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Activity of the Oral Neuraminidase Inhibitor A-322278 against the Oseltamivir-Resistant H274Y (A/H1N1) Influenza Virus Mutant in Mice[▽]

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The new oral neuraminidase (NA) inhibitor A-322278 was evaluated in mice infected with influenza A/H1N1 wild-type virus or the oseltamivir-resistant (H274Y mutant) virus. A-322278 decreased mortality rates and lung virus titers significantly more than oseltamivir in mice infected with the NA H274Y mutant when therapy was started 4 h before or even 48 h after infection.

The development of new antiviral agents and innovative approaches for the control of seasonal influenza epidemics and eventual pandemics remains an important priority. The neuraminidase (NA) inhibitors target the active site of the NA enzyme, whose activity is essential for release of influenza virions from host cells and their spread throughout the respiratory mucus. Two commercially available NA inhibitors, inhaled zanamivir and oral oseltamivir, have demonstrated clinical benefits in the prevention and treatment of seasonal influenza virus infections (1), whereas others are at some stage of development. Among the agents under development is a pyrrolidine-based compound from Abbott Laboratories (A-315675) that showed in vitro activity similar to that of zanamivir and oseltamivir when tested against influenza A (N1, N2, and N9 subtypes) and B viruses (5, 9). In addition, A-315675 retains activity against influenza A/H1N1 viruses containing the oseltamivir resistance NA mutations H274Y and N294S, as well as NA enzymes of A/H3N2 viruses containing the oseltamivir resistance NA mutations E119V and N294S. Slight increases in A-315675 50% inhibitory concentrations (IC_{50}s) were found for influenza A/Turkey/Minnesota/833/80 (H4N2) and A/Japan/305/57 (H2N2) viruses containing the NA R292K mutation (only six- and eightfold, respectively) in contrast to the larger increases in oseltamivir IC_{50}s (>1,600- and 15,000-fold, respectively) (12).

On the other hand, limited data are available with regard to the in vivo efficacy of A-322278, the oral prodrug of A-315675. In an immunocompromised murine model, A-322278 showed an efficacy similar to that of oseltamivir in reducing viral replication, decreasing weight loss, and prolonging survival after infection with the wild-type (WT) A/Japan/305/57 (H2N2) virus (8).

During the 2007 to 2008 influenza season, a significant rise in the frequency of influenza A/H1N1 virus strains carrying the oseltamivir resistance NA mutation H274Y was reported worldwide in untreated patients (10, 13). The aim of this study was to investigate the efficacy of A-322278 given prophylactically or therapeutically in BALB/c mice infected with recombinant A/WSN/33 (H1N1) viruses with or without the oseltamivir resistance mutation H274Y.

The recombinant WT and NA H274Y mutant viruses were rescued using a reverse genetics system (2). Groups of twelve 6- to 8-week-old BALB/c mice (Charles River, LaSalle, QC, Canada) were used in this study. Animals were randomized on the basis of their weight (18 to 20 g), housed four per cage, and kept under conditions which prevented cage-to-cage infections. Mice were infected intranasally under isoflurane anesthesia with 7×10^3 PFU of recombinant viruses in 30 μl of phosphate-buffered saline. Daily treatments with oseltamivir or A-322278 at concentrations of 1 or 10 mg/kg of body weight/day were given by oral gavage for 5 days. Treatment regimens were initiated either 4 h before or 48 h after viral challenge. Mice were monitored daily for body weight loss, and mortality was recorded over a period of 14 days. For the determination of lung viral titers, subgroups of three mice were sacrificed on day 4 postinfection (p.i.), approximately 6 h after treatment, and their lungs were removed aseptically and homogenized in 1 ml of sterile phosphate-buffered saline. Lung homogenates were then centrifuged at $600 \times g$ for 10 min, and supernatants were titrated in Madin-Darby bovine kidney cells by using a standard plaque assay. Viral RNA was also isolated from lung homogenates for reverse transcription-PCR amplification of the hemagglutinin (HA) and NA genes, followed by determination of their sequences. A one-way analysis of variance was done to compare the weight loss and lung viral titers between different treatment regimens.

The mortality rates (100%), mean weight losses on day 5 (17 and 22%), mean days of death (5.8 and 5.1 days), and lung viral titers on day 4 (2×10^6 and 1×10^6 PFU/lung) were similar for untreated BALB/c mice infected with 7×10^3 PFU of the WT or infected with the recombinant NA H274Y mutant (A/H1N1) virus. In groups of mice infected with the WT virus, prophylaxis with 1 mg/kg of either oseltamivir or A-322278 completely prevented mortality (Table 1). For treatments initiated 48 h after virus challenge, only the 10-mg/kg concentrations of oseltamivir and A-322278 were associated with 100% survival. Lung viral titers, determined on day 4 p.i., significantly decreased by approximately 2 $\log_{10}$ with all A-322278 regimens

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TABLE 1. Effect of NA inhibitor treatment on mice infected with recombinant WT (A/H1N1) influenza virus[a]

Regimen	Mean % of wt loss on day 5	Significance of wt loss vs the untreated group (*P* value)	Mortality rate (%)	Mean day of death	Lung viral titer ± SD on day 4 (PFU/lung)[b]	Significance of lung viral titer vs the untreated group (*P* value)
Untreated	17.4	NA	100	5.8	$1.9 \times 10^6 \pm 0.1 \times 10^6$	NA
1 mg/kg oseltamivir 4 h preinfection	2.3	<0.01	0	NA	$3.9 \times 10^4 \pm 1 \times 10^4$	<0.001
1 mg/kg A-322278 4 h preinfection	1.4	<0.01	0	NA	$6 \times 10^4 \pm 2 \times 10^4$	<0.001
1 mg/kg oseltamivir 48 h p.i.	3.9	<0.01	11	5	$5.2 \times 10^4 \pm 1.9 \times 10^4$	<0.001
1 mg/kg A-322278 48 h p.i.	9.1	<0.05	11	5	$3.2 \times 10^4 \pm 1.3 \times 10^4$	<0.001
10 mg/kg oseltamivir 48 h p.i.	1.2	<0.001	0	NA	$5.2 \times 10^3 \pm 1.0 \times 10^3$[c]	<0.001
10 mg/kg A-322278 48 h p.i.	9.5	<0.05	0	NA	$3.0 \times 10^4 \pm 1.7 \times 10^4$	<0.001

[a] NA, not applicable.
[b] Lung viral titers were done in triplicate. SD, standard deviation.
[c] $P < 0.05$ versus 10 mg/kg A-322278 48 h p.i.

and by 2 to 3 $\log_{10}$ with oseltamivir regimens, compared to those of untreated mice. At a concentration of 10 mg/kg initiated 48 h p.i., the reduction in lung viral titers was greater for oseltamivir than for A-322278 ($P < 0.05$), but there were no significant differences between the two drugs at the concentration of 1 mg/kg, initiated either 4 h preinfection or 48 h p.i. (Table 1).

Oseltamivir prophylactic and therapeutic regimens were associated with no significant reductions in mortality or weight loss compared to that of untreated mice infected with the H274Y mutant, although there was a significant reduction in lung viral titers (approximately 1 $\log_{10}$) for all oseltamivir regimens (Table 2). In contrast, prophylactic administration of A-322278 at 1 or 10 mg/kg completely prevented mortality and decreased lung viral titers by close to 2 $\log_{10}$ in mice infected with the H274Y mutant. When administered as treatment, only the 10-mg/kg dose of A-322278 significantly reduced mortality from 100% to 25%, and this reduction was associated with a 2-$\log_{10}$ reduction in lung viral titers compared to that of untreated mice. The reduction in lung viral titers was significantly greater for A-322278 than for oseltamivir for all prophylactic and therapeutic regimens (Table 2). Analysis of lung homogenate supernatants collected on day 4 revealed no alterations in HA and NA viral sequences compared to those of inoculated viruses.

Development of resistance to oseltamivir remains an important concern, particularly among immunocompromised patients (4, 7), in those infected with avian A/H5N1 viruses (6), and, more recently, in otherwise healthy subjects infected with A/Brisbane/59/2007-like (H1N1) viruses harboring the H274Y mutation (10, 13). Such mutant viruses remain susceptible to zanamivir in vitro (13), although the bioavailability of inhaled zanamivir in peripheral lungs may not always be adequate, notably in young children in whom failure of zanamivir therapy has been observed (4, 11). Thus, new oral agents are urgently needed as an alternative to oseltamivir.

The present animal study confirms the previously reported in vitro activity of A-315675 (the active compound of A-322278) against the oseltamivir-resistant NA H274Y mutant (3). Although A-322278 was more active than oseltamivir in both prophylactic and therapeutic settings in mice infected with the H274Y mutant, its activity was slightly less important than oseltamivir in mice infected with the WT virus, notably when drug administration was begun 48 h after infection. We arbitrarily used the same concentrations for the two drugs in this pilot work (1 and 10 mg/kg/day), and thus further studies

TABLE 2. Effect of NA inhibitor treatment on mice infected with recombinant H274Y mutant (A/H1N1) influenza virus[a]

Regimen	Mean % of wt loss on day 5	Significance of wt loss vs the untreated group (*P* value)	Mortality rate (%)	Mean day of death	Lung viral titer ± SD on day 4 (PFU/lung)[b]	Lung viral titer vs the untreated group (*P* value)
Untreated	22.2	NA	100	5.1	$1.0 \times 10^6 \pm 0.3 \times 10^6$	NA
1 mg/kg oseltamivir 4 h preinfection	23.7	>0.05	100	5	$1.9 \times 10^5 \pm 1.3 \times 10^5$	<0.001
1 mg/kg A-322278 4 h preinfection	10.8[c]	<0.01	0	NA	$6.9 \times 10^4 \pm 0.7 \times 10^4$[d]	<0.001
10 mg/kg A-322278 4 h preinfection	5	<0.001	0	NA	$3.5 \times 10^4 \pm 0.4 \times 10^4$	<0.001
1 mg/kg oseltamivir 48 h p.i.	20.1	>0.05	100	5.2	$2.3 \times 10^5 \pm 0.9 \times 10^5$	<0.001
1 mg/kg A-322278 48 h p.i.	19	>0.05	75	5.4	$8.3 \times 10^4 \pm 0.5 \times 10^4$[e]	<0.001
10 mg/kg oseltamivir 48 h p.i.	21.5	>0.05	75	5.1	$1.4 \times 10^5 \pm 0.6 \times 10^5$	<0.001
10 mg/kg A-322278 48 h p.i.	15.6	<0.05	25	5	$1.3 \times 10^4 \pm 0.3 \times 10^4$[f]	<0.001

[a] NA, not applicable.
[b] Lung viral titers were done in triplicate. SD, standard deviation.
[c] $P < 0.001$ versus 1 mg/kg oseltamivir at 4 h preinfection.
[d] $P < 0.001$ versus 1 mg/kg oseltamivir at 4 h preinfection.
[e] $P < 0.001$ versus 1 mg/kg oseltamivir at 48 h p.i.
[f] $P < 0.001$ versus 10 mg/kg oseltamivir at 48 h p.i.

are needed to determine the best therapeutic regimens, notably in the case of A-322278.

Finally, we confirmed that the A/H1N1 H274Y mutant virus is as virulent as the WT virus, at least in this mouse model and in the A/WSN/33 background (2), which correlates with its natural occurrence and high transmissibility in humans during the 2007 to 2008 influenza season. Of note, A-322278 appears to be somewhat less effective in protecting mice challenged with the NA H274Y mutant virus than those infected with the WT virus. As we have previously reported similar 50% lethal dose values for the two viruses in mice (i.e., 10^3 PFU) (2), it is possible that such differences in activity could be attributable to low-level cross-resistance between the two drugs that is not measurable in in vitro assays. Clinical trials of A-322278 are warranted in the context of an eventual pandemic and in consideration of the limited alternatives to oseltamivir.

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