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Impact of Mutations at Residue I223 of the Neuraminidase Protein on the Resistance Profile, Replication Level, and Virulence of the 2009 Pandemic Influenza Virus

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Amino acid substitutions at residue I223 of the neuraminidase (NA) protein have been identified in 2009 pandemic influenza (pH1N1) variants with altered susceptibilities to NA inhibitors (NAIs). We used reverse genetics and site-directed mutagenesis to generate the recombinant A/Québec/144147/09 pH1N1 wild-type virus (WT) and five (I223R, I223V, H275Y, I223V-H275Y, and I223R-H275Y) NA mutants. A fluorimetry-based assay was used to determine 50% inhibitory concentrations (IC_{50}s) of oseltamivir, zanamivir, and peramivir. Replicative capacity was analyzed by viral yield assays in ST6GalI-MDCK cells. Infectivity and transmission of the WT, H275Y, and I223V-H275Y recombinant viruses were evaluated in ferrets. As expected, the H275Y mutation conferred resistance to oseltamivir (982-fold) and peramivir (661-fold) compared to the drug-susceptible recombinant WT. The single I223R mutant was associated with reduced susceptibility to oseltamivir (53-fold), zanamivir (7-fold) and peramivir (10-fold), whereas the I223V virus had reduced susceptibility to oseltamivir (6-fold) only. Interestingly, enhanced levels of resistance to oseltamivir and peramivir and reduced susceptibility to zanamivir (1,647-, 17,347-, and 16-fold increases in IC_{50}s, respectively) were observed for the I223R-H275Y recombinant, while the I223V-H275Y mutant exhibited 1,733-, 2,707-, and 2-fold increases in respective IC_{50}s. The I223R and I223V changes were associated with equivalent or higher viral titers *in vitro* compared to the recombinant WT. Infectivity and transmissibility in ferrets were comparable between the recombinant WT and the H275Y or I223V-H275Y recombinants. In conclusion, amino acid changes at residue I223 may alter the NAI susceptibilities of pH1N1 variants without compromising fitness. Consequently, I223R and I223V mutations, alone or with H275Y, need to be thoroughly monitored.

In the last 2 decades, neuraminidase (NA) inhibitors (NAIs) have provided a valuable approach for the prevention and treatment of epidemic and pandemic influenza infections. Although many investigational compounds are currently under development, oral oseltamivir, inhaled zanamivir, and, in some countries, intravenous peramivir and inhaled laninamivir are the only NAIs licensed for clinical use against influenza viruses, including the 2009 pandemic (pH1N1) influenza A strain (5, 6, 9, 24). However, as for other antivirals, extensive use of NAIs may lead to the emergence and dissemination of drug-resistant variants that may compromise their clinical usefulness. Moreover, as the different NAIs target the active site of the NA enzyme with similar mechanisms of action, NA mutations conferring cross-resistance may also occur, which constitutes a serious clinical problem, especially in immunocompromised individuals.

Almost 600 cases of oseltamivir-resistant pH1N1 viruses were reported worldwide over a period of 2 years after the onset of the pandemic in April 2009 (37). These viruses harbor a histidine-to-tyrosine mutation at position 275 (H275Y in N1 numbering) of the NA protein, which has also been shown to confer a lesser but still moderate degree of resistance to peramivir, while remaining susceptible to zanamivir (22, 28). In addition, pH1N1 clinical isolates harboring different amino acid substitutions at residue I223 of the NA protein have been detected in patients with or without previous exposure to NAIs. In that regard, a isoleucine-to-valine (I223V) mutation was detected along with H275Y in respiratory samples from two U.S. campers receiving prophylaxis with oseltamivir (7). These oseltamivir-resistant double mutants retained susceptibility to zanamivir. In other studies, the isoleucine-to-arginine (I223R) mutation has been shown to cause a moderate degree of resistance to oseltamivir and a low level of resistance to zanamivir (11, 19, 34). Furthermore, Nguyen and colleagues reported the presence of a highly oseltamivir- and peramivir-resistant I223R-H275Y double mutant virus isolated from an immunocompromised child (26). The double I223R-H275Y mutant had also reduced susceptibility to zanamivir. In the same study, the authors commented on a clinical isolate with the isoleucine-to-lysine (I223K) mutation, leading to a resistance phenotype comparable to that of the single I223R virus.

As a framework residue of the hydrophobic pocket of the active site of the influenza NA protein, I223 is largely conserved among influenza viruses (8). Despite increased clinical reports, the impact of mutations at residue I223 alone or in combination with H275Y on viral fitness of pH1N1 viruses has not yet been thoroughly determined. To address this issue, we generated recombinant pH1N1 viruses containing I223R and I223V NA mutations with or without H275Y changes and compared their NAI resistance phenotypes and *in vitro* replicative capacities. Furthermore,

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TABLE 1 Profiles of susceptibility of recombinant wild-type and mutant pH1N1 viruses to neuraminidase inhibitors

NA mutation(s)[a]	IC_{50} (nM) (fold increase)[b]		
	Oseltamivir	Zanamivir	Peramivir
None (WT)	0.46 ± 0.01 (1)	0.15 ± 0.01 (1)	0.06 ± 0.00 (1)
H275Y	451.90 ± 26.01 (982)	0.14 ± 0.01 (1)	39.66 ± 5.98 (661)
I223V	2.63 ± 0.01 (6)	0.35 ± 0.02 (2)	0.15 ± 0.01 (3)
I223R	24.48 ± 2.12 (53)	1.10 ± 0.10 (7)	0.60 ± 0.06 (10)
I223V-H275Y	797.40 ± 51.00 (1,733)	0.32 ± 0.01 (2)	162.39 ± 10.24 (2,707)
I223R-H275Y	757.39 ± 68.65 (1,647)	2.32 ± 0.10 (16)	1,040.80 ± 170.69 (17,347)

[a] All mutations are in N1 numbering.
[b] Values are means ± standard deviations from three independent experiments; increase was determined by comparing the value for the mutant to that for the wild-type (WT) virus (set at 1).

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we assessed virulence and transmissibility of some selected mutants in a well-validated ferret model.

MATERIALS AND METHODS

Generation of recombinant viruses. A reverse genetics system using pLLBA and pLLBG plasmids (20) was recently developed for the influenza A/Québec/144147/09 pH1N1 virus (GenBank accession numbers FN434457.1 to FN434464.1) (28). The pLLBA plasmid containing the NA gene was used for the introduction of the I223R, I223V, or H275Y mutation by using appropriate primers and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Secondary I223R or I223V mutations were then incorporated in the pLLBA-NA_{275Y} mutant plasmid as described above. All recombinant plasmids were sequenced to ensure the absence of undesired mutations. The eight bidirectional plasmids were cotransfected into 293T human embryonic kidney cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Supernatants were collected 72 h posttransfection and used to inoculate ST6GalI Madin-Darby canine kidney cells overexpressing the α-2,6 sialic acid receptor (ST6GalI-MDCK cells kindly provided by Y. Kawaoka, University of Wisconsin, Madison, WI). The recombinant wild-type (WT) virus as well as the three single (I223R, I223V, and H275Y) and two double (I223R-H275Y and I223V-H275Y) mutants were subsequently sequenced and then titrated by standard plaque assays in ST6GalI-MDCK cells (14).

NA inhibition assays. The drug resistance phenotype was determined by NA inhibition assays using the 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO) substrate (29), with minor modifications. Briefly, recombinant viruses were standardized to an NA activity level 10-fold higher than that of the background, as measured by the production of a fluorescent product from the MUNANA substrate. The drug resistance phenotype was determined by the extent of NA inhibition after incubation with serial 3-fold dilutions of the drugs (final concentrations ranging from 0 to 1,800 nM), including oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Stevenage, United Kingdom), and peramivir (BioCryst, Birmingham, AL). The 50% inhibitory concentration (IC_{50}) for each drug was calculated from the dose-response curve (4). Mutant viruses were considered to have reduced susceptibility to NAIs if they showed a 5- to 10-fold increase in their IC_{50}s compared with that of the recombinant WT. Viruses were considered resistant to a drug if their IC_{50} was increased ≥10-fold compared to that of the recombinant WT (23, 25). All linear and nonlinear regression tests to determine IC_{50}s were performed using GraphPad Prism software, version 5.

***In vitro* replication kinetics.** ST6GalI-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell and then incubated at 37°C for 1 h in a 5% CO_2 incubator. The cells were subsequently washed with phosphate-buffered saline (PBS), overlaid with modified Eagle medium (MEM) (GIBCO; Invitrogen, Burlington, Ontario, Canada) containing 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma, Oakville, Ontario, Canada), and incubated at 37°C with 5% CO_2. Supernatants were collected at 12, 24, 36, 48, 60, and 72 h postinfection (PI) and titrated by plaque assays in ST6GalI-MDCK cells. The mean viral plaque area of each virus 60 h after infection was determined from ≥20 plaques with ImageJ software, version 1.41 (Wayne Rasband, National Institutes of Health, Bethesda, MD). The presence of the desired mutations was confirmed by sequencing of the NA gene of viruses recovered at 72 h PI.

Ferret studies. Three groups of four seronegative (900- to 1,500-g) male ferrets (Triple F Farms, Sayre, PA) housed in individual cages were lightly anesthetized with isoflurane and received, by intranasal instillation, 250 μl of PBS containing 4.5 log $TCID_{50}$/ml of the recombinant pH1N1 WT, H275Y, or I223V-H275Y virus. To evaluate direct-contact transmissibility, inoculated-contact animal pairs were established by placing a naïve ferret into each cage 24 h after inoculation of the index ferret (12). To evaluate airborne transmissibility, naïve ferrets were housed in a separate special cage with a perforated Plexiglas wall to prevent direct contact between animals but to allow airflow from an index ferret to its adjacent naïve neighbor (13). All ferrets were weighed daily and monitored for clinical signs of sneezing, dyspnea, and level of activity. Nasal wash samples were collected from animals on days 2, 4, and 6 PI, with 5 ml of PBS containing 2% bovine serum albumin and immediately stored at −80°C. Virus titers in nasal wash samples were determined by plaque assays in ST6GalI-MDCK cells. The presence of the desired mutations was confirmed by sequencing of the NA gene of viruses recovered from nasal wash samples on day 6 PI. Serum samples were collected from each ferret before intranasal infection (day 0) and on day 14 PI to evaluate levels of specific antibody against the A/Québec/144147/09 pH1N1 strain using standard hemagglutination inhibition (HAI) assays. Animals were sacrificed on day 14 PI. All procedures were approved by the Institutional Animal Care Committee at the Armand Frappier Institute (Laval, QC, Canada) and Laval University according to the guidelines of the Canadian Council on Animal Care.

Molecular modeling. Molecular models of the mutants were generated using the program O (18) and subsequently analyzed with the PyMOL Molecular Graphics system (Delano Scientific, Palo Alto, CA).

Statistical analyses. Viral titers and plaque areas obtained from *in vitro* replicative capacity assays as well as nasal washes of ferrets were compared by one-way analysis of variance (ANOVA), with Tukey's multiple comparison posttest.

RESULTS

Susceptibility of recombinant viruses to NAIs. In this study, a recombinant WT pH1N1 virus and its five (I223R, I223V, H275Y, I223R-H275Y and I223V-H275Y) variants were generated. All six recombinants were produced with similar viral titers (≥10^6 PFU/ml), and comparison of nucleotide sequences confirmed the absence of unintended mutations in both the HA and NA genes. Susceptibility profiles of the recombinant viruses to oseltamivir, zanamivir, and peramivir are summarized in Table 1. As expected, the recombinant WT virus was sensitive to the three NAIs tested,

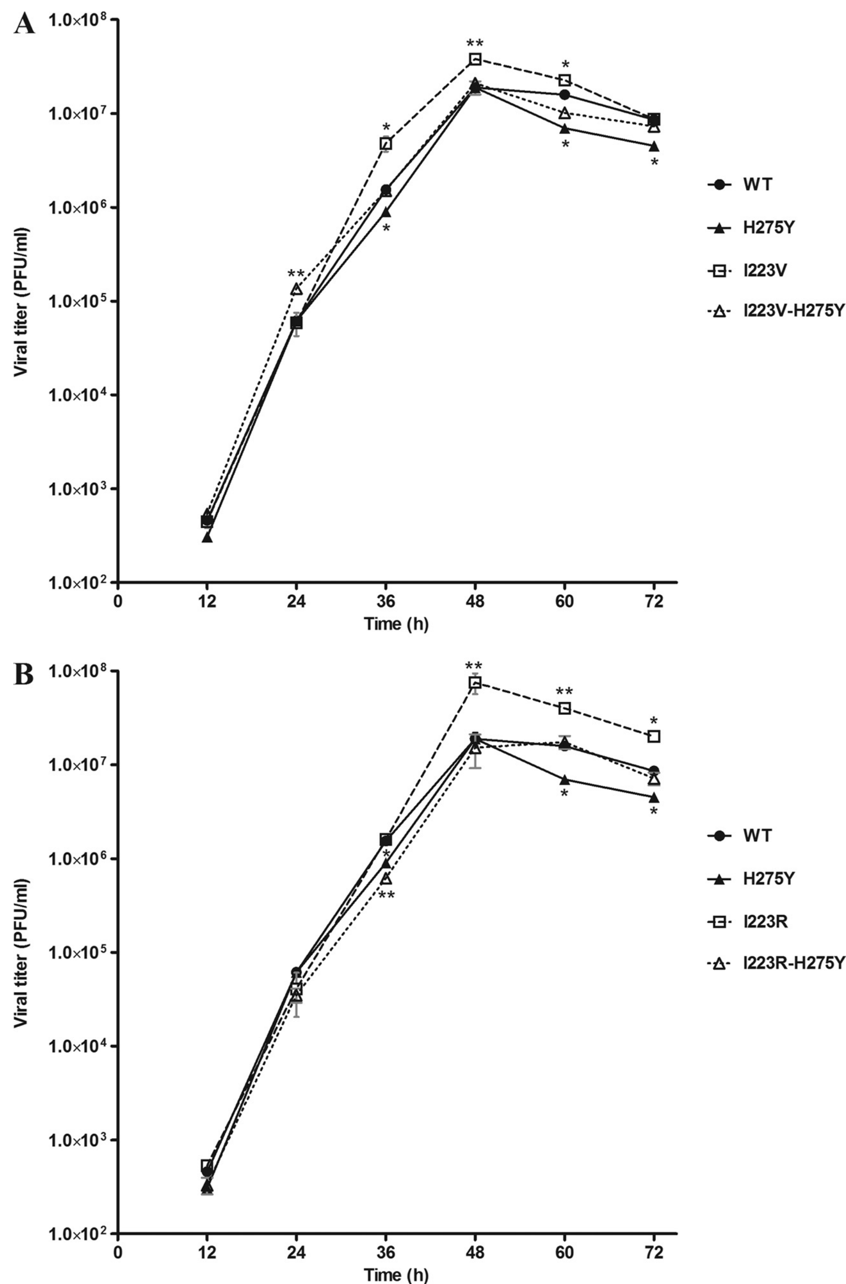

FIG 1 Replication kinetics of recombinant pH1N1 viruses *in vitro*. Confluent ST6Gal1-MDCK cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.001 PFU/cell. Supernatants were harvested at 12, 24, 36, 48, 60, and 72 h postinfection and titrated by standard plaque assays. (A) Wild-type (WT), I223V, H275Y, and I223V-H275Y viruses. (B) WT, I223R, H275Y, and I223R-H275Y viruses. Means and standard deviations from three experiments are presented. * and **, $P < 0.05$ and $P < 0.01$ for differences in viral titers between mutant and WT viruses.

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while the H275Y mutant was resistant to oseltamivir and peramivir (mean 982- and 661-fold increases in IC_{50}s, respectively) but susceptible to zanamivir. Only oseltamivir susceptibility was affected by the I223V mutation, but to a low level (6-fold increase in IC_{50} compared to the recombinant WT). However, the combination of this substitution with H275Y in the I223V-H275Y double mutant virus increased the oseltamivir and peramivir resistance phenotypes 2- and 4-fold relative to those observed in the single H275Y virus, respectively, for a total increase of 1,733- and 2,707-fold compared with the recombinant WT. The I223R mutation conferred moderate resistance to oseltamivir and reduced susceptibility to zanamivir and peramivir (53-, 7-, and 10-fold increases in IC_{50}, respectively, relative to that of the recombinant WT). These IC_{50}s were further increased in the I223R-H275Y double mutant, which showed 1,647-, 16-, and 17,347-fold-higher IC_{50}s than the recombinant WT.

***In vitro* replicative capacity of recombinant pH1N1 viruses.** In replicative-capacity experiments, the peak of replication was reached at 48 h PI by all recombinant pH1N1 viruses, with viral titers ranging from 1.89×10^7 to 7.53×10^7 PFU/ml (Fig. 1). The

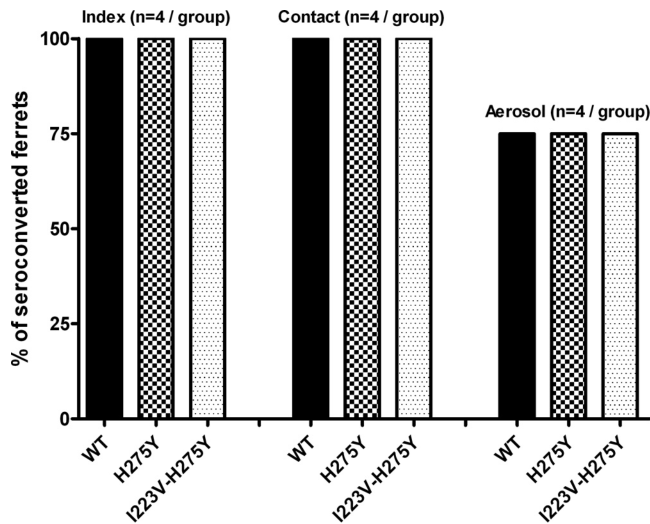

FIG 2 Seroconversion rates in ferrets infected with recombinant wild-type (WT), H275Y, and I223V-H275Y pH1N1 viruses. Anti-A/Québec/144147/09 pH1N1 virus seroconversion rates of index, direct-contact, and aerosol-contact ferrets were determined by hemagglutination inhibition assay (HAI). Seroconversion was defined as a >4-fold increase in reciprocal geometric mean hemagglutination inhibition titers on day 14 PI relative to titers on day 0 (at which time point all ferrets were seronegative).

recombinant WT and the single H275Y mutant had comparable viral titers at 48 h PI, whereas significant reductions were observed for the latter at 36, 60, and 72 h PI ($P < 0.05$). Viral titers obtained with the recombinant I223V virus were significantly higher than those of the recombinant WT at 36 ($P < 0.05$), 48 ($P < 0.01$), and 60 ($P < 0.05$) h PI (Fig. 1A). Similarly, the I223R mutation was associated with significantly higher titers compared to the recombinant WT at 48 ($P < 0.01$), 60 ($P < 0.01$) and 72 ($P < 0.05$) h PI (Fig. 1B). The I223R and I223V substitutions seemed to improve growth of the H275Y mutant, as titers of both I223R-H275Y and I223V-H275Y double mutants were comparable to those of the recombinant WT at 48, 60, and 72 h PI. No significant differences in mean plaque areas were observed among any of the recombinant viruses, with values ranging from 0.350 to 0.404 mm^2 at 60 h PI.

Characteristics of recombinant pH1N1 viruses in ferrets. Following intranasal inoculation of the recombinant WT, H275Y, and I223V-H275Y viruses, there were no significant differences in body weight loss among the three groups of animals at any time points, and only minimal clinical signs were observed (data not shown). All index and direct-contact ferrets seroconverted to A/Québec/144147/09 pH1N1 when tested 14 days after infection or contact, with reciprocal geometric mean HAI titers of >1,280, compared to <10 on day 0 (Fig. 2). Accordingly, pH1N1 viruses could be recovered in nasal wash samples on days 2 to 6 PI from all index cases and direct contact ferrets. For index cases, mean viral titers in nasal wash samples from both the H275Y and I223V-H275Y groups were comparable to those of the recombinant WT group (Fig. 3A), ranging from 1.7×10^5 to 3.0×10^6 PFU/ml (day 2), 8.7×10^3 to 2.5×10^4 PFU/ml (day 4), and 1.9×10^3 to 2.7×10^3 PFU/ml (day 6 PI). Similar findings were obtained in direct-contact groups (Fig. 3B), ranging from 1.5×10^1 to 3.0×10^1 PFU/ml on day 2, 1.8×10^5 to 2.3×10^6 PFU/ml on day 4, and 1.9×10^4 to 3.7×10^4 PFU/ml on day 6 PI. On the other hand, when aerosol transmission was evaluated, three of four animals in each group seroconverted (Fig. 2), with reciprocal geometric mean HAI titers of >1,280 in all cases (compared to <10 on day 0), except for one ferret of the I223V-H275Y group, which had an HAI titer of 80. Interestingly, only 1 animal per group showed detectable nasal wash viral titers, reaching maxima of 5.2×10^3 PFU/ml (recombinant WT), 9.6×10^3 PFU/ml (H275Y), and 5.0×10^3 PFU/ml (I223V-H275Y) on day 6.

Molecular modeling. From the computer-generated molecular model (Fig. 4), it could be inferred that the I223R mutation is likely to cause its effect through steric clashes. The extended side chain of an arginine would come very close to the hydrophobic moiety of oseltamivir, thus disrupting its binding. Peramivir has a similar bulky hydrophobic group in the same position as that of oseltamivir, and thus it is expected that the I223R and H275Y mutations would have the same detrimental effect on binding of peramivir and oseltamivir. For zanamivir, however, the corresponding glycerol moiety sits slightly higher up in the binding site and thus is less disrupted. In addition, any disruption of binding may be offset by potential hydrogen bonds between the glycerol hydroxyls and the nitrogens in the arginine side chain. The I223V mutation does not significantly affect zanamivir binding, but the effect on oseltamivir is more important due to the mutation reducing the hydrophobicity of the active site, hence decreasing the favorable interactions with oseltamivir. Because oseltamivir is still likely to bind in its standard way, the I223V mutation causes a less pronounced effect than the I223R.

DISCUSSION

The active site of the influenza NA is constituted by 8 functional residues (R118, D151, R152, R224, E276, R292, R371 and Y406) and 11 framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294 and E425), all in N2 numbering (8). Amino acid mutations at framework residues such as I222 (I223 in N1 numbering) may interfere with the correct binding of NAIs, thus disrupting the natural susceptibility of influenza viruses to these antiviral agents (1). Previous studies demonstrated that influenza variants that are NAI resistant due to framework NA substitutions usually retain susceptibility to at least one of the three commercially available NAIs (oseltamivir, zanamivir, and peramivir) (27). Here, we report that mutations at residue I223, in particular the I223R change, may confer reduced susceptibility to many NAIs associated with increased replicative capacities *in vitro*. In addition, when combined with the well-known H275Y mutation, the I223R and I223V substitutions potentiate the oseltamivir and peramivir resistance phenotypes and restore the viral fitness of the H275Y mutant.

The I223V mutation combined with the H275Y was previously induced by serial *in vitro* passages of the influenza A/Texas/36/91 (H1N1) virus under oseltamivir pressure (36). Some influenza A/H5N1 and B strains with different substitutions at residue I223 have also been reported (16, 31). Moreover, our group has previously described the recovery of an oseltamivir-resistant influenza A/H3N2 virus harboring the I222V (I223V in N1 numbering) mutation in combination with E119V from an immunocompromised patient undergoing oseltamivir therapy (3). In these studies, the I223V substitution was found to alter the resistance phenotype, with a negative effect on replication kinetics of A/H5N1 viruses but improved viral fitness in the A/H3N2 subtype (3, 16, 32, 36). The impact of amino acid changes at residue I223 has

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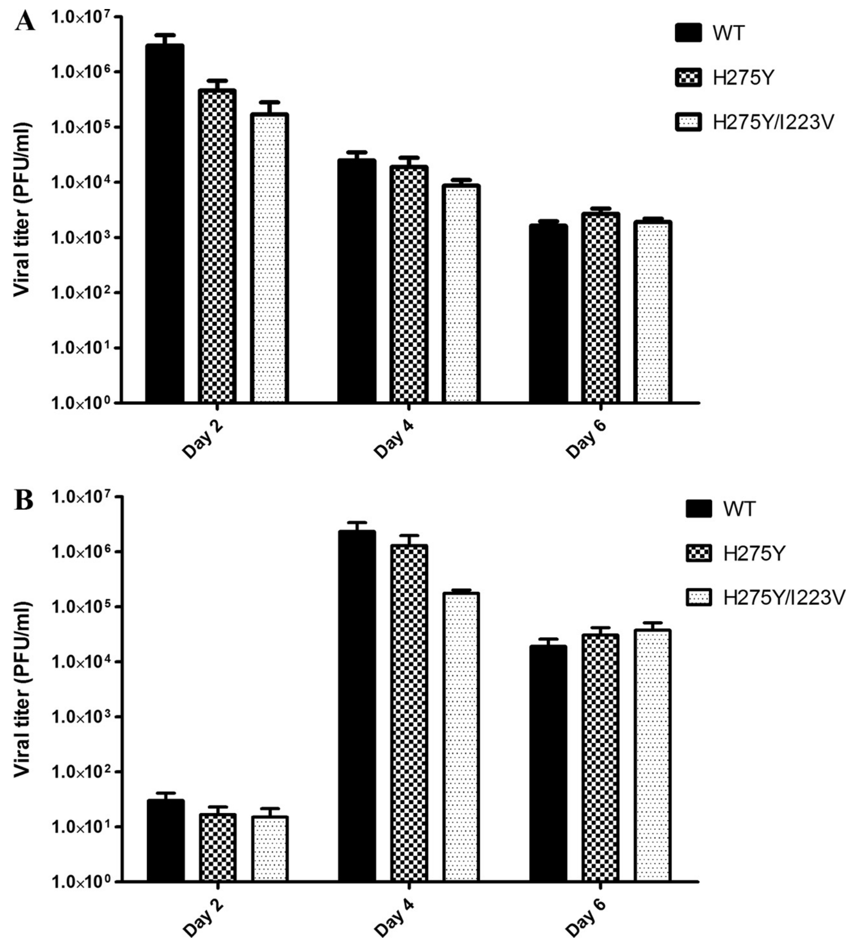

FIG 3 Nasal wash viral titers in ferrets infected with recombinant wild-type (WT), H275Y, and I223V-H275Y pH1N1 viruses. Groups of four ferrets were infected with 250 μl of PBS containing 4.5 log $TCID_{50}$/ml of the recombinant pH1N1 WT, H275Y, or I223V-H275Y virus. Inoculated-contact pairs were established 24 h postinfection. Mean viral titers and standard deviations in nasal wash samples of index (A) and direct-contact (B) ferrets were determined by standard plaque assays in ST6Gal1-MDCK cells.

recently elicited a more serious concern in the context of the 2009 pandemic H1N1 virus. Indeed, I223R, -V, and -K changes have been identified in pH1N1 clinical isolates with reduced susceptibility to zanamivir and/or significantly increased oseltamivir and peramivir resistance phenotypes, when combined with the H275Y mutation (7, 11, 19, 26, 34). In one case, pH1N1 viruses with both I223V and H275Y mutations were detected in two symptomatic adolescents who were cabinmates at a summer camp in the United States and were receiving oseltamivir prophylaxis (7). Potential transmission of the I223V-H275Y double mutant between the two campers or after exposure to a third unknown ill person was suggested but could not be proven.

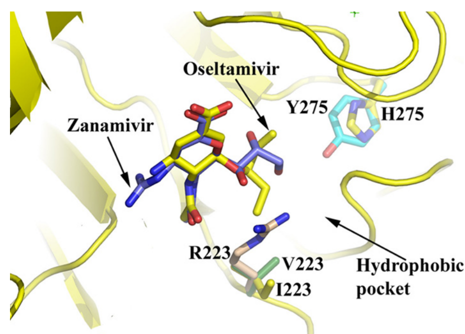

FIG 4 Molecular modeling of the I223R, I223V, and H275Y NA mutations. The schematic representation of the active site of the NA with both NAIs shows oseltamivir in yellow and zanamivir in blue. The location of amino acids at positions 223 and 275 are highlighted in stick representation and colored to distinguish different mutations.

As the previous pH1N1 mutant strains could contain additional changes in the NA gene or elsewhere in the viral genome that could influence viral fitness, we generated recombinant viruses to dissect the role of the single mutations at residue I223, alone and in combination with the well-described H275Y mutation. The five recombinants generated in this study were produced at comparable viral titers ($\geq 10^6$ PFU/ml), and despite minor differences, all of them proved to be replication competent in ST6GalI-MDCK cells, excluding any major negative effects of the mutations tested on viral fitness *in vitro*. As previously reported by our group (28), the single I223V mutation conferred only a minor reduction in oseltamivir susceptibility, but its combination with H275Y significantly enhanced the oseltamivir- and peramivir-resistant phenotype of the latter mutant. The moderate degree of

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resistance to oseltamivir and the reduced susceptibility to zanamivir and peramivir due to the I223R substitution described in this study (53-, 7-, and 10-fold increases in $IC_{50,}$ respectively, versus the recombinant WT) are in accordance with previous findings originating from clinical isolates collected from immunocompetent and immunocompromised patients who were treated with NAIs or not treated (11, 19, 34, 35). Furthermore, the phenotype of reduced susceptibility to multiple drugs obtained for the double I223R-H275Y mutant confirmed data from a recent report involving an immunocompromised girl (26).

Unlike functional NA mutations such as R292K, which severely impair viral fitness of resistant mutants, NAI-resistant influenza variants containing framework substitutions may conserve their virulence and replicative properties (15, 38). To address whether this would be the case for I223R and I223V changes, we assessed replicative capacities both *in vitro* in ST6GalI-MDCK cells and *in vivo* using the well-established ferret model. In contrast to a previous report that showed reduced viral titers and mean plaque sizes for the I223R mutant (19), we found that both single I223R and I223V mutants had improved replication *in vitro* compared to the recombinant WT virus. In fact, the addition of the I223V or I223R substitution to the H275Y in the double recombinant mutant restored the slightly reduced fitness of the single H275Y virus. Based on these *in vitro* data and the possible transmission of a I223V-H275Y virus between two summer campers (7), we selected the WT, H275Y, and I223V-H275Y recombinants for assessing virulence and transmission in the ferret model. In line with previous reports (17, 21), ferrets infected with pH1N1 variants did not evidence symptoms of severe disease. Interestingly, we found no significant differences in either seroconversion rates or nasal wash viral titers among the three viruses tested, showing comparable viral fitness and transmission efficiency. Even if a reduction in transmissibility by respiratory droplets compared to direct contact was observed, the three different recombinant viruses tested seemed to be equally affected. Although our group previously reported a reduction in airborne transmission of an influenza A/Québec/147365/09 pH1N1 virus with the H275Y mutation (GenBank accession numbers FN434448.1 to FN434451.1 and FN434453.1 to FN434455.1) compared to a WT isolate (13), whole-genome sequence comparison between the clinical strain previously evaluated and the present recombinant A/Québec/144147/09 virus shows many substitutions in the PB1, PA, HA, M1, and NS1 protein sequences that may account for this differential effect.

The reduced number of animals per group is one major limitation of this study. As a result, we cannot completely rule out a positive or negative effect of the I223V mutation on the transmissibility of pH1N1 viruses. Furthermore, we did not evaluate viral fitness in the ferret model of the I223R and I223R-H275Y mutants associated with a phenotype of reduced susceptibility to multiple drugs, although a recent study showed no marked differences in viral fitness and transmissibility between a single I223R mutant virus and the WT counterpart (35).

There is no general consensus on the definition of drug resistance based on IC_{50}s obtained by NAI assays. As previously mentioned and based on reports by Mishin et al. (23) and the Global Neuraminidase Inhibitor Susceptibility Network (25), the criteria defined in the present study set the cutoff for resistance to a $\geq$10-fold increase in IC_{50} compared to the recombinant WT. However, the significance of these increases in IC_{50} is not known, as clinical success or failure will also depend on the drug concentration at the site of viral replication. In that regard, following oral administration with the standard 75 mg twice-daily treatment, the average minimum plasma concentration of oseltamivir carboxylate is approximately 330 nM, which is significantly higher than the 50% inhibitory concentration for viral NA (10). Furthermore, a trial by Shelton et al. (30) reported median pulmonary zanamivir concentrations of 326 ng/ml in healthy adults treated with the regular 10-mg inhaled dose. Altogether, these clinical data suggest that only important increases in IC_{50}s (at least 100-fold or higher) would be associated with resistance to NAIs in the clinical setting. In line with this statement, we and others previously confirmed that parenteral peramivir conserves prophylactic activity against seasonal A/H1N1 variants with the H275Y mutation in mice, despite the peramivir-resistant phenotype demonstrated in NAI assays (2, 33).

In summary, I223R and I223V mutations may alter the susceptibility of pH1N1 viruses to one or all NAIs currently in use, without significantly compromising viral fitness. Importantly, such mutations have the potential to improve the fitness of the oseltamivir-resistant H275Y viruses and, at least in some cases, can be efficiently transmitted. Surveillance of the possible emergence and dissemination of multidrug-resistant variants in the human population due to amino acid changes at residue I223 should be increased, as well as efforts to broaden the spectrum of available anti-influenza agents.

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