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Short communication

Characterization of drug-resistant recombinant influenza A/H1N1 viruses selected *in vitro* with peramivir and zanamivir

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Abstract

There is a limited information with regard to the neuraminidase (NA) mutations conferring resistance to peramivir and zanamivir in the influenza N1 background. In this study, an influenza A/WSN/33 (H1N1) recombinant virus was passaged under peramivir or zanamivir pressure. The peramivir-selected variant had a H274Y mutation in the neuraminidase (NA) gene conferring resistance to peramivir and oseltamivir but susceptibility to zanamivir. The zanamivir-selected variant had a massive deletion in the region encoding the NA active center and an A200T hemagglutinin mutation. This variant exhibited reduced susceptibility to zanamivir with a drug-dependent phenotype.

Keywords: Influenza; Neuraminidase inhibitors; Resistance

The catalytic site of the influenza neuraminidase (NA) enzyme is constituted of eight functional amino acids (aa) surrounded by 11 framework residues, which are conserved in influenza A and B viruses (Colman et al., 1993). Two commercially available NA inhibitors (NAIs) (inhaled zanamivir and oral oseltamivir) have demonstrated excellent *in vitro* activity against influenza viruses of all types and subtypes (Li et al., 1998; Von Itzstein et al., 1993). These agents also provide clinical benefits in the prevention and treatment of influenza infections (Moscona, 2006). Other agents, such as peramivir (BCX-1812) (Babu et al., 2000) and A-315675 (Kati et al., 2002) were also shown to efficiently inhibit the influenza NA but they are not commercially available. Although oral administration of peramivir was not associated with significant benefits in clinical trials, a single intramuscular injection successfully treated influenza infections in a mouse model (Bantia et al., 2006). Influenza resistance to NAIs has been shown to be drug specific due to structural differences among the various derivatives of 2,3-dehydro-2-deoxy-*N*-acetyl neuraminic acid (DANA). Zanamivir has a single substitution of the O-4 hydroxyl group with a guanidium group whereas oseltamivir has several differences including substitution of an amino group at the 4′ position and a hydrophobic pentyl ether replacing the glycerol side chain at the 6′ position (Zurcher et al., 2006). Peramivir, which is based on a cyclopentane ring, has features of the two other compounds with a guanidium group at the 4′ position of DANA and a hydrophobic group at the 6′ position (Zurcher et al., 2006). The active site of the NA needs some modifications in order to bind the hydrophobic side chains of oseltamivir and peramivir, which is not the case for zanamivir.

In vitro selection of resistant variants using different NAIs (oseltamivir, zanamivir, peramivir and A-315675) revealed the development of mutations at codons E119 and R292 within the NA of influenza A viruses of the N2 and N9 subtypes (Gubareva, 2004). The E119V and R292K A/H3N2 variants have also been recovered from oseltamivir-treated individuals (Ison et al., 2006; Whitley et al., 2001). *In vitro* passages of influenza A/H1N1 viruses in presence of oseltamivir resulted in the emergence of the H274Y NA mutation (Wang et al., 2002). This mutation was also identified in A/H1N1 variants from oseltamivir-treated individuals (Gubareva et al., 2001; Weinstock et al., 2003; Whitley et al., 2001) and in A/Hanoi/30408/2005 (H5N1) variants isolated from a Vietnamese girl after oseltamivir prophylaxis (Le et al., 2005). To date, there have been no data on the *in vitro* selection of NA mutations conferring resistance to zanamivir or peramivir in recombinant viruses of the N1 subtype. Moreover, zanamivir-resistant influenza A viruses have not been reported in the clinic. This lack of resistance could be explained by the

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fact that zanamivir is close to the natural substrate and thus drug-resistance mutations can also compromise the binding to sialic acid (Zurcher et al., 2006). The goal of our study was to select and characterize drug-resistant A/H1N1 viruses with the particularity of initiating the passages with one recombinant strain (A/WSN/33) but using two drugs which have different interactions with the NA active center (zanamivir and peramivir) for selection. We hypothesize that different viral mutations would be selected, which could have important implications in the case of a pandemic caused by a virus of the N1 subtype (e.g. H5N1) and in the context of the emergence of oseltamivir-resistant viruses (Le et al., 2005).

A recombinant influenza A/WSN/33 (H1N1) virus previously generated by a reverse genetics system (Abed et al., 2004) was used in limiting-dilution passaging experiments (McKimm-Breschkin et al., 1998) to ensure homogeneous viral genomic background. The initial passage was performed by infecting Madin Darby canine kidney (MDCK) cells with the recombinant virus at a MOI of 1 in presence of 10 nM of peramivir or 30 nM of zanamivir. The drug concentration was increased slowly with some passages being performed at the same level to increase replication. The A/WSN/33 virus was also passaged in the absence of drug as a control. Plaque-purified variants (PPVs) were selected at different passages for further characterization.

The resistance phenotype of PPVs against NAIs was analysed by NA inhibition assays using the methylumbelliferone *N*-acetylneuraminic acid (MUNANA) as the substrate and an equivalent of 800–1200 fluorescent units of NA enzyme (Potier et al., 1979). Virus yields were determined at 72 h after infection of MDCK cells with an MOI of 0.001 in presence of different concentrations (1–100,000 nM) of zanamivir. Viral RNA was isolated from supernatants of infected cell cultures and reverse-transcribed before PCR amplification of the entire HA and NA genes using primers selected from the respective 3′ and 5′ noncoding regions. PCR products were purified and sequenced. The replicative capacity of the peramivir-resistant variant was evaluated by virus yield assays as previously described (Abed et al., 2004).

After 16 passages in presence of peramivir, an A/WSN/33 (H1N1) variant with good cytopathic effect (i.e. complete cell lysis) was plaque purified in presence of 100 nM of drug and subjected to two additional passages in presence of 100 nM of peramivir (P18-pera). Sequence analysis of P18-pera revealed no amino acid substitutions in the HA gene whereas the NA gene contained a H274Y mutation. The P18-pera variant was highly resistant to both peramivir and oseltamivir whereas it remained susceptible to zanamivir in NA inhibition assays (Table 1). In replicative capacity experiments, P18-pera showed a $\geq$1 log decrease in virus titers compared to the virus passaged without drug (Fig. 1).

The recombinant A/WSN/33 virus was subjected to 14 passages in presence of zanamivir and then plaque-purified in presence of 1000 nM of drug (P14-zana). The P14-zana virus showed poor growth in MDCK cells and had undetectable NA activity which precluded NA inhibition testing. Amplification of the NA gene of P14-zana revealed truncated PCR products of 600–1200 bp size. The HA gene of this variant had a full-length size and contained an A200T substitution (H3 numbering). This variant was submitted to seven additional passages and the P21-zana variant had a defective NA gene of little less than 600 bp (in addition to the full-length NA) whose sequence analysis revealed an important deletion in the region coding for the active center (Fig. 2) associated with the HA A200T mutation. Again, NA inhibition assays could not be performed with this variant because of undetectable NA activity. The P21-zana variant grew somewhat more efficiently in MDCK cells and exhibited a reduced susceptibility to zanamivir in virus yield assays with a drug dependence phenotype as demonstrated by increased virus titers in presence of up to 1 μM of zanamivir (Fig. 3).

Several *in vitro* studies on the mechanisms of resistance of influenza A and B viruses to NAIs have been performed so far although limited data exist on resistance mechanisms to zanamivir and peramivir in influenza viruses of the N1 subtype (Blick et al., 1995; Barnett et al., 1999; Gubareva et al., 1996, 1997; Molla et al., 2002; Staschke et al., 1995). In this study, the influenza A/WSN/33 (H1N1) recombinant virus was passaged under the pressure of each of these two NAIs to specifically verify the type of mutations selected by these drugs with different chemical structures. Indeed, in order to bind peramivir (but not zanamivir), the NA active site is modified by the creation of a hydrophobic pocket where the glycerol binds, by generating an

Table 1
Susceptibility of the wild-type (WT) influenza A/WSN/33 (H1N1) recombinant virus and the peramivir-selected variant (P18-pera) as assessed by NA inhibition assays

Recombinant influenza viruses	IC50 values (nM) by neuraminidase inhibition assay[a]		
	Zanamivir	Oseltamivir	Peramivir
WT	1.35 ± 0.18 (1)	1.1 ± 0.2 (1)	0.11 ± 0.01(1)
P18-pera	1.8 ± 0.09 (1.3)	679.5 ± 44.5 (617)	36.5 ± 7.8 (331)

Numbers in parentheses indicate fold differences compared to wild-type virus.
[a] Experiments were performed in triplicate with the IC50 values reported as means ± S.D.

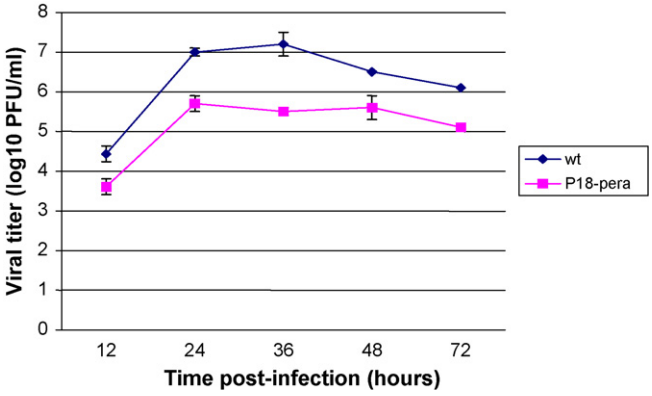

Fig. 1. Replication of the recombinant wild type (WT) A/WSN/33 (H1N1) virus and the P18-peramivir (pera) variant *in vitro*. MDCK cells were infected at a multiplicity of infection of 0.001. At the indicated times post-infection, supernatants were collected, and virus titers were determined in MDBK cells using a standard plaque assay. The values are means of three experiments with the standard deviations indicated.

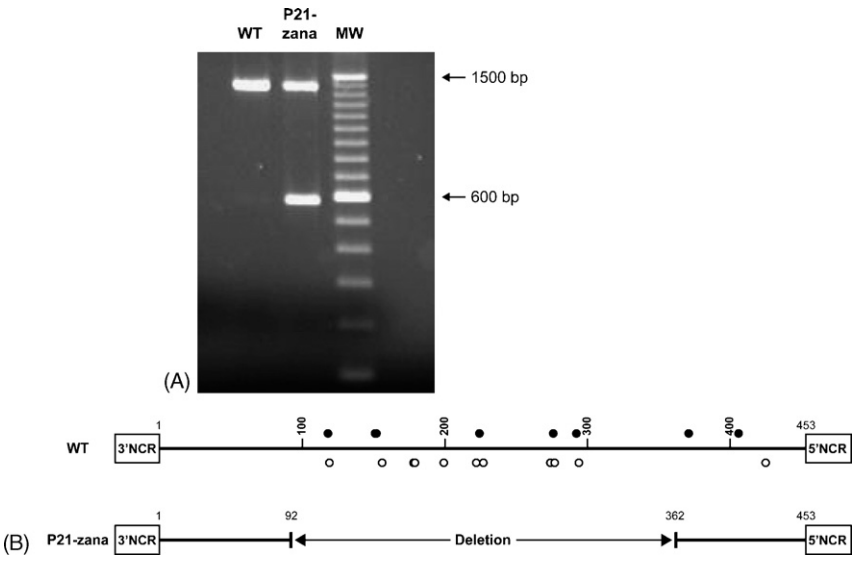

Fig. 2. Characteristics of the neuraminidase (NA) genes of the wild type (WT) A/WSN/33 (H1N1) virus and the P21-zanamivir (zana) variant. (A) PCR products, obtained with primers selected from the 3′ and 5′ noncoding regions (NCRs) of the NA gene, were separated on a 1% electrophoresis gel in presence of a 100-bp molecular weight (MW) marker. (B) Schematic representation of the deduced aa sequences of the full-length (453 aa) WT and the deleted (183 aa) NA proteins. The 8 catalytic and 11 framework residues of the NA active site are indicated by black and empty circles, respectively.

internal salt link between E276 and R224 (Smith et al., 2002; Varghese et al., 1998).

A peramivir-resistant variant obtained after 18 passages (final concentration of 100 nM) was found to contain the H274Y mutation within the NA gene. The P18-pera variant showed the same resistance phenotype (resistance to peramivir and oseltamivir and susceptibility to zanamivir) and replicative properties compared to our reverse genetics-rescued A/WSN/33 H274Y mutant (Abed et al., 2004). The H274Y NA mutation was previously generated *in vitro* using the influenza A/WS/33 virus with oseltamivir pressure (Wang et al., 2002). The corresponding H273Y mutation was also selected under peramivir pressure in the influenza B/Yamagata/16/88 virus and was associated with resistance to peramivir and oseltamivir in plaque reduction assays (Baum et al., 2003). Furthermore, this mutation is the most frequently encountered variation in oseltamivir-treated individuals infected with viruses of the N1 subtype (H1N1 and H5N1) (Gubareva et al., 2001; Le et al., 2005; Whitley et al., 2001; Weinstock et al., 2003). The H274 codon is a framework residue conserved among influenza A and B viruses (Colman et al., 1993). It was suggested that the replacement of histidine with a larger residue (tyrosine) at position 274 would prevent the reorientation of glutamic acid at codon 276 thus preventing the formation of a salt bridge with arginine 224 (Ives et al., 2002).

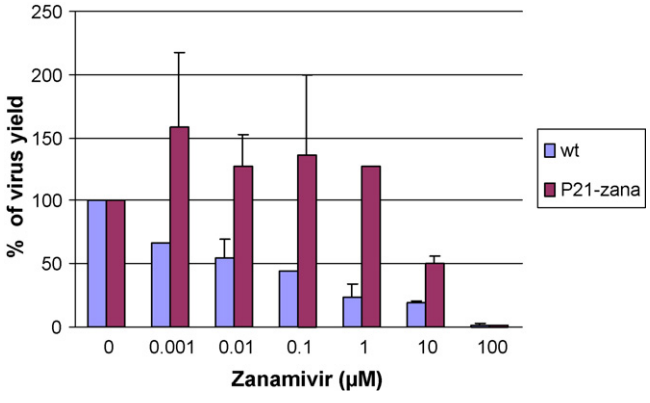

Fig. 3. Resistance phenotype of the wild type (WT) A/WSN/33 (H1N1) virus and the P21-zanamivir (zana) variant as assessed by a virus yield assay. MDCK were infected with the WT and the P21-zana viruses at an MOI of 0.001 in presence of 0.001–100 μM of zanamivir. Supernatants were harvested at 72 h post-infection for determination of virus titers by standard plaque assay in MDBK cells. The mean percentages of virus titers compared to the respective viral yield in the absence of drug from three experiments ± S.D. are indicated.

In contrast to the P18-pera variant, we found no aa substitutions in the NA gene of zanamivir-selected variants. However, RT-PCR testing revealed the presence of defective NA genes in variants selected at passages 14 and 21. The accumulation of defective NA genes, previously identified in influenza A/Charlottesville/31/95 or A/Texas/39/91 (H1N1) variants selected *in vitro* or in vivo with peramivir or oseltamivir, has been suggested as a NAI-resistance marker (Nedyalkova et al., 2002). Also, defective NA mutants with massive deletions in the internal catalytic region of the NA have been selected in presence of exogenous NA in the culture medium (Liu and Air, 1993). Notably, these variants could infect MDCK cells with normal CPE as found in our zanamivir-selected viruses (Hughes et al., 2000). Since HA mutations within or near the receptor binding site (RBS) resulting in weaker cell attachment have arisen during *in vitro* selection with NAIs (McKimm-Breschkin et al., 1996; Gubareva, 2004), we also postulate a role for the HA mutation (A200T) found in our drug-dependent variant although this substitution is not part of the RBS.

Thus, our study confirms the diversity of resistance mechanisms to NAIs and indicates that, besides aa substitutions in or near the receptor binding site of the HA protein and in framework or catalytic residues of the NA enzyme, the accumulation of defective RNA segments may be another important mecha-

nism of resistance to NAIs. Furthermore, our data suggest that zanamivir but not peramivir would retain *in vitro* activity against oseltamivir-resistant H274Y variants of the N1 subtype.

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