

An I436N substitution confers resistance of influenza A(H1N1)pdm09 viruses to multiple neuraminidase inhibitors without affecting viral fitness

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Abstract

The resistance of influenza viruses to neuraminidase (NA) inhibitors (NAIs; i.e. oseltamivir, zanamivir, peramivir and laninamivir) can be associated with several NA substitutions, with differing effects on viral fitness. To identify novel molecular markers conferring multi-NAI resistance, the NA gene of oseltamivir-resistant (H275Y, N1 numbering) 2009 pandemic influenza [A(H1N1)pdm09] virus was enriched with random mutations. This randomly mutated viral library was propagated in Madin–Darby canine kidney (MDCK) cells under zanamivir pressure and gave rise to additional changes within NA, including an I436N substitution located outside the NA enzyme active site. We generated four recombinant A(H1N1)pdm09 viruses containing either wild-type NA or NA with single (I436N or H275Y) or double (H275Y-I436N) substitutions. The double H275Y-I436N mutation significantly reduced inhibition by oseltamivir and peramivir and reduced inhibition by zanamivir and laninamivir. I436N alone reduced inhibition by all NAIs, suggesting that it is a multi-NAI resistance marker. I436N did not affect viral fitness *in vitro* or in a murine model; however, H275Y and I436N together had a negative impact on viral fitness. Further, I436N alone did not have an appreciable impact on viral replication in the upper respiratory tract or transmissibility in ferrets. However, the rg-H275Y-I436N double mutant transmitted less efficiently than either single mutant via the direct contact and respiratory droplet routes in ferrets. Overall, these results highlight the usefulness of a random mutagenesis approach for identifying potential molecular markers of resistance and the importance of I436N NA substitution in A(H1N1)pdm09 virus as a marker for multi-NAI resistance.

INTRODUCTION

The first pandemic of the 21st century was caused by influenza A(H1N1)pdm09 virus in 2009. This virus became dominant and replaced the previously circulating seasonal A(H1N1) viruses. Most people younger than 65 years were immunologically naïve to the newly introduced virus [1], and the vaccine used for former seasonal A(H1N1) viruses was no longer effective. For these reasons, antiviral drugs, specifically the neuraminidase (NA) inhibitors (NAI) oseltamivir (OS) and zanamivir (ZA), were used more prominently to treat influenza virus-infected patients in 2009 than in previous years. During the pandemic, NAIs were the only anti-influenza drugs recommended for prophylaxis and

treatment of influenza A(H1N1)pdm09 virus infections. Unlike seasonal A(H1N1) viruses (97% of which were found to be OS-resistant variants between 2008 and 2009) [2], A(H1N1)pdm09 strains appear to have only a 0.5–3.4% rate of resistance to OS [3–8]. However, the increased use of NAIs, specifically OS, could result in the increased emergence and global spread of OS-resistant A(H1N1)pdm09 variants in the future.

The NAIs were developed by mimicking sialic acid (i.e. they possess similar binding motifs) and thus resistant phenotypes often have changes within the catalytic and/or framework NA residues [9, 10]. As a result of its prevalent use in clinical management, OS resistance-associated NA

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Abbreviations: BALF, bronchial alveolar lavage fluid; DC, direct contact; DMEM, Dulbecco's modified Eagle's medium; p.c., post-contact; p.i., post-inoculation; FBS, foetal bovine serum; HI, haemagglutinin inhibition; LAN, laninamivir; MDCK, Madin–Darby canine kidney; MEM, minimal essential medium; MUNANA, 2'-(4-methylumbelliferyl)- α -D-N-acetylneurameric acid sodium salt hydrate; NA, neuraminidase; NAI, neuraminidase inhibitor; OS, oseltamivir; PE, peramivir; RG, reverse genetics; RM, random mutant; RD, respiratory droplet; SN, serum neutralization; WT, wild-type; ZA, zanamivir. Supplementary material is available with the online version of this article.

substitutions have been observed more frequently than mutations for resistance to other NAIs. The OS resistance mutations include H275Y in A(H1N1)pdm09 viruses [10–12], and E119V [13, 14] and R293K in A(H3N2) influenza viruses [14, 15]. The other NA substitutions in influenza A and B viruses, including I223R/V/T, R152K, D199N and R368K, confer reduced susceptibility to other NAIs [9, 10, 16], and these changes are within the NA active site. Although most resistance-conferring NA substitutions are directly associated with the enzyme active site, recent surveillance and laboratory studies have reported that certain NAI resistance-conferring substitutions, such as Q136K/L, A247T and I427T, [17–19], are outside the active residues.

Although OS and ZA are modifications of DANA (2,3-dehydro-2-deoxy-N-acetylneuraminic acid) and imitate sialic acid [20], the difference between the side-chains of OS and ZA structures assumes that changes conferring cross-resistance to both NAIs will be rare. Nevertheless, the subsequent use of OS and ZA in immunocompromised patients has often resulted in the development of multiple NAI (multi-NAI) resistance, which is associated with I223R or E119D substitutions combined with H275Y in A(H1N1)pdm09 viruses [21–23]. In addition, the results of an *in vitro* study also showed that A(H1N1)pdm09 and A(H5N1) viruses carrying double E119D/G/A-H275Y substitutions had multi-NAI resistance, although most of these variants showed diminished viral fitness [24]. The results of recent surveillance and experimental studies demonstrated that the I427T substitution confers multi-NAI resistance (decreased susceptibility to ZA) in the A(H1N1)pdm09 virus, despite being outside the active site, although again, this variant has impaired viral fitness *in vitro* and *in vivo* [18, 25]. The rapid genetic evolution caused by the error-prone nature of RNA viruses and selection resulting from the continuous use of OS and ZA to treat those with influenza virus infections will likely result in the generation of multi-NAI-resistant variants; identifying such variations will aid antiviral surveillance and future drug optimization. Thus, we used an *in vitro* random mutagenesis approach to identify NA changes that potentially confer multi-NAI-resistance in A(H1N1)pdm09 virus and compared the fitness of multi-NAI-resistant variants to that of NAI-susceptible wild-type (WT) virus *in vitro* and *in vivo*.

RESULTS

Substitutions selected by ZA and susceptibility to NAIs

To identify NA changes that may confer multi-NAI resistance, random mutations were introduced into the NA gene of an OS-resistant A/California/04/2009 (H1N1)pdm09 virus via gene-fragmented random mutagenesis (GFRM). The H275Y change was conserved to retain the OS-resistant phenotype (Fig. 1). Two sets of random-mutant (RM) N1 plasmid libraries were used: region 1 (RM-R1, from 102 to 819 nt) and region 2 (RM-R2, from 823 to 1410 nt). The RM virus libraries were then generated in the genetic

background of A/Puerto Rico/8/1934 (H1N1) virus (PR8) and either RM-R1 or RM-R2 by using a reverse-genetics (RG) system. The generated RM virus libraries (from the rescue supernatants) were passaged in the presence of increasing concentrations of ZA in Madin–Darby canine kidney (MDCK) cells. The NA gene of 2–4× passaged and selected viruses was fully sequenced to identify potential resistance mutations. In addition to H275Y, two changes within the N1 genes, N200K or I436N, were identified. Therefore, the WT and five mutant viruses possessing either mutant NAs with single (N200K, I436N and H275Y) or double (N200K-H275Y and H275Y-I436N) substitutions were generated in the genetic background of influenza A (H1N1)pdm09 virus using RG to verify the effect of these changes on the susceptibility to NAIs [OS, ZA, peramivir (PER) and laninamivir (LAN)] (Table 1). The IC₅₀ of the A (H1N1)pdm09 mutants with the N200K substitution, rg-N200K and rg-N200K-H275Y, demonstrated similar NAI susceptibilities to those of their corresponding parental viruses, rg-WT and the rg-H275Y mutant, respectively. Thus, these mutant viruses were excluded from further studies. The rg-virus carrying the I436N single substitution exhibited reduced inhibition to all NAIs tested. Moreover, the A(H1N1)pdm09 rg-H275Y-I436N double mutant, which was selected by ZA, exhibited highly reduced inhibition by OS and PER and reduced inhibition by ZA and LAN; the rg-H275Y single mutant was susceptible to ZA and LAN (Table 1). To further investigate the impact of the NA substitutions on virus properties, we compared the NA activity and enzyme kinetics of our rg-variants to those of rg-WT virus. Although the enzymatic activity of the rg-H275Y, rg-I436N and rg-H275Y-I436N viruses was lower than that of the rg-WT virus, the single rg-I436N variant had the highest activity among the variants (Fig. 2). Collectively, these results indicate that the novel I436N substitution can be selected by ZA *in vitro* in A(H1N1)pdm09 virus and that it conferred resistance to all four NAIs.

Growth of multi-NAI-resistant variants in eggs and MDCK cells

To evaluate the impact of NA substitutions leading to multi-NAI-resistance on viral replication efficiency, we determined the growth properties of rg-I436N, rg-H275Y, rg-H275Y-I436N mutants and rg-WT virus in eggs and MDCK cells. Five 11-day-old embryonic chicken eggs were inoculated with 1×10^2 TCID₅₀ ml⁻¹ of each virus and incubated at 37 °C for 48 h. The yields of both rg-H275Y and rg-H275Y-I436N mutants (7.0 and 6.9 log₁₀TCID₅₀ ml⁻¹, respectively) were slightly lower than rg-WT virus (7.3 log₁₀TCID₅₀ ml⁻¹), although the single rg-I436N mutant (7.4 log₁₀TCID₅₀ ml⁻¹) was similar to that of rg-WT virus (7.3 log₁₀TCID₅₀ ml⁻¹) (Fig. 3a). The MDCK cells were inoculated with the rg-WT and mutant viruses at a multiplicity of infection (m.o.i.) of 0.0001 and incubated at 37 °C for 72 h. The replication kinetics of the single mutant viruses were similar to those of the rg-WT virus (Fig. 3b). However, the double mutant virus had significantly delayed growth until 48 h post-infection (p.i.) ($P < 0.01$). These results suggest that

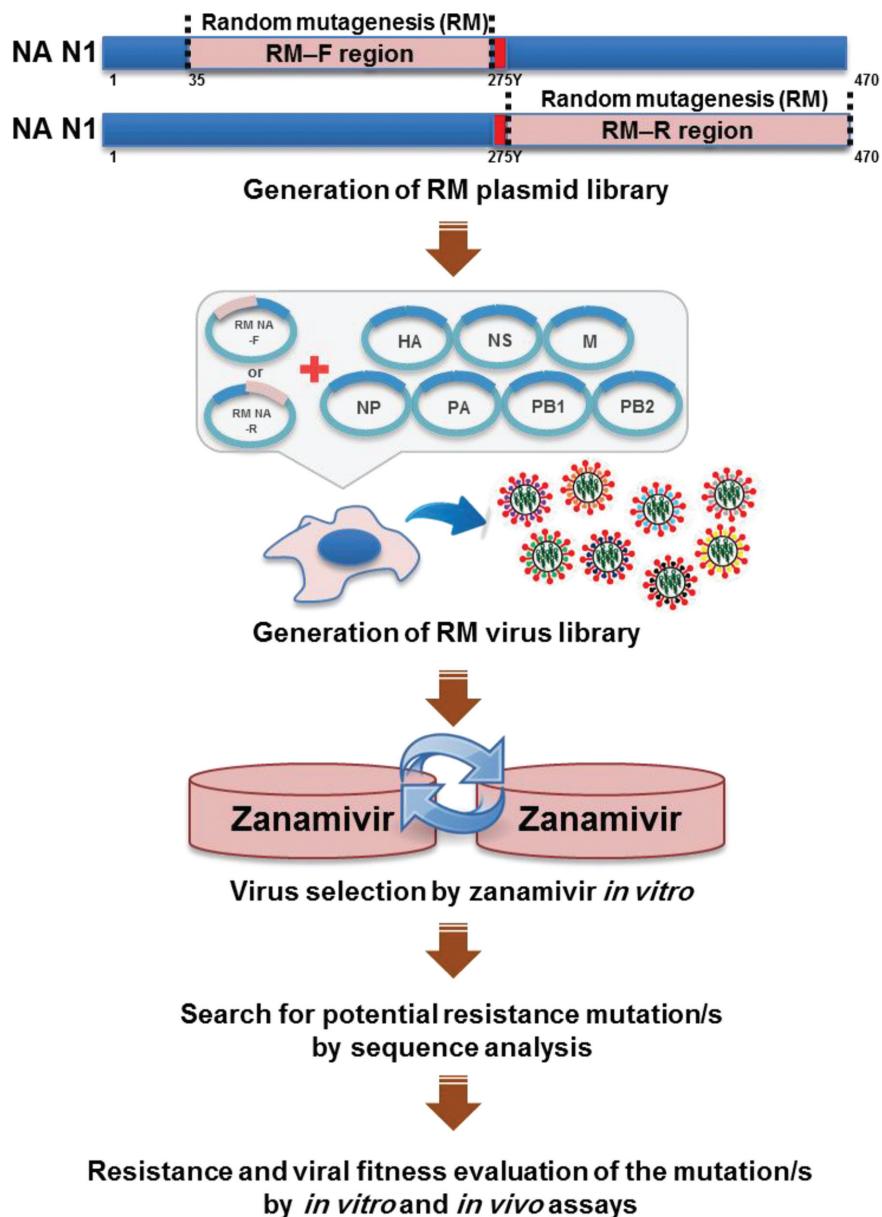


Fig. 1. Schematic overview of library screening method. Two sets of N1 plasmid libraries possessing random mutations in the catalytic domain (except the Y275 residue, which was conserved) were generated. Next, random mutant virus libraries were recovered by using a reverse-genetics technique, and ZA-resistant variants were selected *in vitro*. The NA genes of the viruses growing in the presence of ZA were sequenced. Substitutions found within the NA gene were used to generate recombinant A(H1N1)pdm09-like viruses, and the impact of these alterations on NAI susceptibility was evaluated.

the single I436N NA substitution did not affect virus replication efficiency *in vitro*, although the combined H275Y-I436N substitutions resulted in impaired growth.

Pathogenicity of the multi-NAI-resistant variants in mice

To determine whether the I436N and H275Y-I436N substitutions alter virus pathogenicity *in vivo*, we used a BALB/c mouse model to compare the lethality and viral replication

efficiency of the NAI-resistant viruses with those of rg-WT virus. The weight loss and survival rates of mice ($n=10$ /group) infected with rg-H275Y, rg-I436N and rg-H275Y-I436N viruses were similar to those for mice infected with the rg-WT virus (Fig. 4a, b). Although it was not the case for the rg-H275Y-I436N mutant, rg-WT and single NAI-resistant rg-viruses were detected in the lungs of mice ($n=3$ /group/time point) up to 7 days p.i. At 2 days p.i., the viral titres of the three viruses were similar ($7.2\text{--}7.3 \log_{10} \text{TCID}_{50}$

Table 1. NAI susceptibility of recombinant A(H1N1)pdm09 influenza viruses carrying single or double NA substitutions

Influenza A(H1N1)pdm09 virus*	Mean IC ₅₀ ±SD (nM)†				Susceptibility phenotype (fold change‡) in NA inhibition assay§			
	OS	ZA	PER	LAN	OS	ZA	PER	LAN
rg-WT	0.65±0.15	0.35±0.05	0.10±0.00	0.07±0.01	S (1.0)	S (1.0)	S (1.0)	S (1.0)
rg-N200K	0.55±0.05	0.40±0.10	0.10±0.00	0.07±0.01	S (0.8)	S (1.1)	S (1.0)	S (1.0)
rg-I436N	20.00±4.30	25.00±5.40	2.00±0.60	1.38±0.24	RI (30.8)	RI (71.4)	RI (20.0)	RI (19.7)
rg-H275Y	310.45±0.95	0.80±0.00	28.45±0.75	0.15±0.07	HRI (477.6)	S (2.3)	HRI (284.5)	S (2.1)
rg-N200K-H275Y	325.50±24.50	0.56±0.36	31.06±2.16	0.30±0.20	HRI (500.8)	S (1.6)	HRI (310.6)	S (4.3)
rg-H275Y-I436N	3127.50±398.50	12.60±1.30	1045.90±83.10	1.52±0.40	HRI (4811.5)	RI (36.0)	HRI (10459.0)	RI (21.7)

*Amino acid numbering is based on that of N1 NA [33].

†Values are means ±SD from two independent experiments.

‡Fold change relative to the mean IC₅₀s of the WT virus.§The criteria recommended by the World Health Organization Antiviral Working Group for interpreting data for the susceptibility of influenza A viruses to NAIs: S, susceptible or normal inhibition (<10-fold increase in IC₅₀ over that of WT); RI, reduced inhibition (10- to 100-fold increase in IC₅₀ over that of WT); HRI, highly reduced inhibition (>100 fold increase in IC₅₀ over that of WT).

NA, neuraminidase; WT, wild-type; OS, oseltamivir; ZA, zanamivir; PER, peramivir; LAN, laninamivir.

ml⁻¹), whereas the double rg-variant had a lower peak titre (6.4 log₁₀TCID₅₀ ml⁻¹) than the rg-WT virus (Fig. 4c). In addition, bronchial alveolar lavage fluid (BALF) samples were harvested from infected mice (*n*=3/group/time point) at 1 and 4 days p.i., and the concentration of infiltrated cells was measured. Prior to 4 days p.i., the numbers of cells in BALF of virus-infected mice were 6.1- to 8.7-fold higher than those of PBS-infected control mice and similar between rg-WT and rg-NAI-resistant variants. However, at 4 days p.i., the cell counts were 30 % lower in mice infected with the double rg-H275Y-I436N variant compared to mice infected with rg-WT or single rg-variants (Fig. 4d). Overall, these results indicate that viruses carrying only the I436N

NA substitution possess undiminished pathogenicity in mice compared to the rg-WT virus. The double rg-H275Y-I436N variant, however, had impaired replication efficiency in the lungs of infected mice.

Pathogenicity and transmissibility of the multi-NAI-resistant variants in ferrets

We next wished to determine the impact of NAI resistance-associated substitutions on virus pathogenicity and transmissibility in a ferret model [26, 27]. We determined the transmission using both direct contact (DC) and respiratory droplet (RD) routes. Groups of three naïve ferrets were infected with 5.0×10^{5.0} TCID₅₀ of rg-WT, rg-I436N and

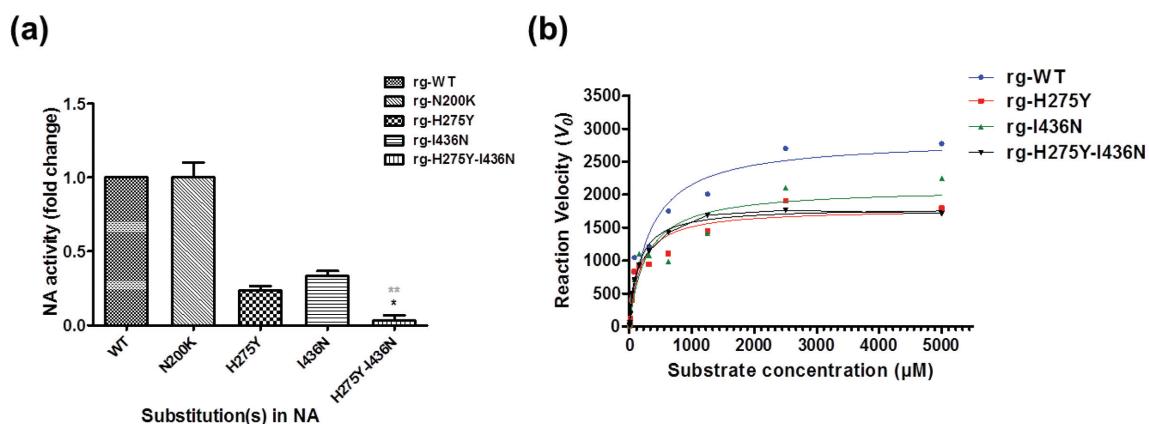


Fig. 2. NA activity and enzyme kinetics of recombinant NAI-resistant A(H1N1)pdm09 viruses. (a) NA activity was determined by using the fluorogenic substrate MUNANA. The NA activity was determined in triplicate, and significant differences are indicated as follows: *P<0.01 for the comparison of mutants to the rg-H275Y virus and **P<0.005 for the comparison of mutants to the rg-I436N virus. (b) NA enzyme kinetics were standardized to equivalent virus infectious doses of 10^{6.0} TCID₅₀ ml⁻¹. The MUNANA was used at final concentrations ranging from 0 to 5000 μM. Fluorescence was measured every 60 s for 60 min at 37 °C using respective excitation and emission wavelengths of 360 and 460 nm.

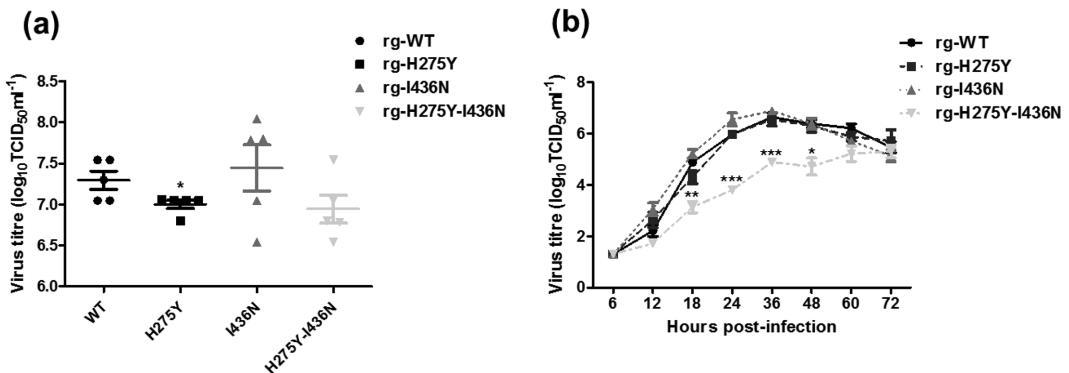


Fig. 3. Replicative efficiency of recombinant NAI-resistant A(H1N1)pdm09 viruses *in vitro*. (a) Five 10-day-old eggs were injected with $10^{2.0}$ TCID₅₀ of each virus variant, including rg-WT virus, and incubated at 37 °C for 48 h. The virus-containing allantoic fluid was titrated by performing TCID₅₀ assays in MDCK cells. (b) MDCK cells were infected with virus variants at an m.o.i. of 0.0001 TCID₅₀/cell. Supernatants were harvested at 12, 24, 36, 48, 60 and 72 h p.i. and titrated by performing TCID₅₀ assays in MDCK cells. * $P<0.05$ and ** $P<0.01$ for the comparison of NAI-resistant viruses to WT virus.

rg-H275Y-I436N viruses and an additional two naïve ferrets per group were used for DC and RD transmissibility evaluation. All of the viruses were detected in the upper respiratory tract of the virus-inoculated donor ferrets by 4 days p.i., with similar growth kinetics and viral peak titres (4.0–5.0 log₁₀ TCID₅₀ ml⁻¹; Fig. 5). Similar clinical disease signs,

including sneezing, minor weight loss (less than 10 %) and increased temperature (0.5–1.0 °C from their initial temperature), were observed (Fig. S1, available in the online version of this article). All virus-inoculated donor ferrets seroconverted to the infecting virus at 14 and 19 days p.i. (Table S1). Virus was not detected in the 6 day p.i. lung

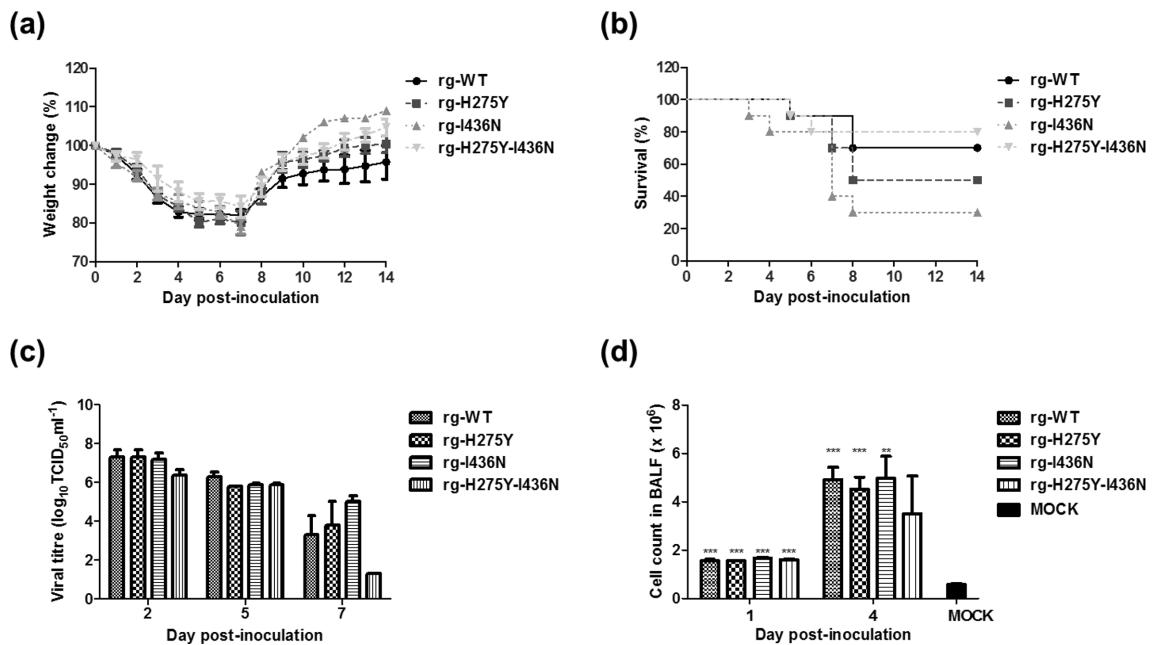


Fig. 4. Pathogenicity of recombinant NAI-resistant A(H1N1)pdm09 viruses in mice. BALB/c mice ($n=10$ /group) were inoculated intranasally with $3.0 \times 10^{4.0}$ TCID₅₀ of rg-NAI-resistant viruses and rg-WT. Mean body weight loss (a) and mortality (b) were monitored for 14 days p.i. The lungs ($n=3$ /group) were collected at 2, 5 and 7 days p.i., and viral titres (c) were determined by performing TCID₅₀ assays in MDCK cells. Samples of BALF (d) were collected from mice ($n=4$ /group) at 1 and 4 days p.i., and the total number of cells in each sample was measured by using an automatic cell counter. Control mice ($n=4$ /group) were inoculated with PBS and used to determine the basal cell count in BALF. * $P<0.05$ and ** $P<0.01$ for comparison of the rg-NAI-resistant viruses to the rg-WT virus. The dotted line indicates the limit of virus detection (1.7 log₁₀ TCID₅₀ ml⁻¹).

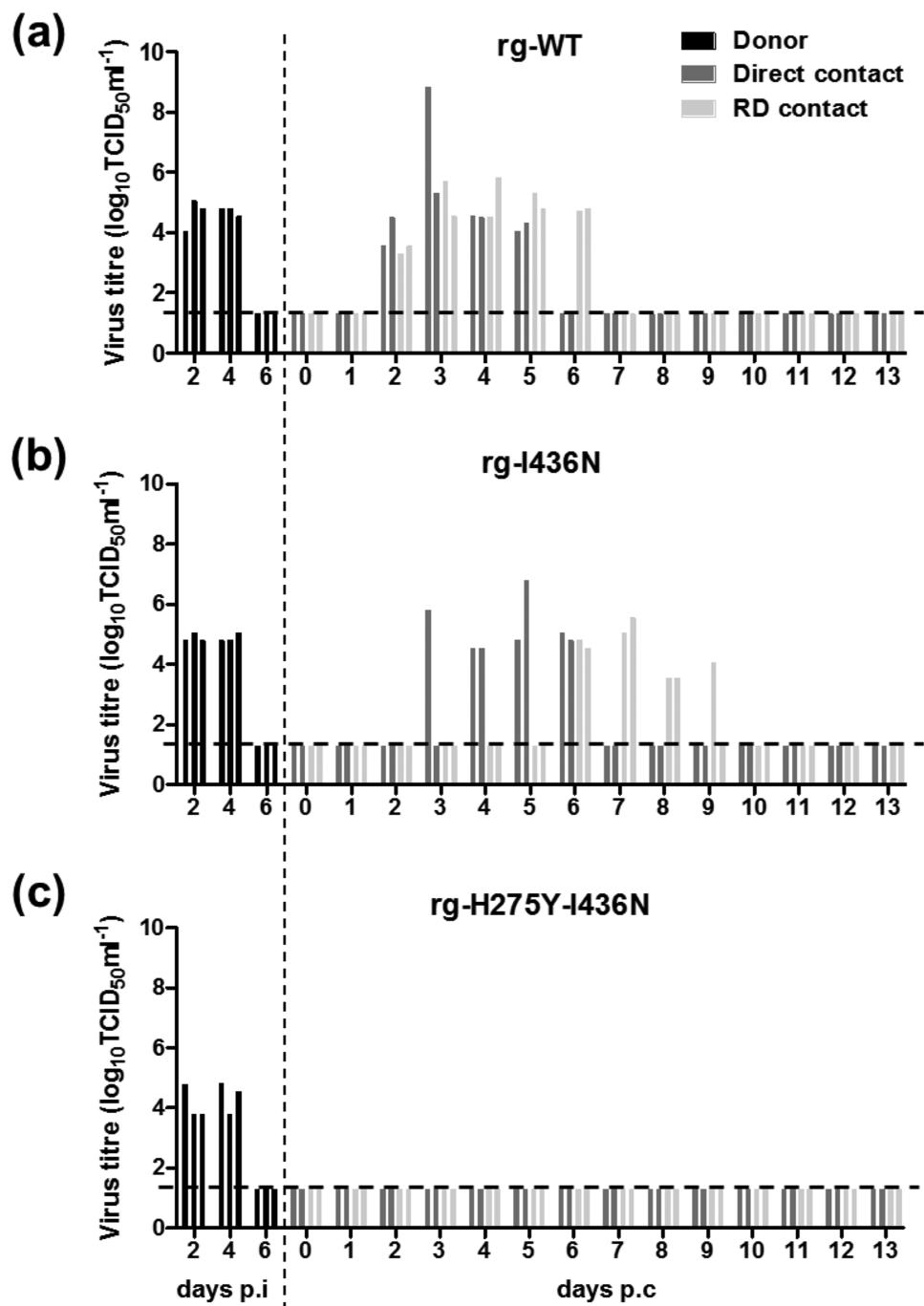


Fig. 5. Replication and transmission of recombinant NAI-resistant A(H1N1)pdm09 viruses in ferrets. Ferrets ($n=3$ /group) were inoculated intranasally with $5.0 \times 10^{5.0}$ TCID₅₀ solution of rg-WT (a), rg-I436N (b), or rg-H275Y-I436N viruses (c). Virus titres in nasal wash samples collected from donor ($n=3$), direct contact ($n=2$) and respiratory droplet (RD; $n=2$) contact ferrets were measured by performing TCID₅₀ assays in MDCK cells. The bars represent the viral titres of individual ferrets. p.i., post-inoculation; p.c., post-contact.

samples collected from any infected ferrets (data not shown). Similar histopathological findings were observed among the groups, including moderate-to-severe peribronchial inflammation and the presence of interstitial immune-cell infiltrates (Fig. S2). The rg-WT and rg-I436N mutant

viruses were transmitted to all DC and RD contact ferrets, which shed virus in the upper respiratory tracts for 4 to 5 days, although the onset of rg-I436N virus transmission via the RD route was delayed by 4 days compared to that of rg-WT virus (Fig. 5a, b). The double rg-H275Y-I436N

mutant virus was not detected in nasal washes from DC or RD contact ferrets (Fig. 5c), although seroconversion was observed in DC ferrets (Table S1). These results indicate that an A(H1N1)pdm09 virus carrying a single I436N substitution retains its viral transmissibility (i.e. transmission via the DC and RD routes); however, the introduction of a second H275Y mutation leads to attenuation and loss of transmission fitness.

DISCUSSION

Here, we applied the gene-fragmented random mutagenesis approach, which is a powerful tool for the identification of molecular markers of antiviral drug resistance among influenza viruses [19, 28]. We identified a novel I436N substitution in an A(H1N1)pdm09 NA via ZA selection in MDCK cells that confers multi-NAI resistance. Importantly, viruses carrying this single I436N substitution possess similar fitness to the NAI-susceptible virus; this conclusion is based on the evaluation of enzyme kinetics parameters, virus replication capacity in eggs and MDCK cells, pathogenicity in mice and ferrets, and transmissibility in ferrets. To address the potential risk of an I436N mutant that might pose a threat to public health, its antigenicity was compared to that of WT-pH1N1 and the current H1N1 vaccine strain used widely in vaccination by performing both haemagglutinin inhibition (HI) and serum neutralization (SN) assays (Table S2). There is no significant difference in the antigenicity among the viruses, including the I436N mutant, showing that the I436N mutant can be covered by the current vaccine, through which the majority of people have the antibody.

Although most NAI-resistant substitutions occur either in catalytic or in framework residues close to the NA enzyme active site, the I436N mutation does not. A recent study revealed that an I427T substitution in A(H1N1)pdm09 virus (also outside the NA active residues) reduced susceptibility to multiple NAIs [18]. However, this change altered viral fitness, as shown by reduced replication *in vitro* and lower pathogenicity in mice compared to the NAI-susceptible virus [18]. While the I436N change on its own did not significantly alter the fitness of the virus, the addition of the H275Y mutation did. On its own, the H275Y mutation had an intermediate effect. The I436 residue is not located in NA active sites [29] and thus may not show a direct effect on the affinity of the virus to NAIs (Fig. 6), and we propose that this is why the single I436N mutant retains its fitness. The decreased susceptibility of A(H1N1)pdm09 virus carrying the I436N substitution is potentially associated with the addition of a potential N-linked glycosylation site at asparagine at position 436 [30]. The N146 residue, which is close to the I436 residue (Fig. 6), possesses *N*-acetyl-*D*-glucosamine, and the N-linked glycosylation at N436 may affect the N-glycosylated N146 residue, causing a structural alteration of the 146–152 loop (Fig. 6), in which 151D and/or 152R interact with all NAIs tested. This could result in a reduced susceptibility to all NAIs [31]. In addition, the rg-N146R-I436N double mutant which has lost N-linked

glycosylation at the R146 residue but maintains the glycosylation at the N436 residue exhibits normal inhibition to all NAIs (Table S3), supporting the contention that the addition of N-glycosylation at the N436 residue causes the potential steric clash with the N-glycosylation at the N146 residue, resulting in the structural alteration of the 146–152 loop. However, the precise mechanism should be verified in a structural crystallography study.

NA subtypes are divided into two groups based on phylogenetic and structural distance: group 1 (containing N1, N4, N5 and N8) and group 2 (containing N2, N3, N6, N7 and N9) [32]. The I436N substitution may confer resistance in group 1 NA influenza viruses. SNP analyses conducted in the Influenza Research Database revealed that more than 99 % of group 1 NA glycoproteins possess isoleucine at position 436, with group 2 NA glycoproteins having valine at that position (Table S4). Similarly, the residue S247, which is conserved in group 1 NA, is associated with NAI resistance when converted to N247. Meanwhile A247T, where the alanine is conserved in group 2 NA, conferred NAI resistance [19, 33].

Although there is no evidence of naturally circulating influenza viruses with the I436N substitution (Table S5), the potential occurrence of this NAI-resistant variant cannot be ruled out. Based on *in vitro* and *in vivo* viral fitness, the single I436N substitution does not have a significant impact on viral replication and transmission properties in ferrets, and nor did we observe a reversion after sequential passages in MDCK cells and mice (Table S6), indicating the high genetic stability of this substitution in the A(H1N1)pdm09 virus background. Further, a competitive growth assay showed a comparable population between rg-WT and I436N mutant viruses after three sequential passages in MDCK cell (Fig. S3). It is not clear why the resistant variant has not been found in nature. Fewer than 1 % of human N1 subtype sequences ($n=92$, out of 16 600) deposited in the IRD contain an amino acid other than isoleucine (I) at position 436 (Table S5). The minor variant sequences might be associated with NAI resistance, although this requires further verification. On the other hand, unlike most minor amino acid variations at 436 (V and T; Table S5), which result from a transition mutation, a more unlikely transversion mutation due to the different number of ring structures is needed for an I436N change (AUC to AAC; U→A) in the NA gene of the pH1N1 virus, which decreases its mutation frequency in nature. It is also possible that the lower frequency of ZA prescription (compared to OS prescription) to treat influenza virus infections could be one of the reasons, as the I436N mutation was selected by ZA and the highest level of IC₅₀ was also shown in the NA inhibition assay with ZA (mean IC₅₀, 25.0 nM and 1.38–20.0 nM in ZA and the other NAIs, respectively). The results of a previous study that screened for NAI resistance markers in the NA glycoproteins of avian influenza viruses also suggested that novel resistance mutations were identified more frequently through ZA rather than OS selection [19, 28]. The

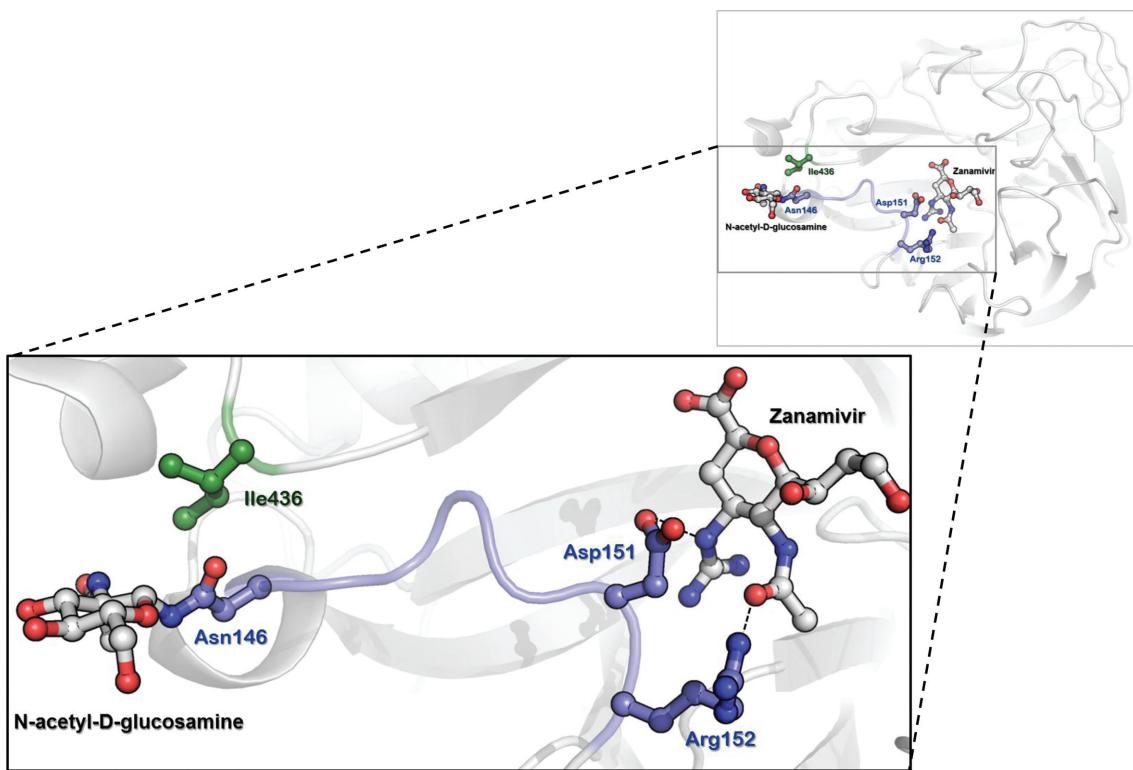


Fig. 6. Structure of the complex between A(H1N1)pdm09 virus NA and ZA (Protein Data Bank accession code 4B7Q). Residue I436 (Ile436), which confers multi-NAI resistance, is shown in green, while the loop that is possibly altered by the substitution I436N (Ile436Asn) is shown in blue and ZA is shown in white. N-acetyl-D-glucosamine covalently linked to residue N146 (Asn146) is also shown in white. The interactions among D151 (Asp151), R 152 (Arg152) and ZA are shown with black dashed lines.

I436N substitution in the N1 gene could be elicited when Y275 is present in the NA gene of the pH1N1 virus. The random mutant virus libraries in the WT pH1N1 background were also generated and passaged in the presence of ZA, but failed to identify any mutations (data not shown). Thus, the continuous use of ZA in the clinical setting due to the continuous circulation of the H275Y variant increases the potential to elicit the I436N mutation in A(H1N1)pdm09 viruses.

Monitoring for already known NAI resistance sites is necessary, but monitoring for residues that are difficult to predict, such as changes outside the NA enzyme active sites, must also be considered. The finding presented here, that I436N substitution in the NA gene of the A(H1N1)pdm09 virus could be a potential marker for multi-NAI resistance, provides additional insight for antiviral resistance monitoring.

METHODS

Compounds

The NAIs oseltamivir carboxylate (ethyl[3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylate) and zanamivir (2,4-dideoxy-2,3-didehydro-4-

guanidineosialic acid) were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). The peramivir ([1S,2S,3R,4R,1S]-3-[1-acetylamino-2-ethyl] butyl-4-[aminoiminomethyl]amino-2-hydroxycyclopentane-1-carboxylic acid) was provided by GreenCross Corp. (South Korea) and the laninamivir (5-(Acetylamino)-4-[(aminoiminomethyl)amino]-2,6-anhydro-3,4,5-trideoxy-7-O-methyl-D-glycero-D-galacto-Non-2-enonic Acid) was purchased from BOC Sciences (New York, USA). The compounds were dissolved in distilled water and stored in aliquots at -20°C until use.

Cells

MDCK and human embryonic kidney (HEK-293T) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The MDCK cells were maintained in minimum essential medium (MEM; Corning, Allendale, NJ, USA) supplemented with 5% foetal bovine serum (FBS) and 1% antibiotics. HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Omega Scientific, Tarzana, CA, USA) and 1% antibiotics (antibiotic/antimycotic, Gibco, Big Cabin, OK, USA). The cells were incubated at 37°C in 5% CO_2 until use.

Generation of randomly mutated NA plasmid and virus libraries, and selection of variants resistant to NAI

Random mutations were produced within the catalytic domain of the NA plasmid of A/California/04/2009 (H1N1) pdm09 virus by using GeneMorph II Random Mutagenesis kits (La Jolla, CA, USA) according to the manufacturer's instructions as previously reported [34]. The catalytic region was divided into two regions – region 1 (RM-R1, from 102 to 819 nt) and region 2 (RM-R2, from 823 to 1410 nt) – by generating two Mega-primers and then amplifying two randomly mutated plasmid libraries (Fig. 1). The remaining seven plasmid genes of A/Puerto Rico/8/1934 (H1N1, PR8) virus were used as the genetic background of the variant libraries. Two random-mutant virus libraries were generated by using two NA random mutant plasmids libraries, as recommended by the results of a previous study [19]. Briefly, mixtures of 2 µg of each NA plasmid of A(H1N1) pdm09 and 1 µg of the seven remaining plasmids of PR8 virus were transfected into a HEK-293T/MDCK cell mixture (3:1) prepared in six-well plates by using TransIT-LT1 reagent (Mirus Bio., Madison, WI, USA). The supernatant was replaced with serum-free Opti-MEM with antibiotics at 18 h post-transfection and then Opti-MEM with L-tosylamide 2-phenylethyl chloromethyl ketone TPCK-treated trypsin (final concentration, 1 µg ml⁻¹) was added at 40 h post-transfection. The resistant variants were selected under ZA treatment after infection of the variant libraries in MDCK cells at 4 days post-transfection. After two to four blind passages of the variant libraries in increasing concentrations (2 to 10 µM) of ZA, the NA genes of the passaged viruses were fully sequenced to identify any substitutions. The procedures for variant screening were performed three times. Mutations and substitutions identified within the NA genes were introduced into the genetic background of the A (H1N1)pdm09 virus to generate recombinant viruses by using a reverse-genetics technique. All recombinant viruses were sequenced to confirm that no additional mutations were present and used for further studies, including NAI sensitivity assays and *in vitro* and *in vivo* viral fitness characterizations.

Susceptibility to neuraminidase inhibitors

The phenotype of resistance to NAIs was identified by NA inhibition assays as previously reported [35]. Briefly, the recombinant viruses were standardized to NA activity 10-fold higher than that of the background and then incubated with fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneurameric acid sodium salt hydrate (MUNANA) (Sigma-Aldrich, Inc., St Louis, MO, USA) substrate. The drug concentration (concentration range, 5 × 10⁻⁷ µM to 50 µM) that inhibited 50 % of the NA enzymatic activity (IC₅₀) was calculated from the dose-response curve using GraphPad Prism 5.0 software (La Jolla, CA, USA). The susceptibility of the recombinant viruses to NAIs was categorized by the criteria recommended by the WHO Antiviral Working Group and based on the fold change in IC₅₀

compared with the susceptible virus: normal inhibition, <10 fold; reduced inhibition, 10- to 100-fold; highly reduced inhibition, >100 fold.

NA enzyme activity and kinetics

A fluorometric assay was used to determine the NA activity levels of the recombinant viruses [35]. We measured the NA enzyme kinetics with MUNANA, with a final substrate concentration ranging from 0 to 5000 mM. All recombinant viruses were standardized to an equivalent dose of 10^{6.0} TCID₅₀ ml⁻¹ [35]. The reaction was conducted at 37 °C in a total volume of 100 µl, and the fluorescence of the released 4-methylumbelliflferone was measured every 60 s for 60 min in a Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT, USA) using excitation and emission wavelengths of 360 and 460 nm, respectively. The enzyme kinetic data were fit to the Michaelis-Menten equation by using nonlinear regression (GraphPad Prism 5; GraphPad, San Diego, CA, USA) to determine the maximum velocity (V_{max}) of substrate conversion.

Virus replication efficiency in eggs and MDCK cells

To evaluate the growth of the recombinant viruses in embryonated chicken eggs (eggs), 1 × 10² TCID₅₀ ml⁻¹ of each virus was inoculated into five 11-day-old eggs and incubated at 37 °C for 48 h. The virus infectious titres were determined by performing the TCID₅₀ assay in MDCK cells. To determine the viral replication efficiency in MDCK cells, the cells were inoculated with recombinant viruses at an m. o.i. of 0.0001. After incubation at 37 °C for 1 h, the supernatants were removed, and the cells were washed thrice with sterile PBS and overlaid with infection medium and 1 µg ml⁻¹ TPCK-treated trypsin. At 6, 12, 18, 24, 36, 48, 60 and 72 h after virus inoculation, the cell culture supernatants were harvested for virus titration. The viral titres at each time point were determined in MDCK cells by performing the TCID₅₀ assay.

Virus pathogenicity in BALB/c mice

Groups of 6-week-old female BALB/c mice (Samtaco, South Korea) (n=10/group) were lightly anaesthetized with 0.1 ml of a mixture of zoletil (30 mg kg⁻¹) and xylazine (10 mg kg⁻¹) administered intraperitoneally and then inoculated intranasally with 3.0 × 10^{4.0} TCID₅₀ of recombinant viruses carrying single or double NA substitutions. The animals were monitored daily for clinical signs of disease, body weight changes and survival for 14 days p.i. Mice that lost more than 25 % of their initial body weight were euthanized. To examine the viral yield, additional groups of mice (n=9/group) were inoculated by using the same method and then the lungs (n=3/group) were harvested at 2, 5 and 7 days p.i. The viral titres of the lung samples were measured in MDCK cells using the TCID₅₀ assay in MDCK cells.

Virus pathogenicity and transmissibility in the ferret model

Young adult female ferrets aged 4 to 5 months (ID Bio Corp., Republic of Korea) were lightly anaesthetized with

isoflurane and inoculated intranasally with $5.0 \times 10^{5.0}$ TCID₅₀ of recombinant virus (rg-WT, rg-I436N and rg-H275Y-I436N). At 1 day p.i., two additional naïve ferrets were cohoused in cages with the infected ferrets, and two other naïve ferrets were placed into cages adjacent to the infected ferrets, separated by two stainless steel grids at a distance of 5 cm to prevent DC while permitting RD. To determine the viral titres in the upper respiratory tract of the inoculated ferrets, the ferrets were anaesthetized by intramuscular injection of a zoletil (20 mg kg⁻¹) and xylazine (0.5 mg kg⁻¹) mixture, and nasal wash samples were collected at 2, 4, 6, 8 and 10 days p.i. from infected ferrets and daily from direct contact and respiratory droplet contact ferrets for 13 days. The nasal wash samples collected from the ferrets were processed to determine viral titration by performing TCID₅₀ assays in MDCK cells. Body weight and temperature changes were evaluated relative to starting weight and temperature (day 0).

Statistical analysis

The differences of NA activity, *in vitro/in vivo* viral titres, survival and cytokine levels between wild-type and mutant viruses were compared using a *t*-test. Differences were considered to be significant at *P*<0.05, and were analysed using the Prism 5.0 program (GraphPad Software).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

All animal experiments (CBNUA-989-16-01 and CBNUA-976-16-02) and the experimental protocol (16-RDM-028) were approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee of Chungbuk National University and conducted in accordance with, and in adherence to, the relevant policies on animal handling as mandated in the Guidelines for Animal Use and Care of Chungbuk National University, Republic of Korea. All NA-resistant virus experiments were performed in an animal biosafety level 3 facility approved by the Korea Center for Disease Control and Prevention (K-CDC).

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