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# In vitro generation and characterisation of an influenza B variant with reduced sensitivity to neuraminidase inhibitors

Ai Lee Cheam [a,b], Ian G. Barr [a], Alan W. Hampson [a], Jennifer Mosse [b], Aeron C. Hurt [a,*]

[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**

A contemporary influenza type B virus was passaged in vitro in the presence of increasing concentrations of the neuraminidase inhibitors, zanamivir and oseltamivir carboxylate (0.1–1000 μM over nine passages). After the fifth passage in the presence of zanamivir (10 μM), the virus acquired a Glu 119 Asp neuraminidase mutation (influenza A N2 subtype numbering) in the enzyme active site. After a further three passages, in which growth occurred in 100 μM of zanamivir, a Gln 218 Lys mutation (A (H3) numbering) in the HA1 domain of the haemagglutinin was found. In a fluorescence-based neuraminidase inhibition assay, viruses with the Glu 119 Asp NA mutation had a 32,000-fold reduction in sensitivity to the NA inhibitor zanamivir compared to the wild-type virus, while the mutation resulted in a 105-fold reduction in sensitivity to oseltamivir carboxylate. Viruses grown in the presence of 1000 μM oseltamivir carboxylate did not acquire any neuraminidase mutations but did have a His 103 Gln substitution (A (H3) numbering) in the HA1 region of the haemagglutinin which was demonstrated to significantly reduce receptor binding strength in vitro. Tissue culture assays demonstrated that the HA mutation caused a seven-fold reduction in sensitivity to oseltamivir carboxylate, and a 90-fold reduction in sensitivity to zanamivir.


The neuraminidase (NA) inhibitors, zanamivir and oseltamivir, are sialic acid analogues that potently and specifically inhibit influenza virus replication by binding competitively to the enzyme active site of the NA, thus, preventing normal function (Gubareva et al., 2000). To date, very few NA inhibitor-resistant clinical isolates have been identified, however, sequential in vitro passage of influenza viruses in cell culture in the presence of a NA inhibitor has yielded resistant variants that have mutations in the NA, haemagglutinin (HA), or both (McKimm-Breschkin, 2000). NA mutations arising under the selective pressure of the NA inhibitors in vitro, generally involve amino acid substitutions in the normally conserved residues of the active site, affecting the binding affinity of the drugs (McKimm-Breschkin, 2000). HA mutations selected in the presence of the NA inhibitors often occur in or near the receptor binding site, decreasing the binding affinity of the HA to cell receptors, resulting in the virus being less dependent on NA for release from infected cells (Gubareva et al., 1996; McKimm-Breschkin et al., 1996; Penn et al., 1996; McKimm-Breschkin et al., 1998).

The majority of previous studies of NA inhibitor-resistant viruses selected in vivo or in vitro have involved influenza type A strains, and have identified amino acid mutations at a number of normally conserved residues of the NA gene (McKimm-Breschkin, 2000). However, there is less information regarding NA inhibitor resistance in influenza B viruses. While an Arg 152 Lys NA mutation in a virus isolated from an immunocompromised child undergoing zanamivir treatment has been shown to confer resistance (Gubareva et al., 1998), only three studies have reported the in vitro generation of resistant influenza B viruses. In two of the studies a Glu 119 Gly NA mutation was identified after passaging in the presence of zanamivir (Barnett et al., 1999; Staschke et al., 1995) while, in the third study, a His 273 Tyr NA mutation was selected by sequential passaging in another NA inhibitor, peramivir (Baum et al., 2003). To date there are no reports of selection of resistance in influenza B viruses by in vitro passage in oseltamivir carboxylate. Unlike the older laboratory strains used in the previously reported studies, this study reports the levels of resistance acquired by a contemporary influenza B virus following sequential passaging in the presence of zanamivir and oseltamivir carboxylate.

Influenza virus B/Taiwan/4/2002, a B/Sichuan/379/99-like isolate collected through the WHO global influenza surveillance program, was selected for use in this study because

* Corresponding author. Tel.: +61 3 9389 1392; fax: +61 3 9389 1181.
*E-mail address:* aeron.hurt@influenzacentre.org (A.C. Hurt).

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of its high titre and its high sensitivity to both NA inhibitor drugs. Zanamivir was used directly from the blister packaging of Relenza™ (5 mg zanamivir and 20 mg lactose, Glaxo Smithkline). Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir, was kindly provided by Professor Noel Roberts (Roche Products, Welwyn Garden City, UK). Virus propagation was carried out at 35 °C in MDCK cells in maintenance media as previously described (Hurt et al., 2004). To minimise the probability of selecting HA mutants with weak affinity for cell receptors, the viruses were allowed to adsorb for only 15 min at 35 °C before removal of the inoculum and the addition of media. The starting virus, which had been previously passaged five times in MDCK cells in the absence of drug, was propagated in confluent MDCK cells using a combination of low multiplicities of infection (0.0001, 0.001, and 0.01 PFU per cell) and increasing drug concentrations (ranging from 0.01 to 1000 μM). Virus growth was monitored daily by observing cytopathic effect and by haemagglutination of turkey erythrocytes. Viruses that had grown to a titre of at least 2HA per 50 μl at the lowest multiplicity of infection and highest drug concentration were harvested, centrifuged at 2000 rpm for 5min to remove cellular debris, and then used to infect a new cell monolayer in the presence of a further range of drug concentrations. During the seventh passage of B/Tawian/4/2002 in zanamivir and oseltamivr carboxylate, viruses were purified by limit dilution passaging in the presence of 100 μM of their respective NA inhibitor. Viruses were then passaged a further two times, again in a range of increasing drug concentrations, before the final passage (ninth) of the zanamivir and oseltamivir carboxylate selected viruses (designated Zan P9 and OC P9 respectively) which grew at 1000 μM (Tables 1 and 2 describe the drug concentrations present at each passage level during the growth of the viruses). Viruses from each passage level were also grown in the absence of drug to remove residual inhibitor to allow NA inhibition and HA elution assays to be performed. HA and NA sequences of viruses before and after the inhibitor-free passages were compared to ensure that the passage in the absence of drug did not allow resistant viruses to revert back to inhibitor sensitivity. B/Taiwan/4/2002 was also passaged in parallel in the absence of drug to confirm that the mutations observed were not due simply to the MDCK passaging protocol.

NA enzyme inhibition was assayed by a flourescence-based assay utilising 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA), and followed the fluorescence-based assay protocol 1 (FA1) method as described previously (Hurt et al., 2004). Haemagglutination and red blood cell (RBC) elution assays followed the methods of (Bantia et al., 1998), except 1% turkey erythrocytes were substituted for fowl erythrocytes, and zanamivir and oseltamivir carboxylate concentrations started at 1 and 10 μM respectively, rather than 12 μM. RNA isolation, RT-PCR and sequencing methods followed those previously described (Hurt et al., 2004). Subsequent to the passage protocol described above the susceptibility of selected passage levels of B/Tawian/4/2002 to zanamivir and oseltamivir carboxylate in MDCK cells was investigated. Selected viruses were passaged in the presence of a range of drug concentrations (0.01 nM–1000 μM) after which the infectivity (measured by $TCID_{50}$; Tannock et al., 1989) of each resulting virus was investigated to determine the drug concentration required to reduce the yield of infectious virus by 50% (tissue culture $IC_{50}$).

When the virus was passaged in the presence of increasing concentrations of zanamivir, it was found that following the fifth passage (Zan P5) at 10 μM, the neuraminidase 50% inhibitory concentration ($IC_{50}$) value for both zanamivir and oseltamivir carboxylate increased significantly (Table 1), but then remained constant following subsequent passages at increased drug concentrations. Based on the mean neuraminidase $IC_{50}$ results for Zan P5–Zan P9 the reduction in

Table 1
NA inhibitor sensitivity (neuraminidase $IC_{50}$) and amino acid substitutions of each passage level of B/Tawian/4/2002 following growth in the presence of increasing concentrations of zanamivir

| Passage level | Zanamivir concentration (μM) | Neuraminidase $IC_{50}$ (nM) | | Amino acid substitutions | |
|---|---|---|---|---|---|
| | | Zanamivir | Oseltamivir carboxylate | HA | NA |
| Starting virus | 0 | 1.2 | 24.2 | – | – |
| Zan P1 | 0.1 | 1.1 | 19.4 | – | – |
| Zan P2 | 1 | 1.1 | 23.7 | – | – |
| Zan P3 | 1 | 0.9 | 19 | – | – |
| Zan P4 | 10 | 2.2 | 23 | – | [a] |
| Zan P5 | 10 | 39,200[b] | 2,830[b] | – | Glu 119 Asp |
| Zan P6 | 100 | 43,400[b] | 2,640[b] | – | Glu 119 Asp |
| Zan P7 | 100 | 35,800[b] | 2,230[b] | [a] | Glu 119 Asp |
| Zan P8 | 500 | 36,100[b] | 2,420[b] | Gln 218 Lys | Glu 119 Asp |
| Zan P9 | 1000 | 40,000[b] | 2,480[b] | Gln 218 Lys | Glu 119 Asp |

[a] Passage level contained a mixed population of virus consisting of some mutated strains.

[b] $P < 0.00001$, determined using linear contrasts in an analysis of variance, contrasting each passage level with all previous passage levels. The contrasts were evaluated using *t*-tests.

Table 2
NA inhibitor sensitivity (neuraminidase $IC_{50}$) and amino acid substitutions of each passage level of B/Tawian/4/2002 following growth in the presence of increasing concentrations of oseltamivir carboxylate

| Passage level | Oseltamivir carboxylate concentration (μM) | Neuraminidase $IC_{50}$ (nM) | | Amino acid substitutions | |
|---|---|---|---|---|---|
| | | Zanamivir | Oseltamivir carboxylate | HA | NA |
| Starting virus | 0 | 1.2 | 24.2 | – | – |
| OC P1 | 1 | 1.7 | 23.7 | – | – |
| OC P2 | 1 | 2.5 | 25.6 | – | – |
| OC P3 | 10 | 2.6 | 21.4 | – | – |
| OC P4 | 1 | 2.3 | 21.1 | – | – |
| OC P5 | 100 | 2.2 | 24.8 | His 103 Gln | – |
| OC P6 | 100 | 1.5 | 25.4 | His 103 Gln | – |
| OC P7 | 100 | 2.1 | 22.4 | His 103 Gln | – |
| OC P8 | 500 | 2.4 | 24.6 | His 103 Gln | – |
| OC P9 | 1000 | 2.6 | 23.2 | His 103 Gln | – |

sensitivity to zanamivir was 32,000-fold and to oseltamivir carboxylate was 105-fold compared with the starting virus (Table 1).

The reduction in sensitivity to zanamivir demonstrated by the Zan P5–Zan P9 viruses was significantly greater than previously reported for either influenza A or B viruses (McKimm-Breschkin, 2000) and was associated with a Glu to Asp mutation of the 119 residue (influenza A N2 numbering (Shaw et al., 1982)), which lies in a highly conserved pocket within the active site of the NA enzyme (Varghese et al., 1992). This amino acid is known to be one of two conserved amino acid residues that interact with the guanidine group of zanamivir to provide tight and specific interaction between the enzyme active site and the inhibitor (Von Itzstein et al., 1993; Varghese et al., 1995).

Although mutation at the Glu 119 residue has previously been reported for resistant influenza B viruses selected in vitro (Barnett et al., 1999), on both occasions the Glu residue has been replaced with Gly (Staschke et al., 1995). The Glu119Asp mutation has not been previously reported in NA inhibitor resistant influenza B viruses, however, of the four Glu 119 replacement amino acids that have been reported in influenza A strains (aspartate, alanine, glycine and valine), substitution with aspartate generated the greatest level of zanamivir resistance (McKimm-Breschkin, 2000).

Whereas in previous studies mutations in the HA usually occur prior to mutations in the NA (McKimm-Breschkin, 2000), in this study a Gln 218 Lys HA mutation (influenza A H3 numbering (Krystal et al., 1983)) was observed in the zanamivir selected viruses three passages after the Glu 119 Asp NA mutation had become established (Table 1). Results from a HA elution assay demonstrated that viruses containing the additional Gln 218 Lys HA mutation had a reduced binding strength to turkey RBCs (complete RBC elution after 6 h) compared with strains without the HA mutation (no RBC elution after 18 h) (Table 3). However, in the presence of either inhibitor, strains with the Gln 218 Lys HA mutation failed to elute from RBC's after 18 h at the lowest concentration tested. The location of the HA mutation, which has not been reported previously in NA inhibitor resistant viruses, does not appear to map closely to any of the amino acid residues thought to be involved in receptor binding for influenza A (Krystal et al., 1983).

In contrast to the findings following passaging in zanamivir, virus passaged in the presence of oseltamivir carboxylate neither acquired mutations in the NA, nor demonstrated increased resistance to either drug in the NA inhibition assay (Table 2). However, the OC P5 virus, which grew in the presence of 100 μM of oseltamivir carboxylate, was found to have a His 103 Gln HA mutation (A (H3) numbering (Krystal et al., 1983)) (Table 2). This HA mutation significantly reduced the receptor binding strength of the virus, allowing it to elute from turkey RBCs in only 30 min in the absence of drug compared with more than 18 h for the wildtype virus (Table 3). Even in the presence of increasing levels of drug, viruses with this HA mutation were able to elute from RBC's indicating that the mutant had very weak binding affinity (Table 3). The His 103 Gln

Table 3
Virus elution time (hours) from turkey red blood cells

| | Without drug | Zanamivir (nM) | | | | Oseltamivir carboxylate (nM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2.5 | 5 | 160 | 625 | 20 | 40 | 1250 | 5000 |
| B/Tawian/4/2002 (Wild-type) | >18 | >18 | >18 | >18 | >18 | >18 | >18 | >18 | >18 |
| Zan P6 (NA mutation –Glu 119 Asp) | >18 | >18 | >18 | >18 | >18 | >18 | >18 | >18 | >18 |
| Zan P9 (NA mutation –Glu 119 Asp), (HA mutation–Gln 218 Lys) | 6 | >18 | >18 | >18 | >18 | >18 | >18 | >18 | >18 |
| OC P9 (HA mutation – His 103 Gln) | 0.5 | 2 | 4 | 8 | 18 | 2 | 4 | 8 | 18 |

Table 4
NA inhibitor sensitivity (tissue culture $IC_{50}$) of selected passage levels of B/Tawian/4/2002 in MDCK culture

| Virus | Amino acid substitutions | | Tissue culture $IC_{50}$ (μM) | |
|---|---|---|---|---|
| | HA | NA | Zanamivir | Oseltamivir carboxylate |
| Starting virus | – | – | 0.8 | 8.3 |
| OC P9 | His 103 Gln | – | 74.6 | 58.8 |
| Zan P5 | – | Glu 119 Asp | 79.4 | 9.3 |
| Zan P8 | Gln 218 Lys | Glu 119 Asp | 238.1 | 684.2 |

mutation has not been previously demonstrated to play a role in receptor binding (Krystal et al., 1983).

The zanamivir and oseltamivir carboxylate drug concentrations required to reduce the infectious yield of four selected passage levels by 50% were also determined (Table 4). The tissue culture $IC_{50}$ of the B/Tawian/4/2002 starting virus demonstrated a greater sensitivity to zanamivir (0.8 μM) than to oseltamivir carboxylate (8.3 μM). This difference was similar to the trend observed in the neuraminidase $IC_{50}$ assay. The Glu 119 Asp NA mutation that was first observed in Zan P.5, occurred following growth at a drug concentration (10 μM zanamivir) (Table 1) approximately 10-fold higher than the zanamivir tissue culture $IC_{50}$ of the starting virus (0.8 μM). Similarly, for the oseltamivir carboxylate grown viruses, the His 103 Gln HA mutation at OC P5 (Table 2) did not arise until the drug concentration (100 μM) greatly exceeded the oseltamivir carboxylate tissue culture $IC_{50}$ of the starting virus (8.3 μM). This demonstrated that growth of the starting virus in a drug concentration only slightly higher than its tissue culture $IC_{50}$ (e.g. 1 μM of zanamivir or 10 μM of oseltamivir carboxylate) did not create sufficient selection pressure, however, a 10-fold increase in concentration did result in the generation of resistant mutants.

The tissue culture $IC_{50}$ of Zan P5 following the Glu 119 Asp NA mutation, increased by approximately 100-fold to 79.4 μM. However, the pressure of two subsequent passages at 100 μM did not fully select for a further mutation (Zan P.7 contained a mixed population of virus with some mutated strains). It was not until the drug concentration was increased to 500 μM (approximately six-fold higher than the tissue culture $IC_{50}$) that the Gln 218 Lys HA mutation appeared, resulting in a three-fold increase in tissue culture $IC_{50}$ to 238.1 μM. While the zanamivir tissue culture $IC_{50}$ increased following the Glu 119 Asp NA mutation, the oseltamivir carboxylate tissue culture $IC_{50}$ of the starting virus was not significantly different to that of the Zan P5 virus with the NA mutation. This differs from the NA enzyme inhibition assay results where the oseltamivir carboxylate neuraminidase $IC_{50}$ increased by 105-fold as a result of this mutation. While mutations at the 119 residue have been previously shown to cause considerable resistance to zanamivir, the same mutations have demonstrated only partial or no resistance to oseltamivir carboxylate (McKimm-Breschkin, 2000).

Unlike the enzymatic NA inhibition assay, measurement of the susceptibility of viruses to the NA inhibitors in cell culture gives some insight into role of the HA, as well as the NA, in conferring resistance to NA inhibitor drugs. While the 119 NA mutation in the zanamivir grown viruses did not increase the oseltamivir carboxylate tissue culture $IC_{50}$ significantly, the HA mutation that arose in Zan P8, increased the oseltamivir carboxylate tissue culture $IC_{50}$ by over 70-fold. The strong affect of the HA mutation that occurred following growth in oseltamivir carboxylate (His 103 Gln) increased tissue culture $IC_{50}$ values for zanamivir by over 90-fold and over seven-fold for oseltamivir. Of note is the similarity in the zanamivir tissue culture $IC_{50}$ values of the Zan P5 (119 NA mutation) and the OC P9 (103 HA mutation) in the infectivity assay (79.4 μM versus 74.6 μM), compared to the 15,000-fold difference in neuraminidase $IC_{50}$ between the same viruses in the NA enzyme inhibition assay (39,000 nM versus 2.6 nM). This highlights the impact that HA mutations can have in NA inhibitor resistance and therefore, the need to perform cell culture based assays, as well as NA enzyme inhibition assays, when assessing the susceptibly of viruses to the NA inhibitors.

The in vitro generation of mutants from this study and others has demonstrated the ability of influenza viruses to become resistant to the NA inhibitors following serial MDCK passaging in the presence of drug. However, to better assess the risks that in vitro generated NA inhibitor-resistant variants may pose if they were to arise in the human population, in vivo studies using animal models are necessary. Of the in vitro selected NA inhibitor viruses that have been previously tested in vivo, the majority have demonstrated a severely reduced capacity for infection, suggesting that these strains may not survive in a clinical situation (McKimm-Breschkin, 2000).

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