H, m), 3.44-3.39 (1H, m), 2.85-2.77 (1H, m), 2.44-2.39(1H, m), 2.35 (6H, s), 1.83 (3H, s), 1.47-1.42 (4H, m), 0.88-0.78 (6H, m); ^{13}C NMR (100 MHz, MeOD) δ (ppm): 170.0, 168.5, 167.7, 165.7, 154.6, 153.9, 138.8, 138.1, 126.6, 126.3, 81.7, 75.1, 61.0, 53.2, 30.5, 26.2, 23.1, 21.2, 14.5, 9.6, 9.4; HR-MS (ESI) *m/z*: calcd for $C_{24}H_{34}N_4O_5$ $[M+H]^+$ 459.2607, found 459.2642.

(3R,4R,5S)-4-acetamido-5-(2-(4-methylbenzoyl)guanidino)-3-(pentan-3-yloxy)cycloh ex-1-ene-1-enecarboxylic acid (**29**).

White solid, 73%. ^{1}H NMR (400 MHz, MeOD) δ (ppm): 7.87 (2H, d, J = 8.0 Hz), 7.36 (2H, d, J = 8.0 Hz), 6.80 (1H, s), 4.28-4.20 (1H, m), 4.19-4.09 (1H, m), 4.02-3.95 (1H, m), 3.48-3.47 (1H, m), 2.94 (1H, dd, J = 17.4 Hz, 4.8 Hz), 2.45 (3H, s), 2.43-2.36 (1H, m), 1.97 (3H, s), 1.59-1.53 (4H, m), 0.98-0.90 (6H, m); ^{13}C NMR (100 MHz, MeOD) δ (ppm): 172.5, 169.0, 166.8, 154.9, 144.3, 138.4, 130.7, 129.8, 129.5, 128.7, 83.0, 75.9, 61.4, 54.0, 31.2, 26.7, 26.3, 22.5, 14.2, 9.6, 9.4; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_5$ $[M+H]^+$ 445.2451, found 445.2468.

(3R,4R,5S)-4-acetamido-5-(2-(4-methoxylbenzoyl)guanidino)-3-(pentan-3-yloxy)cycl ohex-1-ene-1-enecarboxylic acid (**30**).

White solid, 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 12.62 (1H, s), 8.75 (1H, s), 8.07 (1H, s), 7.74 (2H, d, J = 7.3 Hz), 7.04 (2H, d, J = 7.3 Hz), 6.69 (1H, s), 4.19-4.13 (1H, m), 4.13-3.95 (1H, m), 3.87-3.80 (1H, m), 3.38 (3H, s), 3.44-3.40 (1H, m), 2.82-2.78 (1H, m), 2.31-2.21(1H, m), 1.82 (3H, s), 1.47-1.39 (4H, m), 0.87-0.78 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.0, 168.4, 167.7, 153.9, 138.3, 132.9, 130.7, 129.1, 125.7, 114.2, 81.6, 75.0, 56.0, 52.6, 48.4, 30.6, 26.2, 25.8, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{32}N_4O_6$ $[M+H]^+$ 461.2400, found 461.2422.

(3R,4R,5S)-4-acetamido-5-(2-(4-nitro-benzoyl)guanidino)-3-(pentan-3-yloxy)cyclohe x-1-ene-1-enecarboxylic acid (**31**).

White solid, 40%. ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 12.74 (1H, s), 9.34 (1H, d, J = 7.9 Hz), 9.22 (2H, s), 8.42 (2H, d, J = 8.7 Hz), 8.30 (2H, d, J = 8.7 Hz), 8.21 (1H, d, J = 8.7 Hz), 6.72 (1H, s), 4.24-4.22 (1H, m), 4.16-4.07 (1H, m), 4.01-3.94 (1H, m), 3.44-3.41 (1H, m), 2.78 (1H, dd, J = 17.4 Hz, 4.5 Hz), 2.43-2.36 (1H, m), 1.85 (3H, s), 1.53-1.38 (4H, m), 0.88-0.79 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 170.3, 167.5, 150.8, 137.4, 130.4, 128.9, 124.4, 81.8, 74.6, 52.7, 50.6, 29.6, 26.2, 25.7, 23.2, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{22}H_{29}N_5O_7$ $[M+H]^+$ 476.2145 $[M+Na]^+$ 498.1965, found 476.2190.

(3R,4R,5S)-4-acetamido-5-(2-(3-(pyridin-4-yl)propionyl)guanidino)-3-(pentan-3-ylox y)cyclohex-1-ene-1-enecarboxylic acid (**32**).

White solid, 63%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.45 (1H, s), 8.40 (1H, d, J = 3.9 Hz), 8.20 (1H, s), 8.04 (1H, d, J = 8.2 Hz), 7.66 (1H, d, J = 7.9 Hz), 7.32-7.28 (1H, m), 6.63 (1H, s), 4.15-4.10 (1H, m), 3.99-3.92 (1H, m), 3.89-3.81 (1H, m), 3.41-3.33 (1H, m), 2.86 (2H, t, J = 7.1 Hz), 2.75 (2H, t, J = 7.1 Hz), 2.70-2.67 (1H, m), 2.35-2.26 (1H, m), 1.80 (3H, s), 1.50-1.40 (4H, m), 0.86-0.76 (6H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 175.8, 170.2, 168.7, 164.8, 154.5, 150.0, 147.9, 136.3, 130.2, 123.9, 115.0, 81.7, 74.7, 53.0, 50.0, 37.9, 29.9, 27.3, 26.2, 25.7, 23.1, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{23}H_{33}N_5O_5$ $[M+H]^+$ 460.2560, found 460.2547.

(3R,4R,5S)-4-acetamido-5-(2-isonicotinoylguanidino)-3-(pentan-3-yloxy)cyclohex-1-ene-1-enecarboxylic acid (**33**).

White solid, 42%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.67 (2H , s), 8.14 (1H, s), 7.92-7.88 (3H, m), 6.69 (1H, s), 4.40-4.33 (1H, m), 4.15-4.10 (1H, m), 3.92-3.84 (1H, m), 3.43-3.38 (1H, m), 2.84-2.71 (1H, m), 2.33-2.11 (1H, m), 1.82 (3H, s), 1.50-1.42 (4H, m), 0.89-0.80 (6H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 170.2, 167.7, 163.5, 161.8, 150.3, 138.0, 129.5, 122.9, 122.6, 81.6, 75.6, 53.3, 49.5, 31.4, 26.1, 25.7,

23.3, 9.9, 9.5; HR-MS (ESI) *m/z*: calcd for $C_{21}H_{29}N_5O_5$ $[M+H]^+$ 432.2247, found 432.2242.

4.3 Determination of IC_{50} of NA inhibitors

A/PuertoRico/8/1934(H1N1), A/HongKong/498/97(H3N2) and A/PuertoRico/8/1934(H259Y, NA resistant strain) were obtained from Wuhan Institute Of Virology, Chinese Academy of Sciences, stored at -80°C. The substrate used in the enzyme inhibition assay, 2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid sodium salt hydrate (4-MU-NANA), was purchased from Sigma. Alamar-Blue® was purchased from Invitrogen. Oseltamivir carboxylate (OC) was obtained from Wuhan Institute Of Virology, Chinese Academy of Sciences.

The NA inhibition assay was performed according to the modified method.[28] The substrate, 2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid sodium salt hydrate (MUNANA), was cleaved by NA to yield a quantifiable fluorescent product. The tested compounds were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer. For the NA inhibition assay, 30 μL of the diluted virus supernatant was first incubated with 10 μL of compounds at different concentrations in a 96-well plate at 37°C for 10 min. Next, 20μM MUNANA dissolved in 33 mM 2-[N-morpholino]ethanesulfonic acid (pH 6.5) and 4 mM $CaCl_2$, at 37°C for 1 h. The reaction was terminated by adding 0.14 M NaOH in 83% ethanol. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 485 nm using a multi-label plate reader (Wallac Envision, PerkinElmer, MA, USA). OC was used as a positive control.

4.4 Docking studies.

Molecular docking studies with flexible ligand and rigid receptor were performed with the AutoDockVinaprogram.[29] Structures of the proteins and ligand were prepared using AutoDockTools GUI for docking preparation. The pictures were generated

using Pymol and Discover Studio Visualizer 4.0 software. For wild type H1N1, crystallographic structure in complex with oseltamivir (PDB ID: 2HU4) were used for docking. The volume chosen for the grid box was 16 Å ×16 Å ×18 Å, center point (x = 0.509, y = 83.305, z = 110.903). Exhaustiveness was increase to 20, and 9 ligand poses were generated.

4.5 Cytotoxicity assay

Monolayers of MDCKs in 96-well culture plates were incubated with the compounds at different concentrations for 72 h. Then, Alamar-Blue® (Invitrogen) was added. After incubation for 2 h at 37°C, the plates were read in a spectrophotometer at Ex= 570 nm and Em= 595 nm. The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression analysis of the dose-response curves generated from the data.

4.6 Determination of EC_{50} of NA inhibitors

Monolayers of MDCK cells in 96-well plates were infected with influenza virus for 1 h at 37°C. Cells were washed to remove residual viruses and various concentrations of the compounds were added. After 36 h, viruses in the supernatant were harvested and secondly infected the new monolayers of MDCK cells in 96-well plates for 1h. Then cells were washed to remove residual viruses and the tested compounds. Then new culture medium was added and monolayers of MDCK cells in 96-well plates were infected with influenza (the harvested supernatant) for hours at 37°C. After hours (A/PuertoRico/8/1934(H1N1) for 12h, A/Hong Kong/498/97(H3N2) for 24h, and A/PuertoRico/8/1934(H259Y, NA resistant strain) for 20h), viruses in the supernatant were harvested and determined as catalog 4.3. OC was used as a positive control. Linear regression of the dose-response curve was performed to determine the 50% inhibitory effect on viral replication (EC_{50}) for the tested and reference

compounds.

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Supplementary Material

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

Scheme 2. Synthesis of compound **16c**.

Scheme 3. Synthesis of compound **16**.

Copies of ^{1}H and ^{13}C NMR spectra for compounds **6-18** and **31-33**.

RP-HPLC Data (C_{18} analytical column) for compounds **6-18** and **31-33**.

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Graphical Abstract

Discovery of acylguanidine oseltamivir carboxylate derivatives as potent neuraminidase inhibitors

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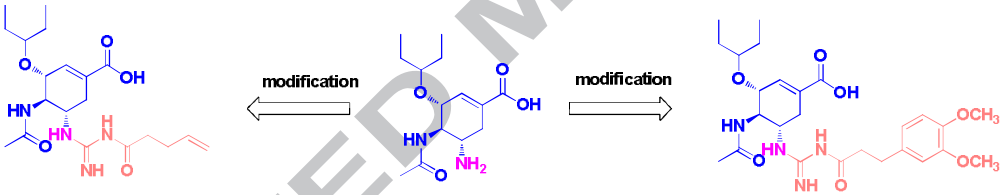

9	oseltamivir carboxylate	17
IC_{50} = 8.1 nM (NA, H1N1)	IC_{50} = 0.76 nM (NA, H1N1)	IC_{50} = 1.43 nM (NA, H1N1)
IC_{50} = 5.4 nM (NA, H3N2)	IC_{50} = 0.80 nM (NA, H3N2)	IC_{50} = 3.64 nM (NA, H3N2)
IC_{50} = 30.5 nM (NA, H1N1-H259Y)	IC_{50} = 169.3 nM (NA, H1N1-H259Y)	IC_{50} = 14.47 nM (NA, H1N1-H259Y)