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Short Communication

Replication inhibition activity of carbocycles related to oseltamivir on influenza A virus *in vitro*

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ABSTRACT

We have recently demonstrated that newly synthesized oseltamivir derivatives that contain a substituted triazole ring at the C-5 amino group interact with the 150 cavity found specifically in the group-1 neuraminidase (NA) subtypes of influenza A virus. These compounds exhibited *in vitro* inhibition activity of a group-1 NA enzyme incorporated in virus-like particles (VLPs). In the current study, we tested these nine triazole-containing carbocycles as well as an amino- and a guanidino-substituted derivative in virus replication inhibitory assays *in vitro*. None of the triazole-containing carbocycles significantly inhibited influenza A virus replication in MDCK cells with either a virus strain containing a group-1 or a group-2 subtype NA. In contrast, the amino- and guanidino-substituted derivatives clearly inhibited the cytopathic effect or spread of virus infection detected by immunostaining in MDCK monolayers as well as progeny virus release; these compounds were also reported to have shown the highest inhibition of group-1 NA in the context of VLPs. These results, together with the structures of these compounds, suggest that hydrogen-bonding interactions between the polar amino or guanidino functions and complementary groups in the neuraminidase active site (*e.g.* Asp151, Glu 119) may be essential for strong inhibition of the neuraminidase enzyme and, in turn, the inhibition of influenza A virus replication.

Influenza viral neuraminidase (NA) is an enzyme that cleaves the receptor molecule for influenza A virus (IFV-A), sialic acid, on host cells, and is necessary to release newly produced virions from the infected cells (Crusat and de Jong, 2007). Because of the crucial role in the viral replication process, compounds that inhibit this enzymatic activity, neuraminidase inhibitors (NAIs), have been developed as anti-influenza drugs and have played a key role in reducing the impact of influenza epidemics (Crusat and de Jong, 2007; Memoli et al., 2008; WHO, 2010). Currently, two NAIs, oseltamivir (Tamiflu) and zanamivir (Relenza) (Fig. 1) are widely used in clinical settings and are commercially available worldwide. Of them, oseltamivir is available by oral administration while zanamivir is not (Hayden, 2009). Since these NAIs bind to the active site of the enzyme it was expected that drug-resistant mutants would not cause a major problem; the mutants that are not bound by the NAI compounds would lose the enzyme activity or have at least weaker enzymatic activity and be less fit for virus replication and transmission (Crusat and de Jong, 2007). So far, this seems to be true for zanamivir; very few zanamivir resistant IFV-A have been reported (Dapat et al., 2010; Hurt et al., 2009; Okomo-Adhiambo et al., 2010; van der Vries et al., 2010). However, in the case of oseltamivir that has been in more widespread use, the percentage of resistant mutants among the clinical isolates is alarmingly increasing (Okomo-Adhiambo et al., 2010; Ujike et al., 2010). To maintain the current control schemes for IFV epidemics, therefore, it is essential to develop new NAIs that are efficacious against the emerging resistant mutants.

We have been developing novel NAI molecules based on the three-dimensional structure of NA. Recent fine structural analyses revealed that there is a large cavity, which was referred as the 150-cavity, adjacent to the active site in the group-1 NA subtype but not in the group-2 NA (Fig. 2)(Russell et al., 2006). Group-1 NA includes subtypes N1, N4, N5 and N8 while group-2 includes subtypes N2, N3, N6, N7 and N9 (Fouchier et al., 2005). To design new NAI molecules, we took advantage of this adjacent cavity and generated new compounds that not only bind to the active site but also occupy the 150-cavity by incorporating various substituted triazole rings in place of the C-5 amino group of oseltamivir (Mohan et al., 2010). These newly generated triazole series of compounds (**1**–**9**, Fig. 3) showed NA inhibition activities with virus-like particles (VLPs) at K_i values in the 10^{-5}–10^{-8} M range. In contrast, the double bond isomer of oseltamivir (**10**, Fig. 3), a known inhibitor that preserves the free amino group, and a new candidate containing a guanidine function (**11**, Fig. 3) are better inhibitors, with

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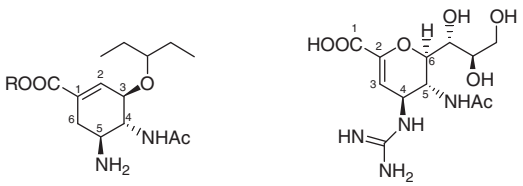

R = Et, Osletamivir
R = H, Osletamivir carboxylate (active metabolite)

Zanamivir

Fig. 1. Structures of oseltamivir and zanamivir.

K_i values of 1.5×10^{-9} and 4.6×10^{-10} M, respectively. In this report, we further explored the potential of these compounds as alternative anti-influenza virus drug candidates by examining the inhibition activity of virus replication *in vitro*.

The compounds tested in this study were described in detail previously (**1**–**11**, Fig. 3) (Mohan et al., 2010). We tested eleven compounds in parallel, including the amino- (the double bond isomer of oseltamivir carboxylate, **10**) and guanidino- (**11**) substituted compounds. Influenza A virus strains Puerto Rico/8/32 (H1N1, PR8) and Hong Kong/1/68 (H3N2, HK1), both of which are mouse-adapted, were obtained from Dr. E. Brown (University of Ottawa) (Brown et al., 2001). MDCK cells were maintained in Dulbecco's minimum essential medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, Invitrogen). After the infection the monolayers were maintained in DMEM with 0.00075% trypsin (Difco) and antibiotics without FBS (DMEM-trypsin). Tissue culture infectious dose 50 ($TCID_{50}$) of the IFV stocks were determined by inoculating 100 μl of 10-fold virus stocks dilutions in 96 well plates. $TCID_{50}$ was calculated from the results of 8 wells for each dilution. In the case of the HK1 strain, the infection of IFV was visually determined by the cytopathic effect (CPE) under a light microscope on 3 days post infection (dpi). In the case of strain PR8, which does not produce obvious specific CPE on MDCK monolayers, the infected monolayers were fixed by methanol–acetone (1:1) and immunostained by PR8-specific chicken antiserum (Charles River) and FITC-labeled anti-chicken IgY (Sigma) on

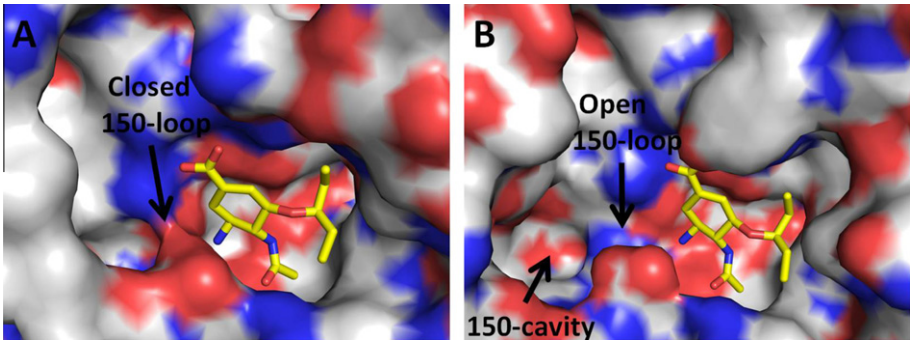

Fig. 2. Active site comparison of group-2 and group-1 enzymes: oseltamivir carboxylate bound in the active site of (A) the N2 subtype (150 loop closed) and (B) the N8 subtype (150 loop open). Reproduced from the respective PDB files, 2QWK and 2HT7.

Compound	Ki (M)
1	4.6×10^{-7}
2	7.2×10^{-8}
3	1.3×10^{-7}
4	4.8×10^{-6}[b]
5	1.1×10^{-5}[b]
6[a]	1.2×10^{-6}
7[a]	5.8×10^{-6}
8[a]	1.9×10^{-5}
9[a]	6.0×10^{-5}
10	1.5×10^{-9}
11	4.6×10^{-10}
zanamivir	1.6×10^{-10}

[a]tested as arginine salt
[b]IC_{50} values

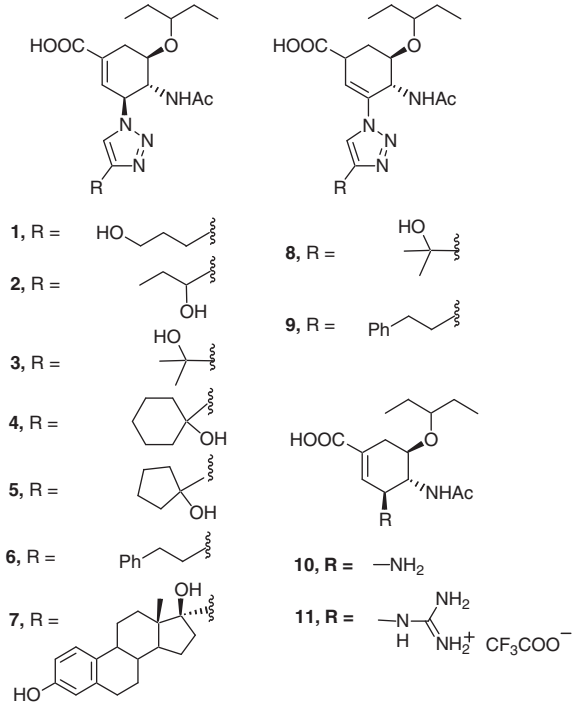

Fig. 3. Structures and inhibitory activities of compounds used in this study (**1**–**11**). The *Ki* values were measured with VLPs containing N1 subtype of influenza A virus neuraminidase (Mohan et al., 2010).

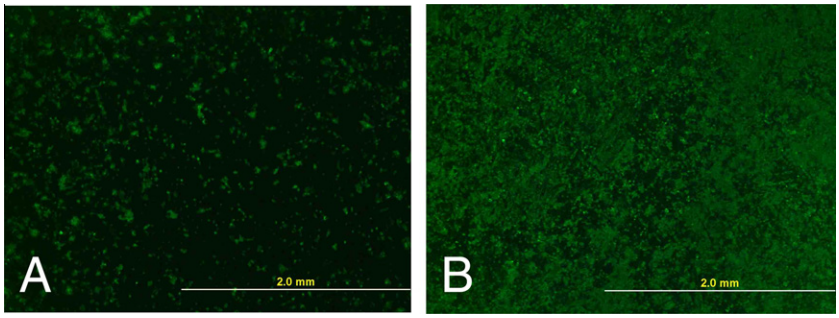

Fig. 4. Typical results of replication inhibition of PR8 strain. A; inhibition positive (compound **11** at 2×10^{-6} M), B; inhibition negative (compound **10** at 2×10^{-6} M).

2 dpi. The monolayers that showed positive staining were considered as infection positive.

To test the replication inhibition efficacy, the compounds were dissolved in sterile water and then diluted in DMEM-trypsin. Each diluted test compound (50 μl) was added to each well of MDCK monolayers in 96 wells and then followed by 50 μl of virus suspension (50 $TCID_{50}$) prepared in DMEM-trypsin. The test was performed in quadruplicate and certain experiments were repeated in similar quadruplicate settings. The inhibition effect was regarded as positive when the virus spread was not observed by the same method as used for the determination of $TCID_{50}$. In the case of strain PR8, the lack of spread of positive staining rather than the lack of individual cell staining was considered as effective inhibition due to the nature of the inhibition mechanism of these compounds (Fig. 4).

As shown in Table 1, compound **11** showed the highest efficacy of inhibition to PR8 (N1), as predicted from the lowest K_i in the NA inhibition assay using VLPs (Mohan et al., 2010). The required concentration for the inhibition was 2×10^{-6} M, which is approximately 10^4 times higher concentration compared to its K_i value. The double bond isomer of oseltamivir carboxylate (compound **10**) inhibited PR8 replication at 1×10^{-4} M, which is almost 10^5 times higher than its K_i value. Other than these two compounds, compound **7** showed an inhibitory effect at 1×10^{-4} M in one experiment but it required 5×10^{-4} M (next higher dilution tested, data not shown) in a similar experiment; thus, the required concentration of compound **7** for the inhibition in this experimental setting was considered between 1 and 5×10^{-4} M. In addition, compound **4** showed an inhibitory effect at 5×10^{-4} M in one experiment but the effect was incomplete at the same concentration in a repeated experiment.

The inhibition of replication was tested with another strain HK1, whose NA subtype is N2 that belongs to the group 2 NA. As shown in Table 1, only compound **7**, **10** and **11** showed significant inhibitory effects, as in the case for PR8. With HK1, compound **11** showed an inhibitory effect as low as 8×10^{-8} M, while compound **10** required 4×10^{-7} M. These inhibitory concentrations for HK1 were 25 and 250 times lower, respectively, than those required for PR8. Compound **7** showed an inhibitory effect at 1×10^{-4} M, that is, a similar concentration required for inhibition of PR8. Other than these three, compounds **1**, **4**, **5** and **6** showed inhibitory effects at 5×10^{-4} M in one experiment (data not shown).

We further tested whether compounds **10** and **11** inhibit the release of progeny infectious virus in the culture supernatants. Quadruple monolayers in 96 well plates were infected with 50$TCID_{50}$ virus and incubated for 4 days in the existence of various concentrations of either compounds **10** or **11**, as described above. The culture supernatants were pooled and the virus titers were determined by either $TCID_{50}$ or plaque assays for PR8 and HK1 strains, respectively. To minimize the compound carryover existing in the supernatants, the infected monolayers were washed twice with PBS after absorption for one hour in both experiments.

Both compounds reduced virus titers in the culture supernatants dose-dependently (Fig. 5). Compound **11** decreased virus titers in the supernatants of PR8 and HK1 strains to less than one-thousandth and one-ten thousandth of the control, respectively, at a concentration of 10^{-6} M. Compound **10** reduced the titer of PR8 to less than one-thousandth of the control at a concentration of 10^{-4} M while it reduced the titer of HK1 strain approximately to one-hundredth at a concentration of 10^{-6} M. These results demonstrate that compounds **10** and **11** significantly decrease the progeny virus release from the infected cells, as expected

Table 1
Inhibitory effect of the new compounds for PR8 (H1N1) and HK1 (H3N2).

Conc. (M)/compound	1		4		6		7		10		11[a]	
IFV strain	PR8	HK1	PR8	HK1	PR8	HK1	PR8	HK1	PR8	HK1	PR8	HK1
$5\ (2.5) \times 10^{-4}$	–	±	+	–	–	±	+	+	+	+	+	+
1×10^{-4}	–	–	±	–	–	–	+	+	+	+	+	+
2×10^{-5}	–	–	–	–	–	–	–	–	±	+	+	+
2×10^{-6}	–	ND[b]	–	ND	–	ND	–	ND	–	+	+	+
4×10^{-7}	–	ND	–	ND	–	ND	–	ND	–	+	±	+
8×10^{-8}	–	ND	–	ND	–	ND	–	ND	–	±	–	+
1.6×10^{-8}	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	±
3.2×10^{-9}	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	±
6.4×10^{-10}	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	ND	–

Only compounds that showed any inhibitory effect are shown.
Test monolayers were infected at 50 $TCID_{50}$.
+, inhibition positive; ±, incomplete inhibition; –, inhibition negative
[a] 2.5×10^{-4} was the highest concentration tested.
[b] Not done

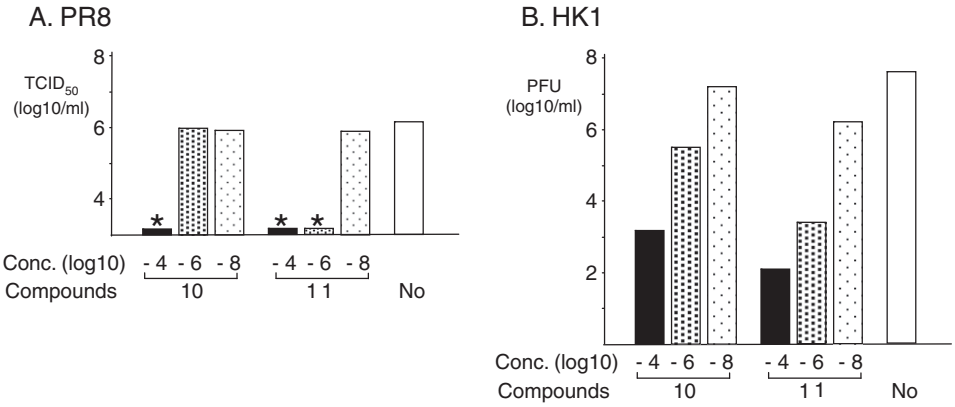

Fig. 5. Virus titers in the culture supernatants of cells treated with compounds **10** and **11**. The virus titers were determined either by $TCID_{50}$ (PR8, A) or plaque (HK1, B) assays. Culture supernatants from three concentrations, 10^{-4}, 10^{-6} and 10^{-8} M, for each compound were tested. For the $TCID_{50}$ assays, 50 μl of diluted supernatants were inoculated to each of 96 wells. The inoculums were removed after an absorption period (1 h) and the monolayers were washed with PBS twice before being fed with fresh medium. For the plaque assays, monolayers, in 6 well plates were infected for one hour and washed with PBS twice after this absorption period before being fed with DMEM-trypsin containing 1% agar (Agar Noble, USB). Asterisks above the bars in A indicate the titer was less than 2×10^3 $TCID_{50}$.

from their inhibitory effect on the spread of infection in monolayers.

Compound **11**, a guanidino-substituted compound, showed replication inhibitory effects for both strains at less than μM, which is similar to the available anti-influenza NAI drugs, zanamivir and oseltamivir (Yen et al., 2005), whereas the double bond isomer of oseltamivir carboxylate (compound **10**) also showed inhibition at the μM level for strain HK1 but required 50 times higher concentration for inhibition of PR8. It is known that there are significant strain variations in the sensitivity for NAIs (Govorkova et al., 2001; Sidwell and Smee, 2000). Compared to the K_i values obtained in the NA inhibition assay using virus-like particles (Mohan et al., 2010), these concentrations were approximately 10^3–10^4 times higher.

Several other compounds showed weak replication inhibitory effects at a higher concentration but it was not always the case that compounds which showed lower K_i values for NA activity also showed better inhibitory efficacy in this replication inhibition assay. For example, compound **2**, which showed the third lowest K_i value in the NA inhibition assay ($K_i = 7.2 \times 10^{-8}$ M) next to compound **10** and **11** (Mohan et al., 2010), did not show any replication inhibitory effect.

Although the triazole series of compounds were designed to better bind to group-1 NA (including N1) than group-2 NA (including N2), and in fact shown to occupy the 150-cavity and inhibit group-1 NA better (Mohan et al., 2010), these compounds were still not potent enough inhibitors to exhibit a significant effect in the virus replication inhibition assay. In contrast, the amino- (compound **10**) and guanidino- (compound **11**) substituted compounds were efficacious in the virus replication inhibition, with the latter showing similar activities as oseltamivir. It is likely that the hydrogen-bonding interactions between the amino or guanidino function with the active site amino acids, Asp151 and Glu 119 (Calligari et al., 2009) are absent in the complexes of the triazole compounds (**1**–**9**) with the neuraminidase enzymes. Therefore, the next-generation candidates should incorporate not only the cavity-filling group but also a polar group such as the guanidino function to engage the complementary H-bonding sites on the enzyme.

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