Generation and Characterization of Recombinant Pandemic Influenza A(H1N1) Viruses Resistant to Neuraminidase Inhibitors

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Background. Neuraminidase inhibitors (NAIs) play a key role in the management of influenza epidemics and pandemics. Given the novel pandemic influenza A(H1N1) (pH1N1) virus and the restricted number of approved anti-influenza drugs, evaluation of potential drug-resistant variants is of high priority.

Methods. Recombinant pH1N1 viruses were generated by reverse genetics, expressing either the wild-type or any of 9 mutant neuraminidase (NA) proteins (N2 numbering: E119G, E119V, D198G, I222V, H274Y, N294S, S334N, I222V-H274Y, and H274Y-S334N). We evaluated these recombinant viruses for their resistance phenotype to 4 NAIs (oseltamivir, zanamivir, peramivir, and A-315675), NA enzymatic activity, and replicative capacity.

Results. The E119G and E119V mutations conferred a multidrug resistance phenotype to many NAIs but severely compromised viral fitness. The oseltamivir- and peramivir-resistance phenotype was confirmed for the H274Y and N294S mutants, although both viruses remained susceptible to zanamivir. Remarkably, the I222V mutation had a synergistic effect on the oseltamivir- and peramivir-resistance phenotype of H274Y and compensated for reduced viral fitness, raising concerns about the potential emergence and dissemination of this double-mutant virus.

Conclusions. This study highlights the importance of continuous monitoring of antiviral drug resistance in clinical samples as well as the need to develop new agents and combination strategies.

Antiviral therapy plays an important role in the management of influenza outbreaks and pandemics, with both prophylactic and therapeutic indications. Two classes of drugs have been approved for clinical use against influenza: the adamantanes and the neuraminidase inhibitors (NAIs). The global circulation of adamantane-resistant virus variants has led to the use of NAIs as the anti-influenza agents of choice [1, 2]. Apart from the 2 commercially available compounds oseltamivir and zanamivir, experimental NAIs such as the cyclopentane analogue peramivir and the pyrrolidine-based agent A-315675 have been tested with promising results, with the former being approved in Japan [3–5]. NAIs target the active site of influenza A and B neuraminidase (NA) enzyme, preventing cleavage of terminal sialic acid residues on the membrane of the infected cell and thus hampering viral propagation. Several subtype-specific mutations in framework or catalytic residues of NA that confer resistance to these drugs have been described in vitro and in vivo [6–11]. In influenza A(H3N2) viruses, oseltamivir-resistant variants with the R292K substitution have been reported in the clinic [6, 12]. Furthermore, mutation E119V has also been detected in drug-resistant strains from patients treated with oseltamivir [6, 12, 13]. Another substitution at position 119 (E119G) has been described in viruses of the N2 subtype after serial passages in the presence of zanamivir [14]. In contrast, the predominant mutation conferring resistance to oseltamivir in the A(H1N1) subtype is H274Y (N2 numbering), reported after in
vitro passages as well as in clinical isolates [7, 8, 15]. During the 2008–2009 influenza season, > 99% of the influenza A/Brisbane/59/07(H1N1)-like strains isolated in North America and Europe carried the H274Y mutation [16]. In the A(H5N1) subtype, the H274Y substitution has also been reported in treated patients [17, 18], whereas in vitro assays have documented the NAI-resistant E119G and D198G mutants [18, 19]. More recently, the oseltamivir-resistant N294S mutation has been identified in viruses of the A(H3N2), A(H1N1), and A(H5N1) subtypes [3, 12, 18, 20]. Another naturally occurring substitution (Q136K) has been associated with reduced sensitivity to zanamivir in A(H3N2) and A(H1N1) viruses [21, 22]. A study performed by our group with recombinant NA proteins of the N1 subtype has shown that the E119V mutation may confer cross-resistance to the 4 tested NAIs (oseltamivir, zanamivir, peramivir, and A-315675) [23].

In April 2009, a novel triple-reassortant swine-origin influenza A(H1N1) virus emerged to cause the first influenza pandemic of the 21st century. As of May 2010, 214 countries had reported laboratory-confirmed cases of pandemic influenza A(H1N1) (pH1N1), with at least 18,138 deaths [24]. Although the pH1N1 virus is naturally susceptible to NAIs, ~300 cases of oseltamivir-resistant pH1N1 have been reported worldwide (1%–1.5% of tested cases), all of them containing at least the H274Y mutation [25].

Given the fact that pH1N1 continues to be the predominant influenza virus circulating worldwide, and considering the restricted number of anti-influenza drugs available, evaluation of the potential drug-resistant variants is of high priority. In this study, we generated recombinant pH1N1 viruses using a recently described reverse-genetics system to assess the impact of several NA mutations, previously reported in NAI-resistant influenza viruses, on the resistance phenotype, NA activities, and in vitro replicative capacities.

METHODS

Generation of recombinant pandemic influenza A(H1N1) viruses
All 8 genes of the first pH1N1 virus isolated in Québec City, Canada, on 3 May 2009 (A/Québec/144147/09, GenBank accession numbers FN434457–FN434464) were amplified by reverse-transcription polymerase chain reaction, and each segment was cloned into either pLLBA or pLLBG bidirectional expression/translation vectors as described by Liu et al [26]. We incorporated 7 single mutations (N2 numbering: E119G, E119V, D198G, I222V, H274Y, N294S, and S334N) into the pLLBA plasmid containing the NA segment using appropriate primers and the QuikChange site-directed mutagenesis kit (Stratagene). We used the plasmid containing the H274Y-NA gene to introduce a second mutation (I222V or S334N) for the rescue of double mutants. All plasmids were sequenced to ensure the absence of undesired mutations. The 8 plasmids were cotransfected into 293T human embryonic kidney cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Supernatants were collected 72 h after transfection and then used to inoculate ST6Gal I Madin-Darby canine kidney (MDCK) cells overexpressing the α2,6-sialic acid receptors (MDCK α2,6 cells kindly provided by Dr Y. Kawaoka, Department of Pathological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI) to amplify the rescued viruses. The recombinant wild-type (WT) virus and each of the 9 mutants were subsequently sequenced and titrated by standard plaque assays in MDCK α2,6 cells [27].

Drug-susceptibility assays
The drug-resistance phenotype was determined by NA-inhibition assays as described by Potier et al [28], with minor modifications. Recombinant viruses were standardized to an NA activity 10-fold greater than that of the background and then incubated with serial 3-fold dilutions of the drugs, including oseltamivir carboxylate (Hoffmann-La Roche), zanamivir (GlaxoSmithKline), peramivir (BioCryst), and A-315675 (Abbott Laboratories). The final concentrations of the drugs ranged from 0 to 1800 nM. Methylumbelliferyl-N-acetylneuraminic acid (MUNANA, Sigma) was used as the fluorogenic substrate, and the half-maximal inhibitory concentration (IC50) was determined from the dose-response curve [29]. A mutant virus was considered to have reduced susceptibility to NAIs if it showed a 5- to 10-fold increase in IC50 value compared with that of the WT virus. A virus was considered resistant to a drug if its IC50 value was 10-fold greater than that of the WT virus [30].

In vitro replication assays
Replicative capacities of the recombinant viruses were evaluated by infecting MDCK α2,6 cells with a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU) per cell. Supernatants were collected at 12, 24, 36, 48, 60, and 72 h after infection and titrated by plaque assays. The mean viral plaque area of each virus 60 h after infection was determined from ≥20 plaques with the ImageJ software (version 1.41, developed by Wayne Rasband of the National Institutes of Health).

Enzymatic assays
To measure the NA enzymatic activity, we conducted fluorometric assays using MUNANA as substrate. All recombinant viruses were standardized to an equivalent dose of 105.5 PFU/mL and incubated at 37°C in 50-μL reactions with different concentrations of MUNANA [18]. The final concentration of the substrate ranged from 0 to 3000 μM. Fluorescence was monitored every 90 s for 53 min (35 measures). The Michaelis constant (Km) and the maximum velocity (Vmax) were calculated with the Prism software (version 5, GraphPad) by fitting the data to the Michaelis-Menten equation using nonlinear regression.
Table 1. Susceptibility Profiles of Recombinant Pandemic A/Québec/144147/09(H1N1) Viruses to Neuraminidase Inhibitors

<table>
<thead>
<tr>
<th>NA Mutanta</th>
<th>Oseltamivir IC&lt;sub&gt;50&lt;/sub&gt; ± SD, nM (Ratio)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Zanamivir IC&lt;sub&gt;50&lt;/sub&gt; ± SD, nM (Ratio)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Peramivir IC&lt;sub&gt;50&lt;/sub&gt; ± SD, nM (Ratio)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A-315675 IC&lt;sub&gt;50&lt;/sub&gt; ± SD, nM (Ratio)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.46 ± 0.01 (1)</td>
<td>0.15 ± 0.01 (1)</td>
<td>0.09 ± 0.01 (1)</td>
<td>0.20 ± 0.03 (1)</td>
</tr>
<tr>
<td>E119G</td>
<td>1.34 ± 0.12 (2.9)</td>
<td>124.9 ± 7.6 (832)</td>
<td>4.61 ± 0.97 (51.2)</td>
<td>24.5 ± 1.1 (123)</td>
</tr>
<tr>
<td>E119V</td>
<td>27.7 ± 1.4 (60.2)</td>
<td>85.7 ± 10.6 (571)</td>
<td>2.28 ± 0.08 (25.3)</td>
<td>13.8 ± 1.0 (69)</td>
</tr>
<tr>
<td>D198G</td>
<td>7.70 ± 0.72 (16.8)</td>
<td>0.90 ± 0.06 (6)</td>
<td>0.22 ± 0.01 (2.4)</td>
<td>0.83 ± 0.03 (4.2)</td>
</tr>
<tr>
<td>I222V</td>
<td>2.63 ± 0.01 (5.7)</td>
<td>0.35 ± 0.02 (2.3)</td>
<td>0.17 ± 0.00 (1.9)</td>
<td>0.20 ± 0.00 (1)</td>
</tr>
<tr>
<td>H274Y</td>
<td>451.9 ± 26.0 (982)</td>
<td>0.14 ± 0.01 (0.9)</td>
<td>26.6 ± 5.5 (263)</td>
<td>0.28 ± 0.01 (1.4)</td>
</tr>
<tr>
<td>N294S</td>
<td>96.8 ± 5.4 (208)</td>
<td>0.49 ± 0.02 (3.3)</td>
<td>1.04 ± 0.03 (12)</td>
<td>0.20 ± 0.01 (1.1)</td>
</tr>
<tr>
<td>S334N</td>
<td>0.43 ± 0.03 (0.9)</td>
<td>0.18 ± 0.03 (1.2)</td>
<td>0.08 ± 0.00 (0.9)</td>
<td>0.18 ± 0.01 (0.9)</td>
</tr>
<tr>
<td>I222V-H274Y</td>
<td>797.4 ± 51.0 (1733)</td>
<td>0.32 ± 0.01 (2.1)</td>
<td>119.8 ± 13.8 (1321)</td>
<td>0.39 ± 0.03 (2)</td>
</tr>
<tr>
<td>H274Y-S334N</td>
<td>302.8 ± 43.1 (658)</td>
<td>0.11 ± 0.02 (0.7)</td>
<td>15.99 ± 2.3 (178)</td>
<td>0.27 ± 0.02 (1.4)</td>
</tr>
</tbody>
</table>

**NOTE.** The mean half-maximal inhibitory concentration (IC<sub>50</sub>) values of 3 experiments ± standard deviations (SD) are indicated. NA, neuraminidase; WT, wild-type virus.

a All mutations are presented in N2 numbering.

b Compared with that of the WT virus.

Statistical analyses

In the replicative capacity assays, viral titers and plaque sizes of each mutant were compared with those of the WT by the use of t tests. A probability value of <.05 was chosen to reject the null hypothesis.

RESULTS

The recombinant A/Québec/144147/09 pH1N1 WT virus was successfully generated by reverse genetics. Of note, phylogenetic analysis of the NA and HA genes showed that the A/Québec/144147/09 strain described in this study is closely related to the prototype A/California/07/09 pandemic strain (nucleotide identity of 99.7% and 99.6%, respectively). Using this genetic background, 9 different NA mutant variants (E119G/V, D198G, I222V, H274Y, N294S, S334N, I222V-H274Y, and H274Y-S334N) were also generated (Table 1). NA-inhibition assays showed that the E119G mutation conferred important levels of resistance to zanamivir, peramivir, and A-315675, with 832-, 51-, and 1222V-H274Y, and H274Y-S334N) were also generated (Table 1). NA-inhibition assays showed that the E119G mutation conferred important levels of resistance to zanamivir, peramivir, and A-315675, with 832-, 51-, and 123-fold increases in IC<sub>50</sub> values compared with that of the WT virus. A multidrug resistance phenotype to oseltamivir, zanamivir, peramivir, and A-315675, with 832-, 51-, and 123-fold increases in IC<sub>50</sub> was observed for the E119V mutant. The D198G mutation was associated with a low level of resistance to oseltamivir as well as with a small reduction in zanamivir susceptibility (17- and 6-fold increases in IC<sub>50</sub>). The oseltamivir-resistance phenotype of H274Y and N294S was confirmed with 982- and 208-fold increases in IC<sub>50</sub>, whereas both mutants remained sensitive to zanamivir and A-315675. Resistance to peramivir (263- and 12-fold increases in IC<sub>50</sub>) was also observed for H274Y and N294S, respectively. When compared with the single H274Y mutant, the double I222V-H274Y mutant had increased IC<sub>50</sub> values for oseltamivir and peramivir (1.8- and 5-fold, respectively), whereas the H274Y-S334N mutant had decreased IC<sub>50</sub> values (1.5-fold decreases for both). The single I222V mutation was associated with a minor increase in oseltamivir IC<sub>50</sub> value (6-fold increase compared with that of the WT virus), whereas the single S334N mutation did not seem to contribute significantly to a resistant phenotype (Table 1).

The effect of NA mutations on viral growth was assessed in vitro for all recombinant viruses (Figure 1). Major differences in replication kinetics were observed among the WT and E119G and E119V mutant viruses. Mutant E119V had the greatest replication impairment, with viral titers that were 1.5 log<sub>10</sub> to 3.5 log<sub>10</sub> lower than those of the WT virus at all time points. The replication of E119G was also reduced by ~1 log<sub>10</sub> during the first 48 h compared with that of the WT virus. Whereas the WT virus reached its peak at 48 h after infection, the E119G mutant reached it 12 h later. However, both maximum viral titers were comparable, with 2.4 × 10<sup>7</sup> PFU/mL for the WT and 1.9 × 10<sup>7</sup> PFU/mL for E119G. The H274Y substitution had a reduction in viral replication of ~5 log<sub>10</sub> at 36, 60, and 72 h. In contrast, the I222V mutant showed a significant increase in viral titers during the first 60 h. For the rest of the mutant viruses, the growth curves were not significantly different from that of the WT virus, although some differences could be observed at specific time points (24 h for N294S and I222V-H274Y; 72 h for D198G and S334N). The mean plaque areas of the recombinant viruses were consistent with the viral titers observed in the yield assays (Table 2). The E119G (0.18 ± 0.10 mm<sup>2</sup>) and E119G (3.2 ± 0.14 mm<sup>2</sup>) mutants exhibited plaques significantly smaller than that of the WT virus (0.47 ± 0.25 mm<sup>2</sup>), with no significant differences for the remaining viruses.

We performed analyses of replication kinetics to evaluate the impact of NA mutations on sialidase activities. K<sub>m</sub> and V<sub>max</sub> were the 2 parameters determined for each recombinant virus, the former reflecting the affinity for the substrate and the latter the activity of the enzyme (Table 2). The V<sub>max</sub> ratio comparing
each mutant to the WT virus serves as an indicator of the relative NA activity. Except for the single S334N and double H274Y-S334N mutants, all NA substitutions decreased the affinity of the enzyme, with higher $K_m$ values compared with that of the WT virus. However, except for E119G, E119V, and I222V, all mutations considerably decreased enzymatic activity, supported by lower $V_{max}$ values. A particular combination of low affinity and high NA activity was observed for the E119G and E119V viruses, with 9- and 7.1-fold increases in $K_m$ as well as $V_{max}$ ratios that were 100% and 181%, respectively, of $V_{max}$ for the WT virus. Kinetic parameters were similar for D198G (5.1-fold increase in $K_m$ and 44% $V_{max}$ ratio) and N294S (5.2-fold increase in $K_m$ and 45% $V_{max}$ ratio). The H274Y mutant had the most affected enzymatic activity, with a 25-fold increase in $K_m$ and a $V_{max}$ ratio of only 18%. Substitution I222V resulted in a minor reduction of affinity (1.8-fold increase in $K_m$) with an important increase in enzymatic activity (181% $V_{max}$ ratio). The double I222V-H274Y virus showed an intermediate phenotype between the 2 single mutants (2.4-fold increase in $K_m$ and 47% $V_{max}$ ratio). Even if a minimal effect on enzyme affinity can be attributed to the single S334N and double H274Y-S334N substitutions ($\sim$0.2-fold decrease and 1.3-fold increase in $K_m$, respectively), NA activity was significantly reduced for both mutants (61% and 23% $V_{max}$ ratios, respectively).

**DISCUSSION**

Although many subtype-specific NA mutations conferring resistance to NAIs in influenza A(H1N1), A(H3N2), and A(H5N1) viruses have been previously described [10, 12, 18, 19, 23, 31], differences in viral genetic backgrounds can account for a differential effect of these mutations on antiviral resistance or viral fitness. Hence, it is important to assess the impact of NA substitutions using recombinant pH1N1 viruses to better evaluate the relevance of these potentially emergent drug-resistant variants. So far, the emergence of only oseltamivir- and peramivir-resistant viruses has been reported in pH1N1 influenza. In all cases, the H274Y NA substitution was detected and found responsible for the resistant phenotype, although these viruses remained susceptible to zanamivir [25, 32–36].

Here, we optimized a reverse-genetics system to produce recombinant pH1N1 viruses, expressing either the WT or any of 9 different mutant NA proteins. The effects of 5 framework NA mutations (E119G, E119V, D198G, H274Y, and N294S), previously associated with NAI resistance in different viral backgrounds, were evaluated in vitro regarding drug resistance, enzymatic activity, and viral replicative capacity. Two double mutants (I222V-H274Y and H274Y-S334N) described in patients with oseltamivir-resistant pH1N1 viruses [32] (and unpublished data) were also generated. The single mutants I222V and S334N, even though not yet widely reported, were included in this study to assess their contributions to resistance and viral fitness.

Our results confirm the oseltamivir- and peramivir-resistance phenotype conferred by the N1 subtype–specific H274Y
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Table 2. Viral Plaque Area and Neuraminidase Enzymatic Parameters of Recombinant Pandemic A/Québec/144147/09(H1N1) Viruses

<table>
<thead>
<tr>
<th>NA Mutanta</th>
<th>Mean Plaque Area ± SD, mm²</th>
<th>$K_m$ ± SD, μM (Ratio)b</th>
<th>$V_{max}$ ± SD, U/s (Ratio)c</th>
<th>$V_{max}$ Ratio, % of WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>.47 ± .25</td>
<td>22.5 ± 3.1 (1)</td>
<td>17.5 ± 2.4 (1)</td>
<td>100</td>
</tr>
<tr>
<td>E119G</td>
<td>.32 ± .14 *</td>
<td>202.7 ± 7.0 (9)</td>
<td>17.5 ± 1.9 (1)</td>
<td>100</td>
</tr>
<tr>
<td>E119V</td>
<td>.18 ± .10 **</td>
<td>159.2 ± 11.9 (7.1)</td>
<td>31.6 ± 3.0 (1.8)</td>
<td>181</td>
</tr>
<tr>
<td>D198G</td>
<td>.38 ± .13</td>
<td>115.1 ± 7.9 (5.1)</td>
<td>7.7 ± .7 (1.4)</td>
<td>44</td>
</tr>
<tr>
<td>I222V</td>
<td>.48 ± .28</td>
<td>41.0 ± 3.2 (1.8)</td>
<td>31.6 ± 3.4 (1.8)</td>
<td>181</td>
</tr>
<tr>
<td>H274Y</td>
<td>.40 ± .21</td>
<td>563.5 ± 44.2 (25)</td>
<td>3.1 ± .3 (2.1)</td>
<td>18</td>
</tr>
<tr>
<td>N294S</td>
<td>.49 ± .18</td>
<td>117.8 ± 9.2 (5.2)</td>
<td>7.9 ± .9 (1.5)</td>
<td>45</td>
</tr>
<tr>
<td>S334N</td>
<td>.45 ± .13</td>
<td>18.6 ± 1.5 (.8)</td>
<td>10.6 ± 1.1 (.6)</td>
<td>61</td>
</tr>
<tr>
<td>I222V-H274Y</td>
<td>.48 ± .18</td>
<td>54.5 ± 4.3 (2.4)</td>
<td>8.2 ± .9 (1.5)</td>
<td>47</td>
</tr>
<tr>
<td>H274Y-S334N</td>
<td>.44 ± .17</td>
<td>28.9 ± 2.3 (1.3)</td>
<td>4.0 ± .4 (2.1)</td>
<td>23</td>
</tr>
</tbody>
</table>

NOTE. For viral plaque area, the mean values of 20 measures ± standard deviations (SD) are indicated; for neuraminidase (NA) enzymatic parameters, the mean values of 3 experiments ± SD are indicated. $K_m$, Michaelis constant; $V_{max}$, maximum velocity; WT, wild-type virus.

a All mutations are presented in N2 numbering.
b Compared with that of the WT virus.

c $P < .05$ and **$P < .01$ for differences in plaque area when compared with that of the wild-type virus using the t test.

mutation in the pH1N1 background. This is in accordance with our previous report on oseltamivir resistance in a patient infected with pH1N1 [34] as well as with the study of Memoli et al [35]. Our results also demonstrate that in addition to the H274Y mutation, other NA mutations are replication competent in the pH1N1 background, and some can confer a specific or multi-drug resistance phenotype. However, some mutants, such as E119G and E119V, replicate poorly in cell culture and are thus probably unlikely to become clinically relevant, which is in line with the fact that these mutations have not yet been detected in seasonal A(H1N1) or pH1N1 isolates. In contrast, the I222V-H274Y mutant has the most important potential for dissemination owing to its improved replication and enzymatic kinetics.

The H274Y mutant conferred the highest level of resistance to oseltamivir and peramivir among the recombinant pH1N1 viruses with single mutations. This mutant was also the most affected in terms of NA kinetics due to its 25-fold reduction in affinity for the substrate and its 5-fold reduction in sialidase activity. However, such impaired enzymatic activity caused a minor impact on viral fitness, consistent with the numerous reports of this pH1N1 mutant in both immunocompromised and immunocompetent individuals [25] as well as in limited transmission events [37]. The addition of the I222V mutation significantly increased the levels of resistance to oseltamivir and peramivir in the I222V-H274Y mutant (>1000-fold increases in IC₅₀ for both NAIs compared with the WT virus), even though the single I222V mutant had a marginal effect on oseltamivir resistance (6-fold increase over that of the WT virus). Furthermore, the I222V substitution increased the NA activity and replicative capacity of the double mutant, restoring the fitness and partially compensating for the loss of NA activity due to H274Y. This synergic effect of the I222V mutation on resistance and NA activity has been previously described in A(H3N2) [38] as well as in A(H1N1) and A(H5N1) viruses [19], and this should be a matter of careful monitoring. Interestingly, in a small outbreak wherein transmission of drug-resistant viruses may have occurred, the pH1N1 strains also contained both the H274Y and I222V mutations [32]. In addition, oseltamivir-resistant pH1N1 isolates from Canada that harbor both the H274Y and S334N NA mutations have been identified (GenBank accession number CY060552.1). The role of S334N as a potential compensatory mutation is not as clear as that of I222V. The single S334N mutant remained susceptible to the 4 NAIs tested in this study, whereas the double H274Y-S334N mutant had the same oseltamivir- and peramivir-resistance phenotype as that of the single H274Y virus, although with lower IC₅₀ values. Apart from a reduction in NA activity, no effect on either the affinity of the enzyme or the replicative capacity was observed as a result of this mutation.

The oseltamivir-resistance phenotype of the N294S mutant, previously reported in A(H3N2), A(H5N1), and A(H1N1) viruses [12, 18, 20], was confirmed in the pH1N1 background. Although this mutation also conferred resistance to peramivir in pH1N1, IC₅₀ values are not high enough to disregard in advance the use of peramivir as a therapeutic alternative to oseltamivir in that context. The susceptibility of the N294S mutant to zanamivir and A-315675 was not altered. Mutations in this conserved amino acid reduced the NA activity and affinity for the substrate, but not sufficiently to affect viral fitness. Yen et al [18] described a similar effect of this mutation in A(H5N1) and A(H1N1) viruses, although Collins et al [39] reported a higher NA activity for the N294S mutant compared with the WT virus. Mutation D198G has been described only in A(H5N1) viruses in vitro and was associated with low levels of resistance to oseltamivir and zanamivir, as well as reductions in NA activity and viral fitness [19]. The even lower resistance levels to these 2 NAIs found in our recombinant pH1N1 virus, along with its unaffected replicative capacity, suggest that this amino acid may
not play such an important role in pH1N1. Of note, the D198G mutant described by Hurt et al [19] was obtained after many passages under zanamivir pressure, possibly leading to other mutations in the viral genome.

One of the most frequent NA mutations associated with resistance to oseltamivir in A(H3N2) viruses is the E119V, which retains susceptibility to zanamivir, peramivir, and A-315675 [12, 13, 30, 40]. In line with our previous findings using recombinant proteins of the N1 subtype [3, 23], we found that this substitution also conferred resistance to all NAIs in the H1N1 background; however, this mutant had significant fitness impairment. The E119G mutant, responsible for zanamivir-resistance in A(H3N2) and A(H5N1) viruses [19, 30], also showed a peramivir- and A-315675-resistance phenotype in the pH1N1 background but remained susceptible to oseltamivir. Not surprisingly, this mutation also affected the replicative capacity of the recombinant virus [18, 19] but to a lesser extent than the E119V, eventually reaching maximum viral titers comparable to those of the WT. Interestingly, our enzymatic studies reveal a particular NA activity profile for the E119G/V mutants, not observed in the other recombinant viruses. Whereas both substitutions caused an important reduction in the affinity for the substrate, there was no loss of enzymatic activity, which was in fact increased in the case of the E119V mutant. In an attempt to explain these observations, we should consider that the total NA enzymatic activity of the virus can be affected not only by the inherent effect of the specific amino acid substitutions, but also by differences in the levels of expression of NA proteins on the surface of the viral particles [41]. We suggest that even if a higher NA activity has been usually associated with an improvement in viral fitness, a balance between the sialidase activity ($V_{\text{max}}$) and the affinity for the sialic acid moieties on host cell receptors ($K_m$) may be necessary for optimal virus–cell interactions. As a result, the altered balance of these 2 parameters observed in the E119G and E119V mutants may play a negative role in viral fitness, with E119V being the most seriously affected. However, further studies are needed to confirm this hypothesis.

The fact that we did not study clinically adapted strains that may contain additional compensatory mutations may represent a limitation to this study. However, the uniform background provided by recombinant viruses makes them differ solely in the introduced NA substitutions. This strategy minimizes any possible bias in the interpretation of results as a consequence of additional mutations in the genome, enabling the description of the most comprehensive in vitro resistance study to NAIs in pH1N1 reported so far. It is of note that some other relevant NA mutations (ie, the zanamivir-resistant Q136K mutation [22]) have not been tested and should be considered for future analyses. Finally, animal studies should be conducted to assess the virulence and transmissibility of drug-resistant mutant strains. Such studies would also be invaluable to address the clinical relevance of drug-resistant viruses because increased IC$_{50}$ values may be overcome by higher drug levels, as we recently showed for intravenous peramivir [4].

In conclusion, some NA mutations, such as E119G and E119V, can confer a multidrug resistance phenotype in pH1N1 viruses and may represent a potential problem for immuno-compromised patients. However, poor replication kinetics suggests that their potential for transmission is low. The I222V mutation increases the oseltamivir- and peramivir-resistance phenotype and compensates for the minor reduction in viral fitness of H274Y, raising concern about the potential emergence and sustained community transmission of this double mutant virus. The results obtained in this cell-culture model underscore the need for animal studies to further investigate the effect of these drug-resistant mutants on virulence and especially transmission. Overall, our results highlight the importance of a continuous monitoring of antiviral resistance in clinical samples as well as the need to develop new drugs and combination strategies.

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