Accumulation of Defective Neuraminidase (NA) Genes by Influenza A Viruses in the Presence of NA Inhibitors as a Marker of Reduced Dependence on NA

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With the use of neuraminidase (NA) inhibitors (BCX-1812, oseltamivir, or zanamivir), drug-resistant variants of influenza A viruses were generated that lacked characteristic markers of resistance, such as substitutions in the NA active center or in the hemagglutinin. Drug resistance was associated with the accumulation of defective (Δ) RNA segments encoding NA. This phenomenon could be explained by reduced dependence of the virus on its NA activity. Analysis of the last isolates recovered from 11 volunteers, experimentally infected with influenza virus and treated with BCX-1812, revealed that they maintained full susceptibility to the drug in the NA inhibition assay (50% inhibitory concentration, 0.35–0.5 nM). The presence of ΔRNA segments was detected in 1 of these isolates but was not found in the isolates recovered from placebo recipients (n = 8). Because of a lack of cell culture–based assays for susceptibility testing of human influenza viruses, detection of ΔRNA segments should be considered an additional assay for monitoring of NA inhibitor resistance.

The hemagglutinin (HA) and neuraminidase (NA) proteins of influenza A and B viruses interact with cellular receptors containing terminal neuraminic (sialic) acid residues [1]. HA binds to receptors and initiates viral infection, whereas NA destroys the receptors and liberates the progeny virions. NA activity is also important for preventing the self-aggregation of progeny virions whose surface glycoproteins are sialylated by cellular enzymes [2]. The enzymatic activity of viral NA is the target of a new class of anti-influenza drugs, the NA inhibitors [3]. Two drugs of this class, zanamivir and oseltamivir, are available for use in humans, and others are under investigation [4].

The use of these novel antiviral agents prompts necessary monitoring for drug resistance in clinical settings. However, the lack of a reliable cell culture–based assay for testing the susceptibility of clinical isolates to NA inhibitors impedes the detection of the resistant virus variants [5, 6].

To elucidate the inhibitory effect of NA inhibitors, influenza viruses must change, facilitating the release of virions from infected cells and allowing them to spread within the respiratory tract. Substitutions at conserved residues in the active center of NA have been identified in NA inhibitor–resistant viruses selected in vitro [7–13] or recovered from drug-treated patients [14–17]. Such variants have been detected in NA inhibition assays [18]. Another mechanism of resistance involves substitution(s) in the HA that appear to facilitate release of virus from infected cells by reducing the efficiency of the virus binding to receptors [9, 19–22]. These virus variants have a substantially decreased requirement for NA activity, and they demonstrate cross-resistance to NA inhibitors in cell culture [18]. In addition, some of these mutant cells exhibit drug dependence that is manifested by augmentation of virus yields in the presence of low concentrations of NA inhibitor in vitro [9, 19] and in experimental animals [18]. These variants also demonstrate cross-resistance to NA inhibitors in cell culture.

The reduced binding efficiency of influenza viruses, resistant to NA inhibitors because of HA substitutions, decreases the dependence of these viruses on their own NA activity during the
release of progeny virions from infected cells. In this respect, they are comparable to the propagation of influenza A viruses in the presence of exogenous (i.e., bacterial) NA, another situation that diminishes the need for the viral enzyme. Work elsewhere showed that virus variants, selected in the presence of exogenous NA, accumulated defective RNA segments encoding the viral NA [23–25]. They were derived from the progenitor RNA segment in which a massive internal region was deleted, whereas the 5’ and 3’ gene termini were retained. Therefore, these defective segments lacked the region encoding the NA active center. Although defective viral RNAs seem to arise because of errors made by the viral RNA polymerase [26], their accumulation appears to result from a diminished need for viral NA enzymatic activity. Because of these observations, we wanted to investigate whether the reduced binding efficiency of NA inhibitor–resistant viruses would lead to accumulation of defective (Δ) RNA segments encoding NA in vitro and in infected humans. Consequently, we examined the genomes of the resistant viruses adapted to grow in the presence of the NA inhibitor and assayed samples from experimentally infected humans given the neuraminidase inhibitor BCX-1812.

Materials and Methods

Compounds. The NA inhibitors oseltamivir carboxylate (GS-4071) and BCX-1812 (RWJ-270201) were provided by BioCryst. They were resuspended in distilled water and stored at −20°C before they were further diluted for use in the enzyme assay.

Viruses and cells. Clinical isolates of influenza A (H1N1) virus were recovered from patients at the University of Virginia Health Sciences Center during the 1994–1995 influenza season. Clinical isolates were propagated twice in Madin-Darby canine kidney (MDCK) cells by using a standard procedure involving Eagle MEM containing 10% fetal bovine serum [27, 28]. A/turkey/Minnesota/833/80 (H4N2) and its zanamivir-resistant variant (resistant mutant [RM]–1) [9] were obtained from the repository at St. Jude Children’s Research Hospital (Memphis).

Virus isolates were recovered from volunteers experimentally infected with A/Texas/36/91 (H1N1) and treated with either BCX-1812 or a placebo [29]. The treatment was started 24 h after virus inoculation and continued for 5 days. The last isolates recovered from the nasal washes of the BCX-1812 recipients (n = 11) and the placebo recipients (n = 8) who shed the virus for ≥5 days were propagated in MDCK cells in the absence of drug and stored at −70°C until used for the analysis.

Influenza virus propagation in MDCK cells in the presence of NA inhibitor. To generate influenza virus resistant to the NA inhibitor BCX-1812 in vitro (the 31-RM variant), the clinical isolate A/Charlottesville/31/95 (H1N1) was passaged 18 times in MDCK cells in the presence of increasing concentrations of BCX-1812 (0.3–300 μM) or in the absence of the drug (as a control) by a procedure described elsewhere [9]. Viral infection of monolayers of MDCK cells was done at a low MOI (~0.001 TCID50/cell). The NA inhibitor was added to media after virus adsorption for 1 h at room temperature. Virus yields harvested after 72 h of infection at 34°C were used for the next round of propagation. Similarly, the virus isolate A/Charlottesville/28/95 (H1N1) was propagated 5 times in the presence of increasing concentrations (0.1–2000 μM) of oseltamivir carboxylate (GS4071) to obtain the resistant variant 28-RM.

Plaque assay. Viral resistance to NA inhibitors was evaluated on monolayers of MDCK cells by a plaque reduction assay described elsewhere [9]. Confluent monolayers were inoculated with virus diluted in Eagle MEM to give ~50 plaques per well. After the virus adsorption to cells was completed and the residual virus was removed, 10-fold dilutions (0.1–100 μg/mL) of the NA inhibitor were added in the agar overlays. The size and number of plaques formed in the absence of the NA inhibitor were used as controls.

NA inhibition assay. Viruses (grown in the absence of an NA inhibitor) from clarified cell culture supernatants were used as a source of NA activity in the modified fluorometric assay of Potier et al. [30] for measuring influenza NA activity and its inhibition by NA inhibitor. The assay measures 4-methylumbelliferone released from the fluorogenic substrate 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Sigma) by the influenza virus enzyme. The reaction was done in 0.33 mM 2-(N-morpholino)ethanesulfonic acid containing 4 mM CaCl2 at the final substrate concentration of 100 μM, as described elsewhere [31].

Reverse-transcriptase (RT) polymerase chain reaction (PCR) amplification of the NA and HA genes. Viral RNAs were extracted from the virus-containing cell supernatants with Trizol (Life Technologies) and used with a universal primer (5’-AGACAAAGCAGG-3’) for the synthesis of cDNA according to procedures described elsewhere [15]. Two overlapping fragments of the full-length RNA segment 6 encoding NA were amplified by use of the primer pair N1.2 (5’-CAGACACATTCAGACTC-3’) and N1.1439R (5’-GCTATAAACAGTAGTATG-3’) and a second primer pair, N1.600 (5’-TGCTAACAATCGGAATTTCC-3’) and N1.1439R (5’-AACGGACTACCTGTCAATGG-3’). The PCR products were purified with the use of a QIAquick PCR purification kit (Qiagen) and subjected to sequence analysis.

To identify RNA segments in the 31-RM variant and clinical isolates, we used PCR amplification with primers complementary to the 3’ and 5’ termini of the NA gene (N1.2 and N1.1439R). The products of the reactions were analyzed in a 1% agarose gel stained with ethidium bromide according to standard procedures. PCR products that were smaller than the full-length RNA segment (the ΔRNA) were extracted from the gel according to the manufacturer’s protocol (QIAquick gel extraction kit; Qiagen) and subjected to sequence analysis.

The HA gene was analyzed by a procedure similar to that for the NA gene, the only difference being the use of primers H1.11 (5’-GGGGGAAAAATAAAGAAACCC-3’), H1.1101R (5’-TCCATCTATCATTCCAGTCC-3’), H1.913 (5’-GCTATAACAGTAGTTCC-3’), and H1.1725R (5’-GAAATTCGTTCTCAGATGG-3’).

Sequence analysis of the HA and NA genes. Corresponding PCR products were sequenced by use of Taq Dye Terminator chemistry and then analyzed on an ABI 373 DNA sequencer (Applied Biosystems), according to the manufacturer’s instructions, at the Center of Biotechnology at the University of Virginia. Sequencer 4.0 software (Gene Codes) was used for the analysis and translation of nucleotide data. The HA and NA sequences of A/Charlottesville/31/95 (H1N1) and A/Charlottesville/28/95 (H1N1) were submitted
to the GenBank database (accession nos. AF398868, AF398869, AF398870, AF398872, AF398873, AF398874, AF398875, and AF398878).

Results

Generation of a BCX-1812–resistant mutant and analysis of its HA and NA sequences. The variant of A/Charlottesville/31/95 (H1N1), selected after 18 passages in the presence of BCX-1812 (designated 31-RM), was analyzed for drug resistance by a plaque reduction assay, and these results were compared with those for the wild-type and MDCK-passaged counterparts. The 31-RM variant was highly drug resistant because no reduction in plaque size was observed when BCX-1812 was present in the agar overlay (table 1). In contrast, the plaque size of the 2 counterpart viruses was reduced by 50% at >1000-fold lower concentration of the inhibitor (table 1). Moreover, the 31-RM variant was cross-resistant to the NA inhibitors zanamivir and oseltamivir carboxylate in cell culture (IC50 [drug concentration that inhibits 50% of enzyme activity or reduces plaque size], >100 µg/mL). However, when the NA of the 31-RM variant was examined in the NA inhibition assay, no increase in the IC50 values was detected (table 1). Therefore, its enzyme remained fully susceptible to the drug.

Substitutions in either HA or NA or in both surface glycoproteins are the recognized molecular markers of resistance of influenza viruses to NA inhibitors. Therefore, we compared the sequences of the HA and NA genes of the 31-RM variant with those for the wild-type and MDCK-passaged counterparts. The 31-RM variant contained 2 substitutions, Asn58→Asp and Ile211→Thr (table 1). Neither of these substitutions involved the conserved residues that form the active center of NA [2]. The absence of substitutions in the NA active center of the 31-RM variant is consistent with the retention of NA sensitivity to BCX-1812, as assessed in the NA inhibition assay.

Detection of defective NA genes in the virus passaged in the presence of NA inhibitors. To test our hypothesis—namely, that reduced virus susceptibility to NA inhibitors and, therefore, reduced virus dependence on NA activity—would lead to accumulation of ΔRNA segments, we examined the genomes of the drug-resistant viruses. To distinguish between a full-length RNA segment 6, which encodes NA, and ΔRNA segments that have an internal deletion, we amplified cDNA by PCR with primers complementary to the 3’ and 5’ termini of the RNA segment. The PCR products obtained from the cDNAs of the wild-type and MDCK-passaged viruses were the same size as that predicted for the full-length segment (~1400 bp; figure 1A, lanes 1 and 2). In contrast, when the cDNA of the 31-RM variant was amplified, the band corresponding to the full-length segment was very faint or not detected on the gels (figure 1A, lane 3). Instead, a prominent, smaller band (~600 bp) was present. As we hypothesized, sequence analysis of this smaller PCR product confirmed that it arose from an NA-encoding RNA segment that had undergone a massive internal deletion.

We then tested whether defective copies of the RNA segment encoding HA could be detected in the 31-RM variant. Defective HA genes were not detected with primers complementary to the

Table 1. Characterization of the influenza virus variants selected in vitro in the presence of neuraminidase (NA) inhibitors.

<table>
<thead>
<tr>
<th>Virus strain, variant</th>
<th>NA inhibitor</th>
<th>Plaque size reduction, IC50 (µM)</th>
<th>NA inhibition, IC50 (nM)</th>
<th>Substitution(s) in HAa</th>
<th>Substitution(s) in NAb</th>
<th>Detection of ΔRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Charlottesville/31/95 (H1N1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>None</td>
<td>~0.3</td>
<td>0.3</td>
<td>None</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>MDCK passed</td>
<td>None</td>
<td>~0.3</td>
<td>0.2</td>
<td>None</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>31-RM</td>
<td>BCX-1812</td>
<td>&gt;300</td>
<td>0.1</td>
<td>None</td>
<td>Asn58→Asp, Ile211→Thr</td>
<td>Yes</td>
</tr>
<tr>
<td>A/Charlottesville/28/95 (H1N1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
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<td>ND</td>
<td>0.06</td>
<td>None</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>MDCK passed</td>
<td>None</td>
<td>ND</td>
<td>0.06</td>
<td>HA1; Ser140→Pro</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>28-RM</td>
<td>Oseltamivir carboxylate</td>
<td>ND</td>
<td>0.5</td>
<td>HA1; Ser140→Pro</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>A/turkey/Minnesota/833/80 (H4N2)a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>None</td>
<td>~0.001</td>
<td>8.0</td>
<td>None</td>
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<td>No</td>
</tr>
<tr>
<td>MDCK passed</td>
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<td>&lt;0.01</td>
<td>7.0</td>
<td>HA2; Gly114→Lys</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td>RM-1</td>
<td>Zanamivir</td>
<td>~10</td>
<td>6.0</td>
<td>HA2; Gly75→Glu, Arg249→Lys</td>
<td>Yes</td>
<td></td>
</tr>
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</table>

NOTE. Plaque reduction and NA inhibition assays were done in the presence of the NA inhibitor used for selection of resistant variant in Madin-Darby canine kidney (MDCK) cells. IC50 values (drug concentration that inhibits 50% of enzyme activity or reduces plaque size) are averages from at least 3 experiments. HA, hemagglutinin; ND, not determined; RM, resistant mutant; ΔRNA, defective RNA segments encoding NA.

a H3, HA subtype numbering.
b N2, NA subtype numbering.
c Data for A/turkey/Minnesota/833/80 and its RM-1 variant have been reported elsewhere [9, 10, 22].
and 5’ termini of the HA gene (figure 1B), whereas full-size HA products were detected readily. Therefore, accumulation of ΔRNA segments encoding NA—but not HA—was observed in the drug-resistant 31-RM variant.

Next we examined how withdrawal of the NA inhibitor affected the detection of the RNA segments in the viral genome. The 31-RM variant was propagated for 5 additional passages in the absence of the NA inhibitor and then reexamined. The PCR product corresponding to the full-length RNA segment was readily visible on the gel (figure 1C). Therefore, propagation of 31-RM in the absence of the NA inhibitor increased the proportion of full-length RNA segments encoding NA.

To determine whether accumulation of the ΔRNA segments was limited to the particular virus or was the result of the particular NA inhibitor that we used, we passaged the clinical isolate A/Charlottesville/28/95 (H1N1) in a manner similar to that of the 31-RM variant in the presence of the NA inhibitor oseltamivir carboxylate. Viruses were harvested after each of the 5 subsequent passages and analyzed. No ΔRNA segments encoding mutant NA were detected in the virus, before and after 5 passages in the absence of the NA inhibitor (figure 1D, lane 6). The emergence of ΔRNA segments was detected after the first passage of the virus in the presence of the NA inhibitor (figure 1D, lane 1). The ΔRNA segments were ~250–700 nt. The accumulation of a single dominant ΔRNA segment became prominent after 4 passages, when the PCR product for the full-length RNA segment was no longer visible on the gel (figure 1D, lanes 5 and 6). The virus harvested after 5 passages in the presence of the NA inhibitor was designated as 28-RM, and the HA and NA sequences of this virus were compared with those of the MDCK-passaged counterpart. No changes in these 2 sequences of the viruses were detected, although both of the viruses contained an HA substitution that was absent in the wild-type HA (table 1).

In addition, we examined the virus variant of A/turkey/Minnesota/833/80 (H4N2), which we previously selected in MDCK cells in the presence of zanamivir and the properties of which have been described elsewhere [9, 10, 22] (table 1). In light of the results of the plaque reduction assay, the virus variant selected after 8 passages (designated RM-1) had reduced susceptibility to zanamivir, but there were no substitutions in the active center of its NA. RT-PCR analysis of the NA gene revealed the presence of 2 ΔRNA segments (~390 bp and 380 bp) (table 1).
Therefore, passage of 3 strains of influenza A viruses in the presence of an NA inhibitor (BCX-1812, oseltamivir carboxylate, or zanamivir) resulted in the accumulation of ΔRNA segments.

Examination of influenza virus isolates recovered from volunteers treated with an NA inhibitor. The activity of NA is essential for efficient viral propagation in vivo [23, 25]. Therefore, we wanted to examine whether accumulation of viral ΔRNA segments could be detected in humans treated with an NA inhibitor. For this purpose, we analyzed virus isolates recovered from volunteers experimentally infected with influenza A (H1N1) virus and treated with BCX-1812 [29].

When tested in the NA inhibition assays, drug susceptibility was similar among the viruses recovered from both groups of subjects and among the challenge virus (IC₅₀, 0.35–0.5 nM). Full-length RNA segments were detected in all isolates (figure 2A and 2B). However, the isolate recovered from 1 subject who received BCX-1812 contained an additional band of a smaller size, indicating the presence of a ΔRNA segment (figure 2B, lane 7). The sequence analysis of this ΔRNA segment revealed that, as in the viruses generated in vitro, it contained the sequences of the 5' and 3' ends (the sequence from nt 1–127 connected to the sequence from nt 1027–1461) but lost the internal part of the segment encoding NA (900 nt shorter than the wild-type RNA segment). To confirm the presence of the ΔRNA segment in the virus before its propagation in cell culture, we performed RT-PCR amplification of viral RNA extracted directly from the clinical sample. The size of the ΔRNA segment detected in the virus of the original clinical sample was similar to that of the virus grown in MDCK cells (~550 bp). When the individual virus clones recovered from the nasal wash were subject to RT-PCR, only 1 of 8 clones contained the ΔRNA segment (not shown). The virus titers in this subject’s nasal washes recovered on days 1–6 of shedding were 10⁴, 10⁴.8, 10³, 10³, 10³.8, and 10¹ TCID₅₀/mL, respectively. No virus was detected on day 7 after infection. Therefore, the virus containing the ΔRNA segments was cleared.

Next, we examined how early during the treatment course the ΔRNA segments could be detected in the clinical samples recovered from this NA inhibitor recipient. The ΔRNA segments were detected in the viruses recovered on days 2, 5, and 6 of...
Discussion

In the present study, we describe the rapid accumulation of defective RNA segments encoding NA as a result of the passage of influenza virus in the presence of several NA inhibitors. The ΔRNA segments were detected in influenza A viruses carrying the NA of N1 and N2 subtypes. In addition, we identified ΔRNA segments in clinical samples recovered from a subject experimentally infected with influenza A virus and treated with an NA inhibitor.

Detection of ΔRNA segments in clinical samples demonstrates the need for additional studies to evaluate the incidence of their emergence in patients who receive NA inhibitors for natural influenza A virus infections. If the majority of influenza A viruses resistant to NA inhibitor acquired reduced dependence of their NA enzymatic activity and all of them accumulated ΔRNA segments encoding NA, detection of such defective genes directly in clinical samples could be a useful test for emerged resistance. Although the presented results indicate existence of the discrete mechanism of influenza virus resistance to NA inhibitors that does not involve substitutions in the NA active site or in HA, they do not reveal the molecular basis of this mechanism.

The genome of influenza A virus consists of 8 RNA segments of negative polarity, of which RNA segment 6 encodes a single protein, NA [1]. The mechanism leading to the accumulation of defective RNA segments may be associated with the formation of virus aggregates in the presence of the NA inhibitors [32]. Therefore, coinfection by virions carrying a complete genome and virions carrying any defective RNA segment could allow assembly of the defective RNA segments into the genomes of the progeny virions. If a defective segment were small, it would have a replicative advantage; thus, the number of the progeny virions carrying the defective segment would increase with passage. Accumulation of defective RNAs in the genome of influenza viruses also may be a result of virus propagation at a high MOI (von Magnus effect) [26, 33]. In the present study, we did not control the MOI, although we used high dilutions of the virus yields for subsequent passage of the virus. Either of the described feasible mechanisms of accumulation of defective genes is based on coinfection of the same cell, with virions containing a complete set of segments and virions carrying the defective ones. Of importance, these mechanisms do not account for the absence of defective RNA segments encoding HA in the genome of the virus variants selected in the presence of NA inhibitor in our study.

Of note, the number of ΔRNA segments was not necessarily greater than the number of full-length RNA segments in the genomes of the 31-RM (figure 1A, lane 3) or 28-RM (figure 1D, lane 6) variants, as well as in the clinical isolate (figure 2R, lane 7). The virions carrying ΔRNA segments could possibly exist as a small proportion of the total virus load, and the overwhelming abundance of the products synthesized from the ΔRNA segments is likely to be a result of the more efficient amplification of a short (rather than long) template by RT and Taq polymerase.

We believe that the emergence of the ΔRNA segments encoding NA was caused by the virus’s reduced dependence on its own enzyme. This property was acquired by the virus variants during propagation in the presence of an NA inhibitor and was manifested by resistance to the drug in cell culture. Sequence analysis of the NA of the 31-RM variant revealed 2 substitutions: one (Asn58→Asp) resulted in abolishment of a glycosylation site in the stalk region, whereas the other (Ile211→Thr) led to the creation of a potential glycosylation site in the head region of the molecule. Because of the location with respect to the enzyme active site, either of the substitutions is unlikely to be involved in emergence of viral resistance to NA inhibitors. The fact that the second NA inhibitor–resistant variant, 28-RM, did not contain any substitutions in its NA supports such a conclusion. An NA-independent mechanism of emerged resistance was supported by the results of the NA inhibition assays, which demonstrated unaltered susceptibility to BCX-1812. The observed cross-resistance of the BCX-1812–selected virus to 2 other NA inhibitors in cell culture also supports its NA-independent mechanism of drug resistance [18]. It seems unlikely that the emergence of resistant viruses with reduced efficiency of binding would have a consequence for the clinical use of BCX-1812. In our experiments in a ferret model, we demonstrated that the virus carrying ΔRNA segments encoding NA has reduced infectivity and virulence (unpublished data).

Results of several studies support the possible role of the HA substitutions in the development of resistance to zanamivir and oseltamivir carboxylate [13–15, 19]. It appears that HA substitutions, by reducing dependence of the virus on the enzymatic activity of NA, could serve as prerequisites for the changes in the NA enzyme itself [18]. Confirmation of the role of HA substitutions for emerged resistance to NA inhibitors is difficult [18]. Of importance, propagation of egg-adapted human or avian influenza viruses in MDCK cells [34–37] could be responsible for at least some of the changes identified in the HA of the NA inhibitor–resistant variants in previous studies [7, 12, 19, 38], including ours [9, 10, 22]. We also reported selection of HA variants in humans experimentally infected with egg-adapted influenza A virus [15].

In contrast to egg-grown influenza viruses, human influenza viruses passaged in MDCK cells typically show few HA
sequence variations [39, 40]. For this reason, in the present study, we used clinical isolates A/Charlottesville/28/95 and A/ Charlottesville/31/95, which were isolated and propagated exclusively in MDCK cells. Compared with the HA sequences of viruses passed in parallel in the absence of NA inhibitor, the HA sequences of viruses passed in the presence of NA inhibitor contained no substitutions. Thus, the molecular basis of the viral resistance lies outside the HA sequence. Overall, we did not detect any of the characteristic changes described previously for NA inhibitor–resistant viruses, such as amino acid substitutions in either or both of the surface glycoproteins, HA and NA.

In the reports by Tai et al. [12] and Staschke et al. [7], the NA inhibitor–resistant virus variants had substitutions in NA in but not in HA. The substitutions in NA that confer drug resistance to these virus variants at the same time compromise the function of the enzyme [7, 10, 41, 42]. This situation leaves unanswered the question of compensatory changes for the reduced NA activity of these resistant variants.

The virus variant could acquire the ability to replicate in the presence of the NA inhibitor because of the reduced efficiency of binding. We speculate that changes in genes other than the HA gene could be responsible for this feature. For example, it was shown that virus interactions with cellular receptors could be influenced by the M1 protein [43]. Our findings, together with those of others, necessitate further investigation of the mechanism(s) by which influenza virus compensates for the reduced NA activity in the presence of NA inhibitors.

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