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Short communication

Neuraminidase inhibitor drug susceptibility differs between influenza N1 and N2 neuraminidase following mutagenesis of two conserved residues

Hui-Ting Ho [a,b], Aeron C. Hurt [a,b,*], Jenny Mosse [b], Ian Barr [a,b]

[a] *World Health Organisation Collaborating Centre for Reference and Research on Influenza, 45 Poplar Road, Parkville, Victoria 3052, Australia*
[b] *Monash University, School of Applied Sciences, Churchill, Victoria 3842, Australia*

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Abstract

Neuraminidase (NA) inhibitors are a class of antivirals designed to target the conserved residues of the influenza NA active site. While there are many conserved residues in the NA active site that are involved in NA inhibitor binding, only a few have been demonstrated to confer resistance. As such, little is known regarding the potential of the other conserved residues in the NA active site to cause NA inhibitor resistance. Two conserved residues (E227 and E276) of an N1 NA that have not previously been associated with resistance to NA inhibitors were investigated. Site-directed mutagenesis was used to generate three alternative amino acids at each residue. Reverse genetics was used to generate recombinant mutant viruses which were characterized for growth, NA activity and NA inhibitor sensitivity. Of the six recombinant viruses expressing NA with mutations at either E227 or E276, only the E227D and E276D viruses were able to grow without supplementary NA activity, and all mutant viruses had a significant reduction in NA activity.

The E227D virus demonstrated significantly reduced sensitivity to zanamivir while the E276D virus did not demonstrate any significant changes in NA inhibitor sensitivity. Interestingly, the resistance profiles of E227D and E276D in N1 NA were significantly different from these sites that have been reported for N2 NA.

This study confirmed the essential role of NA active site residues in viral fitness, and identified clear differences in the role of residues E227 and E276 in NA inhibitor resistance with N1 and N2 neuraminidases.

Keywords: Influenza; Neuraminidase inhibitors; Conserved residues; N1; Mutagenesis

Neuraminidase (NA) inhibitor drugs zanamivir (Relenza) and oseltamivir (Tamiflu) target the conserved residues of the influenza NA active site of both influenza A and B viruses preventing virus release from host cells (Gubareva, 2004). As a consequence of mutations at certain key residues in the NA active site, NA inhibitor (NAI) resistance has been found to occur. While the NA inhibitors interact with many residues in the NA active site, mutations in only a few conserved residues (E119G/A/D/V, R292K, N294S (N2 viruses); H274Y (N1 viruses); R152K, D198N/E (type B viruses)) are known to confer resistance to the NA inhibitors (Gubareva, 2004; Hurt et al., 2006a,b).

In contrast, relatively little is known regarding the potential of mutations at other conserved NA active site residues to confer NAI resistance. Studies investigating the mutagenesis of conserved NA residues of influenza A virus N1 and N2 NA using reverse genetics have been reported previously, although all but one of these studies focused on the mutagensis of NA residues previously identified to confer NAI resistance (Abed et al., 2004; Yen et al., 2005; Zurcher et al., 2006). The single study that investigated the role of conserved NA residues in an N2 subtype influenza virus that have not previously been associated with NAI resistance, demonstrated varied degrees of NAI resistance associated with these conserved sites (Yen et al., 2006). No such study however has been reported for an N1 subtype influenza virus. Here, we describe a mutagenesis study of two conserved NA residues E227 (a framework residue) and E276 (a catalytic residue) of N1 NA and identify clear NAI susceptibility differences between the N1 and N2 neuraminidases following mutagenesis of these sites. Neither of these residues has previously been reported to be associated with NA inhibitor resistance

* Corresponding author at: WHO Collaborating Centre for Influenza, 45 Poplar Rd., Parkville, Victoria 3052, Australia. Tel.: +61 3 9389 1392; fax: +61 3 9389 1881.
E-mail address: Aeron.Hurt@influenzacentre.org (A.C. Hurt).

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under drug selective pressure *in vitro* or *in vivo*, although both have direct interactions with NA inhibitors. We therefore created substitutions at these NA residues based on the hypothesis that mutations at these sites may alter NA inhibitor binding affinity causing a reduction in drug sensitivity.

Site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Strategene, CA) was used to mutate the residues at either E227 or E276 to three alternative amino acids in the N1 gene. Both residues were substituted with the most conserved amino acid (D) (i.e. E227D and E276D) and two other less conservative mutations (E227G and E227V; E276Q and E276K). The E227 amino acid mutations were chosen as these have been observed at residue E119, another framework residue that plays a role in NAI resistance (Hurt et al., 2006a,b). The E276 amino acid mutations (E276Q and E276K) were chosen as they could arise from a single base mutation in the codon and hence may be more likely to occur spontaneously. In addition the amino acids Q and K have a similar sized side chain to E. As a positive control a previously described NAI resistance mutation (H274Y), was introduced into the NA gene. The eight-plasmid reverse genetics system was used to generate recombinant viruses (with the NA gene from A/New Caledonia/20/99 (H1N1) virus and remaining genes from A/Puerto Rico/8/1934 (A/PR/8/34)) each carrying one of the six different NA mutations, using methods described previously (Hoffmann et al., 2000). A recombinant virus carrying the wild-type A/New Caledonia/20/99 N1 NA (no introduced mutations) and a recombinant virus with the engineered H274Y mutation (know to confer oseltamivir resistance) were included as controls. All recombinant viruses were characterised in terms of their growth, stability, NA activity and susceptibility to the NA inhibitors zanamivir and oseltamivir carboxylate (the active form of the pro-drug oseltamivir phosphate). Zanamivir was used directly from the blister packaging of Relenza (5 mg zanamivir and 20 mg lactose) (GlaxoSmithKline), while oseltamivir carboxylate (GS 4071) was kindly provided by James Smith, Hoffman-La Roche.

Growth and genetic stability of the E227D/G/V or E276D/Q/K mutant recombinant viruses, was assessed following three passages in MDCK cells in the presence and absence of *C. perfringens* NA (Sigma, St. Louis, MO) (Table 1). The initial generation of virus by reverse genetics was perfromed in the absence of *C. perfringens* NA. Only the mutant recombinant viruses with the most conserved NA mutation (E227D and E276D) were able to grow to an HA titre of >16HA in the absence of *C. perfringens* NA, while the other mutants required supplementary *C. perfringens* NA for efficient growth (Table 1).

NA activity of the recombinant viruses following the first MDCK passage was determined using a fluorescence-based assay (Hurt et al., 2004) and expressed as a percentage of the NA activity of the wild-type virus with the equivalent HA titre (Table 1). There is no ideal method for the quantitation of NA protein in viruses to compare NA activity levels, but previously used methods such as standardising on either M1, or NP protein (Yen et al., 2005, 2006) were not possible in this study due to low volumes and low viral titres of the viruses. It was found however that the relative NA activity for the recombinant H274Y virus, which in this study was 35% of the wild-type enzyme activity, was comparable to levels reported previously using another alternative method, which was based on expression of NA protein in mammalian cells (Wang et al., 2002). In addition we found a strong correlation between the two viruses with the lowest NA activity (E227G and E276K) being the only strains that 'reverted back' to the wildtype genotype following either the first (E227G) or second (E276K) MDCK passage, giving further confidence in the NA activity comparisons. The two viruses with the most conserved substitutions (E227D and E276D) had the highest NA activity (Table 1), and were the only mutant viruses from which NAI susceptibility could be determined, due to the other mutants having NA activity levels below the level of detection of the assay. Using a chemiluminescence-based NA inhibition assay (Buxton et al., 2000), the E227D virus had a significant reduction in sensitivity to zanamivir (126-fold increase in IC_{50}) and a slight

Table 1
Growth, genetic stability and NA activity of wildtype and recombinant A(H1N1) influenza viruses expressing NA mutations at E227 or E276

Virus	Percentage NA activity of wild-type at 1st MDCK passage (%)[a]	Growth of the virus in MDCK cells[b]		Genetic stability following multiple MDCK passage[c] AA (codon)	
		−	With *C. perfringens* NA	−	With *C. perfringens* NA
Wild type	100 ± 9	+++	+++	E (GAG)	E (GAG)
H274Y	46 ± 7	+++	+++	Y (TAT)	Y (TAT)
E227D	2.9 ± 0.8	+++	+++	D (GAC)	D (GAC)
E227G	0.5 ± 0.2	+	++	E (GAG)[d]	E (GAG)[d]
E227V	1.2 ± 0.3	+	++	V (GTG)	V (GTG)
E276D	6.7 ± 1.2	+++	+++	D (GAC)	D (GAC)
E276Q	1.0 ± 0.4	+	++	Q (CAG)	Q (CAG)
E276K	0.5 ± 0.1	−	++	E (GAG)[d]	E (GAG)[d]

[a] Assay performed on 1st MDCK passage level of virus, sequence analysis confirmed presence of mutant residue. Value presented as mean ± 1 S.D. based on two separate assays. The percentage NA activity of each recombinant virus was calculated based on the NA activity of wild-type virus with the same HA titre.

[b] Growth of virus in MDCK cells in the absence (−) or the presence of 2 mU of *C. perfringens* NA/mL is expressed as "−" no growth; "+" growth was compromised and could not grow to a detectable HA titre but could be detected by rapid test (ESPLINE Influenza A&B-N, Fujirebio Inc.); "++" could only grow to a low HA titre (<16HA); "+++" could grow to a higher HA titre (>16HA).

[c] Genetic stability of each recombinant viruses is indicated by the maintenance of the NA mutation (based on sequence analysis) for at least three passages in MDCK cells in the absence (−) or presence of 2 mU of *C. perfringens* NA.

[d] Reverted back to wild-type. AA, amino acid.

Table 2
Sensitivity of wildtype and recombinant A(H1N1) influenza viruses to zanamivir and oseltamivir carboxylate

Virus	Zanamivir		Oseltamivir carboxylate	
	IC_{50} (nM)	Fold[a]	IC_{50} (nM)	Fold[a]
Wild-type	0.5 ± 0.1	1.0	0.4 ± 0.1	1.0
H274Y	0.4 ± 0.1	0.8	**149.1 ± 87.8**	**372.8**
E227D	**63.1 ± 24.5**	**126.2**	1.1 ± 0.4	2.8
E276D	0.4 ± 0.2	0.8	0.4 ± 0.1	1.0

Values in bold are those demonstrating a reduction in NA inhibitor sensitivity greater than five-fold.
Mean ± 1 S.D. calculated on IC_{50} values from two separate assays.
[a] Fold difference in IC_{50} compared with wild-type recombinant virus.

alteration in sensitivity to oseltamivir carboxylate (∼threefold increase in IC_{50}) compared to the wildtype (Table 2). In contrast, the E276D virus demonstrated no reduction in sensitivity to either zanamivir or oseltamivir carboxylate compared to the wildtype (Table 2). The NAI susceptibility of the recombinant H274Y virus from this study was similar to that reported previously in a wildtype A(H1N1) H274Y mutant (Monto et al., 2006), confirming the applicability of using a reverse genetics approach to investigate NAI susceptibility. Sequence analysis revealed no HA mutations in the HA1 gene of any of the recombinant viruses following passage.

The framework residue E227 plays an important role in stabilizing the structure of the NA active site and has an indirect interaction with the substrate (sialic acid) in a N2 NA (Varghese et al., 1992). While the virus carrying the E227D mutation in the N1 NA generated in this study was able to grow efficiently in cell culture, a virus with the same mutation in N2 NA (also derived by site-directed mutagenesis and reverse genetics) was found to have severe growth defects (Yen et al., 2006). These differences suggest that the shortened side chain resulting from the E → D substitution at E227 has a minor impact in N1 NA in providing a framework for the active site (although probably with reduced strength) but has a severe effect on the structure and function of the N2 NA. In a N9 NA, a E227D mutation had a very similar reduction in NA activity (2.5% relative enzymatic activity of wildtype) to the N1 in this study, while other residue substitutions at the E227 site of the N9 NA resulted in a more significant enzymatic impact resulting in no detectable NA activity (Goto et al., 1997). The E227D mutation in N1 NA generated in our study demonstrated reduced sensitivity to zanamivir, possibly due to the shortened side chain at this residue resulting in a reduced binding affinity with the guanidinium group of zanamivir (Smith et al., 2002). Previous studies investigating the E227D mutation in N2 (Yen et al., 2006) and N9 (Goto et al., 1997) were unable to determine NAI sensitivity due to either the instability of the virus or insufficient enzyme activity.

The E276D virus generated in our study was found to have no significant changes in sensitivity to zanamivir or oseltamivir carboxylate even though it was able to grow well in cell culture. The recent study by Yen et al. (2006), which generated the same mutation in N2 NA, reported that the recombinant virus carrying this mutation was able to grow efficiently in cell culture and demonstrated reduced sensitivity to both oseltamivir carboxylate (∼15-fold) and zanamivir (∼160-fold). Structural differences between N1 and N2 NA can again explain these observations. Structural analysis by Russell et al. (2006) recently revealed that the Y347 residue in N1 NA makes an additional hydrogen bond to the carboxylate group of oseltamivir that cannot be made by the equivalent Q347 residue in N2 NA. The added binding strength via residue 347 in N1 NA may explain why a loss of interaction following the E276D mutation has little affect on the sensitivity to oseltamivir carboxylate, compared to that observed in N2 NA. The study by Russell et al. also revealed that the 270-loop in N1 NA makes a tighter turn than the equivalent loop in N2 NA, and that there is a conserved Y252 residue in N1 NA that is not present in N2 NA, which makes hydrogen bonds to the 273 and 274 residues in the loop that are absent in N2 NA. Therefore, it may be possible that the E276D mutation has a more pronounced effect on the conformation around the loop in N2 NA, leading to the loss of interaction with zanamivir and subsequently the reduced susceptibility of the virus with this mutation to zanamivir as observed by Yen et al. (2006). A more detailed crystal structural analysis however would be required to fully elucidate the exact role of this mutation.

Our study has extended the understanding of the role of conserved residues E227 and E276 in NAI resistance in N1 NA. Further investigations of the zanamivir resistant E227D mutant remain necessary to determine the fitness of the virus *in vivo* and the potential clinical significance this mutant may have if circulating in the human population. We have identified clear differences in the role of residues E227 and E276 in NAI resistance between N1 and N2 NA, further indicating that NAI resistance in influenza viruses is subtype specific. With the serious outbreak of highly pathogenic influenza A(H5N1) strains worldwide, it is particularly important to further investigate the potential of other conserved NA residues of N1 NA in conferring NAI resistance.

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