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PII: S0223-5234(18)30443-4

DOI: 10.1016/j.ejmech.2018.05.030

Reference: EJMECH 10441

To appear in: *European Journal of Medicinal Chemistry*

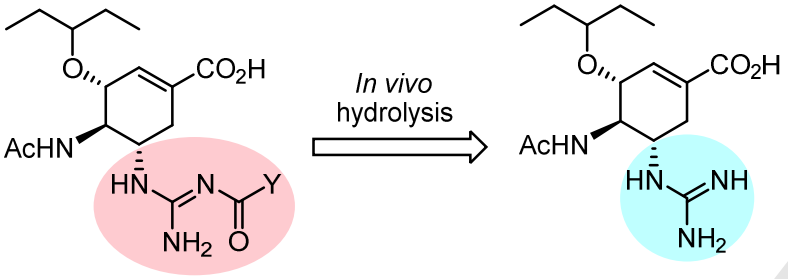
Received Date: 13 January 2018

Revised Date: 5 May 2018

Accepted Date: 20 May 2018

Please cite this article as: P.-H. Hsu, D.-C. Chiu, K.-L. Wu, P.-S. Lee, J.-T. Jan, Y.-S.E. Cheng, K.-C. Tsai, T.-J. Cheng, J.-M. Fang, Acylguanidine derivatives of zanamivir and oseltamivir: Potential orally available prodrugs against influenza viruses, *European Journal of Medicinal Chemistry* (2018), doi: 10.1016/j.ejmech.2018.05.030.

Graphical abstract



Acylguanidine prodrug
Y = hydrophobic group
Improved bioavailability

Guanidine active drug
Guanidino group
Enhanced ionic interactions

Acylguanidine Derivatives of Zanamivir and Oseltamivir: Potential Orally Available Prodrugs against Influenza Viruses

Peng-Hao Hsu,[a] Din-Chi Chiu,[a] Kuan-Lin Wu,[a] Pei-Shan Lee,[a] Jia-Tsrong Jan,[b] Yih-Shyun E. Cheng,[b] Keng-Chang Tsai,[c,d] Ting-Jen Cheng,[b] and Jim-Min Fang[a,b,]*

[a]Department of Chemistry, National Taiwan University, Taipei 106, Taiwan

[b]The Genomics Research Center, Academia Sinica, Taipei 115, Taiwan

[c]National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei 112, Taiwan

[d]The Ph.D. Program for Medical Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei 110, Taiwan.

* Corresponding author. J.-M.F.: Department of Chemistry, National Taiwan University, Taipei, 106, Taiwan. *E-mail address*: jmfang@ntu.edu.tw.

Abstract:

Zanamivir (ZA) and guanidino-oseltamivir carboxylic acid (GOC) are very potent inhibitors against influenza neuraminidase (NA). The guanidinium moiety plays an important role in NA binding; however, its polar cationic nature also hinders the use of ZA and GOC from oral administration. In this study, we investigated the use of ZA and GOC acylguanidine derivatives as possible orally available prodrugs. The acylguanidine derivatives were prepared by coupling with either *n*-octanoic acid or (*S*)-naproxen. The lipophilic acyl substituents were verified to improve cell permeability, and may also improve the bioavailability of acylguanidine compounds. In comparison, the acylguanidines bearing linear octanoyl chain showed better NA inhibitory activity and higher hydrolysis rate than the corresponding derivatives having bulky branched naproxen moiety. Our molecular docking experiments revealed that the straight octanoyl chain could extend to the 150-cavity and 430-cavity of NA to gain extra hydrophobic interactions. Mice receiving the ZA octanoylguanidine derivative survived from influenza infection better than those treated with ZA, whereas the GOC octanoylguanidine derivative could be orally administrated to treat mice with efficacy equal to oseltamivir. Our present study demonstrates that incorporation of appropriate lipophilic acyl substituents to the polar guanidine group of ZA and GOC is a feasible approach to develop oral drugs for influenza therapy.

Keywords: Acylguanidine, Zanamivir, Oseltamivir, Neuraminidase inhibitor, Influenza.

Research highlights

• ZA and GOC acylguanidines are potent influenza neuraminidase (NA) inhibitors.

• Alkyl chain of acylguanidine improves lipophilicity and fits NA 150 & 143-cavities.

• ZA and GOC acylguanidines as prodrugs undergo hydrolysis in physiological conditions.

• ZA octanoylguanidine is better than ZA to treat influenza-infected mice in low dosage.

• GOC octanoylguanidine treats mice well by oral administration against influenza.

Abbreviations:

Arg, arginine; Boc, *tert*-butoxycarbonyl; $(Boc)_2O$, di(*tert*-butyl) dicarbonate; CC_{50}, half maximal cytotoxicity concentration; CPE, cytopathic effect; DIEA, diisopropyl ethylamine; DMSO, dimethylsulfoxide; EC_{50}, half maximal effective concentration; ESI, electrospray ionization; Gln, glutamine; GOC, guanidino-oseltamivir carboxylic acid; HA, hemagglutinin, HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; IC_{50}, half maximal inhibitiory concentration; LC−MS, liquid chromatography−mass spectrometry; $LD_{50,}$ median lethal dose; MDCK, Madin–Darby canine kidney; MUNANA, 2-(4-methylumbelliferyl)-α-D-*N*-acetyl-neuraminic acid; NA, neuraminidase; OC, oseltamivir carboxylic acid; OS, oseltamivir; PBS, phosphate buffered saline; PDB, Protein Data Bank;

PE, peramivir; Pro, proline; Ser, serine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Thr; threonine; TPG, tamiphosphor guanidine; Val, valine; ZA, zanamivir.

1. Introduction

Influenza is a long-standing problem that has caused seasonal epidemics and occassional pandemics to claim a great number of lives. Influenza viruses are classified into A, B and C groups according to their nucleoproteins and matrix proteins. The most virulent group A viruses are further divided into various subtypes depending on their hemagglutinin (HA) and neuraminidase (NA) on the membrane of viruses. Influenza virus proliferate itself by invading host cells to produce its progeny, in which HA is responsible for specific binding of influenza virus to the sialoprotein on the membrane of host cell, whereas NA is responsible for cleaving the terminal sialic acid from the surface glycoprotein of host cell to release progeny virus. Because different subtypes of NA still keep relatively conservative active sites, [1] it is advantageous to design NA inhibitors as effective anti-influenza drugs. [2–5] The NA inhibitors zanamivir (**1**, ZA), [6, 7] oseltamivir (**2a**, OS) [8–10] and peramivir (**3**, PE) [11–13] are currently used in therapies of influenza infection. Tamiflu^{TM}, the phosphate salt of OS, is manufactured as an orally available prodrug, which is hydrolyzed by endogenous esterase to release the corresponding carboxylic acid (**2b**, OC) as the active gradient. Emergence of the OS-resistant influenza viruses, such as the H1N1 H275Y strain, is a serious concern in clinic treatment. ZA and PE are not treated as oral drugs, mainly because they carry the hydrophilic charged carboxyl and guanidino groups, rendering low bioavailability and fast renal elimination. Although GOC (**4**), the guanidino analog of OC, shows higher NA inhibitory

activity, [14] incorporation of the guanidino group greatly reduces its bioavailability (< 5%), [15] Therefore, GOC and its ethyl ester derivative have not been developed for therapeutic use in spite of their preferable NA inhibitory activities.

Derivatization of ZA and GOC, including modification of the guanidino group, has been explored to improve their usefulness in treatment of influenza. [5, 16–19] For example, the ZA derivatives having an extended (piperazinocarbonyl)propyl substituent at the internal *N*-position of the guanidino group was found to exhibit a potent anti-influenza activity against H1N1 virus with EC_{50} = 0.77 μM. [20] This ZA derivative renders extra interactions in the 150-cavity [3] of group-1 neuraminidases as the guanidino group is decorated, and the hydrophobic alkyl substituent may also improve pharmacokinetics properties. In another study, [21] the guanidino group of ZA is modified with appropriate acyl substituents at the external *N*-position to attain extra bindings in the 150-cavity of H1N1 virus. Furthermore, a library of 1,2,3-triazole-containing acylguanidine derivatives of ZA is constructed to seek for potent NA inhibitors. [22] Recently, some GOC acylguanidines are found to possess higher activities than OC against wild-type H1N1 and OS-resistant H259Y viruses. [23]

From a different angle of view, acylguanidine compounds are liable to hydrolysis in acidic or basic conditions, [24–26] and even by bioactivation. [27] Therefore, the acylguanidine derivatives of ZA and GOC may act as prodrugs if they can be hydrolyzed in body to give the parental guanidine drugs. In such case, modification with hydrophobic acyl

substituent may enhance the drug lipophilicity for better membrane permeability and oral administration. [19] To illustrate this concept, a thrombin inhibitor containing guanidino group has been prepared as the acylguanidine derivatives to uphold membrane permeability and drug absorptivity. [27] In one of our previous studies, [28] we also observed that acylguanidine **6** effectively protected Madin–Darby canine kidney (MDCK) cells from the infection of H1N1 viruses with EC_{50} of 0.8 nM, similar to tamiphosphor guanidine (**5**, EC_{50} = 3.8 nM), even **6** has poor inhibitory activity against NA (IC_{50} > 100 μM). This result suggests the possible conversion of acylguanidine **6** to the parental drug **5** in the cell-based assay.

Although some ZA and GOC acylguanidine derivatives have been revealed to possess good NA inhibitory activity, [21–23] further experiments are required to elucidate whether they can act as prodrugs or not. In this study, we prepared the ZA and GOC acylguanidine derivatives **7a**, **7b**, **8a** and **8b** (Fig. 1), and then investigated their anti-influenza activities and susceptibility to hydrolysis in different conditions. Due to steric effect, compounds **7b** and **8b** carrying an α-naphthylpropanoyl group are predicted to be less sensitive to hydrolysis than their counterparts **7a** and **8a** bearing *n*-octanoyl group. [27]

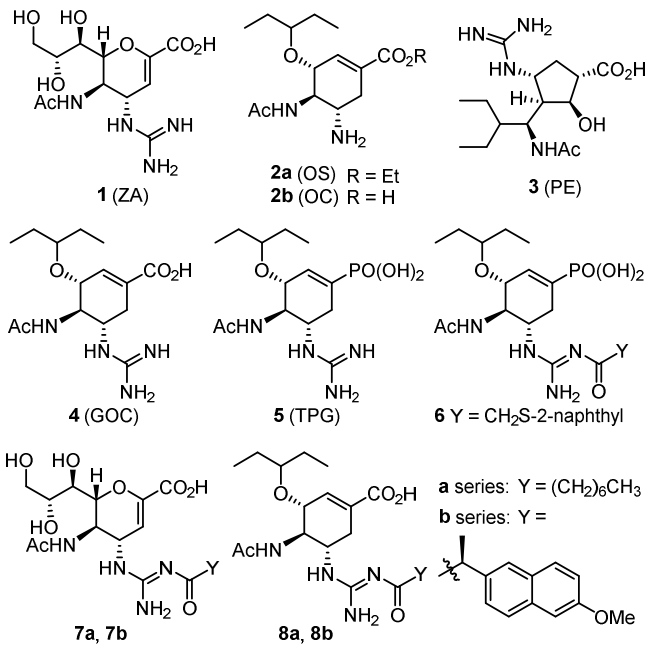


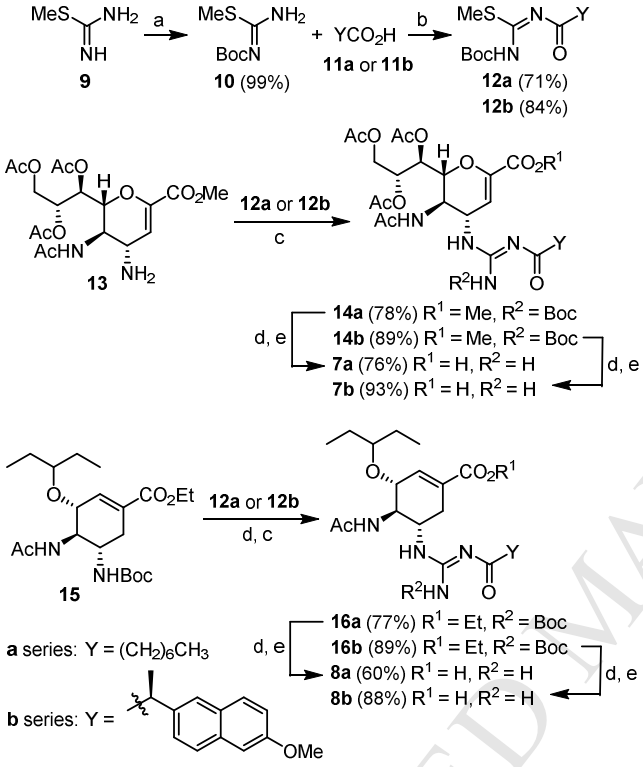
Fig. 1. Chemical structures of influenza neuraminidase inhibitors and acylguanidine derivatives.

2. Results and discussions

2.1. Chemical synthesis

Mono-Boc protected *S*-methylisothiourea (**10**) was prepared, [23, 29] and reacted with *n*-octanoic acid and (*S*)-naproxen, respectively, in the presence of HBTU and DIEA to give the corresponding acylisothiourea compounds **12a** and **12b** (Scheme 1). Using sialic acid as the starting material, compound **13** was prepared according to the previously reported method. [30] Using $HgCl_2$ as an activator, the coupling reactions of amine **13** with **12a** and **12b** were carried out to give **14a** and **14b** in 78% and 89% yields, respectively. Compounds **14a** and **14b** were treated trifluoroacetic acid to remove the Boc protecting group, and the ethyl ester

was subsequently saponified at room temperature for 30 min to give the desired products **7a** and **7b**, respectively, without hydrolysis of the acylguanidine group.



Scheme 1. Synthesis of acylguanidine derivatives **7a**, **7b**, **8a** and **8b**. *Reagents and conditions*: (a) $(Boc)_2O$, $NaHCO_3$, THF/H_2O (1:1), rt, 14 h; (b) HBTU, DIEA, CH_2Cl_2, rt, 14 h; (c) $HgCl_2$, Et_3N, CH_2Cl_2, rt, 6 h; (d) TFA, CH_2Cl_2, rt, 1 h; (e) KOH, CH_3CN/H_2O (1:1), rt, 30 min. Boc = *tert*-butoxycarbonyl, $(Boc)_2O$ = di(*tert*-butyl) dicarbonate, DIEA = diisopropyl ethylamine, HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid, THF = tetrahydrofuran.

For preparation of the GOC acylguanidines, Tamiflu capsule was treated with $(Boc)_2O$ in

the presence of $NaHCO_3$ to afford the Boc-protecting oseltamivir **15**. [31, 32] The Boc group was removed, and the intermediate aminium salt underwent the Hg(II)-promoted coupling reactions with **12a** and **12b** to afford compounds **16a** and **16b**, respectively. The desired acylguanidines **8a** and **8b** were obtained from **16a** and **16b**, respectively, after removal of the Boc group and saponification of the ester.

2.2. Neuraminidase inhibition and cell-based assay

With ZA and GOC acylguanidines **7a**–**8b** in hand, we then evaluated their NA inhibition and anti-influenza activities (Table 1). The ZA acylguanidines **7a** and **7b** showed the NA inhibitory activities against wild-type (WSN H1N1) virus and the H275Y mutant with IC_{50} values of 180 and 290 nM, respectively, inferior to ZA by approximately 160 fold. Acylguanidines **7a** and **7b** were nontoxic to MDCK cells (CC_{50} > 100 μM), but efficiently protected MDCK cells from WSN virus infection with EC_{50} values of 90 and 140 nM, respectively. As the EC_{50} values were even lower than their IC_{50} values, we speculated that acylguanidines **7a** (ZA-oct) and **7b** (ZA-nap) might be hydrolyzed, at least in parts, to yield the parental ZA. The GOC acylguanidines **8a** and **8b** also displayed remarkable cellular protection against WSN virus with EC_{50} values of 1 and 2 nM, respectively, even though they are inferior NA inhibitors than GOC. This result might be also accounted for the increased lipophilicity of **8a** and **8b** to facilitate cell absorption.

Table 1. Neuraminidase inhibition (IC_{50}), anti-influenza activity (EC_{50}) and calculated lipophilicity.

Compound[a]	WSN		H275Y		Lipophilicity	
	IC_{50}[b] (nM)	EC_{50}[c] (nM)	IC_{50}[b] (nM)	EC_{50}[c] (nM)	cLogP[d]	cLogD[d]
1 (ZA)	1 ± 0[e]	27 ± 11	2 ± 0[e]	150 ± 20	–4.13	–5.77
2b (OC)	1 ± 0[e]	16 ± 4	370 ± 100	26000 ± 11000	0.43	–1.84
4 (GOC)	1 ± 0[e]	7 ± 4	2 ± 1	2100 ± 900	0.09	–1.55
7a (ZA-oct)	180 ± 50	90 ± 15	290 ± 78	480 ± 30	–1.27	–2.92
7b (ZA-nap)	200 ± 50	140 ± 10	260 ± 70	900 ± 210	–0.99	–2.63
8a (GOC-oct)	8 ± 1	1 ± 0[e]	18 ± 5	6600 ± 2600	2.95	1.30
8b (GOC-nap)	53 ± 9	2 ± 1	190 ± 50	19000 ± 8000	3.23	1.59

[a] All the test compounds are nontoxic to MDCK cells (CC_{50} > 100 μM).

[b] IC_{50} value is the half maximal inhibitory concentration against the neuraminidase of influenza virus A/WSN/1933 (H1N1) or the H275Y mutant.

[c] EC_{50} value is half maximal effective concentration for protection of MDCK cytopathic effects due to influenza virus infection.

[d] Octanol−water partition coefficient calculated using MarvinSketch 17.29

[e] The values were between 0.1 to 0.4, which were round-up in order to standardize all the numbers to the same standard deviation significant digits.

Similar to the previous studies, [21–23] acylguanidine derivatives (e.g. **7a**−**8b**) generally displayed weaker NA inhibitory activities than their parental anti-influenza agents (Table 1). Some anti-influenza activity could be reverted by hydrolysis of acylguanidine to the parental

guanidine as inferred from the cell-based assay. The EC_{50} values in Table 1 might just reflect the combined effect of the acylguanidine and its hydrolysis product. It was interesting to note that GOC-oct **8a** still had sufficient binding strength with the neuraminidase of WSN virus (IC_{50} = 7.9 nM) and exhibited high protective effect on MDCK cells (EC_{50} = 0.5 nM) against the infection of WSN virus, presumably a consequence of enhanced permeability of the acylguanidine combined with the partial hydrolysis to more active guanidine **4** (GOC). In contrast, the binding strength of **8a** with H275Y mutant was weaker, and no better anti-influenza activity than GOC was observed. Following the similar trend, the anti-influenza activity of GOC-nap **8b** against wild-type and H275Y mutant was correlated to its binding strength with NA.

2.3. Cell permeability study

To validate the assumption that the lipophilic acyl group could bolster up cell permeation, we measured the cell permeability of **4** and **8a**. We first identified the characteristic fragments at *m*/*z* = 180 for guanidine **4** and at *m*/*z* = 365 for acylguanidine **8a** (Fig. S1). The calibration curves for concentrations of **4** and **8a** were established, respectively, on the basis of the ion currents (expressed as integral area) of the characteristic fragments in LC−MS spectra (Fig. S2). Thus MDCK cells were incubated with 100 μM of **4** or **8a** for 48 h, and the cell lysates were prepared for the quantification of **4** and **8a** in the cells by LC−MS analyses. The

experiments showed that the cell lysate of the cells treated with **8a** contained 1225 nM of **8a** and 130.4 nM of the hydrolysis product **4**, equivalent to a sum of 1355.4 nM **8a** entering the MDCK cells after a 48-hour incubation (Fig. S3). In comparison, the parallel experiment showed that the cell lysates of the cells treated with **4** only contained 47.6 nM of **4**. Thus, acylguanidine **8a** has better cell permeability than guanidine **4** by 28 fold.

2.4. Stability of acylguanidine compounds in buffer and plasma

According to our cell permeability study, approximately 10% of **8a** was hydrolyzed at 48 h of incubation to release more active NA inhibitor **4**. To have a deeper insight into the attributes of acylguanidines **7a**–**8b**, we also examined their hydrolytic behaviors in different media (Table 2). These compounds were stable in acidic media; no appreciable degradation occurred after incubation for 96 h at pH 1.3 or 4.1 (entries 1 and 2). The acylguanidine derivatives were slowly hydrolyzed in neural phosphate buffer, whereas the hydrolytic degradation of octanoylguanidine compounds **7a** and **8a** accelerated in basic conditions (entries 5 and 6). For unknown reason, the naproxen-derived acylguanidine **8b** could sustain longer in basic borate or glycine buffers ($t_{½}$ = 76.7−81.4 h) than that in neutral condition ($t_{½}$ = 64.9 h). The stability experiments also revealed that octanoylguanidines (**7a** and **8a**) were more easily hydrolyzed than the naproxen-derived acylguanidines (**7b** and **8b**). This result is in agreement with the previous finding that shows acylguanidine with α-substituent will slow

down the hydrolysis rate. [27] The similar trend was observed in hydrolysis of acylguanidines in rat plasma and rabbit serum (entry 7). Compound **7a** in rat plasma was hydrolyzed with a half-life at 29.5 h, compared with 67.7 h of **7b**. The LC–MS analysis showed a signal at *m*/*z* 333 corresponding to the protonated molecule ion [M + H] of ZA, indicating that ZA was the hydrolysis product of **7a** (Fig. S4). Both **8a** and **8b** were rather reluctant to hydrolysis in rabbit serum; thus 55% of **8a** and 76% of **8b** remained after incubation for 96 h at 37 °C.

Table 2. Stability tests of acylguanidine compounds.

entry	Media	$t_{1/2}$ (h)[a]			
		7a[b]	**7b**[b]	**8a**[b]	**8b**[b]
1	HCl/KCl solution (50 mM, pH 1.3)	> 96[c]	ND[d]	> 96[c]	> 96[c]
2	acetate buffer (50 mM, pH 4.1)	> 96[c]	ND[d]	ND[e]	ND[e]
3	phosphate buffer (50 mM, pH 7.4)	39.2	ND[d]	ND[e]	64.9
4	phosphate buffer (10 mM, pH 7.4)	56.8	67.2	ND[e]	69.7
5	borate buffer (50 mM, pH 9.1)	26.1	ND[d]	18.1	76.7
6	glycine buffer (50 mM, pH 9.2)	ND[d]	ND[d]	22.4	81.4
7	animal plasma[f]	29.5	67.7	≈ 96[g]	> 96[c]

[a] Half-life was determined by HPLC analysis of the remaining acylguanidine compound. The calculation was based on pseudo-first order rate law.

[b] Three duplicate experiments were performed for **7a** and **7b**, whereas single measurement was taken for **8a** and **8b**.

[c] More than 75% of the indicated compound remained after incubation for 96 h at 37 °C.

[d] ND: Not determined. The hydrolysis tests were not performed.

[e] ND: Not determined. The hydrolysis tests were not performed due to incomplete dissolution of the substrates.

[f] Experiments of **7a** and **7b** was performed in rat plasma, whereas that of **8a** and **8b** was conducted in rabbit serum.

[g] Only 55% of **8a** remained after incubation for 96 h at 37 ^{o}C.

2.5. Molecular modeling

To address the distinction of IC_{50} values between **a**- and **b**-series acylguanidines, we conducted molecular docking experiments on the GOC acylguanidines **8a** and **8b** to compare their binding modes in the open-form NA of H1N1 virus (Fig. 2). In addition to the key interactions of the C1-carboxy, C3-pentoxy, C4-acetamido and C5-gaunidino groups in the NA active site, [33] the carbonyl group of the acylguanidine moiety also generates a hydrogen bond with Arg-118. The similar hydrogen-bond interaction is also shown in the binding modes of other GOC and ZA acylguanidines. [21–23] The straight hydrocarbon chain of **8a** extends to the 150-cavity [3] and 430-cavity [34] to gain hydrophobic interactions with Val-116, Gln-136, Ser-145 and Thr-439. In contrast, the bulky naproxen moiety of **8b** cannot access the 150-cavity, and would rather points away from the NA pocket. Moreover, the hydrophobic interactions induced by the naphthyl ring with Arg-430 and Pro-431 in the 430-cavity are significantly less robust than those possessed by compound **8a**. Taken together, the molecular docking experiments are in agreement with the results of NA inhibition assays, i.e. **7a** and **8a** bearing a linear octanoyl substituent at the guanidine moiety can fit the

open-form NA to attain better inhibitory activity than **7b** and **8b**.

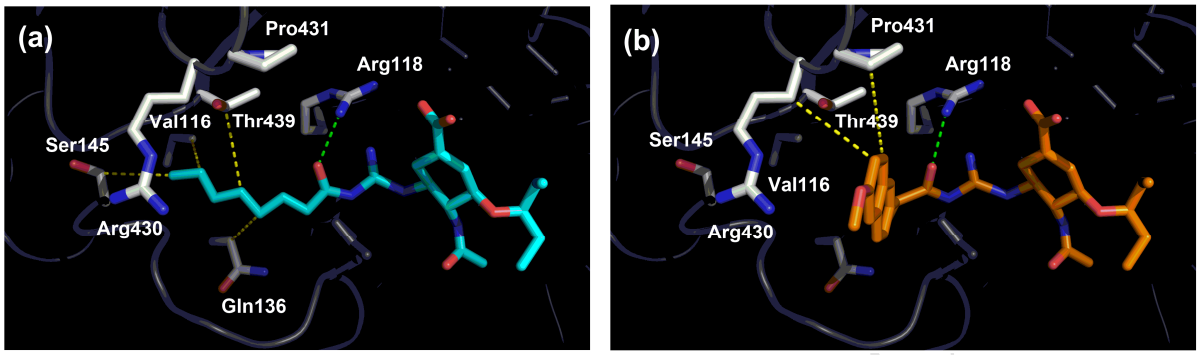


Fig. 2. Schematic representations of molecular modeling of compounds **8a** (a) and **8b** (b) in the open-form neuraminidase (N1 subtype, PDB code: 2HU0).

2.6. Mice experiments

ZA requires a dose of 400 μg/kg/day (1.2 μmol/kg/day) by intranasal administrations twice (with half of the dosage at each administration) for treatment of influenza infected patients. Taking 10 times metabolic rate of mouse, the mice experiment was first conducted using the dosage of 12 μmol/kg/day by intranasal instillation. Phosphate buffered saline (PBS) was utilized as negative control. Fig. 3a shows that the acylguanidine **7a** (ZA-oct) was as effective as ZA in protection of mice (100% survival) from the infection of H1N1 virus, but acylguanidine **7b** (ZA-nap) was somewhat inferior with 70% survival rate at day 21. When mice were treated with a low dosage of 2.4 μmol/kg/day, compound **7a** showed somewhat better efficacy than ZA (Fig. 3b). The in vitro stability tests suggested that the half-life of

compound **7a** is approximately 30 h (Table 2). Compared to the reported half-life of zanamivir of 2−5 h [35], it is possible that the introduction of lipophilic group helps the stability of **7a**. Also, the cell permeability tests implied that higher amount of compound with lipophilic group is available to the cells compared to the parental compound. Therefore, the observed virus challenge results are ascribed to higher availabilities and stabilities, combined to the antiviral effects of the ZA derivative and ZA. This mechanism is similar to that proposed for laninamivir octanoate, which is a long-acting NA inhibitor. [36]

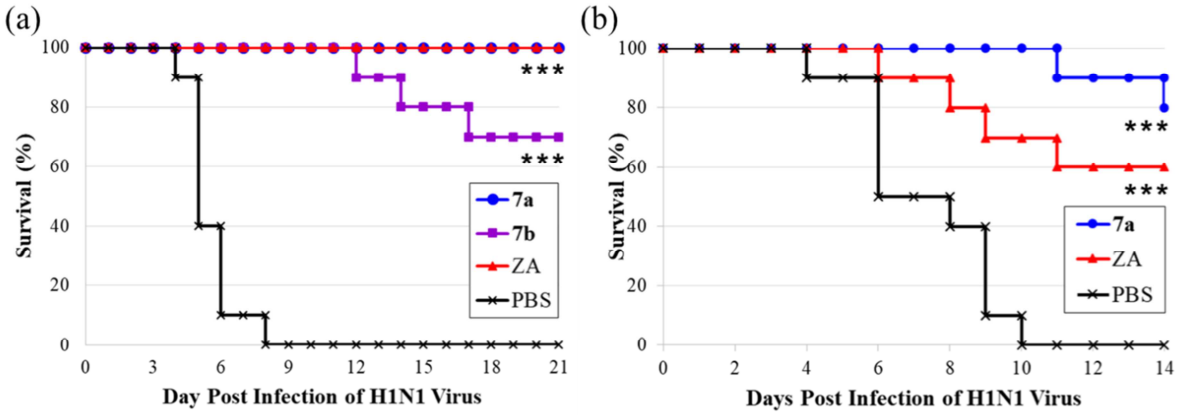


Fig. 3. Survival rates of virus-infected mice on treatment with ZA acylguanidines. The administered dosage of each compound was 12 μmol/kg/day (a) and 2.4 μmol/kg/day (b). Compounds were administered by intranasal instillation and continued twice daily for 5 days. BALB/c mice were intranasally challenged with 10 LD_{50} of A/WSN/1933 (H1N1) viruses. The animal experiments were conducted with 10 mice per group. ***P<0.0001.

Compared to cLogP of 1.16 for OS, GOC acylguanidine **8a** is predicted to have a suitable lipophilicity (cLogP = 2.95) for oral administration in mice experiment. OS requires a dose of 3 mg/kg/day (9.6 μmol/kg/day) for influenza treatment. Fig. 4 shows that **8a** and OS have equivalent efficacy by oral administration at 48 μmol/kg/day dosage to protect mice from the infection of WSN virus. This experiment suggests the possible development of GOC acylguanidine as an orally available drug in influenza therapy.

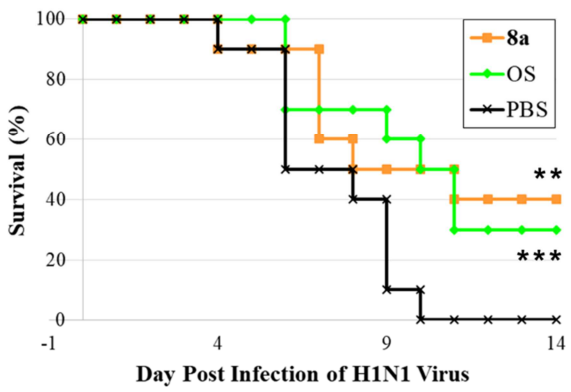


Fig. 4. Survival rates of virus-infected mice on treatment with GOC acylguanidine **8a**. Compounds were administered by oral gavage and continued twice daily for 5 days. The administered dosage of each compound was 48 μmol/kg/day. BALB/c mice were intranasally challenged with 10 LD_{50} of A/WSN/1933 (H1N1) viruses. The animal experiments were conducted with 10 mice per group. ***P<0.0001. **P<0.001.

3. Conclusion

In this study, we designed and synthesized the ZA and GOC acylguanidine derivatives **7a**,

7b, **8a** and **8b**. Similar to the previous studies, [21–23] these acylguanidine derivatives generally displayed weaker NA inhibitory activities than their parental anti-influenza agents (ZA or GOC, Table 1). Nonetheless, the GOC octanoylguanidine derivative **8a** still possess high inhibitory activity with IC_{50} of 8 nM and EC_{50} of 1 nM against wild-type H1N1 virus. By conducting cell permeability experiments, we ensured that **8a** is 28-fold more readily uptaken by MDCK cells compared to the parental agent, and approximately 10% of **8a** can be hydrolyzed to produce **4**. Our experiments also indicated that the acylguanidines were prone to hydrolysis in neutral and basic buffer solutions as well as in animal plasma at physiological condition, leading to liberation of the parental anti-influenza agents ZA and GOC (Table 2). The hydrolysis of acylguanidines **7a** and **8a** was appreciably faster than their counterparts **7b** and **8b**. Taken together, the ZA and GOC acylguanidine derivatives are not only active to NA but also act further as prodrugs by hydrolysis in cell to generate the parental guanidines (ZA and GOC). Incorporation of appropriate lipophilic acyl substituents to the polar guanidine group of ZA and GOC can improve the cell permeability and render the possible oral administration for treatment of influenza.

The molecular docking experiments revealed that the straight octanoyl chain of **8a** could extend to the 150-cavity and 430-cavity of NA to gain extra hydrophobic interactions. In comparison, the GOC acylguanidine **8b** showed a weaker affinity to NA by 7 fold because the bulky branched naproxen moiety could not be incorporated into the 150-cavity. Following the

similar trend, the ZA octanoyl derivative **7a** also showed better NA inhibitory activity than the naproxen derivative **7b**. By intranasal administration, the octanoylguanidine derivative **7a** (ZA-oct) secured higher mice survival than ZA in treatment with H1N1 influenza, but **7b** (ZA-nap) was less effective (Fig. 3). By oral gavage, mice receiving **8a** (GOC-oct) showed comparable survival rate to those treated with OS (Fig. 4).

5. Experimental section

5.1. General part

All the reagents and solvents were reagent grade and were used without further purification unless otherwise indicated. All solvents were ACS grade unless indicated otherwise. CH_2Cl_2 was distilled from CaH_2. All non-aqueous reactions were conducted in oven-dried glassware under a slight positive pressure of argon or nitrogen unless otherwise noted. Reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) on silica gel using phosphomolybdic acid as visualizing agents. Silica gel (Merck, 0.040–0.063 mm) and LiChroprep RP-18 (Merck, 0.040–0.063 mm) were used for column chromatography. Melting points were recorded on a Yanaco or Electrothermal MEL-TEMP 1101D apparatus and are not corrected. Optical rotations were measured on digital polarimeter of Japan JASCO Co. DIP-1000. $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. Infrared (IR) spectra were recorded on a Thermo Nicolet iS5 FT-IR spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker DPX-400 (400 MHz) or Bruker AVIII-400 (400 MHz) spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to internal standards: $CHCl_3$ (δ_H = 7.24), $CDCl_3$ (δ_C = 77.0 for the central line of triplet), HDO (δ_H = 4.81), CH_3OD (δ_H = 3.31), CD_3OD (δ_C = 49.0), $(CH_3)_2SO$ (δ_H = 2.50) and

$(CD_3)_2SO$ (δ_C = 39.5). The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet), dd (double of doublets) and br (broad). Coupling constants (*J*) are given in Hz. The ESI−MS experiments for compound characterization were conducted on a Bruker Daltonics BioTOF III high-resolution mass spectrometer. High-performance liquid chromatography (HPLC) was performed on Agilent 1100 Series instrument equipped with a degasser, a Quat pump, and a UV detector. LC–MS experiments for compound characterization were performed on Agilent 1200 Series instrument and Agilent 6320 Ion Trap LC–MS System.

5.2. Material and methods

Influenza virus A/WSN/1933 (H1N1) was obtained from Dr. Shin-Ru Shih at Chang Gung University in Taiwan, and influenza virus A/Udorn/1972 (H3N2) was from Dr. Jia-Tsrong Jan (The Genomics Research Center, Academia Sinica). WSN 274Y was selected with Tamiflu from influenza virus A/WSN/1933 (H1N1) in our lab. All Viruses were cultured in the allantoic cavities of 10-day-old embryonated chicken eggs for 72 h, and purified by sucrose gradient centrifugation. Madin–Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection (Manassas, Va), and were grown in DMEM (Dulbecco's modified Eagle medium, GibcoBRL) containing 10% fetal bovine serum (GibcoBRL) and penicillin-streptomycin (GibcoBRL) at 37 ^oC under 5% CO_2.

5.3. Synthesis and characterization of compounds

5.3.1. *Tert-Butyl [amino(methylthio)methylene]carbamate (**10**).* [29]

To a solution of *S*-methylisothiourea hemisulfate salt (**9**) (1085 mg, 7.80 mmol) and $NaHCO_3$ (655 mg, 7.80 mmol) in H_2O (15 mL) and THF (15 mL) was added di(*tert*-butyl) dicarbonate (1283 mg, 5.88 mmol) dropwise. The mixture was stirred at room temperature

under argon for 14 h, and then concentrated under reduced pressure. The residue was partitioned between EtOAc and H_2O. The organic phase was dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 1:4) to afford the desired product **10** (1104 mg, 99%). $C_7H_{14}N_2O_2S$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.39 (3 H, s), 1.45 (9 H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 172.3, 160.4, 79.1, 27.5, 12.7; HRMS (ESI) calcd for $C_7H_{15}N_2O_2S$: 191.0854, found: *m/z* 191.0848 $[M + H]^+$.

*5.3.2. Tert-Butyl [(methylthio)(octanamido)methylene]carbamate (**12a**)*

To a solution of octanoic acid (158 μL, 1.00 mmol) in anhydrous CH_2Cl_2 (5 mL) was added HBTU (417 mg, 1.10 mmol), DIEA (192 μL, 1.10 mmol) and compound **10** (209 mg, 1.19 mmol). The mixture was stirred at room temperature under argon for 14 h, and then concentrated under reduced pressure. The residue was diluted with EtOAc and washed with 1 M $HCl_{(aq)}$, saturated $NaHCO_{3(aq)}$ and brine. The organic phase was dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 1:19) to afford the desired product **12a** (169 mg, 71% yield). $C_{15}H_{28}N_2O_3S$; white solid; mp 68.0–69.2 ^oC; TLC (EtOAc/hexane = 1:9) R_f = 0.45; IR υ_{max} (neat) 2930, 1748, 1567 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz, containing two tautomers (3:1)) δ 12.41 (0.75 H, s), 12.12 (0.25 H, s), 2.50–2.29 (5 H, m), 1.71–1.58 (2 H, m), 1.48 (9 H, s), 1.37–1.19 (8 H, m), 0.84 (3 H, t, *J* = 5.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 171.1, 170.9, 160.6, 80.6, 77.2, 37.0, 31.3, 28.7, 27.7 (3 ×), 24.3, 22.3, 14.1, 13.7; HRMS (ESI) calcd for $C_{15}H_{29}N_2O_3S$: 317.1899 found: *m/z* 317.1897 $[M + H]^+$.

*5.3.3. Tert-Butyl [[2-(6-methoxynaphthalen-2-yl)propanamido](methylthio)methylene]carbamate (**12b**)*

To a solution of (*S*)-naproxen (**11b**) (115 mg, 0.50 mmol) in anhydrous CH_2Cl_2 (5 mL) was added HBTU (247 mg, 0.65mmol), DIEA (130 μL, 0.75mmol), and compound **10** (114 mg, 0.6 mmol). The mixture was stirred at room temperature under argon for 14 h, and then

concentrated under reduced pressure. The residue was diluted with EtOAc and washed with 1 M $HCl_{(aq)}$, saturated $NaHCO_{3(aq)}$, and brine. The organic phase was dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 1:9) to afford the desired product **12b** (169 mg, 84%, containing tautomers (11:9)). $C_{21}H_{26}N_2O_4S$; colorless syrup; TLC (EtOAc/hexane = 1:9) R_f = 0.27; $[\alpha]^{24}_D$ –18.4 (c = 1.0, CH_2Cl_2); IR υ_{max} (neat) 2933, 1747, 1557 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 12.31 (0.55 H, s), 12.19 (0.45 H, s), 7.69 (3 H, m), 7.38 (1 H, br), 7.15–7.07 (2 H, m), 4.02–3.90 (1 H, br), 3.87 (3 H, s), 2.30 (3 H, s), 1.62 (3 H, d, J = 6.8 Hz), 1.37 (9 H, br); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 186.3, 172.1, 171.0, 170.2, 169.4, 160.2, 157.4, 157.2, 150.3, 150.1, 136.4, 133.8, 133.6, 133.2, 128.9, 128.6, 127.4, 126.4, 126.2, 125.9, 125.3, 118.,8 118.4, 105.2, 82.7, 82.3, 80.5, 77.2, 53.7, 50.9, 47.6, 27.4, 17.5, 17.0, 14.1, 13.9; HRMS (ESI) calcd for $C_{21}H_{27}N_2O_4S$: 403.1692, found: *m/z* 403.1700 $[M + H]^+$.

*5.3.4. Methyl 5-acetamido-6-(1,2,3-triacetoxypropyl)-4-[2-(tert-butoxycarbonyl)]-3-octanoyl]guanidino-4,5-dihydro-6H-pyran-2-carboxylate (**14a**)*

Amine **13** was prepared from sialic acid according to the previously reported method. [30] To a solution of amine **13** (138 mg, 0.32 mmol) in CH_2Cl_2 (5 mL) were added compound **12a** (100 mg, 0.32 mmol), $HgCl_2$ (95 mg, 0.35 mmol), and Et_3N (49 μL, 0.35 mmol). The mixture was stirred at room temperature under argon for 6 h, filtered through Celite, and concentrated under reduced pressure. The residue was diluted with EtOAc and washed with 1 M $HCl_{(aq)}$, saturated $NaHCO_{3(aq)}$, and brine. The organic phase was dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 1:1 to 3:2) to afford the coupling product **14a** (172 mg, 78%). $C_{32}H_{50}N_4O_{13}$; white foam; TLC (EtOAc/hexane = 3:2) R_f = 0.45; $[\alpha]^{24}_D$ +58.6 (c = 1.0, CH_2Cl_2); IR υ_{max} (neat) 3269, 2930, 1746, 1657 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, two tautomers (17:3)) δ 12.21 (0.15 H, s), 12.19 (0.85 H, s), 9.12 (0.85 H, d, J =

8.4 Hz), 8.48 (0.15 H, d, *J* = 8.4 Hz), 6.00 (0.85 H, d, *J* = 8.8 Hz), 5.91 (0.15 H, d, *J* = 2.4 Hz), 5.86 (0.85 H, d, *J* = 2.4 Hz), 5.76 (0.15 H, d, *J* = 9.2 Hz), 5.46–5.38 (1 H, m), 5.33–5.25 (1 H, m), 5.20–5.05 (1 H, m), 4.71–4.60 (1 H, m), 4.36–4.20 (2 H, m), 4.20–4.10 (1 H, m), 3.84–3.73 (3 H, m), 2.41–2.30 (2 H, m), 2.13–2.00 (9 H, m), 1.88–1.80 (3 H, m), 1.67–1.57 (2 H, m), 1.51–1.41 (9 H, m), 1.35–1.19 (8 H, m), 0.85 (3 H, t, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz, two tautomers (17:3)) δ 187.6, 174.4, 170.6, 170.5, 170.2, 170.1, 170.0, 169.89, 169.86, 162.9, 161.4, 156.6, 156.1, 152.5, 144.4, 144.3, 109.8, 109.7, 83.2, 79.3, 77.1, 71.4, 67.7, 62.0, 52.0, 49.3, 49.1, 46.8, 46.7, 41.1, 37.5, 31.4, 31.2, 29.0, 28.8, 28.52, 28.46, 27.8, 27.6, 25.3, 24.2, 22.6, 22.3, 22.2, 20.5, 20.4, 13.72, 13.68; HRMS (ESI) calcd for $C_{32}H_{51}N_4O_{13}$: 699.3453, found: *m/z* 699.3453 $[M + H]^+$.

5.3.5. *Methyl 5-acetamido-6-(1,2,3-triacetoxypropyl)-4-[2-(tert-butoxycarbonyl)-3-(2-(6-methoxynaphthalen-2-yl)propanoyl)guanidino-4,5-dihydro-6H-pyran-2-carboxylate (**14b**)*

According to the procedure similar to that for compound **14a**, the reaction of amine **13** (180 mg, 0.42 mmol) in CH_2Cl_2 (5 mL) with compound **12b** (169 mg, 0.42 mmol) in the presence of $HgCl_2$ (125 mg, 0.46 mmol) and Et_3N (64 μL, 0.46 mmol) afforded the coupling product **14b** (293 mg, 89%). $C_{38}H_{48}N_4O_{14}$; white foam; TLC (EtOAc/hexane = 3:2) R_f = 0.26; $[\alpha]^{24}_D$ +49.0 (*c* = 1.0, CH_2Cl_2); IR υ_{max} (neat) 3280, 2933, 1748, 1606 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz, two tautomers (3:2)) δ 12.24 (0.6 H, s), 11.97 (0.4 H, s), 8.98 (0.6 H, d, *J* = 8.4 Hz), 8.39 (0.4 H, d, *J* = 7.2 Hz), 7.77–7.62 (3 H, m), 7.46 (0.4 H, d, *J* = 8.4 Hz), 7.33 (0.6 H, d, *J* = 8.4 Hz), 7.18–7.07 (2 H, m), 6.12 (0.6 H, d, *J* = 8.8 Hz), 5.87–5.78 (0.4 H, m), 5.42–5.37 (0.6 H, m), 5.30–5.23 (1 H, m), 5.23–5.18 (0.4 H, m), 5.09–4.97 (1 H, m), 4.69–4.59 (1 H, m), 4.29–4.05 (3 H, m), 3.95–3.82 (3 H, m), 3.82–3.66 (4 H, m), 2.18–1.97 (9 H, m), 1.89–1.78 (3 H, m), 1.62–1.52 (3 H, m), 1.43–1.28 (9 H, m); ^{13}C NMR ($CDCl_3$, 100 MHz, two tautomers (3:2)) δ 187.6, 175.2, 170.6, 170.2, 170.09, 170.06, 169.8, 169.6, 169.5, 162.5, 161.3, 161.2,

157.4, 157.1, 156.5, 156.1, 152.1, 144.0, 143.9, 137.2, 133.6, 133.5, 133.1, 128.9, 128.7, 128.6, 128.4, 127.3, 126.6, 126.3, 126.2, 125.6, 125.4, 118.7, 118.6, 109.8, 105.3, 105.1, 83.2, 79.0, 77.2, 76.8, 71.3, 71.0, 71.0, 67.6, 67.3, 61.9, 54.74, 54.69, 51.82, 51.76, 50.7, 49.2, 47.8, 46.3, 46.2, 27.5, 27.3, 22.5, 22.1, 20.4, 20.3, 17.8, 16.8; HRMS (ESI) calcd for $C_{38}H_{49}N_4O_{14}$: 785.3245, found: *m/z* 782.3236 $[M + H]^+$.

5.3.6.

*5-Acetamido-4-(3-octanoylguanidino)-6-(1,2,3-trihydroxypropyl)-4,5-dihydro-6H-pyran-2-carboxylic acid (**7a**)*

To a solution of compound **14a** (58 mg, 0.083 mmol) in CH_2Cl_2 (1 mL) was added TFA (1 mL). The mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was dissolved in CH_3CN (1 mL), and then added 1 M $KOH_{(aq)}$ (1 mL). The mixture was stirred at room temperature for 30 min, acidified by adding TFA, and concentrated under reduced pressure. The residue was purified by reversed-phase chromatography (RP-18; elution with gradients of MeOH in H_2O (0−60%)) to afford the desired compound **7a** (29 mg, 76%). The purity of **7a** was 97.2% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm particle size), t_R = 8.5 min [MeOH/H_2O = 2:3 to 7:3 for 20 min then 7:3 to 100% H_2O for 20 min] at a flow rate of 1.0 mL/min. $C_{20}H_{34}N_4O_8$; white solid (hygroscopic); $[\alpha]^{24}_D$ +70.3 (*c* = 1.0, DMSO); IR υ_{max} (KBr) 3398, 2929, 1696, 1637 cm^{-1}; ^1H NMR (CD_3OD/CF_3CO_2D = 2:1, 400 MHz) δ 5.92 (1 H, d, *J* = 2.8 Hz), 4.72–4.62 (1 H, m), 4.51 (1 H, d, *J* = 8.8 Hz), 4.40–4.30 (1 H, m), 3.92–3.80 (2 H, m), 3.78–3.69 (2 H, m), 2.43 (2 H, t, *J* = 7.2 Hz), 2.00 (3 H, s), 1.68–1.55 (2 H, m), 1.37–1.18 (8 H, m), 0.83 (3 H, t, *J* = 6.4 Hz); ^{13}C NMR (CD_3OD/CF_3CO_2D = 2:1, 100 MHz) δ 176.9, 174.4, 164.6, 154.9, 146.3, 106.3, 76.7, 70.7, 69.6, 63.8, 50.4, 37.2, 31.9, 29.12, 29.07, 24.5, 22.7, 21.8, 13.3; HRMS (ESI) calcd for $C_{20}H_{35}N_4O_8$: 459.2455, found: *m/z* 459.2449 $[M + H]^+$.

5.3.7.

*5-Acetamido-4-(3-(2-(6-methoxynaphthalen-2-yl)propanoyl)guanidino-6-(1,2,3-trihydroxypropyl)-4,5-dihydro-6H-pyran-2-carboxylic acid (**7b**)*

According to the procedure similar to that for compound **7a**, compound **14b** (293 mg, 0.38 mmol) was treated with TFA in CH_2Cl_2 to remove the Boc group and subsequently saponified with 1 M $KOH_{(aq)}$ in CH_3CN to give the desired compound **7b** (189 mg, 93%). The purity of **7b** was > 99% as shown by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 μm particle size), t_R = 7.8 min [MeOH/H_2O = 2:3 to 7:3 for 20 min then 7:3 to 100% H_2O for 20 min] at a flow rate of 1.0 mL/min. $C_{26}H_{32}N_4O_9$; white solid (hygroscopic); $[\alpha]^{24}_D$ +28.9 (*c* = 1.0, DMSO); IR υ_{max} (KBr) 3415, 2938, 1691, 1605 cm^{-1}; ^1H NMR (CD_3OD/CF_3CO_2D = 2:1, 400 MHz) δ 7.69–7.60 (3 H, m), 7.37–7.25 (2 H, m), 7.10–7.03 (1 H, m), 5.86 (1 H, s), 4.62–4.55 (1 H, m), 4.50–4.42 (1 H, m), 4.32–4.25 (1 H, m), 3.95–3.78 (6 H, m), 3.73–3.66 (2 H, m), 1.92 (3 H, s), 1.47 (3 H, d, *J* = 7.2 Hz); ^{13}C NMR (CD_3OD/CF_3CO_2D = 2:1, 100 MHz) δ 177.8, 174.3, 164.5, 158.6, 155.1, 146.3, 134.8, 134.7, 134.6, 129.6, 129.5, 128.0, 126.9, 125.9, 119.5, 106.1, 105.9, 76.7, 70.7, 69.6, 63.8, 54.9, 50.3, 21.8, 17.6; HRMS (ESI) calcd for $C_{26}H_{33}N_4O_9$: 545.2248, found: *m/z* 545.2249 $[M + H]^+$.

5.3.8. *Ethyl 4-acetamido-5-(2-(tert-butoxycarbonyl)-3-octanoyl)guanidino-3-(pentyl-3-oxy)cyclohex-1-ene-1-carboxylate (**16a**)*

To a suspension of Tamiflu capsule (985 mg, 2.40 mmol) and $NaHCO_3$ (1007 mg, 11.99 mmol) in THF/H_2O (10 mL/10 mL) was added $(Boc)_2O$ (720 μL, 3.13 mmol). The mixture was stirred at room temperature for 5 h. The organic layer was collected, and the aqueous layer was washed with EtOAc. All organic phase was combined, dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 3:7 to 4:6) to afford the Boc-protecting oseltamivir **15** (907 mg, 92%). [31]

To a solution of compound **15** (128 mg, 0.31 mmol) in CH_2Cl_2 (2 mL) was added TFA (2 mL). The mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure to give the corresponding amine. The amine product was dissolved in CH_2Cl_2 (4 mL), and compound **12a** (98 mg, 0.31 mmol), $HgCl_2$ (92 mg, 0.34 mmol), and Et_3N (216 μL, 1.55 mmol) were added. The mixture was stirred at room temperature under argon for 6 h, filtered through Celite, and concentrated under reduced pressure. The residue was diluted with EtOAc and washed with 1 M $HCl_{(aq)}$, saturated $NaHCO_{3(aq)}$, and brine. The organic phase was dried over $MgSO_4$, filtered, and purified by silica gel chromatography (EtOAc/hexane = 2:3 to 1:1) to afford the coupling product **16a** (141 mg, 77%). $C_{30}H_{52}N_4O_7$; white foam; TLC (EtOAc/hexane = 1:1) R_f = 0.48; $[\alpha]^{24}_D$ –65.1 (*c* = 1.0, CH_2Cl_2); IR υ_{max} (neat) 3280, 2929, 1722, 1614 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, two tautomers (4:1)) δ 12.18 (0.8 H, s), 12.12 (0.2 H, s), 9.19 (0.8 H, d, *J* = 8.4 Hz), 8.65 (0.2 H, d, *J* = 7.6 Hz), 6.75 (0.2 H, s), 6.71 (0.8 H, s), 6.42–6.31 (1 H, m), 4.39–4.25 (1 H, m), 4.14–3.89 (4 H, m), 3.27 (1 H, quin, *J* = 5.6 Hz), 2.71 (1 H, dd, *J* = 17.6, 5.2 Hz), 2.32–2.19 (3 H, m), 1.82 (0.6 H, s), 1.80 (2.4 H, s), 1.61–1.48 (2 H, m), 1.48–1.31 (13 H, m), 1.27–1.10 (11 H, m), 0.84–0.69 (9 H, m); ^{13}C NMR ($CDCl_3$, 100 MHz, two tautomers (4:1)) δ 187.6, 174.3, 170.2, 170.1, 165.8, 165.7, 163.2, 156.4, 155.9, 152.5, 137.6, 137.1, 128.8, 128.5, 82.9, 82.6, 82.2, 79.4, 75.3, 75.2, 60.69, 60.66, 53.6, 53.0, 47.9, 41.2, 37.6, 31.5, 31.3, 30.0, 29.5, 29.1, 29.0, 28.62, 28.59, 28.0, 27.9, 27.7, 25.88, 25.85, 25.5, 24.3, 23.0, 22.9, 22.4, 22.3, 13.9, 13.82, 13.77, 9.4, 9.3, 9.14, 9.08; HRMS (ESI) calcd for $C_{30}H_{53}N_4O_7$: 581.3914, found: *m/z* 581.3912 $[M + H]^+$.

*5.3.9. Ethyl 4-acetamido-5-(2-(tert-butoxycarbonyl)-3-(2-(6-methoxynaphthalen-2-yl)propanoyl)guanidino-3-(pentyl-3-oxy)cyclohex-1-ene-1-carboxylate (**16b**)*

According to the procedure similar to that for compound **16a**, compound **15** (157 mg, 0.38 mmol) was treated with TFA to give the corresponding amine, which reacted with **12a**

(151 mg, 0.38 mmol) in the presence of $HgCl_2$ (114 mg, 0.42 mmol) and Et_3N (265 μL, 1.90 mmol) to afford the coupling product **16b** (225 mg, 89%). $C_{36}H_{50}N_4O_8$; white foam; TLC (EtOAc/hexane = 1:1) R_f = 0.40; $[\alpha]^{24}_D$ –101.1 (*c* = 1.0, CH_2Cl_2); IR υ_{max} (neat) 3287, 2933, 1722, 1606 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz, two tautomers (3:2)) δ 12.28 (0.4 H, s), 12.02 (0.6 H, s), 9.09 (0.4 H, d, *J* = 8.4 Hz), 8.52 (0.6 H, d, *J* = 8.0 Hz), 7.67–7.57 (3 H, m), 7.41 (0.6 H, d, *J* = 9.6 Hz), 7.29 (0.4 H, d, *J* = 9.6 Hz), 7.07–6.98 (2 H, m), 6.70 (0.4 H, s), 6.67 (0.6 H, s), 6.34 (0.4 H, d, *J* = 8.8 Hz), 5.37 (0.6 H, d, *J* = 9.2 Hz), 4.31–4.01 (3.4 H, m), 3.95–3.85 (1 H, m), 3.82–3.69 (4 H, m), 3.51–3.46 (0.6 H, m), 3.28–3.22 (0.4 H, m), 2.98–2.92 (0.6 H, m), 2.72–2.62 (1 H, m), 2.32–2.22 (1 H, m), 1.80 (1.2 H, s), 1.53 (3 H, d, *J* = 7.2 Hz), 1.45–1.33 (9.4 H, m), 1.30–1.13 (8.4 H, s), 0.84–0.73 (4.2 H, m), 0.63 (1.8 H, t, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz, two tautomers (3:2)) δ 187.8, 175.1, 170.1, 169.9, 165.5, 162.9, 157.5, 157.2, 156.7, 156.1, 152.3, 137.6, 137.5, 137.3, 133.8, 133.7, 133.0, 129.0, 128.8, 128.7, 128.6, 128.4, 127.4, 126.8, 126.3, 126.2, 125.64, 125.55, 118.8, 118.7, 105.3, 83.1, 82.6, 82.1, 79.2, 76.1, 75.1, 60.6, 60.5, 54.93, 54.85, 53.9, 53.3, 51.0, 48.2, 48.1, 47.8, 29.7, 27.8, 27.6, 25.8, 25.6, 25.5, 25.3, 22.8, 22.5, 17.8, 17.0, 13.9, 9.2, 9.0, 8.8; HRMS (ESI) calcd for $C_{36}H_{51}N_4O_8$: 667.3707, found: *m/z* 667.3702 $[M + H]^+$.

*5.3.10. 4-Acetamido-3-(pentyl-3-oxy)-5-(3-octanoylguanidino)cyclohex-1-ene-1-carboxylic acid (**8a**)*

To a solution of compound **16a** (47 mg, 0.081 mmol) in CH_2Cl_2 (1 mL) was added TFA (1 mL). The mixture was stirred at room temperature for 1 h, and then concentrated under reduced pressure. The residue was dissolved in CH_3CN (1 mL) and then added 1 M $KOH_{(aq)}$ (1 mL). The mixture was stirred at room temperature for 30 min, acidified by adding TFA, and concentrated under reduced pressure. The residue was purified by reversed-phase chromatography (RP-18; elution with gradients of MeOH in H_2O (0−75%)) to afford the desired compound **8a** (22 mg, 60%). The purity of **8a** was > 99% as shown by HPLC on an

HC-C18 column (Merck, 4.6 × 100 mm, 2 µm particle size), t_R = 11.1 min [MeOH/(0.1% TFA aqueous solution) = 3:2] at a flow rate of 0.5 mL/min. $C_{23}H_{40}N_4O_5$; white foam; $[\alpha]^{24}_D$ –9.6 (*c* = 1.0, MeOH); IR υ_{max} (neat) 3273, 2931, 1683 cm^{-1}; ^1H NMR (CD_3OD/CF_3CO_2D = 2:1, 400 MHz) δ 6.88 (1 H, s), 4.27–4.20 (1 H, m), 4.16–4.01 (2 H, m), 3.39 (1 H, quin, *J* = 5.6 Hz), 2.84 (1 H, dd, *J* = 17.6, 4.0 Hz), 2.50–2.35 (3 H, m), 1.98 (3 H, s), 1.67–1.55 (2 H, m), 1.55–1.42 (4 H, m), 1.36–1.18 (8 H, m), 0.92–0.78 (9 H, m); ^{13}C NMR (CD_3OD/CF_3CO_2D = 2:1, 100 MHz) δ 176.8, 174.1, 168.6, 154.5, 138.0, 128.8, 83.7, 74.8, 54.1, 50.4, 37.2, 31.9, 29.4, 29.2, 29.1, 26.4, 26.0, 24.6, 22.7, 21.9, 13.3, 8.9, 8.8; HRMS (ESI) calcd for $C_{23}H_{41}N_4O_5$: 453.3077, found: *m/z* 453.3074 $[M + H]^+$.

5.3.11.

*4-Acetamido-3-(pentyl-3-oxy)-5-(3-(2-(6-methoxynaphthalen-2-yl)propanoyl)guanidino)cyclohex-1-ene-1-carboxylic acid (**8b**)*

According to the procedure similar to that for compound **8a**, compound **16b** was treated with TFA in CH_2Cl_2 to remove the Boc group and subsequently saponified with 1 M $KOH_{(aq)}$ in CH_3CN to give the desired compound **8b** (159 mg, 88%). The purity of **8b** was 97.3% as shown by HPLC on an HC-C18 column (Merck, 4.6 × 100 mm, 2 µm particle size), t_R = 8.9 min [MeOH/(0.1% TFA aqueous solution) = 3:2] at a flow rate of 0.5 mL/min. $C_{29}H_{38}N_4O_6$; amorphous colorless solid; $[\alpha]^{24}_D$ –17.8 (*c* = 1.0, MeOH); IR υ_{max} (neat) 3274, 2934, 1684 cm^{-1}; ^1H NMR (CD_3OD/CF_3CO_2D = 2:1, 400 MHz) δ 7.71–7.61 (3 H, m), 7.36–7.25 (2 H, m), 7.13–7.05 (2 H, m), 6.85 (1 H, s), 4.18–4.10 (1 H, m), 4.10–3.97 (2 H, m), 3.92–3.85 (1 H, m), 3.82 (3 H, s), 3.36–3.28 (1 H, m), 2.82–2.70 (1 H, m), 2.37 (1 H, dd, *J* = 17.6, 6.8 Hz), 1.86 (3 H, s), 1.49 (1 H, d, *J* = 7.2 Hz), 1.47–1.31 (4 H, m), 0.80 (3 H, t, *J* = 7.6 Hz), 0.74 (3 H, t, *J* = 7.2 Hz); ^{13}C NMR (CD_3OD/CF_3CO_2D = 2:1, 100 MHz) δ 177.5, 174.0, 168.5, 154.5, 137.8, 134.83, 134.76, 134.6, 129.7, 129.5, 128.7, 128.1, 126.9, 125.8, 119.6, 105.9, 83.6, 74.5, 54.9, 53.7, 50.0, 29.0, 26.3, 26.0, 21.7, 17.4, 8.82, 8.79; HRMS (ESI) calcd for

$C_{29}H_{39}N_4O_6$: 539.2870, found: *m/z* 539.2872 $[M + H]^+$.

5.4. Determination of influenza virus $TCID_{50}$

The $TCID_{50}$ (50% tissue culture infectious dose) was determined by serial dilution of the influenza virus stock onto 100 μL MDCK cells at 1×10^5 cells/mL in 96-well microplates. The infected cells were incubated at 37 ^oC under 5.0% CO_2 for 48 h and added to each wells with 100 μL per well of CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega). After incubation at 37 ^oC for 15 min, absorbance at 490 nm was read on a plate reader. Influenza virus TCID50 was determined using Reed–Muench method.

5.5. Determination of neuraminidase activity by fluorescent assay

The neuraminidase activity was measured using diluted allantoic fluid harvested from influenza A/WSN/1933 (H1N1) infected embryonated eggs. A fluorometric assay was used to determine the NA activity with the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma). The fluorescence of the released 4-methylumbelliferone was measured in Envision plate reader (Perkin-Elmer, Wellesley, MA) using excitation and emission wavelengths of 365 and 460 nm, respectively. Neuraminidase activity was determined at 200 μM of MUNANA. Enzyme activity was expressed as the fluorescence increase during 15 min incubation at room temperature.

5.6. Determination of IC_{50} values

NA inhibition was determined by mixing inhibitor and neuraminidase for 10 min at room temperature followed by the addition of 200 μM of substrate. Inhibitor IC_{50} values were determined from the dose-response curves by plotting the percent inhibition of NA activity versus inhibitor concentrations using Graph Pad Prism 4.

5.7. Determination of EC_{50} and CC_{50} values

The anti-flu activities of neuraminidase inhibitors were measured by the EC_{50} values that were the concentrations of NA inhibitor for 50% protection of the virus infection-mediated cytopathic effects (CPE). Fifty microliter of diluted influenza virus at 100 $TCID_{50}$ were mixed with equal volumes of NA inhibitors at varied concentrations. The mixtures were used to infect 100 μL of MDCK cells at 1×10^5 cells/mL in 96-wells. After 48 h incubation at 37 ^oC under 5.0% CO_2, the CPE were determined with CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay reagent as described above. Inhibitor EC_{50} value were determined by fitting the curve of percent CPE versus the concentrations of NA inhibitor using Graph Pad Prism 4.

5.8. Cell permeability study

MDCK cells (2.5×10^6 cells) were grown overnight on 10-cm cell culture dishes (Greiner Bio-One) at 37 ^oC under 5% CO_2. After washed with PBS buffer, the cells were treated with 70 μL DMSO (MOCK) or 70 μl of 10 mM test compound (**4** or **8a**, 100 μM as the final concentration) in 7 mL 2% fetal bovine serum (FBS, Gibco) for 48 h. The cells were washed with PBS buffer twice, collected, and lysed by RIPA buffer (Thermo Fisher Scientific). The cell lysates (34 μL) of the cells treated with the test compounds were mixed with 66 μL of methanol and the mixtures were incubated at −80 °C for 30 min. The precipitated proteins were removed by filtration using Amicon® Ultra Filters (molecular cutoff of 10 KDa, Merck) and the filtrate was then subjected to LC−MS analysis to determine the concentrations of guanidine (**4**) and acylguanidine (**8a**). The calibration curves were established by using the cell lysates of the MOCK spiked with the test compound (**4** or **8a**) at final concentrations of 300, 30, 3, 0.3, and 0.03 μM (34 μL) followed with the methanol precipitation and filtration as described above. The quantitation of **4** or **8a** in the samples were performed with

LC−ESI−MS/MS analysis by using an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies) equipped with a Famos auto sampler (LC Packings) and a Velos Pro mass spectrometer (Thermo Fisher Scientific) equipped with standard electrospray ionization (ESI) ion source (Genomics Research Center, Academia Sinica). The samples were run on an XBridge BEH C18 Column (1.0 mm × 150 mm, 3.5 µm, Waters) with a linear gradient from 2% acetonitrile in 0.1% formic acid to 98% acetonitrile in 0.1% formic acid in 15 min with a flow rate of 100 µL/min. The target *m*/*z* values (327 and 453 for the protonated ions of **4** and **8a**, respectively) were sequentially isolated for collision-induced dissociation (CID) with the rapid scan mode. The signals in the range of *m*/*z* 100−500 were collected in a full-scan mode with enhanced resolution for quantitation.

5.9. Stability test

Stock solution (20 mM) of tested sample in DMSO was prepared and diluted to 1 mM with appropriate buffer or plasma. The reaction mixture was shaken at 1000 rpm at 37 ^{o}C. At different time intervals, 50 µL of reaction mixture was taken to quantitate the concentration of tested sample by HPLC on an HC-C18 column (Agilent, 4.6 × 250 mm, 5 µm particle size or Merck, 4.6 × 100 mm, 2 µm particle size) using aqueous solution or mixed solvents A/B, whereas solvent A was MeOH and solvent B was H_2O or 0.1% TFA aqueous solution, at a flow rate of 0.5 or 1.0 mL min^{-1} with detection at 214 or 254 nm wavelength. A plot of the concentration of tested sample versus the reaction time was drawn based on HPLC results, and the half-life of hydrolysis was calculated by fitting the plot with the pseudo-first order rate law.

5.10. Molecular modeling

The model of a specific compound in complex with the NA was constructed through docking this compound to the crystallographic structure of N1 neuraminidase (PDB codes:

3B7E for wild-type and 3CL0 for H275Y mutant). [37] By modifying the 3D structure of zanamivir (**1**, from PDB code: 3B7E) and oseltamivir carboxylate (**2b**, from PDB code: 2HU4) with SYBYL 8.0 program (Tripos Associates, St. Louis, MO), the 3D structure of compounds (**8a** and **8b**) was built up. GOLD 4.0.1 was used to dock the compound onto the protein with flexible docking option turned on. [38, 39] Kollmann-all atom charges [40] were assigned to the protein atoms, and Gasteiger–Hückel charges [41] were assigned to ligand atoms using the SYBYL 8.0 program. Initial 1000 independent genetic algorithm cycles of computation were carried out with ligand torsion angles varying between −180 and 180^{o}. The search efficiency was set at 200% to ensure the most exhaustive search for the docking conformational space. All other parameters were kept the same as the default settings. The docking processes were distributed to a 40-processor Linux cluster with Intel(R) Xeon(TM) CPU 3.00 GHz CPUs. The resultant ligand−protein complex structures were ranked with the GOLDSCORE scoring function to determine the top 1000 hits.

5.11. Mice experiments

Female BALB/c mice (4–5 weeks old) were anesthetized with zoletil and inoculated intranasally with 25 μL of infectious influenza virus. Synthetic compounds and ZA or OS were administered to groups of 10 mice at specific dosages by intranasal instillation (for ZA, **7a** and **7b**) or oral administration (for GOC, **8a** and **8b**) twice daily for 5 days. Control (placebo) mice received PBS on the same schedule. Four hours after the first dose of drug, mice were inoculated with 10 MLD_{50} of influenza virus [A/WSN/1933 (H1N1)]. Mice were observed daily for 14 days for survival. Survival curves were generated and compared by the Log-rank (Mantel−Cox) test using Prism 5.0b.

5.12. Ethical regulation of laboratory animals

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee, Academia Sinica, Taiwan. The work was done in the BSL-3 Laboratory of Genomics Research Center, Academia Sinica, Taiwan.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank Academia Sinica and the Ministry of Science & Technology for financial support (MOST 103-2325-B-002-041 and 104-0210-01-09-02).

Supplementary data

Cell permeability study, half-life measurements and LC−MS analyses of hydrolysis products, ^{1}H and ^{13}C NMR spectra and HPLC diagrams of compounds. Supplementary data related to this article can be found at https://

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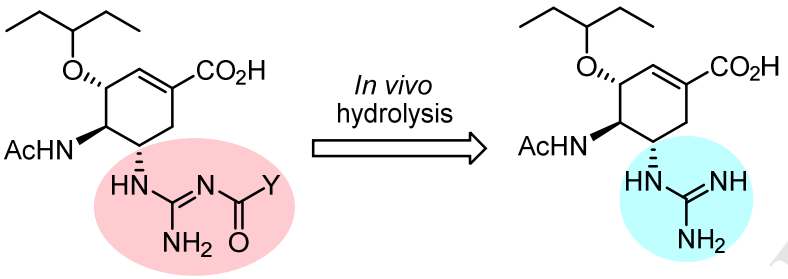
Graphical abstract



Acylguanidine prodrug
Y = hydrophobic group
Improved bioavailability

Guanidine active drug
Guanidino group
Enhanced ionic interactions

Legend of Tables, Figures and Schemes

Table 1. Neuraminidase inhibition (IC_{50}), anti-influenza activity (EC_{50}) and calculated lipophilicity.

Table 2. Stability tests of acylguanidine compounds.

Fig. 1. Chemical structures of influenza neuraminidase inhibitors and acylguanidine derivatives

Fig. 2. Schematic representations of molecular modeling of compounds **8a** (a) and **8b** (b) in the open-form neuraminidase (N1 subtype, PDB code: 2HU0).

Fig. 3. Survival rates of virus-infected mice on treatment with ZA acylguanidines. The administered dosage of each compound was 12 μmol/kg/day (a) and 2.4 μmol/kg/day (b). Compounds were administered by intranasal instillation and continued twice daily for 5 days. BALB/c mice were intranasally challenged with 10 LD_{50} of A/WSN/1933 (H1N1) viruses. The animal experiments were conducted with 10 mice per group. ***P<0.0001.

Fig. 4. Survival rates of virus-infected mice on treatment with GOC acylguanidine **8a**. Compounds were administered by oral gavage and continued twice daily for 5 days. The administered dosage of each compound was 48 μmol/kg/day. BALB/c mice were intranasally challenged with 10 LD_{50} of A/WSN/1933 (H1N1) viruses. The animal experiments were conducted with 10 mice per group. ***P<0.0001. **P<0.001.

Scheme 1. Synthesis of acylguanidine derivatives **7a**, **7b**, **8a** and **8b**.

Research highlights

• ZA and GOC acylguanidines are potent influenza neuraminidase (NA) inhibitors.

• Alkyl chain of acylguanidine improves lipophilicity and fits NA 150 & 143-cavities.

• ZA and GOC acylguanidines as prodrugs undergo hydrolysis in physiological conditions.

• ZA octanoylguanidine is better than ZA to treat influenza-infected mice in low dosage.

• GOC octanoylguanidine treats mice well by oral administration against influenza.