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Short communication

Activity of the neuraminidase inhibitor A-315675 against oseltamivir-resistant influenza neuraminidases of N1 and N2 subtypes

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Abstract

Clinical use of the neuraminidase inhibitor (NAI) oseltamivir has been associated with the emergence of viral resistance resulting from subtype-specific neuraminidase (NA) mutations. In this study, we evaluated the impact of the most frequent oseltamivir-resistant NA mutations including E119V, H274Y, R292K and N294S on the susceptibility profile to a novel NAI (A-315675) using recombinant NA proteins of N1 and N2 subtypes and also selected oseltamivir-resistant influenza H1N1 and H3N2 viruses. In the N1 subtype, recombinant NA proteins containing mutations H274Y and N294S previously associated with resistance to oseltamivir (754- and 197-fold increases in IC_{50} values, respectively, compared to WT) remained susceptible to A-315675 (2.5- and 2-fold increases in IC_{50} values, respectively). In the N2 subtype, NA proteins harboring mutations E119V and R292K conferring high levels of resistance to oseltamivir (1016- and >10,000-fold increases in IC_{50} values, respectively) had IC_{50} values that increased by only 1.5- and 13-fold, respectively, against A-315675. Similar susceptibility patterns to A-315675 were obtained when testing recombinant H1N1 mutant viruses (H274Y and N294S) and clinical H3N2 mutants (E119V). The V116A and I117V mutations, previously associated with oseltamivir resistance in H5N1 viruses, were susceptible to oseltamivir when tested in the H1N1 background suggesting a strain-specific impact of these mutations. These results confirm the potent inhibitory effect of A-315675 against oseltamivir-resistant influenza viruses of the N1 and N2 subtypes and support the clinical development of its bioavailable prodrug A-322278.

Keywords: Influenza; Neuraminidase inhibitors; A-315675; Oseltamivir; Resistance

The development of potent antiviral agents for the control of seasonal influenza epidemics and occasional but devastating pandemics is an important priority. The new class of anti-influenza agents known as neuraminidase inhibitors (NAIs) targets the active site of the neuraminidase (NA) enzyme whose activity is essential for release of influenza virions from host cells and for spread of the virus throughout respiratory mucus. Two commercially available NAIs, i.e. inhaled zanamivir and oral oseltamivir, have demonstrated clinical benefits in the prevention and treatment of seasonal influenza infections (reviewed in Moscona, 2006; Abed and Boivin, 2006) whereas other NAIs are at some stage of development. Peramivir, a cyclopentane analogue compound, has shown potent *in vitro* activity against influenza A and B viruses (Sidwell and Smee, 2002). In clinical trials, peramivir reduced viral titers but did not demonstrate a significant decrease in time to relief of symptoms, probably due to a low oral bioavailability (Barroso et al., 2005). A parenteral formulation of this agent is being investigated currently by BioCryst (Bantia et al., 2006). 5-[(1*R*,2*S*)-1-(Acetylamino)-2-methoxy-2-methylpentyl]-4-[(1*Z*)-1-propenyl]-(4*S*,5*R*)-D-proline (A-315675), a pyrrolidine-based compound from Abbott Laboratories, also demonstrated good *in vitro* activity against influenza A (N1, N2, and N9 subtypes) and B viruses which was similar to that of zanamivir and oseltamivir (DeGoey et al., 2002; Kati et al., 2002). In an immunocompromised murine model of influenza infection, A-322278 (the prodrug of A-315675) (Barnes et al., 2003) demonstrated an efficacy similar to that of oseltamivir in reducing viral replication, decreasing weight loss, and prolonging survival (Ison et al., 2006a).

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In vitro selection of resistant variants using different NAIs revealed that NA mutations at codons E119 and R292 were most frequent in influenza A viruses of the N2 subtype (Gubareva, 2004) whereas a mutation at residue H274 predominated in the N1 subtype (Wang et al., 2002; Baz et al., 2007). Similarly, in the clinic, the E119V and R292K A/H3N2 variants have been recovered from oseltamivir-treated individuals (Ison et al., 2006b; Kiso et al., 2004; Baz et al., 2006; Whitley et al., 2001) whereas the H274Y mutation was identified in A/H1N1 variants from oseltamivir-treated individuals (Gubareva et al., 2001; Weinstock et al., 2003; Whitley et al., 2001) and in A/H5N1 variants isolated from humans (Le et al., 2005; De Jong et al., 2005). A N294S mutation was recently associated with resistance in both N1 (H5N1) (Le et al., 2005) and N2 (H3N2) (Kiso et al., 2004) variants recovered from oseltamivir-treated children. Mutations at codons V116 and I117 were also identified in H5N1 viruses from chickens and were associated with resistance to oseltamivir (11- and 16-fold increase in IC_{50} values, respectively) (Hurt et al., 2007).

Although drug-resistant influenza variants have not been detected before the availability of NAIs (Hayden et al., 2005), the global Neuraminidase Inhibitor Susceptibility Network reported that influenza H1N1 and H3N2 viruses with reduced susceptibility to NAIs emerged at 0.25–0.35% rates during the first 5 years (1999–2004) following the introduction of zanamivir and oseltamivir (Monto et al., 2006; WHO, 2005). Resistance to oseltamivir was infrequently detected in clinical trials (<1% of adults and 4% of children) (Jackson et al., 2000) but appears to be more important in certain settings such as in young hospitalized children (up to 18%) (Kiso et al., 2004; Ward et al., 2005), in immunocompromised patients (Baz et al., 2006; Ison et al., 2006b; Weinstock et al., 2003) and in the context of avian H5N1 infections (Le et al., 2005; De Jong et al., 2005).

The objective of the present study was to evaluate the impact of various oseltamivir-resistant NA mutations of influenza A viruses on the susceptibility profile of the novel NAI A-315675 using recombinant NA proteins of N1 and N2 subtypes as well as selected oseltamivir-resistant influenza H1N1 and H3N2 viruses.

The expression and analysis of recombinant NA proteins were previously described (Goto et al., 1997; Abed et al., 2006). Briefly, 293T (human embryonic kidney) cells were cotransfected with 1 μg of each of the 4 expression plasmids (pCAGGS-PA, -PB1, -PB2, -NP) and the pPOLI transcription plasmid containing the NA gene of A/WSN/33 (H1N1) or A/Sydney/5/97 (H3N2) viruses. At 48 h post-transfection, cells were treated with 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS and harvested. After one wash with PBS, the cells were resuspended in PBS containing 3.5 mM $CaCl_2$ and stored in aliquots of 50 μl at −80 °C.

The recombinant A/WSN/33 viruses containing the V116A and I117V NA mutations were rescued in this study by using reverse genetics as previously described (Abed et al., 2006). The other H1N1 recombinants (H274Y and N294S mutants) were described in previous studies (Abed et al., 2004, 2006). Clinical H3N2 variants (E119V and E119V + I222V mutants) were recovered from an immunocompromised child treated with oseltamivir (Baz et al., 2006).

Table 1
Resistance phenotype to A-315675 of oseltamivir-resistant neuraminidases (NAs) of N1 and N2 subtypes

Recombinant NA	A-315675 IC_{50} in nM (fold increase vs. WT)[a]	Oseltamivir IC_{50} in nM (fold increase vs. WT)[a]
NA of A/WSN/33 (H1N1)		
WT	0.43 ± 0.1	0.75 ± 0.04
H274Y	(2.5×)	(754×)
N294S	(2×)	(197×)
V116A	(14×)	(1×)
I117V	(3×)	(1×)
NA of A/Sydney/5/97 (H3N2)		
WT	0.46 ± 0.22	0.43 ± 0.01
E119V	(2×)	(1028×)
R292K	(13×)	(>10,000×)
N294S	(2×)	(1878×)

[a] The mean IC50 values of three experiments ± standard deviations are indicated.

To determine the resistance phenotype to zanamivir, oseltamivir carboxylate (GS4071) (both synthesized at GlaxoSmithKline, Stevenage, UK), peramivir/BCX-1812 (BioCryst, Birmingham, AL) and A-315675 (Abbott Laboratories), NA inhibition assays were performed by using methylumbelliferyl-*N*-acetylneuraminic acid (MUNANA, Sigma, St. Louis, MO, USA) as a fluorescent substrate and dilutions of samples with a NA activity equivalent to 8–10× fluorescence units compared to the background (Potier et al., 1979).

As shown in Table 1, recombinant N1 mutant proteins H274Y and N294S that were resistant to oseltamivir (754- and 197-fold increases in IC_{50} values compared to WT, respectively) were susceptible to A-315675 (2.5- and 2-fold increases in IC_{50} values, respectively). The V116A mutant was susceptible to oseltamivir but had a reduced susceptibility to A-315675 (14-fold increase in IC_{50} value). The I117V mutant was susceptible to both drugs. In recombinant NAs of the N2 subtype, mutations E119V, R292K and N294S conferring high levels of resistance to oseltamivir (1028-, >10,000- and 1878-fold increases in IC_{50} values compared to WT, respectively) had IC_{50} ratios of 2×, 13× and 2×, respectively, against A-315675. Similar findings were obtained when using recombinant H1N1 mutant viruses (H274Y, N294S, V116V and I117V) (Table 2). Also, clinical H3N2 mutants (E119V and E119 + I222V) from an immunocompromised child were resistant to oseltamivir (105- and 1006-fold increases in IC_{50} values, respectively) but retained susceptibility to A-315675 (IC_{50} ratio of 1× compared to WT).

Antivirals constitute an important option for the management i.e. prevention and treatment of seasonal influenza infections (Abed and Boivin, 2006; Moscona, 2006). In addition, NAIs were shown to retain *in vitro* activity against influenza pandemic candidate strains, including amantadine-resistant H5N1 viruses (Moscona, 2006), although higher doses of oseltamivir (Yen et al., 2005) and zanamivir (Hayden et al., 2005) were required to provide protection against highly virulent H5N1 strains in a mouse model. The World Health Organisation (WHO) recom-

Table 2
Resistance phenotype to A-315675 of oseltamivir-resistant influenza A/H1N1 and A/H3N2 viruses

Influenza virus	A-315675 IC_{50} in nM (fold increase vs. WT)[a]	Oseltamivir IC_{50} in nM (fold increase vs. WT)[a]
A/WSN/33 (H1N1)[b]		
WT	0.3 ± 0.06	1.74 ± 0.01
H274Y	(2×)	(427×)
N294S	(2×)	(113×)
V116A	(18×)	(1.5×)
I117V	(2×)	(2×)
A/California/7/2004 (H3N2)-like[c]		
WT	0.21 ± 0.02	2 ± 0.6
E119V	(1×)	(105×)
E119V + I222V	(1×)	(1006×)

[a] The mean IC50 values of three experiments ± standard deviations are indicated.
[b] Influenza A/WSN/33 (H1N1) viruses were rescued using a reverse genetics system (Abed et al., 2004, 2006).
[c] Influenza A/California/7/2004 (H3N2)-like viruses are clinical isolates previously recovered from an immunocompromised child treated with oseltamivir (Baz et al., 2006).

mends the use of oseltamivir, the most widely used NAI, for early treatment of confirmed or strongly suspected H5N1 infections (WHO, 2006) and this drug is currently stockpiled in many countries in the advent of a pandemic.

As for other antivirals, there is a concern about the emergence of NAI resistance and its potential clinical impact on disease management (Hayden et al., 2005). Rates of oseltamivir resistance of 16% and 18% were recently reported in children infected with H1N1 (Ward et al., 2005) and H3N2 (Kiso et al., 2004) viruses, respectively. At least some NAI-resistant influenza A variants, in particular viruses containing framework NA mutations such as E119V and H274Y, could retain their fitness and transmissibility in animal models although this subject still requires further investigations (Abed et al., 2006; Mishin et al., 2005; Herlocher et al., 2004). More recently, oseltamivir-resistant influenza B viruses containing the D198N and the I222T mutations were recovered from untreated patients and it was suggested that they were transmitted through community contacts (Hatakeyama et al., 2007). Consequently, the search for alternative bioavailable antivirals with different patterns of resistance compared to oseltamivir is of great interest.

In this study, we evaluated the inhibitory effect of a novel NAI (A-315675) against influenza neuraminidases of N1 and N2 subtypes containing various mutations of resistance to oseltamivir. A-315675 was previously found to have a stronger affinity for influenza NA compared to oseltamivir (Kati et al., 2002). Indeed, A-315675 dissociates about 12- and 18-fold more slowly from the NA of influenza A (H3N2) and B viruses, respectively, compared to oseltamivir (Kati et al., 2002). A-315675 has a propenyl group which makes hydrophobic contacts within the NA subsite contrasting with oseltamivir carboxylate which makes hydrophilic hydrogen bonding and electrostatic interactions in the enzyme subsite (Maring et al., 2005). A-315675 possesses similar inhibitory activity than oseltamivir carboxylate against influenza A (N1, N2 and N9 subtypes) and B viruses *in vitro* (Kati et al., 2002). In addition, its oral prodrug (A-322278) demonstrated efficacy similar to that of oseltamivir in reducing viral replication, decreasing weight loss, and prolonging survival in a mouse model (Ison et al., 2006a). *In vitro* generation of resistance to A-315675 and to oseltamivir carboxylate from an influenza A/H1N9 strain resulted in different NA mutations (E119D for A-315675 and E119V for oseltamivir carboxylate) (Molla et al., 2002). Interestingly, the A-315675-resistant variant remained susceptible to oseltamivir carboxylate and vice-versa. In our study, we demonstrated the susceptibility to A-315675 of N1 neuraminidases containing H274Y and N294S mutations previously associated with resistance to oseltamivir in H1N1 and H5N1 viruses. Similarly, the N2 neuraminidases containing the oseltamivir resistance mutations E119V and N294S also retained susceptibility to A-315675 and these results were extended to clinical H3N2 isolates containing two mutations (E119E + I222V) (Baz et al., 2006). The R292K mutation, which is part of the catalytic site of the enzyme, was associated with a significantly lower level of resistance (13×) to A-315675 compared to its level of resistance to oseltamivir carboxylate (>10,000×). In another report, there were also only 6- and 8-fold increases in A-315675 IC_{50} values for influenza A/Turkey/Minnesota/833/80 (H4N2) and A/Japan/305/57 (H2N2) viruses containing the R292K NA mutation contrasting with >1600- and 15,000-fold increases in oseltamivir carboxylate IC_{50} values, respectively (Mishin et al., 2005).

Residues V116 and I117 are conserved among several influenza NAs. These residues are adjacent to one (R118) of three arginine residues that bind the carboxylate of sialic acid substrate (Russell et al., 2006). Mutations V116A and I117V were recently identified in influenza A/Chicken/Vietnam/486A/2004 and A/Chicken/Indonesia/77/2005 H5N1 strains, respectively, and were associated with reduced susceptibility to oseltamivir carboxylate (11- and 16-fold increases in IC_{50} values, respectively) (Hurt et al., 2007). When investigated in the NA of A/WSN/33 (H1N1), the V116A mutant was associated with moderate reduction of susceptibility to A-315675 (14-fold), zanamivir (6-fold) and peramivir (6-fold) whereas this variant retained susceptibility to oseltamivir carboxylate (Table 1 and data not shown). The clinical impact of such low levels of resistance is unknown at the present time. The I117V variant was susceptible to the 4 NAIs tested (Table 1 and data not shown). Different NAs from H1N1 and H5N1 viruses could explain the slight discrepancy in phenotypic results. Also, it should be noted that the A/Chicken/Indonesia/77/2005 (H5N1) I117V variant had an additional NA mutation (I314V) that could have contributed to the NAI resistance profile. Further investigations are thus required to clarify this apparent discrepancy.

In summary, this study confirms the interesting absence of cross-resistance between oseltamivir carboxylate and A-315675. In light of the good oral bioavailability of A-315675 following oral administration of prodrug forms such as A-322278, this feature makes A-315675 a potential option for the control of influenza infections due to oseltamivir-resistant strains.

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