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Synthesis of acylguanidine zanamivir derivatives as neuraminidase inhibitors and the evaluation of their bio-activities†

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A series of acylguanidine-modified zanamivir analogs were synthesized and their inhibitory activities against the NAs of avian influenza viruses (H1N1 and H3N2) were evaluated. In particular, zanamivir derivative 3j, with a hydrophobic naphthalene substituent, exhibits the best inhibitory activity against group-1 NA with an IC_{50} of 20 nM.

Due to its rapid global spread, influenza is one of the most widespread pandemic human diseases. Despite advances in the understanding of the molecular and cellular aspects of influenza, this disease affects millions of people, causing serious public health and economic problems. The influenza virus is an RNA virus of the Orthomyxoviridae family, and the subtypes of influenza virus are classified based on the distinct antigenic properties of two viral surface membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Both glycoproteins are essential for virus infection, and there are currently 17 HA (H1–H17) and 9 NA (N1–N9) serotypes of influenza A virus circulating in the avian population.[1a,b] The NAs are phylogenetically categorized into two groups: group 1 contains the N1, N4, N5, and N8 subtypes, whereas group 2 contains N2, N3, N6, N7, and N9.[2] Furthermore, NA has been targeted in the development of structure-based drug design programs. As a result, two drugs, oseltamivir (**1**, Tamiflu),[3] an orally active NA inhibitor, and zanamivir (**2**, Relenza),[4] administered *via* nasal inhalation, have been developed and are commercially available. However, the serious threat of Tamiflu-resistant viruses[5] increases the urgency of developing a new generation of anti-influenza NA inhibitors.[6a–c]

The analyses of the X-ray crystal structures of NAs have revealed conformational differences among the subgroups with respect to the 150-loop adjacent to the active site of NA:[7] the group-1 NAs adopt an open 150-loop conformation with a 150-cavity near the active site, whereas the group-2 NAs show a closed 150-loop. Moreover, molecular dynamics simulations have suggested that the 150-loop and adjacent binding site loops may be more flexible than observed in the crystal structures.[8] In addition, recent evidence suggests that the 150-cavity exists not only in group-1 NAs but also in group-2 NAs.[9] These findings clearly provide insights that may aid in the design of new NA inhibitors that target the 150-cavity.

Previously, NA inhibitors were designed based on the transition state mimic of a hydrolyzed-sialic acid residue and the structural information of the group-2 NAs.[3,4] Since the discovery of the 150-cavity near the active site of group-1 NAs,[7] many efforts have been made to target the newly found cavity by extending the structures of the existing inhibitors by attaching additional groups with a suitable shape, size, and hydrophobicity.[10a–i] In general, the amine or guanidine functional groups of oseltamivir[10a–d] and zanamivir[10e] provide the chemical foundation for the generation of compounds either by click reaction or amide bond formation. Although several derivatives have been proposed to access the 150-cavity based on computational methods,[10a,c,d,i] the synthetic derivatives seemed not to maintain the same level of affinity for group-1 NAs as the parent compounds did. For example, Mohan *et al.*[10b] employed a click reaction to construct triazole derivatives at the C-3 position of the similar structure of oseltamivir. However, the modification resulted in a decrease in the inhibition potency. In addition, Wen *et al.*[10e] synthesized C-4 modified zanamivir derivatives by employing *N*-alkylation, and the resulting derivatives only showed inhibitory activities at the micromolar level. Recently, von Itzstein *et al.*[10f] modified the well-known

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neuraminidase inhibitor 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (Neu5Ac2en) at its C-3 position with a (*p*-tolyl)allyl group and demonstrated by X-ray crystallography that the inhibitor locks the flexible 150-loop of group-1 NAs. Furthermore, these two synthesized derivatives exhibited the selective inhibition of group-1 NAs, including the NAs of A/Hong Kong/156/97 (H5N1), A/California/07/2009 (H1N1), and A/Paris/0497/2007 (H1N1), over the group-2 NA A/Paris/908/97 (H3N2). However, due to the lack of a guanidine group at the C-4 position of Neu5Ac2en, the activities of the synthesized compounds are far lower ($K_i = 15.3 \times 10^{-5}$ M) than that of zanamivir ($K_i = 0.13 \times 10^{-9}$ M) against the pandemic 2009 A/H1N1 influenza virus.

However, a close examination of the crystal structure of the zanamivir-bound group-1 NA revealed that the C-4 guanidino group of zanamivir is also located near the 150-cavity and can serve as a potential modification site in the design of group-1 NA-specific inhibitors. Inspired by these findings and the identification of viruses less resistant to zanamivir, we modified the C-4 guanidino group of zanamivir and evaluated the activities of the derivatives against NAs. In contrast to the previous findings for zanamivir with an *N*-alkylated guanidino group, the *N*-acylated zanamivir analogs (**3** in Fig. 1) maintained potent inhibitory effects.

To synthesize the C-4 guanidino-modified zanamivir derivatives **3**, zanamivir **2** (Scheme 1) was prepared from sialic acid by following the reported procedures.[11] The initial attempt to synthesize acylguanidines by the direct coupling of the guanidino group in zanamivir with acids using coupling reagents, such as EDC with HOBt or HBTU, HBTU with HOBt, and ByBop, resulted in low yields. To circumvent this problem, as illustrated in Scheme 1, the synthetic route was modified by first forming the acylguanidine derivative **5**, followed by reacting this derivative with the corresponding amine **6**[12] to give the *N*-acylated guanidine **7**. Initially, the N-terminal region of 1*H*-pyrazole-1-carboximidamide (**4**),[13] a guanidinylation reagent, was protected with Boc_2O,[14] followed by deprotonation using NaH and then acylation with *N*-hydroxysuccinimide-activated acid to give **5** as a mixture of two stereoisomers. Notably, the yields of the amide bond formation reaction were moderate (40–60%) due to the decomposition of the activated acid by NaH. In addition, the direct coupling of **4** with acid failed. The guanidinylation of **6** with **5** under slightly basic conditions in THF gave acylguanidine **7** (75–87%), which was deprotected under acidic conditions (TFA in DCM) to afford compound **8** as a single isomer with an excellent yield. Finally, the hydrolysis of **8** with K_2CO_3 gave the zanamivir derivatives **3a–3ac** (Table 1). However, when the acid was protected as the methyl ester, in some cases (such as **3r**), the hydrolysis of the methyl ester resulted in a low yield due to the instability of the acylguanidine group under strong basic conditions, and further attempts to improve the yield were unsuccessful (see Table S1†). Considering the potential instability of the acylguanidine group,[15] the methyl ester-protecting group in **6** was replaced with an ethyl ester, which has been demonstrated to be much more labile than the former under basic deprotection conditions.[16] In addition, the release of ethanol is less toxic *in vivo*, benefiting future prodrug development.[17] Thus, full deprotection was achieved by using molar equivalents of K_2CO_3 in H_2O–methanol (v/v = 1/4) at 0 °C (Table S1, entry 7†) to afford the final products in 70–90% yields.

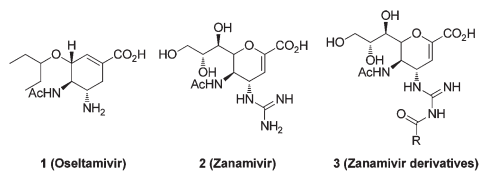
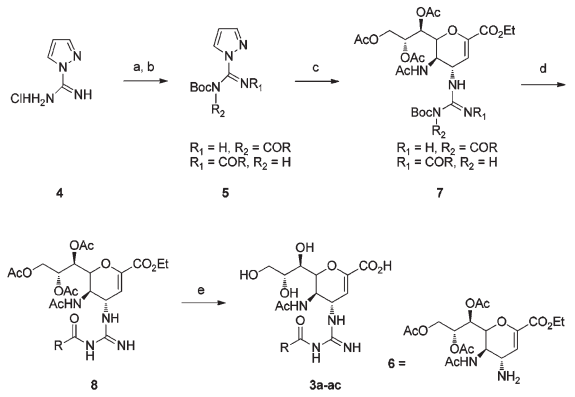


Fig. 1 NA inhibitors of transition state and zanamivir derivatives.

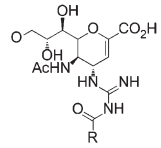


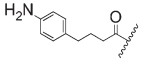
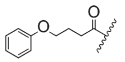
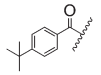
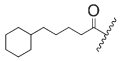
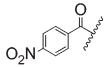
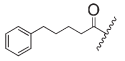
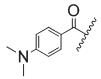
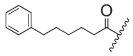
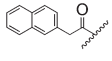
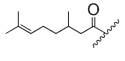
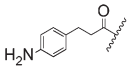
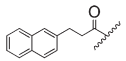
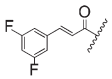
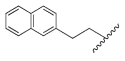
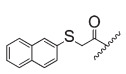
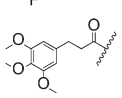
Scheme 1 Synthesis of acylated zanamivir derivatives by modifying the guanidino group of zanamivir. Reagents and conditions: (a) Boc_2O, DIPEA, DCM, DMF, rt, 95%; (b) NaH, RCOOSuc, DCM, rt, 40–60%; (c) **6**, Et_3N, THF, rt, 75%–87%; (d) TFA, DCM, rt, 95%; (e) K_2CO_3, EtOH, H_2O, rt, 70–90%.

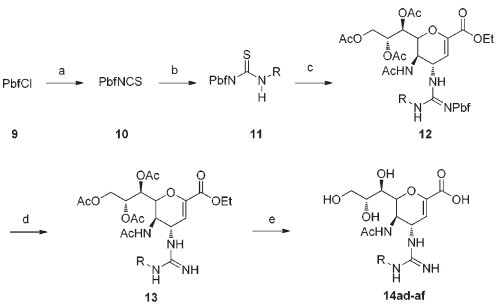
However, the amide bond formed at the guanidino group may affect the ability of the guanidine to form hydrogen bonds with the interacting amino acid residues of NA due to the electron-withdrawing property of the amide group. Thus, the *N*-alkylated guanidino groups of zanamivir derivatives **14ad–14af** were synthesized to investigate the electronic effect of the guanidine on H-bonding interactions. As shown in Scheme 2, the synthesis of **14** was similar to that described in Scheme 1 except that Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-protected thiourea (**11**) was used as a guanidine precursor. Compound **11** was obtained by reacting tetrabutylammonium thiocyanate [$(n\text{-Bu})_4$NSCN] with PbfCl, followed by the addition of an amine.[18] Finally, the guanidine was formed by coupling thiourea **11** with **6** in the presence of EDC and DIEPA[19] to afford **12**. The removal of the Pbf group under acidic conditions (TFA–DCM) was followed by the hydrolysis of the ethyl ester under basic conditions (K_2CO_3–EtOH) to yield the *N*-alkylated guanidino group on zanamivir, derivative **14**, in 73–88% yield.

The inhibitory activities of synthetic compounds **3a–3ac** and **14ad–14af** against the H1N1 and H3N2 NAs were evaluated, and the results are shown in Table 1. In our assay, zanamivir, with an IC_{50} of 0.74 ± 0.03 nM, was used as a positive control. The results indicate that the amide derivatives

Table 1 Inhibitory activities of novel zanamivir derivatives against group-1 (H1N1) and group-2 (H3N2) neuraminidases



Compd	R	H1N1 IC_{50} (nM)	H3N2 IC_{50} (nM)	Compd	R	H1N1 IC_{50} (nM)	H3N2 IC_{50} (nM)	Compd	R	H1N1 IC_{50} (nM)	H3N2 IC_{50} (nM)
Relenza		0.74 ± 0.03		**3k**		275 ± 1	334 ± 63	**3v**		421 ± 9	399 ± 1
3a		>1000	>1000	**3l**		203 ± 10	234 ± 7	**3w**		180 ± 9	173 ± 2
3b		>1000	>1000	**3m**		189 ± 5	211 ± 6	**3x**		397 ± 1	472 ± 4
3c		152 ± 3	147 ± 9	**3n**		462 ± 60	606 ± 17	**3y**		260 ± 21	421 ± 72
3d		>4000	>4000	**3o**		196 ± 2	205 ± 27	**3z**		293 ± 23	360 ± 3
3e		46.1 ± 0.4	44.9 ± 2.7	**3p**		134 ± 17	134 ± 16	**3aa**		775 ± 16	868 ± 38
3f		54.9 ± 0.5	58.3 ± 0.6	**3q**		332 ± 22	472 ± 31	**3ab**		541 ± 24	615 ± 20
3g		98.3 ± 2.9	84.4 ± 8.9	**3r**		176 ± 10	209 ± 8	**3ac**		203 ± 1	196 ± 1
3h		209 ± 18	243 ± 13	**3s**		1200 ± 10	1400 ± 14	**14ad**		39 400 ± 1400	29 600 ± 1100
3i		74.9 ± 0.8	66.9 ± 0.5	**3t**		160 ± 13	182 ± 1	**14ae**		34 644 ± 2786	38 303 ± 859
3j		20.1 ± 0.7	25.5 ± 1.0	**3u**		239 ± 16	337 ± 20	**14af**		>40 000 (43%)	>40 000 (35%)



Scheme 2 Synthesis of *N*-alkylated zanamivir derivatives by modifying the guanidino group of zanamivir. Reagents and conditions: (a) $(n\text{-Bu})_4NSCN$, DCM, rt, 60%; (b) R-NH_2, DCM, rt 78%; (c) **6**, EDC, DIPEA, DCM, rt, 65%–83%; (d) TFA, DCM, rt, 70%–83%; (e) K_2CO_3, EtOH, H_2O, rt, 73–88%.

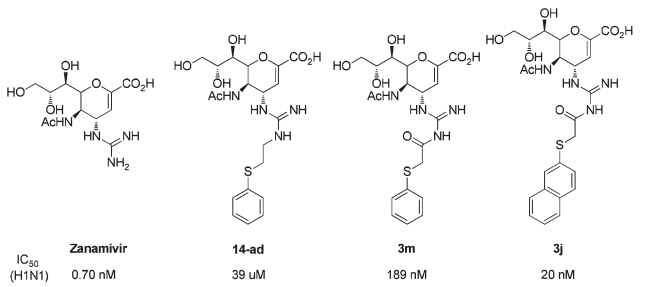


Fig. 2 Structures and inhibition activities of zanamivir and its derivatives.

(**3a–3ac**) are, in general, more potent inhibitors than the *N*-alkylated derivatives (**14ad–14af**) but are slightly less active than the parent compound, zanamivir. Although the inhibitory activity gradually decreased with increasing alkyl chain length in the arylguanidine groups (**3e**, **3l**, **3y**, and **3z**), compound **3ac**, with the longest alkyl chain tested, still maintained inhibitory activity, with an IC_{50} of 203 nM. This result may be due to the increased flexibility of the benzene ring, allowing it to fit into the 150-cavity, when the linker is longer. Notably, the activity seems to be reduced in the absence of a linker between the aromatic ring and the acylguanidine (**3a**, **3b**, and **3d**). However, the presence of a strong electron-withdrawing group (NO_2) on the aromatic ring of **3c** shows an intricate effect on the enhancement of the inhibitory activity (**3c** *vs*. **3a** and **3d**). Moreover, the inhibitory activity is significantly enhanced to 46.1 nM by introducing a methylene unit between the acylguanidine and aromatic ring (**3e** *vs*. **3a**). Although the potency of inhibition decreases slightly to 54.9 nM with the incorporation of a more hydrophobic naphthalene group (**3f**), compound **3j**, with an internal S atom, is the most potent NA inhibitor (IC_{50} = 20.1 nM) among those tested in this study. The results show that the heteroatom, S, in the internal chain and the hydrophobic naphthalene group are important factors to increase the inhibitory activity (**3i** *vs*. **3j**, **3l** *vs*. **3m**, and **3i** *vs*. **3l**). Although the heteroatom located at the *ortho* position of an aromatic ring can increase the inhibitory activity (**3p**), the steric hindrance imposed would likely decrease the inhibitory activity, as for **3e** *vs*. **3h** and **3l** *vs*. **3n–3q**. Substituents at the other positions (**3t** and **3u**) of the benzene ring or the replacement of benzene with acyclic substituents (**3aa–3ab**) or other carbocyclic analogs (**3s** and **3x**) only produce moderate inhibitory activities. In addition, the replacement of the benzene ring (**3y**) with cyclohexane (**3x**) results in a decrease in the inhibition potency.

To further investigate whether the synthesized inhibitors exhibit selectivity among the different NA subgroups, the activities of the inhibitors against the H3N2 NA were evaluated. The results indicate that the zanamivir analogs show inhibitory activities against N2 that are similar to those against N1 (Table 1), although the activity against N2 is lower. Therefore, the acylguanidium moiety of the zanamivir analogs may also participate in an interaction that contributes to the observed inhibitory activities against the neuraminidase of group-2 influenza viruses. Although the 150-loop of the N2 neuraminidase has the closed form, the inhibitor may induce a conformational change in the 150-loop, yielding the open form, when it interacts with NA.[7]

In addition, the *N*-alkylated zanamivir analogs, such as **14ad**, **14ae**, and **14af**, show very low inhibitory activities for N1 (IC_{50} > 30 μM), similar to a previous observation.[10e] Interestingly, the *N*-alkylated zanamivir analog **14ad** exhibits more than 200-fold lower potency relative to acyl zanamivir analogs, such as **3m** (Fig. 2). These results clearly indicate that the change in the electronic properties of the guanidium moiety affects the binding affinity and that the carbonyl function of the acylguanidino group has a prominent role in the NA inhibitory activity.

To better understand the effect of a carbonyl group at the acylguanidino group on activity, computer docking studies of the inhibitors with NA were performed (Fig. 3). GEMDOCK[20] was employed to predict the docked conformations of the inhibitors in the binding cavity of H1N1 neuraminidase based on calculated binding energies. The 3D structures of the inhibitors were generated using ACD/ChemSketch, and the open-form crystal structure of H1N1 neuraminidase (PDB code 3BEQ) was selected for the docking procedure. The binding site of NA was defined to include the residues within a 10 Å radius sphere centered around the 150-loop (residues 147–152) of the H1N1 neuraminidase. The simulation for compound **3j** clearly shows that the hydrophobic substituent of the guanidine interacts with the 150-cavity, as shown in Fig. 3A. In addition, to avoid the steric hindrance involving the Asp151 residue of NA generated by the modification of the guanidine, the docking conformation of compound **3j** is slightly different from the docking mode of zanamivir, as shown in Fig. 3B and 3D. The carboxylic acid of zanamivir forms an H-bond with residue Arg118 of NA, but in **3j**, Arg118 interacts with the carbonyl group of the amide of the guanidine moiety. In addition, the guanidine of zanamivir forms six hydrogen bonds with the residues Glu119 and Asp151, and the carboxylic acid of zanamivir forms five hydrogen bonds with the residues Arg118, Arg292, and Arg371. However, the total number of hydrogen bonds generated by the same groups as above is reduced to seven in the interaction between **3j** with NA. The acylguanidine of **3j** forms three hydrogen bonds with the Arg118 and Asp151

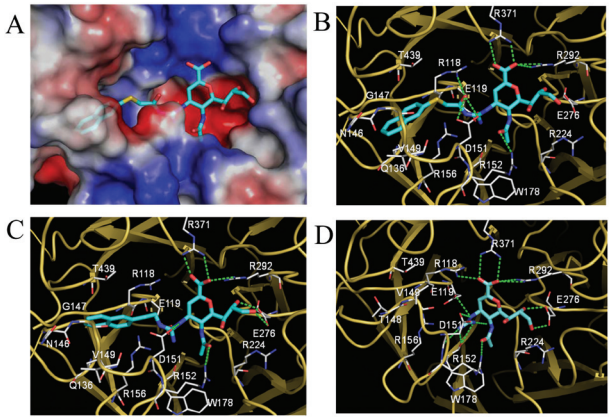


Fig. 3 Computational conformations of the compounds in the open-form of H1N1 neuraminidase. Molecular surface of N1 neuraminidase with bound compound **3j** (A) and the docking results of compounds **3j** (B), **14ad** (C), and zanamivir (D) with neuraminidase in the open form with carbon atoms of inhibitor in cyancolor and protein carbons in light blue color. Oxygen and nitrogen atoms are red and blue, respectively. Hydrogen bonds between the compounds and the protein are represented by green dotted lines.

residues, whereas the carboxylic acid forms four hydrogen bonds with Arg292 and Arg371 (Fig. 3B and 3D). In contrast, the *N*-alkylated guanidino group of compound **14ad** has only one hydrogen bond with NA (Fig. 3C). In addition, the aromatic or heterocyclic group of compound **3** fit into the 150-cavity and interacted with residues Q136, G147, V149, and T439, which form a hydrophobic pocket. Overall, although the modification of the guanidino group of zanamivir results in the loss of four H-bonds, the hydrophobic interaction between the 150-cavity and the hydrophobic substituent compensates for this loss in binding energy, resulting in only a minimal reduction in the binding energy. These results suggest that the addition of a hydrophobic group to zanamivir that fits into the 150-cavity could be a good strategy to enhance the binding affinity. In addition, the modification at the C-3 position of zanamivir seems to be another approach to obtain a more potent inhibitor because the hydrogen bonding between the guanidine group and NA may be retained, and thus the extended group can target the 150-cavity.

Conclusions

In summary, the results show that acylguanidine derivatives of zanamivir are more potent inhibitors than the corresponding *N*-alkylated derivatives and that the carbonyl group plays a dominant role in binding by generating three additional H-bonds with R118 and D151 of NA. Although the modifications at the guanidino group of zanamivir interrupt the H-bonding interactions with NA, the addition of a carbonyl group and a hydrophobic moiety can compensate for the binding energy loss. Among the compounds tested, **3j** displayed the highest potency against both N1 and N2, with IC_{50} values of 20 nM and 25 nM, respectively. Although there is no apparent selectivity among the NAs when targeting the 150-loop, our results may provide an overall advantage of a better oral bioavailability due to the improved hydrophobicity of zanamivir resulting from the addition of hydrophobic groups at its guanidine site. Finally, we anticipate that these findings may provide new insight that will aid in the development of a new generation of anti-influenza drugs.

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