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Effect of influenza H1N1 neuraminidase V116A and I117V mutations on NA activity and sensitivity to NA inhibitors

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ABSTRACT

Neuraminidase inhibitors (NAIs) play a key role in the management of influenza. Given the limited number of FDA-approved anti-influenza drugs, evaluation of potential drug-resistant variants is of high priority. Two NA mutations, V116A and I117V, are found in ~0.6% of human, avian, and swine N1 isolates. Using the A/California/04/09-like (CA/04, H1N1) background, we examined the impact of V116A and I117V NA mutations on NAI susceptibility, substrate specificity, and replicative capacity in normal human bronchial (NHBE) cells and a human respiratory epithelial cell line (Calu-3). We compared the impact of V116A and I117V on the functional properties of NA and compared these mutations with that of previously reported NAI-resistant mutations, E119A, H275Y, and N295S. All NA mutations were genetically stable. None of the viruses carrying NA mutations grew to significantly lower titers than CA/04 in Calu-3 cells. In contrast, V116A, I117V, E119A, and N295S substitutions resulted in significantly lower viral titers (1.2 logs) than the parental CA/04 virus in NHBE cells. V116A conferred reduced sensitivity to oseltamivir and zanamivir (13.7-fold). When MUNANA, 3′SL, and 6′SL substrates were applied, we observed that V116A reduced binding ability for all substrates (13.9-fold) and I117V led to the significantly decreased affinity for MUNANA and 6′SL (4.2-fold). Neither mutation altered the catalytic efficiency (k_{cat}/K_M) in catalyzing 3′SL, but the efficiency in catalyzing MUNANA and 6′SL was significantly decreased: only ~34.7% compared to the wild-type NA. The efficiencies of NAs with E119A, H275Y, and N295S mutations to catalyze all substrates were ~19.4% of the CA/04 NA. Our study demonstrates the direct effect of drug-resistant mutations located inside or adjacent to the NA active site on NA substrate specificity.

1. Introduction

In early April 2009, the unexpected emergence of a unique triple reassortant influenza A/H1N1 (pH1N1) virus containing segments from avian, human, and swine sources prompted a worldwide pandemic. Its novel genomic structure and altered sialyl receptor binding capabilities resulted in higher pathogenicity and more sustained human-to-human transmission as compared to the previously circulating seasonal H1N1 virus (Maines et al., 2009; van den Brandt et al., 2010). By the end of this pandemic, an estimated 60.8 million cases were reported in the United States alone, and around 18,500 laboratory-confirmed deaths had been reported in 195 countries; however, epidemiologic studies estimated the true number of global deaths to be over 200,000 (Dawood et al., 2012; Shrestha et al., 2011). Although the pH1N1 virus retained sensitivity to one of the classes of anti-influenza drugs, neuraminidase (NA) inhibitors (NAIs), preparedness for a potential future influenza pandemic has become an increasingly salient concern given the potential for emergence of NAI-resistant virus mutations.

At present, vaccination remains the most effective method of influenza prophylaxis, but there are often lapses in coverage due to the time needed to produce and distribute an effective vaccine. Therefore, NAIs (oseltamivir, zanamivir, peramivir, and laninamivir), that selectively target the NA enzyme of influenza A and B viruses, serve as an integral “safety net” and play a key role in the management of influenza infection. Given the very limited number of anti-influenza therapeutic agents, including NAIs and the recently FDA-approved baloxavir marboxil (endonuclease inhibitor), the effectiveness of therapeutic and prophylactic NAIs will depend not only on the correct dosage and duration of treatment but also on the sensitivity of the targeted virus strain. The proportion of oseltamivir-resistant pH1N1 viruses has

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remained variable, but relatively low across seasons (~0.5%). Notably, cases with no known exposure to oseltamivir have been documented (Lackenby et al., 2018; Nguyen et al., 2012).

A predominant mutation conferring resistance to oseltamivir and peramivir in the pH1N1 viruses is H275Y (N1 numbering here and throughout) (Lackenby et al., 2018). A previous study using recombinant pH1N1 viruses demonstrated that the N295S mutation can also confer resistance to oseltamivir and peramivir (Pizzorno et al., 2011). Several different amino acid substitutions for I at position 223, a highly conserved framework residue in the NA active site, were reported and included R, V, K, and L. The I223 R/V/K/L mutations are generally associated with mildly decreased susceptibility to oseltamivir and zanamivir in pH1N1 viruses (Nguyen et al., 2012). Moreover, when an I223 substitution is paired with H275Y, this leads to a synergistic enhancement of oseltamivir and peramivir resistance (Pizzorno et al., 2011). The S247N substitution, which confers slightly decreased sensitivity to oseltamivir and zanamivir, was reported in 10–30% of surveillance pH1N1 viruses from the Asia-Pacific region (Hurt et al., 2011). Mutations at framework position E119, E119 A/G/K/V, were shown to confer multidrug resistance phenotype in pH1N1 viruses (Pizzorno et al., 2011; Samson et al., 2014); whereas, S110F and D199G confer slightly decreased sensitivity to oseltamivir and zanamivir (Lackenby et al., 2018; Okomo-Adhiambo et al., 2014).

Mutations at two highly conserved residues adjacent to the active site, V116 and I117, were identified in N1 NAs (Hurt et al., 2012). These mutations were found in ~0.6% of human, avian, and swine isolates of N1 NA subtype, including two V116A and two I117V human cases isolated in recent years. This indicates that mutations at N1 residues, V116 and I117, may occur. However, their impact on NA enzyme properties, including enzyme activity, in homogeneous pH1N1 background has not been examined to date. The objective of the present study was to evaluate, in the A/California/04/09-like (CA/04, pH1N1) genetic background, the impact of two single point NA mutations, V116A and I117V, on viral replication capacity, NA substrate specificity, NA enzyme kinetics, and NAI susceptibility in normal human tracheal/bronchial (NHBE) and lung epithelial (Calu-3) cell lines. We also compared the impact of V116A and I117V on the functional NA properties with that of previously reported NAI-resistant mutations, E119A, H275Y, and N295S.

2. Materials and methods

2.1. Cells, viruses, and compounds

The Madin-Darby canine kidney (MDCK) cell line, human embryonic kidney cell line (293T), and the human lung epithelial cell line (Calu-3) were obtained from the American Type Culture Collection (Manassas, VA) and maintained as described previously (Ilyushina and Donnelly, 2014). Primary NHBE cells were obtained from Lonza (Walkersville, MD) and were grown on membrane supports at the air-liquid interface as described previously (Matrosovich et al., 2004).

Recombinant CA/04-like viruses were generated by DNA transfection of 293T cells (Hoffmann et al., 2000), and the point nucleotide mutations were inserted into the NA gene of the wild-type virus using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Virus stocks were prepared by incubation in 10 day-old embryonated chicken eggs for 48 h at 37 °C. The entire HA and NA genes were sequenced to verify the presence of the desired mutations and absence of additional mutations. All experimental work was performed in a biosafety level-2 (BSL-2) laboratory approved for use of these strains by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Oseltamivir carboxylate (oseltamivir) ([*3R,4R,5S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) was provided by Roche Diagnostics GmbH (Mannheim, Germany). Zanamivir (4-guanidino-Neu5Ac2en) and N-acetylneuraminic acid (NANA) were obtained from Sigma-Aldrich (St. Louis, MO). Laninamivir, 3′-sialyllactose (3′SL), and 6′-sialyllactose (6′SL) were purchased from Carbosynth (Compton, UK).

2.2. Virus sequence analysis

Viral RNAs were isolated from virus-containing cell culture fluid after transfection or after 5 passages in Calu-3 cells by using RNeasy Minikits (Qiagen, Germantown, MD). Samples were reverse transcribed and analyzed by PCR using universal primers specific for influenza gene segments (Hoffmann et al., 2001). Sequencing was performed by the Research Central Facility for Biotechnology Resources at the U.S. Food and Drug Administration, Silver Spring, MD.

2.3. Stability and infectivity of recombinant pH1N1 viruses

The genetic stability of the viruses was monitored by sequencing of the HA and NA genes after transfection and by sequencing of all genes after five passages in Calu-3 cells at a MOI of 0.01 PFU/ml. The infectivity of pH1N1 viruses was determined by plaque assay (Hayden et al., 1980). Briefly, confluent cultures of MDCK cells were incubated at 37 °C for 1 h with 10-fold serial dilutions of each virus. The cells were then washed and overlaid with minimal essential medium containing 0.3% bovine serum albumin, 0.9% Bacto agar, and 1 μg/ml l-(tosylamido-2-phenyl)ethylchloromethylketone (TPCK)-treated trypsin. After 3 days of incubation at 37 °C, the cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the number of plaque-forming units (PFU) per milliliter and plaque size of any 10 plaques were determined using a Finescale magnifying comparator.

2.4. Viral replication kinetics

To determine multistep growth curves for each virus, NHBE and Calu-3 cells were infected with the pH1N1 viruses at an MOI of 0.1 and 0.01 PFU/cell, respectively. After incubation for 1 h, cells were washed, and Calu-3 cells were overlaid with medium containing 0.3% bovine serum albumin and 1 μg/ml TPCK-treated trypsin. NHBE cells were kept under air-liquid interface conditions and no trypsin was added to the cultures because previous studies demonstrated efficient proteolytic activation of influenza viruses by endogenous proteases (Matrosovich et al., 2004). The supernatants were collected at the indicated time points and stored at −70 °C until titration.

2.5. Virus yield reduction assay

The virus yield reduction assay was performed as described previously in 24-well plates containing confluent Calu-3 cells (Ilyushina and Donnelly, 2014). The concentrations of NAIs ranged from 0.001 nM to 100 μM and the compounds were added to the 24-well plates for 2 h. After pretreatment, the cells were overlaid with 2 × drug-containing medium (100 μl/well), infected with influenza virus at a MOI of 0.01 PFU/cell, and incubated for 48 h at 37 °C. Virus yields were determined as the number of PFU/ml in MDCK cells. The drug concentration that caused a 50% decrease in the PFU titer in comparison to control wells without drug was defined as EC_{50}. The results of two independent experiments were averaged.

2.6. NA enzyme inhibition assay

Recombinant pH1N1 viruses were standardized to equivalent NA activity and incubated for 30 min at 37 °C with NAIs or NANA at concentrations of 0.00005–200 mM with MUNANA (Sigma-Aldrich) as a substrate. After 1 h, the reaction was terminated by adding 14 mM NaOH and fluorescence was quantified in a Synergy 2 multimode microplate reader (BioTek Instruments, Winooski, VT). The concentration of NAI that reduced NA activity by 50% relative to a control mixture

with no inhibitor (IC_{50}) was determined by plotting the dose-response curve of inhibition of NA activity as a function of the compound concentration. Values are the mean of two to four independent determinations.

2.7. Virus purification

Allantoic fluid was clarified by low-speed centrifugation. The virus was pelleted and then purified through 27% and 49% (w/v) sucrose cushions. Virus-containing bands were pelleted and stored in phosphate-buffered saline at −70 °C until use. HA and NA content was determined by optical densitometry of the SDS-PAGE gel images using ImageJ software (Schneider et al., 2012; Supplementary Fig. S1) and total protein content was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL).

2.8. NA enzyme kinetics and substrate specificity

The NA enzyme kinetics of influenza pH1N1 viruses was measured by a fluorescence-based assay using the fluorogenic substrate MUNANA (Sigma-Aldrich), based on the method of Potier et al. (1979) as described previously (Marathe et al., 2013; Lee et al., 2018). NA kinetics using 3′SL and 6′SL substrates were determined by a coupled enzyme assay. Viruses standardized to an equivalent NA protein content of 0.3 ng/μl were incubated with 3′SL or 6′SL (final concentration, 0–30 mM) at pH 6.0 with 0.2 M phosphate buffer, excess galactose oxidase, horseradish peroxidase, and Amplex UltraRed reagent (Invitrogen, Carlsbad, CA). The reaction was conducted at 37 °C and the fluorogenic product was measured every 60 s for 90 min using Synergy 2 multimode microplate reader (BioTek Instruments) with excitation and emission wavelengths of 530 and 590 nm, respectively. The kinetic parameters Michaelis-Menten constant (K_M), maximum velocity of substrate conversion (V_{max}), and catalytic efficiency (k_{cat}/K_M) of the NAs were calculated by fitting the data to the appropriate Michaelis-Menten equations by using nonlinear regression in Prism 6.0 software (GraphPad Software, La Jolla, CA).

2.9. Statistical analysis

Virus yield, plaque size and number, IC_{50} values, and NA enzyme kinetic parameters (K_M, V_{max}, and k_{cat}/K_M) of the wild-type and mutant pH1N1 influenza viruses were compared by analysis of variance (ANOVA). Plaque size and numbers before and after passaging in Calu-3 cells were compared by unpaired two-tailed *t*-test. Probability values ≤ 0.05 indicate statistically significant differences.

3. Results

3.1. Generation, genetic stability, and replicative ability of recombinant pH1N1 viruses

We used the eight-plasmid reverse genetics technique to generate recombinant viruses carrying different NA mutations (Table 1, Supplementary Fig. S2). We successfully rescued the recombinant wild-type CA/04 virus and five CA/04-like viruses carrying different NA mutations (CA/04$^{NA-V116A}$, CA/04$^{NA-I117V}$, CA/04$^{NA-E119A}$, CA/04$^{NA-H275Y}$, and CA/04$^{NA-N295S}$). All the viruses grew to comparable titers and formed homogeneous plaques in MDCK cells (diameter: 0.2–0.5 mm), although virus with the H275Y NA mutation formed larger plaques than did the CA/04 virus (Table 1, Supplementary Fig. S3, $P < 0.05$).

To evaluate the genetic stability of the recombinant viruses *in vitro*, we serially passaged each virus five times in Calu-3 cells. Virus yields before passaging and after the fifth passage did not differ significantly, except for the CA/04 and CA$^{NA-H275Y}$ viruses, and all mutants maintained their plaque phenotype (Table 1). Sequence analysis after the fifth passage showed that neither the parental CA/04 virus nor viruses carrying NA mutations at residues 116, 117, 119, 275, or 295 had acquired any additional changes in their genome (data not shown). Thus, our results confirmed that all introduced NA mutations remained genetically stable in the CA/04-virus background.

Table 1
Growth of recombinant pH1N1 viruses before and after passaging in Calu-3 cells.

Viruses	Before passaging		5th passage	
	Virus yield (log_{10} PFU/ml)[a]	Plaque size (mm)[b]	Virus yield (log_{10} PFU/ml)	Plaque size (mm)
CA/04	6.8 ± 0.4	0.2 ± 0.1	5.4 ± 0.3°	0.3 ± 0.1
CA/04$^{NA-V116A}$	6.7 ± 0.2	0.2 ± 0.1	6.2 ± 0.5	0.3 ± 0.1
CA/04$^{NA-I117V}$	7.0 ± 0.4	0.2 ± 0.1	6.6 ± 0.2	0.3 ± 0.1
CA/04$^{NA-E119A}$	6.5 ± 0.2	0.3 ± 0.1	6.0 ± 0.6	0.4 ± 0.1
CA/04$^{NA-H275Y}$	6.3 ± 0.2	0.5 ± 0.1*	5.6 ± 0.3°	0.4 ± 0.1
CA/04$^{NA-N295S}$	7.0 ± 0.2	0.2 ± 0.1	6.5 ± 0.3	0.2 ± 0.1

*, $P < 0.05$ compared with the value for the wild-type virus from the same passage by one-way ANOVA.
°, $P < 0.05$ compared to the virus yield before passaging by unpaired two-tailed *t*-test.
[a] Values are mean log_{10} PFU/ml ± standard deviations (SD) from three independent determinations. The PFU in MDCK cells was determined by plaque assay after 3 days of incubation at 37 °C with 10-fold serial dilutions of virus (Hayden et al., 1980).
[b] Values are mean plaque diameter (mm) ± SD as measured by using the Finescale comparator.

We next examined the replicative ability of the generated recombinant viruses by assaying their virus yields in comparison with those of the wild-type CA/04 virus after multiple replication cycles in Calu-3 and NHBE cells (Fig. 1). None of these variants grew to significantly lower titers than CA/04 in Calu-3 cells, except for CA$^{NA-H275Y}$ at 12 h post-infection ($P < 0.05$, Fig. 1A). In contrast, the yields of the CA/04$^{NA-V116A}$, CA/04$^{NA-I117V}$, CA/04$^{NA-E119A}$, and CA/04$^{NA-N295S}$ mutants were significantly lower than that of the wild-type virus at the 72 h time point in NHBE cells (~1.2 logs, $P < 0.05$, Fig. 1B).

3.2. NAI susceptibility of recombinant pH1N1 viruses and the frequency of emergence of the studied NA mutations

We determined sensitivity of the recombinant viruses to several NAIs (oseltamivir, zanamivir, and laninamivir) and NANA, using an enzyme-based NA inhibition assay (Table 2). The susceptibility of CA/04$^{NA-V116A}$ mutant to oseltamivir and zanamivir was reduced (mean IC_{50} increase: 10.0- and 17.4-fold, respectively) as compared to that of the wild-type CA/04 virus. Viruses carrying the E119A and N295S mutations were more resistant to oseltamivir (39.4-and 59.5-fold increase in mean IC_{50} value, respectively) and the former was also significantly more resistant to zanamivir and laninamivir (133.5–493.7–fold increase in mean IC_{50} values) than was the CA/04 virus. The CA/04$^{NA-H275Y}$ mutant was highly resistant to oseltamivir (mean IC_{50} increase: > 950-fold) as compared to the CA/04 (Table 2).

We next evaluated sensitivity of the CA/04$^{NA-V116A}$ and CA/04$^{NA-I117V}$ mutants to oseltamivir, zanamivir, and laninamivir by virus reduction assay using Calu-3 cells as targets. We observed that both mutants exhibited reduced susceptibility to oseltamivir (mean EC_{50} increase: 15.0- and 23.0-fold, respectively; Fig. 2A) and zanamivir (mean EC_{50} increase: 57.5- and 52.5-fold, respectively; Fig. 2B) compared to the wild-type virus. The EC_{50} values determined by virus reduction assay (Fig. 2) were almost similar to the IC_{50} values determined by the NA inhibition assay (Table 2).

Next, we determined the frequency of emergence of the amino acid

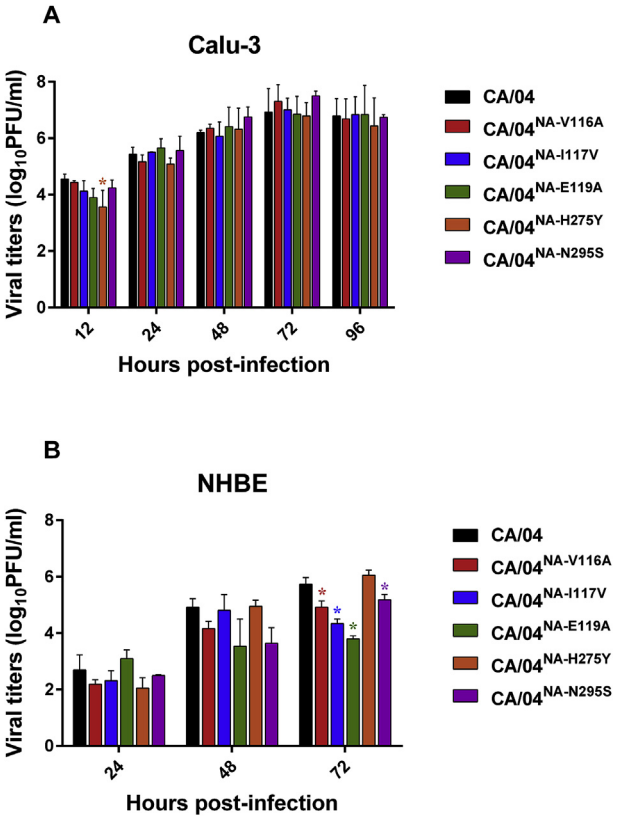

Fig. 1. Replication of the CA/04, CA/04$^{NA-V116A}$, CA/04$^{NA-I117V}$, CA/04$^{NA-E119A}$, CA/04$^{NA-H275Y}$, and CA/04$^{NA-N295S}$ viruses in Calu-3 (A) and NHBE (B) cells. The results are expressed as $\log_{10}$ PFU/ml from three to four independent experiments performed on different days. *$P < 0.05$, compared to the values for the wild-type CA/04 virus.

changes at residues 116, 117, 119, 275, and 295 in influenza N1 isolates of human, avian, and swine origin by analyzing ~19,060 genomic sequences deposited in the Influenza Research Database (www.fludb.org). We found that H275Y was the most predominant drug-resistant mutation in human pH1N1 isolates. The V116A and I117V mutations were found in ~0.6% of human, avian, and swine isolates of N1 NA subtype (Table 3).

3.3. NA enzyme kinetics and NA specificity of recombinant pH1N1 viruses

To determine the effect of the NA mutations on viral functional properties, we characterized their kinetic NA enzymatic parameters, including their Michaelis-Menten constants (K_M), relative NA enzymatic activity (V_{max}), and catalytic efficiency (k_{cat}/K_M), which represent the NA enzyme's ability to convert substrate to product (Table 4 and Fig. 3). When we applied the fluorogenic substrate, MUNANA, we observed that the V116A and I117V mutations increased NA enzyme activity (V_{max} ratio relative to the wild-type virus ≈ 2.3-fold increase). In contrast, the H275Y mutation had the opposite effect on NA enzyme activity (mean V_{max}, 5.0-fold decrease, Table 4 and Fig. 3A). All of the mutant NAs exhibited significantly lower affinity for MUNANA (mean K_M increase, ~5.4-fold) than for wild-type NA. The catalytic efficiencies (k_{cat}/K_M) of the NA proteins harboring the V116A, E119A, H275Y, and N295S mutations were ~26% of the wild-type NA value (Table 4, $P < 0.05$).

When we used 3′SL as the substrate for enzyme kinetic analysis, a different trend was observed. The V116A, E119A, and N295S changes increased relative NA enzyme activity (V_{max} ratio relative to the wild-type virus: ~4.2-fold increase, Fig. 3B) and these mutations resulted in ~17.8-fold decrease in 3′SL-binding ability compared to the wild-type NA. The resulting catalytic efficiencies of the mutants carrying E119A, H275Y, or N295S were significantly decreased compared to CA/04 (~5.6-fold, Table 4, $P < 0.05$). Using 6′SL as the substrate, we observed that the K_M values of all the recombinant viruses, except CA/04$^{NA-H275Y}$, were significantly higher (13.0-fold, $P < 0.05$) than that of the wild-type CA/04. Furthermore, the catalytic efficiencies of all mutant NA proteins were only ~18% of the wild-type NA value (Table 4, $P < 0.05$), indicating that the NAs of all of our pH1N1 mutants were significantly less efficient in catalyzing 6′SL than the wild-type NA.

4. Discussion

NAIs play a major role in the prevention and treatment of seasonal and pandemic influenza virus infections. However, there is always a concern regarding the emergence of NAI resistance and its potential clinical impact on disease management. The identification of novel amino acid substitutions conferring NAI resistance from *in vitro* studies may help to predict possible outcomes of drug-resistance in clinical populations.

Although residues V116 and I117 are highly conserved among several influenza NAs, our analysis of all human pH1N1 strains deposited in the Influenza Research Database revealed that 2 strains from Australia and USA, A/Perth/504/2010 and A/Ohio/30/2018, respectively, contained an I117V mutation. As reported by Hurt et al. (2012), the former virus was isolated from a 5-year-old boy with no history of NAI treatment, had a 4-fold and 3-fold reduction in sensitivity to oseltamivir and zanamivir, respectively, and seemed to have only minor clinical relevance. In addition, two V116A and a number of V116I variants were also detected, indicating that mutations at N1 residues, V116 and I117, may occur. V116A and I117V were shown to confer mild reduction in oseltamivir sensitivity in NAs of pH1N1 and H5N1

Table 2
Sensitivities of recombinant pH1N1 viruses to NAIs and neuraminic acid[a].

Viruses	Oseltamivir		Zanamivir		Laninamivir		NANA	
	IC_{50} (nM)	Fold change	IC_{50} (nM)	Fold change	IC_{50} (nM)	Fold change	IC_{50} (mM)	Fold change
CA/04	1.3 ± 0.2	1.0	0.9 ± 0.1	1.0	0.2 ± 0.1	1.0	10.8 ± 2.0	1.0
CA/04$^{NA-V116A}$	13.1 ± 2.5[c]	10.0 (RI[b])	15.7 ± 2.7[c]	17.4 (RI)	0.7 ± 0.1[c]	3.5 (NI)	72.1 ± 5.5[c]	6.7 (NI)
CA/04$^{NA-I117V}$	6.0 ± 1.1[c]	4.6 (NI)	4.9 ± 0.3[c]	5.4 (NI)	0.2 ± 0.1	1.0 (NI)	22.9 ± 2.3[c]	2.1 (NI)
CA/04$^{NA-E119A}$	51.2 ± 7.9[c]	39.4 (RI)	444.3 ± 64.5[c]	493.7 (HRI)	26.7 ± 3.2[c]	133.5 (HRI)	49.1 ± 2.9[c]	4.6 (NI)
CA/04$^{NA-H275Y}$	1250.7 ± 107.1[c]	962.1 (HRI)	1.4 ± 0.2	1.6 (NI)	0.6 ± 0.1[c]	3.0 (NI)	17.8 ± 4.9	1.7 (NI)
CA/04$^{NA-N295S}$	77.3 ± 9.5[c]	59.5 (RI)	2.9 ± 0.8[c]	3.2 (NI)	0.6 ± 0.1[c]	3.0 (NI)	11.7 ± 2.5	1.1 (NI)

[a] Viral sensitivities were determined in NA inhibition assays. The values were fit to nonlinear regression curves with the variable slope model to determine the IC_{50}, using GraphPad Prism. Results are means ± SD from 2 to 3 independent experiments. IC_{50}-fold changes were calculated relative to the CA/04 wild-type virus.
[b] NI, Normal inhibition (< 10-fold increase in IC_{50} over wild-type); RI, reduced inhibition (10- to 100-fold increase in IC_{50} over wild-type); HRI, highly reduced inhibition (> 100-fold increase in IC_{50} over wild-type) based on WHO AVWG NAI susceptibility proposed criteria.
[c] , $P < 0.05$ compared with the value for the wild-type virus by one-way ANOVA.

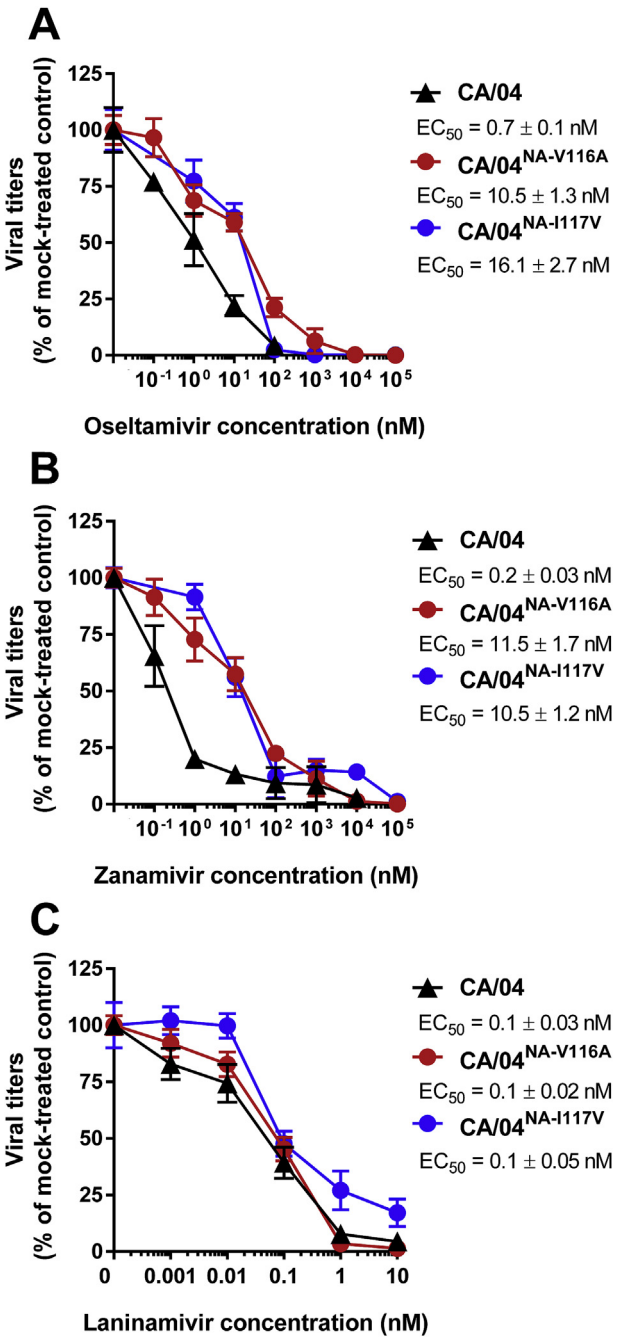

Fig. 2. Antiviral activity of oseltamivir (A), zanamivir (B), and laninamivir (C) against the CA/04, CA/04$^{NA-V116A}$, and CA/04$^{NA-I117V}$ viruses as determined by virus reduction assay in Calu-3 cells.

viruses (Hurt et al., 2007, 2012; Ilyushina et al., 2010), but they had no apparent functional impact on NAI sensitivity in A/WSN/33-like (H1N1) background (Abed et al., 2008). These findings demonstrated that V116A and I117V can have a variable effect on NAI sensitivity in different NA backgrounds.

Here, using homogeneous pH1N1-like background without concomitant HA and/or NA mutations, we demonstrated that the susceptibility of the mutant carrying V116A to oseltamivir and zanamivir was reduced in the NA inhibition assay as compared to the wild-type virus. The CA-NA^{I117V} virus exhibited reduced sensitivity to oseltamivir and zanamivir in virus reduction assay in Calu-3 cells. Although residues V116 and I117 are not in direct structural contact with oseltamivir, they are adjacent to one (R118) of three arginine residues that

Table 3
Emergence of the V116A, I117V, E119A, H275Y, and N295S NA mutations among human pH1N1, avian N1, and swine N1 influenza viruses between 2009 and 2018.

Year of isolation	Host	V116A	I117V	E119A	H275Y	N295S
2009	Human (5377)[a]	0 (0)[b]	0 (0)	0 (0)	82 (1.5)	0 (0)
	Avian (282)	1 (0.4)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (254)	0 (0)	0 (0)	0 (0)	1 (0.4)	0 (0)
2010	Human (1522)	0 (0)	1 (0.1)	0 (0)	65 (4.3)	0 (0)
	Avian (194)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (340)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2011	Human (1025)	0 (0)	0 (0)	0 (0)	31 (3.0)	0 (0)
	Avian (223)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (364)	0 (0)	6 (1.7)	0 (0)	0 (0)	0 (0)
2012	Human (505)	0 (0)	0 (0)	0 (0)	33 (6.5)	0 (0)
	Avian (249)	0 (0)	2 (0.8)	0 (0)	0 (0)	0 (0)
	Swine (414)	0 (0)	4 (1.0)	0 (0)	0 (0)	0 (0)
2013	Human (735)	0 (0)	0 (0)	0 (0)	14 (1.9)	0 (0)
	Avian (187)	0 (0)	1 (0.5)	0 (0)	0 (0)	0 (0)
	Swine (485)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2014	Human (549)	0 (0)	0 (0)	0 (0)	19 (3.5)	0 (0)
	Avian (180)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (379)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2015	Human (620)	0 (0)	0 (0)	0 (0)	13 (2.1)	0 (0)
	Avian (392)	2 (0.5)	3 (0.8)	0 (0)	0 (0)	0 (0)
	Swine (364)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2016	Human (1786)	0 (0)	0 (0)	0 (0)	29 (1.6)	0 (0)
	Avian (98)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (322)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2017	Human (792)	0 (0)	0 (0)	0 (0)	3 (0.4)	0 (0)
	Avian (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (240)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2018	Human (1403)	2 (0.1)	1 (0.4)	0 (0)	8 (0.6)	0 (0)
	Avian (45)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Swine (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Total number of isolates is shown in parenthesis.
[b] Percentage of isolates with identified amino acid substitution is shown in parenthesis.

bind the carboxylate of the NA substrate, sialic acid (Russell et al., 2006). A previous study examined the potential destabilization effect of I117V on NA structural stability (Hurt et al., 2012). It was found that the I117V mutation had only a modest effect on NA activity. This mild destabilization effect was caused mainly by the increase in the NA internal cavity, which could increase flexibility of neighboring residues that form part of the drug-binding framework with further effect on virus NAI susceptibility. Our results also confirmed the oseltamivir-, zanamivir-, and laninamivir-resistance phenotype conferred by the E119A, H275Y, and N295S NA mutations in the pH1N1 background as reported previously by others (Nguyen et al., 2012; Samson et al., 2014; Pizzorno et al., 2011).

We demonstrated that all of the N1 NA mutations that we studied were stably maintained in the pH1N1 background and all mutants grew to titers comparable to the wild-type virus in Calu-3 cells. In contrast, all NA changes, except H275Y, affected the replicative capacity of the recombinant viruses when propagated in NHBE cells, where slightly lower viral titers were observed at 72 h post-infection compared to the wild-type CA/04 virus. Our results are in good agreement with the previously published findings by others that showed that drug-resistant pH1N1 variants containing framework NA mutations retain their fitness *in vitro* (Pizzorno et al., 2011; Wong et al., 2012). Moreover, it is worth noting that contemporary pH1N1 viruses acquired two novel NA permissive mutations (V241I and N369K) that enable these viruses to maintain robust viral fitness when they acquire the NA H275Y oseltamivir resistance substitution (Butler et al., 2014). However, the impact of these permissive mutations on viral fitness of other NAI-resistant mutations is unknown and requires further investigation.

When we applied MUNANA, 3′SL, and 6′SL substrates for enzyme kinetics, we observed that the V116A mutation was associated with reduced binding ability (K_M) for all substrates (13.9-fold). I117V led to

Table 4
NA enzyme kinetics of recombinant pH1N1 influenza viruses with MUNANA, 3′SL, and 6′SL substrates.

Viruses	MUNANA			3′SL			6′SL		
	V_{max} ratio[a]	K_M (μM)[b]	k_{cat}/K_M $(\mu M*sec)^{-1}$	V_{max} ratio	K_M (mM)	k_{cat}/K_M $(\mu M*sec)^{-1}$	V_{max} ratio	K_M (mM)	k_{cat}/K_M $(\mu M*sec)^{-1}$
CA/04	1.0	41.7 ± 6.4	0.7 ± 0.2	1.0	14.3 ± 2.9	1089.6 ± 217.9	1.0	26.7 ± 5.3	104.7 ± 20.9
CA/04$^{NA-V116A}$	2.6	439.0 ± 37.5*	0.2 ± 0.1*	3.5	65.3 ± 13.1*	827.0 ± 165.4	3.8	710.1 ± 142.0*	15.1 ± 3.1*
CA/04$^{NA-I117V}$	2.0	183.4 ± 10.0*	0.4 ± 0.1	1.8	24.7 ± 5.0	1138.5 ± 227.7	1.6	108.0 ± 21.6*	40.6 ± 8.1*
CA/04$^{NA-E119A}$	0.6	250.2 ± 22.6*	0.1 ± 0.03*	6.5	623.8 ± 124.8*	163.5 ± 32.7*	1.3	479.0 ± 95.8*	7.4 ± 1.5*
CA/04$^{NA-H275Y}$	0.2	73.3 ± 6.7*	0.1 ± 0.01*	0.2	24.2 ± 4.9	131.9 ± 26.4*	0.2	20.6 ± 4.1	24.2 ± 4.9*
CA/04$^{NA-N295S}$	1.2	169.0 ± 8.8*	0.2 ± 0.1*	2.8	75.2 ± 15.0*	571.4 ± 114.3*	0.3	88.1 ± 17.6*	8.3 ± 1.7*

*, $P < 0.05$ compared with the value for the wild-type virus by one-way ANOVA.

[a] The V_{max} was calculated using a nonlinear regression of the curve according to the Michaelis-Menten equation (Fig. 3) and then the ratio of the respective viruses' NA V_{max} to the V_{max} of the wild-type CA/04 virus was determined.

[b] The K_M represents the Michaelis-Menten constant at which the reaction rate is half of V_{max}. The enzyme kinetic data were fit to the Michaelis-Menten equation using GraphPad Prism, version 6.0. Values are the means ± 95% confidence interval from 3 to 5 independent determinations.

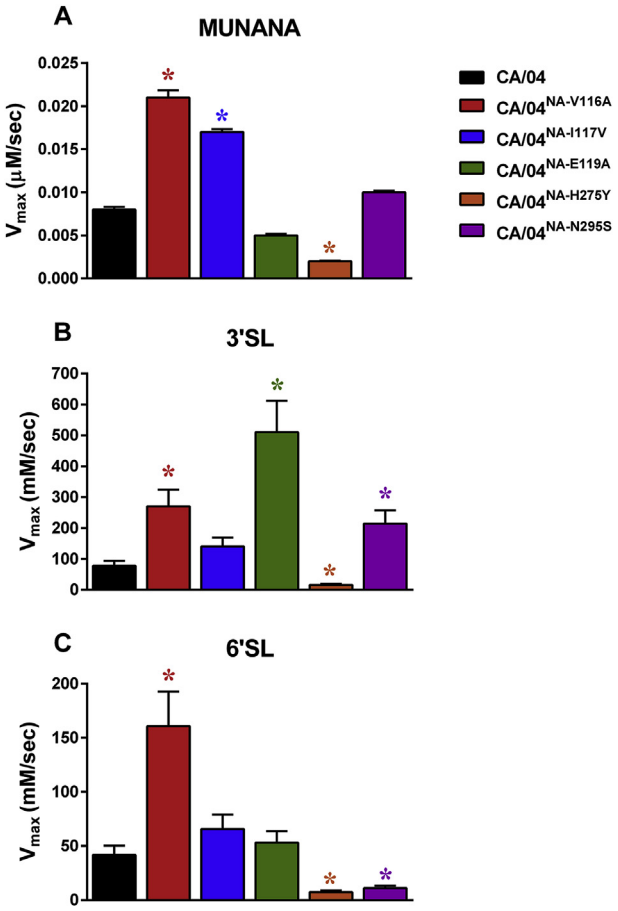

Fig. 3. Maximum velocity (V_{max}) of MUNANA (A), 3′SL (B), and 6′SL (C) conversion of the NAs carrying V116A, I117V, E119A, H275Y, and N295S mutations.

the significantly decreased affinity for MUNANA and 6′SL (4.2-fold). Neither of these mutations affected the catalytic efficiency (k_{cat}/K_M) in catalyzing 3′SL, but the efficiency in catalyzing MUNANA and 6′SL was significantly lower: ~34.7% compared to the wild-type NA. Although H275Y did not affect 3′SL- and 6′SL-binding abilities, the other two framework mutations, E119A and N295S, had the opposite effect and exhibited significantly lower affinity for all substrates in comparison to CA/04. The catalytic efficiencies of the NAs with the E119A, H275Y, and N295S mutations were also significantly lower: ~19.4% of the CA/04 NA.

To our knowledge, our study is the first to demonstrate the direct effect of drug-resistance mutations located inside or adjacent to the NA active site on NA substrate specificity. Since all pH1N1 mutants were significantly less efficient in catalyzing 3′SL and/or 6′SL than the wild-type virus, as determined by k_{cat}/K_M values, this suggests that the replication capacity of NAI-resistant variants may vary in different *in vitro* cell culture systems depending on the cell surface sialyl receptor distribution. This hypothesis may explain the difference in replication capacities of the recombinant pH1N1 viruses observed in Calu-3 versus NHBE. NHBE cells more closely resemble normal human airway epithelium and provide a better *ex vivo* model than Calu-3 cells for evaluating viral fitness of NAI-resistant variants.

In conclusion, our findings demonstrate the importance of continued characterization of NA mutations located near the enzyme active site for drug sensitivity. This experimental approach facilitates identification of novel NA markers of altered NAI susceptibility. Comprehensive characterization of novel NAI-resistance mutations and their impact on drug resistance, substrate specificity, and fitness are integral to establishing an effective anti-influenza surveillance system.

Competing interests

The authors declare that they have no competing interests. This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104539.

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