Resistance profiles for the investigational neuraminidase inhibitor AV5080 in influenza A and B viruses

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Abstract

Neuraminidase inhibitors (NAIs) are recommended for influenza treatment and prevention worldwide. The most widely prescribed NAI is oral oseltamivir, while inhaled zanamivir is less commonly used. Using phenotypic neuraminidase (NA) enzymatic assays and molecular modeling approaches, we examined the ability of the investigational orally-dosed NAI AV5080 to inhibit viruses of the influenza A(H1N1)pdm09, A(H3N2), A(H5N1), and A(H7N9) subtypes and the influenza B/Victoria- and B/Yamagata-lineages containing NA substitutions conferring oseltamivir or zanamivir resistance including: NA-R292K, NA-E119G/V, NA-H274Y, NA-I122L/N, and NA-R150K. Broadly, AV5080 showed enhanced in vitro efficacy when compared with oseltamivir and/or zanamivir. Reduced AV5080 inhibition was determined for influenza A viruses with NA-E119G and NA-R292K, and for B/Victoria-lineage viruses with NA-I122N/L and B/Yamagata-lineage virus with NA-R150K. Molecular modeling suggested loss of the short hydrogen bond to the carboxyl group of AV5080 affected inhibition of NA-R292K viruses, whereas loss of the salt bridge with the guanidine group of AV5080 affected inhibition of NA-E119G. The resistance profiles and predicted binding modes of AV5080 and zanamivir are most similar, but dissimilar to those of oseltamivir, in part because of a guanidine moiety compensatory binding effect. Overall, our data suggests that AV5080 is a promising oral-dosed NAI that exhibited similar or superior in

vitro efficacy against viruses with reduced or highly reduced inhibition phenotypes with respect to currently approved NAIs.

Keywords

AV5080; oseltamivir; zanamivir; influenza virus; neuraminidase; reduced susceptibility; resistance; NAI

Influenza viruses are important human pathogens, causing annual epidemics and significant morbidity worldwide (Adlhoch et al., 2021; Iuliano et al., 2018; Olsen et al., 2021). Vaccination is the most effective influenza control strategy, but efficacy varies from 10% to 60% because of antigenic mismatch between vaccine composition and novel antigenic variants (Centers for Disease Control and Prevention; Tenforde et al., 2021; U.S. Federal Food and Drug Administration). Influenza antivirals provide alternative avenues for disease management and comprise two classes of direct-acting drugs: the neuraminidase (NA) protein inhibitors (NAIs) that target enzymatic activity of surface NA to inhibit virus budding, and a polymerase acidic (PA) cap-dependent endonuclease (CEN) inhibitor baloxavir marboxil. Baloxavir is a new and potent option, but it is not widely used outside Japan (Ison et al., 2021), while the NAIs are widely prescribed worldwide. Currently, there are four NAIs available that are approved in various countries. Oral oseltamivir and inhaled zanamivir have been available worldwide since 1999–2000, intravenous peramivir is approved in China, Japan, South Korea, and the United States, whereas inhaled laninamivir octanoate is approved only in Japan (Beigel and Hayden, 2021). With only one oral NAI available, the development of novel NAIs continues.

AV5080 is an investigational oral NAI (Fig. 1) for which phase 2 clinical study was completed, and phase 3 clinical study has been approved in Russia (ClinicalTrials.gov, 2021). It was designed to have improved activity against the NAs of influenza A and B viruses (IAVs and IBVs), demonstrated 50% inhibitory concentrations (IC_{50}s) at low nM range, and molecular docking showed that AV5080 binding modes within A/Duck/Minnesota/1525/1981 (H5N1) NA active site are similar to oseltamivir and zanamivir (Ivachtchenko et al., 2014).

Although viruses with reduced NAI susceptibility (by phenotypic or genotypic evaluation) have been only rarely detected since 2010 (Govorkova et al., 2022; Takashita et al., 2020), emergence of drug-resistant viruses can affect the clinical management of influenza. Continued preclinical and clinical evaluation of AV5080 in the presence of defined NAI resistance markers is important. Previously, AV5080 showed activity against oseltamivir-resistant influenza A(H1N1)pdm09 with NA-H274Y (N2 numbering) and IBVs with NA-D197E (B numbering) (Ivachtchenko et al., 2014). Here, we comprehensively defined the activity of AV5080 against both naturally occurring and reverse genetics (rg)-derived IAVs and IBVs carrying NA substitutions mediating reduced inhibition (RI) (10- to 100-fold over wildtype [WT] for IAVs; 5- to 50-fold for IBVs) and highly reduced inhibition (HRI) (>100-fold for IAVs; >50-fold for IBVs) by oseltamivir and zanamivir (World Health Organization, 2012). Our virus panel included clinically relevant and naturally occurring A(H1N1)pdm09 and/or A(H3N2) viruses with NA-H274Y(Hurt et al., 2012), NA-E119X (Gaymard et

al., 2016), NA-R294K (Gaymard et al., 2016), as well as recombinant viruses based upon naturally occurring A(H5N1) NA-H274Y and NA-H294S (Le et al., 2005; McKimm-Breschkin et al., 2018), and A(H7N9) NA-R292K (Watanabe et al., 2013). Additionally, rg-generation of clinically relevant NA substitutions in novel subtypes like A(H5N1) NA-E119G or in IBV genotypes including NA-I221X, NA-N294X, and NA-D197E (Abed and Boivin, 2017; Burnham et al., 2014; Farrukee et al., 2015) were also rescued to enhance the panel and provide access to viruses not otherwise readily available for us to test.

Phenotypic NAI susceptibilities were determined using a MUNANA fluorescence-based NA inhibition assay (Govorkova et al., 2013; Potier et al., 1979) to generate IC_{50} values. Virus isolates with naturally occurring NA substitutions were used for A(H1N1)pdm09 and A(H3N2) subtypes. An 8-plasmid reverse-genetics system (Hoffmann et al., 2002a; Hoffmann et al., 2002b) was used to introduce NA substitutions into zoonotic A(H5N1) and A(H7N9) subtypes and Yamagata-lineage and Victoria-lineage IBVs (Pascua et al., 2020; Pascua et al., 2017) (Supplemental Methods).

We first determined oseltamivir, zanamivir, and AV5080 IC_{50} values and fold-changes over WT for NAI-resistant IAVs (Table 1). NA-H274Y in A(H1N1)pdm09 and A(H5N1) caused HRI for oseltamivir but normal inhibition (NI) for zanamivir and AV5080. NA-E119V in A(H3N2) virus (L'Huillier et al., 2015) yielded similar results, causing HRI for oseltamivir but NI for zanamivir and AV5080.However, NA-E119G in A(H5N1) virus caused HRI for zanamivir, while only RI for oseltamivir and AV5080. NA-N294S caused only RI for oseltamivir in A(H5N1), but no negative effects for zanamivir or AV5080. Finally, NA-R292K caused HRI for oseltamivir in A(H3N2) but only RI for zanamivir and AV5080. In all cases where oseltamivir/zanamivir showed HRI/RI, AV5080 showed RI or NI. The exception was NA-R292K in A(H7N9), which caused HRI for oseltamivir and AV5080, but RI for zanamivir. However, IC_{50} fold-changes for oseltamivir vs. AV5080 were dramatically different, with AV5080 nearly 80-fold more effective (Table 1).

Through molecular modeling, we examined structural explanations for these NAI reduced-susceptibility phenotypes (Fig. 2 and detailed explanations in Supplementary Table 1). For oseltamivir, NA-H274Y shifts Glu277 3Å up towards a bulky pentane moiety, causing a tangible clash (distance between the O- of Glu277 and the H of the terminal methyl group of oseltamivir is <1.5 Å), moving it out of the sub-pocket. This is accompanied by the partial loss of xH-π contacts with lone pairs of Asn295 and Arg293, significantly reducing potential binding affinity and allowing no compensatory effect via additional contacts within the binding site. In contrast, the guanidine moiety of zanamivir and AV5080 forms a tight interaction ensemble with the carbonyl groups of Trp179 and Asp151 and with carboxylic fragments of Asp151 and Glu228 by H-bonds and electrostatic interactions (salt bridges). This results in compensatory effects sufficient to retain binding, and likely reflects the efficacy phenotypes. NA-E119G resulted in complete loss of salt bridge contact with AV5080 and zanamivir's guanidine moiety (Fig. 2A). In addition, this space was left unfilled by the amino acid side chain, allowing the guanidine more mobility, potentially deviating from the most efficient binding mode. NA-E119G also decreases oseltamivir affinity, as loss of hydrogen bonding occurs with the drug's amino group. Our models suggest NA-E119V should decrease affinity for all three NAIs tested, as there are salt bridge losses similarly

to those in NA-E119G (Supplementary Table 1). However, we only observed oseltamivir efficacy loss in vitro. AV5080 and zanamivir inhibited normally, possibly because of pose stabilizations by H-π interaction of the valine methyl group protons and their guanidine group (Supplementary Table 1).

For NA-N294S, Asn294 is implicated in stabilizing the enzymes' Ca^{2+}-dependent cluster forming H-bonds with proximal Trp296 and Tyr316. Considering that Arg292 is the next amino acid to Asn294 and that Arg292 plays a significant role in binding (Fig. 2B), NA-N294S may lead to spatial rearrangement of this substitution ensemble. However, the substitution of Asp for Ser at residue 294 presumably maintains cluster stability because of a similar H-bonding interface and may not cause a significant reduction in binding. Nevertheless, our phenotypic data (Table 1) suggest oseltamivir is more adversely affected by such minor shifts than AV5080 or zanamivir. Finally, NA-R292K is a very impactful substitution within the NA catalytic residues, causing cross-resistance to at least three NAIs (oseltamivir, zanamivir, and peramivir) in group 2 IAVs. NA-R292K is a part of a highly conserved arginine triad (Arg118, Arg292, and ArgR371) shared by all sialidases (Colman et al., 1983). Modeling demonstrated reduced affinity of oseltamivir, zanamivir, and AV5080 for NA-R292K, although the salt bridge with acid functionality (a free carboxylic acid-moiety) of the drugs is mainly retained. Moreover, xH-π interactions between Arg lone pairs and protons of a bulky aliphatic substituent in position 3 of the drug core are lost (Fig. 2B). Of the three NAIs tested, oseltamivir was the most negatively affected by NA-R292K because of the lack of a guanidine-related compensatory effect on the NA binding affinity. In contrast, the zanamivir and AV5080 guanidine moieties form tight interaction ensembles with the carbonyl groups of Trp179 and Asp151 and with the carboxylic fragments of Asp151 and Glu228 by H-bonds and electrostatic interactions (salt bridges). The resulting compensatory effect is likely sufficient to retain interactions and mediating their lower IC_{50} values compared to oseltamivir.

We next determined oseltamivir, zanamivir, and AV5080 IC_{50} values and fold-changes over WT and NAI-resistant IBVs (Table 2). RI/HRI for oseltamivir and zanamivir are reported at low incidence in B/Victoria-lineage and B/Yamagata-lineage viruses across influenza seasons (Govorkova et al., 2022). Several NA substitutions in clinical specimens are included in our panel, but changes at the Asp197 and Ile221 residues were most common (Burnham et al., 2014; Govorkova et al., 2022; Takashita et al., 2020). Using a previously generated IBVs with NA substitutions, (Pascua et al., 2020), we assessed the susceptibilities to oseltamivir, zanamivir, and AV5080 of rg-B/Brisbane/60/2008 (B/Victoria-lineage) and rg-B/Phuket/3073/2013 (B/Yamagata-lineage) viruses carrying NA-E105K, NA-G145E, NA-R150K, NA-D197N, NA-I221L/N/T/V, NA-H273Y, NA-N294S, or NA-G407S.

For B/Victoria-lineage viruses, the RI oseltamivir phenotype was determined for viruses with NA-D197N, NA-I221T, and NA-N294S, and the HRI phenotype for viruses with NA-I221L/N, recapitulating previous results (Pascua et al., 2020). NA-R150K detrimentally affected NA activity of rg-B/Brisbane/60/2008 virus such that the virus was successfully rescued, but the IC_{50} values could not be determined from the NA activity of the propagated stocks. For zanamivir, the RI phenotype was exhibited by the presence of NA-I221L/N in rg-B/Brisbane/60/2008 viruses. Notably, AV5080 lost efficacy only against B/Victoria-

lineage viruses carrying NA-I221L or NA-I221N (RI: 8.7-fold and 5.8-fold changes in IC_{50}, respectively). Modeling poses (in detail in Supplementary Table 2) containing NA-I221N or NA-I221L substitutions led to a reduction in Ile221–Arg151–Asp197 cluster stability and resulted in significant clashes with the $-O(CH_2CH_3)_2$ moiety of AV5080 and oseltamivir (Fig. 2C). We propose that the H-bond between Arg151 and the carbonyl oxygen is weakened by these rearrangements.

For B/Yamagata-lineage viruses, a slightly different pattern of oseltamivir susceptibility was observed than for B/Victoria-lineage viruses: the RI phenotype was determined for viruses with NA-D197N, NA-I221L/T, NA-H273Y, and NA-N294S, and the HRI phenotype for viruses with NA-R150K and NA-I221N (Table 2). For zanamivir, the RI phenotype was exhibited by the presence of NA-I221L/N. Additionally, RI by this NAI was observed in the B/Yamagata-lineage virus possessing the NA-R150K substitution. AV5080 lost efficacy only against B/Yamagata-lineage virus carrying NA-R150K, indicating that B/Yamagata-lineage viruses that acquire this marker are potentially resistant to AV5080. This substitution may lead to a loss of contact with the carbonyl oxygen of AV5080, oseltamivir, and zanamivir (Fig. 2D). Moreover, it can influence the stability of the Ile221 and Asp197 cluster; consequently, the interaction between Ile221 and the methyl group of the substituent in position 3 of the drug scaffold may be negatively altered.

In conclusion, the candidate drug AV5080 showed favorable resistance profiles in IAVs and IBVs, including activity similar or superior to oseltamivir against A(H5N1) and A(H7N9) subtypes, which are associated with human zoonotic infections and severe outcomes. CDC interim analyses suggest that patients (outpatient or hospitalized) presenting with infections by these subtypes be administered oseltamivir, although clinical guidance based on randomized clinical trials is lacking. That AV5080 worked as well as or better than oseltamivir against viruses with NAI-resistant substitutions in our study is a powerful preclinical indicator of its efficacy against these and possibly other novel viruses. However, future clinical implementation of AV5080 must be accompanied by active monitoring of treatment-emergent NA substitutions in seasonal and zoonotic subtypes. Overall, the pattern of RI/HRI by AV5080 most resembled that of zanamivir, and in some cases, AV5080 performed better than zanamivir [with NA-E119G in A(H5N1) and NA-I221L/N in B/Yamagata-lineage]. Our docking data demonstrate the importance of guanidine moieties for the binding capacity of both zanamivir and AV5080, which partially explains the similar phenotypes. Zanamivir and AV5080 are analogs of DANA, with a positively charged guanidino group replacing a hydroxyl group linked to C-4, better approximating the influenza NA natural substrate. We propose a guanidine moiety is the most important fragment of AV5080 responsible for sustainability against NA substitutions causing the RI/HRI phenotype. With NA-R292K, which caused the most significant reduction in AV5080 efficacy, switching from Arg to Lys may reduce binding between NA's positively charged amino groups and AV5080. The distance between the two interacting groups increases, leading to H-bond loss and/or an ineffective binding mode (Supplementary Tables 1 and 2). Although phenotypic and structural properties of AV5080 and zanamivir are similar, their routes of administration differ. Zanamivir is administered via aerosol nebulizer rather than orally (as with oseltamivir), potentially limiting its clinical implementation. In contrast, oral administration of AV5080 in mice lethally challenged with A(H1N1) or

A(H3N2) viruses yielded survival outcomes similar to those with oseltamivir (Ivachtchenko et al., 2014). The preclinical efficacy of oral AV5080, combined with its zanamivir-like efficacy in vitro against NAI-resistant viruses, suggests that AV5080 has potential for further development as an NAI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

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HIGHLIGHTS

- AV5080 is a novel neuraminidase inhibitor (NAI) for influenza A and B viruses

- AV5080 exhibits a different resistance profile compared to NAI oseltamivir

- This profile is partially due to AV5080's guanidine moiety compensatory effect(s)
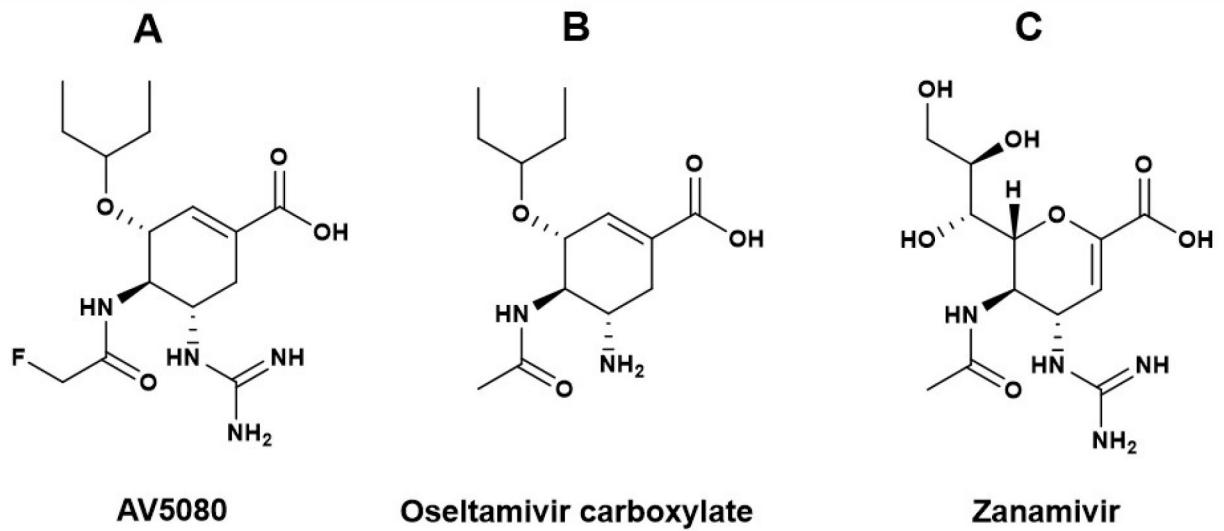
Fig. 1. Chemical structures of anti-influenza NAI drugs:
(A) Investigational drug AV5080, **(B)** oseltamivir carboxylate (the active metabolite of prodrug oseltamivir phosphate), **(C)** zanamivir.
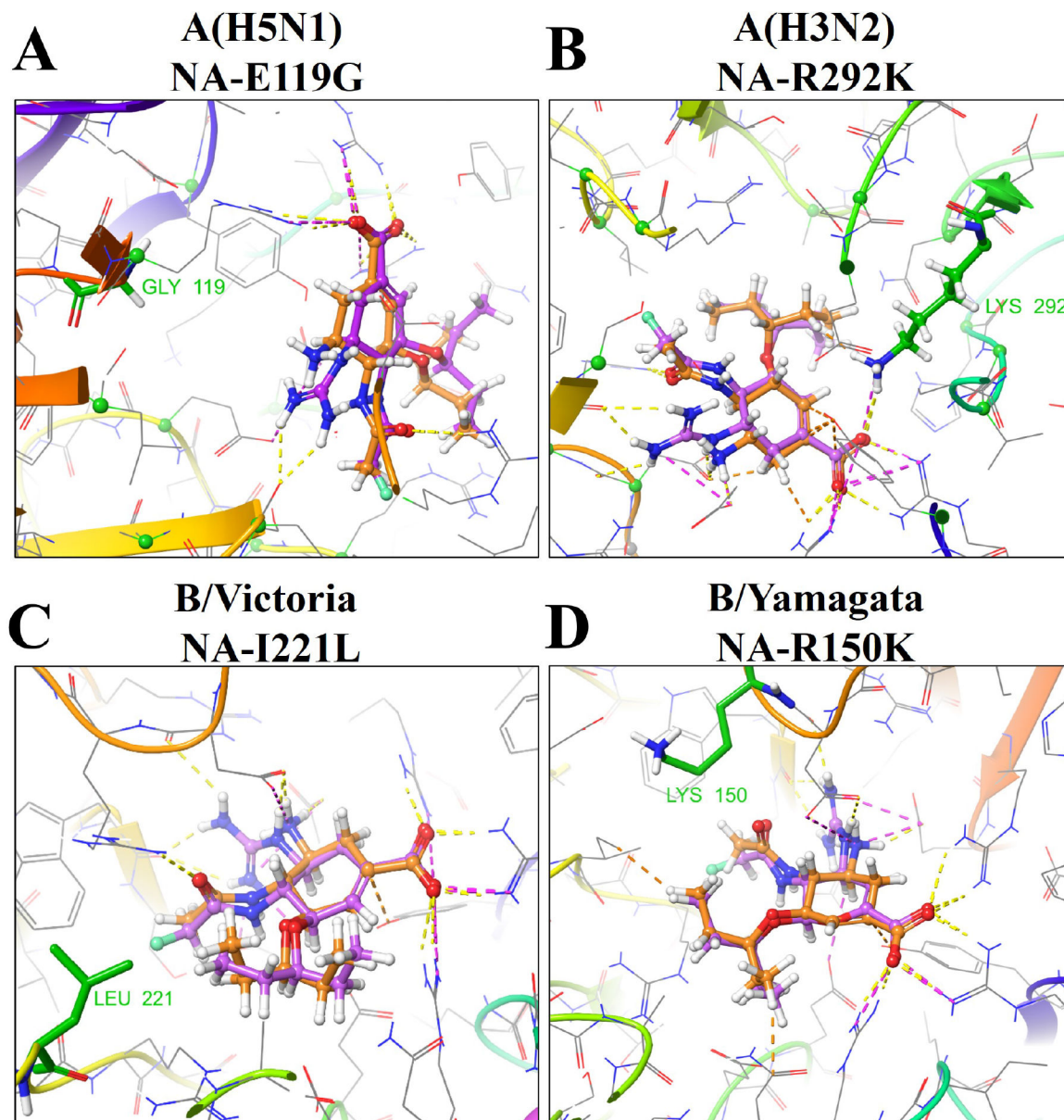
Fig. 2. Binding modes of AV5080 (the carbon backbone is shown in purple) and oseltamivir (the carbon backbone is shown in orange) in the active site of NA of influenza A and B viruses with different NA substitutions (in green).

(A) The model of A/Vietnam/1203/2004 (H5N1) (PDB code: 2HU0) NA-E119G substitution leads to the loss of salt bridge contact between the NA amino acid residue and the guanidine moiety of zanamivir or AV5080. **(B)** The model of A/Tanzania/205/2010 (H3N2) (PDB code: 4GZP) NA-R292K substitution leads to the loss of hydrogen bonding stability and xH-π interactions with AV5080. **(C)** The model of Victoria Lineage B/Lyon/CHU/15.216/2011 (PDB code: 4CPZ) NA-I221L substitution can make the Ile221–Arg151–Asp197 cluster more labile within the NA active site. **(D)** The model of Yamagata lineage B/Brisbane/60/2008 (PDB code: 4CPN) NA-R150K substitution likely causes loss of contact with the carbonyl oxygen of AV5080.

Table 1.

Susceptibility to oseltamivir, zanamivir, and investigational compound AV5080 of IAV carrying NAI resistance–associated substitutions.

Influenza A virus	NA amino acid substitution[a]	Susceptibility to NAIs, mean $IC_{50} \pm SD$ (nM)[b]			NAI susceptibility phenotype (fold-change)[c]		
		Oseltamivir	Zanamivir	AV5080	Oseltamivir	Zanamivir	AV5080
A(H1N1)pdm09							
A/Perth/265/2009	WT	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	NI	NI	NI
A/Perth/261/2009	NA-H274Y	118.8 ± 8.6	0.3 ± 0.0	0.7 ± 0.8	**HRI (396)**	NI (0.6)	NI (7)
A/Denmark/524/2009	WT	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	NI	NI	NI
A/Denmark/528/2009	NA-H274Y	118.5 ± 11.5	0.3 ± 0.0	0.9 ± 0.0	**HRI (592.5)**	NI (1.0)	NI (9.0)
A(H3N2)							
A/Washington/01/2007	WT	0.3 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	NI	NI	NI
A/Texas/12/2007	NA-E119V	96.4 ± 7.0	1.3 ± 0.0	0.7 ± 0.1	**HRI (321.3)**	NI (1.3)	NI (0.9)
A/Bethesda/956/2006	NA-R292K	593.0 ± 16.8	10.6 ± 1.4	6.6 ± 0.3	**HRI (1976.7)**	**~RI (9.6)**	**RI (13.2)**
A/Fukui/20/2004	WT	0.4 ± 0.0	1.5 ± 0.1	1.1 ± 0.0	NI	NI	NI
A/Fukui/45/2004	NA-E119V	68.0 ± 1.1	1.2 ± 0.7	0.6 ± 0.0	**HRI (170.0)**	NI (0.8)	NI (0.6)
A(H5N1)							
rg-A/Vietnam/1203/2004 [d]	WT	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	NI	NI	NI
rg-A/Vietnam/1203/2004-H274Y[d]	NA-H274Y	162.3 ± 6.1	0.6 ± 0.0	1.6 ± 0.6	**HRI (1552.5)**	NI (1.7)	NI (9)
rg-A/Vietnam/1203/2004-N294S[d]	NA-N294S	5.3 ± 0.3	1.4 ± 0.2	0.3 ± 0.0	**RI (50.0)**	NI (4.0)	NI (2.6)
rg-A/Vietnam/1203/2004 [e]	WT	0.3 ± 0.1	0.9 ± 0.2	0.2 ± 0.0	NI	NI	NI
rg-A/Vietnam/1203/2004-E119G[e]	NA-E119G	3.2 ± 0.4	582.2 ± 308.7	3.3 ± 1.3	**RI (13.0)**	**HRI (643.0)**	**RI (17.1)**
A(H7N9)							
rg-A/Anhui/1/2013[f]	WT	0.7 ± 0.0	0.7 ± 0.2	0.4 ± 0.0	NI	NI	NI
rg-A/Anhui/1/2013 × A/Shanghai/1/2013 [g]	NA-R292K	10,820.0 ± 70.7	51.9 ± 9.6	77.4 ± 7.6	**HRI (15457.1)**	**RI (74.1)**	**HRI (193.5)**

[a] Amino acid residue substitution in NA indicated by N2 NA numbering.

[b] Concentration of NAI that reduced viral NA activity by 50% (IC_{50}) relative to viral NA activity without inhibitor in a fluorescence-based assay with MUNANA substrate (100 μM final concentration). Values represent three independent experiments ± SD.

[c] Fold-change relative to the mean IC_{50} of the WT. The criteria recommended by the World Health Organization Antiviral Working Group for interpreting data for the reduced inhibition of IAVs by NAIs are based on the fold-change in IC_{50} compared with that for the susceptible virus: NI, NAI-susceptible IAV < 10-fold; RI, 10- to 100-fold; HRI, >100-fold (World Health Organization, 2012). RI and HRI fold-changes are indicated in boldface.

[d] A recombinant A/Vietnam/1203/2004 (H5N1) influenza viruses carrying either WT NA or NA-H274Y or NA-N294S.

[e] A recombinant A(H5N1) influenza viruses carrying either WT NA or NA-E119G, HA genes from A/Vietnam/1203/2004 (H5N1) virus and the six internal genes from A/Puerto Rico/8/34 (H1N1) virus.

[f] A recombinant A/Anhui/1/2013 (H7N9) influenza virus carrying WT NA.

[g] A recombinant A(H7N9) influenza virus carrying HA gene from A/Anhui/1/2013 (H7N9) virus, NA gene from A/Shanghai/1/2013 (H7N9) virus and the six internal genes from A/Puerto Rico/8/34 (H1N1) virus.

Abbreviations: IC_{50}, 50% inhibitory concentration; NA, neuraminidase; NAI, NA inhibitor; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition; WT, wild-type; rg, recombinant.

Table 2.

Susceptibility to oseltamivir, zanamivir, and investigational AV5080 of recombinant IBV carrying NAI resistance–associated substitutions.

NA amino acid substitution[a]	Susceptibility to NAIs, mean $IC_{50} \pm SD$ (nM)[b]			NAI susceptibility phenotype (fold-change)[c]		
	Oseltamivir	Zanamivir	AV5080	Oseltamivir	Zanamivir	AV5080
rg-B/Brisbane/60/2008 (B/Victoria-lineage)						
WT	8.4 ± 0.8	3.0 ± 0.3	3.1 ± 0.0	NI	NI	NI
NA-G145E	5.1 ± 0.4	1.8 ± 0.2	1.2 ± 0.1	NI (0.6)	NI (0.6)	NI (0.4)
NA-D197N	52.1 ± 2.1	12.4 ± 0.1	8.3 ± 0.7	**RI (6.2)**	NI (4.2)	NI (2.7)
NA-I221L	1247.0 ± 49.5	52.2 ± 11.2	27.1 ± 0.7	**HRI (149)**	**RI (17.7)**	**RI (8.7)**
NA-I221N	468.1 ± 10.3	18.7 ± 1.5	18.0 ± 2.1	**HRI (55.9)**	**RI (6.3)**	**RI (5.8)**
NA-I221T	47.8 ± 9.6	5.5 ± 0.0	6.4 ± 0.7	**RI (5.7)**	NI (1.9)	NI (2.1)
NA-I221V	12.6 ± 3.8	2.90 ± 0.8	6.8 ± 0.4	NI (1.5)	NI (1)	NI (2.2)
NA-H273Y	37.0 ± 1.7	1.5 ± 0.1	1.7 ± 0.0	NI (4.4)	NI (0.5)	NI (0.5)
NA-N294S	115.2 ± 23.6	8.4 ± 1.9	5.3 ± 0.5	**RI (13.8)**	NI (2.8)	NI (1.7)
NA-G407S	27.7 ± 4.3	5.6 ± 0.4	3.7 ± 0.3	NI (3.3)	NI (1.9)	NI (1.2)
rg-B/Phuket/3073/2013 (B/Yamagata-lineage)						
WT	5.7 ± 0.9	1.3 ± 0.2	1.4 ± 0.0	NI	NI	NI
NA-G145E	7.5 ± 2.1	0.7 ± 0.1	0.9 ± 0.0	NI (1.3)	NI (0.5)	NI (0.6)
NA-R150K	910.2 ± 47.5	30.1 ± 0.6	29.0 ± 1.7	**HRI (161.1)**	**RI (23.5)**	**RI (20.7)**
NA-D197N	57.2 ± 6.4	4.1 ± 0.7	3.4 ± 0.2	**RI (10.0)**	NI (3.2)	NI (2.4)
NA-I221L	199.4 ± 41.9	8.4 ± 4.1	2.3 ± 0.2	**RI (35.3)**	**RI (6.6)**	NI (1.6)
NA-I221N	377.0 ± 60.9	17.2 ± 0.9	3.6 ± 0.0	**HRI (66.7)**	**RI (13.2)**	NI (2.6)
NA-I221T	57.0 ± 14.2	2.1 ± 0.2	2.6 ± 0.1	**RI (10.1)**	NI (1.6)	NI (1.9)
NA-I221V	2.7 ± 0.3	3.1 ± 0.2	2.0 ± 0.2	NI (0.5)	NI (2.4)	NI (1.4)
NA-H273Y	34.5 ± 2.9	0.8 ± 0.1	1.0 ± 0.1	**RI (6.1)**	NI (0.6)	NI (0.7)
NA-N294S	62.0 ± 14.5	2.4 ± 0.2	1.9 ± 0.2	**RI (11.0)**	NI (1.9)	NI (1.4)
NA-G407S	13.4 ± 2.5	1.8 ± 0.2	1.2 ± 0.0	NI (2.4)	NI (1.4)	NI (0.9)

[a] Amino acid residue substitution in NA indicated by IBV numbering.

[b] Concentration of NAI that reduced viral NA activity by 50% (IC_{50}) relative to viral NA activity without inhibitor in a fluorescence-based assay with MUNANA substrate (100 μM final concentration). Values represent three independent experiments ± SD.

[c] Fold-change relative to the mean IC_{50} of the rg-WT. The criteria recommended by the World Health Organization Antiviral Working Group for interpreting data for the reduced inhibition of IBVs by NAIs are based on the fold-change in IC_{50} compared with that of the susceptible virus: NI, NAI-susceptible IBV < 5-fold; RI, 5- to 50-fold; HRI, >50-fold (World Health Organization, 2012). RI and HRI fold-changes are indicated in boldface.

Abbreviations: IBV, influenza B virus; HRI, highly reduced inhibition; IC_{50}, 50% inhibitory concentration; NA, neuraminidase; NAI, NA inhibitor; ND, not determined; NI, normal inhibition; RI, reduced inhibition; HRI, highly reduced inhibition; WT, wild-type; rg, recombinant.