Activity of the oral neuraminidase inhibitor A-322278 against the oseltamivir-resistant
H274Y (A/H1N1) influenza mutant in mice.


Mariana Baz\textsuperscript{1}, Yacine Abed\textsuperscript{1}, Benjamin Nehmé\textsuperscript{1}, and Guy Boivin\textsuperscript{1,*}.

\textsuperscript{1}Research Center in Infectious Diseases of the CHUQ-CHUL and Laval University, Québec City, QC, Canada.

Abstract count: 50
Text count: 1220
Inserts: 2 tables

*Corresponding author:

\textbf{Guy Boivin, MD}
CHUQ-CHUL, room RC-709
2705 blvd Laurier, Sainte-Foy,
Québec, Canada G1V 4G2
Tel : (418) 654-2705
Fax : (418) 654-2715
E-mail : Guy.Boivin@crchul.ulaval.ca
Abstract

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

**Key words:** neuraminidase inhibitors, oseltamivir, A-322878, oseltamivir, resistance, influenza.
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 inhibitors (NAIs) target the active site of the neuraminidase (NA) enzyme, whose activity is essential for release of influenza virions from host cells and their spread throughout the respiratory mucus. Two commercially available NAIs, inhaled zanamivir and oral oseltamivir, have demonstrated clinical benefits in the prevention and treatment of seasonal influenza infections (1) whereas others are at some stage of development. Among them, a pyrrolidine-based compound from Abbott Laboratories (A-315675) showed *in vitro* activity similar to that of zanamivir and oseltamivir when tested against influenza A (N1, N2, and N9 subtypes) and B viruses (6, 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. Limited increases in A-315675 IC$_{50}$ values (only 6- and 8-fold, respectively) were found for influenza A/Turkey/Minnesota/833/80 (H4N2) and A/Japan/305/57 (H2N2) viruses containing the R292K NA mutation contrasting with larger increases in oseltamivir IC$_{50}$ values (>1600- 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 wild-type (WT) A/Japan/305/57 (H2N2) virus (8).
During the 2007-2008 influenza season, a significant rise in the frequency of influenza A/H1N1 strains carrying the oseltamivir resistance H274Y NA mutation was reported worldwide in untreated patients (10, 13). The aim of this study was to investigate the efficacy of A-322278 when given prophylactically or therapeutically in BALB/c mice infected with recombinant A/WSN/33 (H1N1) viruses containing or not the oseltamivir resistance mutation H274Y.

The recombinant WT and H274Y NA mutant viruses were rescued using a reverse genetics system (2). Groups of 12 six to eight week-old BALB/c mice (Charles River, Lasalle, QC Canada) were used in this study. Animals were randomized on the basis of their weight (18-20 g), housed four per cage and kept under conditions which prevented cage-to-cage infections. Mice were infected intranasally, under isoflurane anesthesia, with 7 x 10³ plaque forming units (PFUs) of recombinant viruses in 30 µl of phosphate-buffered saline (PBS). Daily treatments with oseltamivir or A-322278 at concentrations of 1 or 10 mg/kg/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 determination of lung viral titers, subgroups of 3 mice were sacrificed on day 4 postinfection (PI) approximately 6 h after treatment and their lungs were removed aseptically and homogenized in 1 ml of sterile PBS. Lung homogenates were then centrifuged at 600 g for 10 min and supernatants were titrated in Madin-Darby bovine kidney (MDBK) cells using a standard plaque assay. Viral RNA was also isolated from lungs homogenates for
reverse transcription-polymerase chain reaction (RT-PCR) amplification of the hemagglutinin (HA) and NA genes followed by their sequence determination. A one-way analysis of variance (ANOVA) was done to compare weight loss and lung viral titers between different treatment regimens.

The mortality rate (100%), mean weight loss on day 5 (17-22%), mean day of death (5.8-5.1 days) and lung viral titers on day 4 (1-2 x 10^6 PFU/lung) were similar for untreated BALB/c mice infected with 7 x 10^3 PFUs of the WT or the recombinant H274Y NA 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 postinfection, significantly decreased by approximately 2 log10 with all A-322278 regimens and by 2-3 log10 with oseltamivir regimens when compared to untreated mice. At a concentration of 10 mg/kg initiated 48 h postinfection, the reduction in lung viral titers was greater for oseltamivir compared to A-322278 (p<0.05) but there was no significant differences between the two drugs at the concentration of 1 mg/kg initiated either 4 h preinfection or 48 h postinfection (table 1).

As expected, oseltamivir prophylactic or therapeutic regimens were associated with no significant reduction in mortality or weight loss compared to untreated mice infected with the H274Y mutant although there was a significant reduction in lung viral titers (approximately 1 log10) 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 log10 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 was associated with a 2 log10 reduction in lung viral titers compared to untreated mice. The reduction in lung viral titers was significantly greater for A-322278 compared to 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 inoculated viruses.

Development of resistance to oseltamivir remains an important concern in particular among immunocompromised patients (4, 7), in the context of infection with avian A/H5N1 viruses (5) and, more recently, in otherwise healthy subjects infected with A/Brisbane/59/2007-like (H1N1) viruses harboring the H274Y mutation (10, 13). Such mutants remain susceptible to zanamivir in vitro (13) although the bioavailability of inhaled zanamivir in peripheral lungs may not be always adequate notably in young children in whom failure of zanamivir therapy was 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 H274Y NA 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 the drug 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 are needed to determine the best therapeutic regimens notably in the case of A-322278.

Finally, we confirmed that the H274Y A/H1N1 mutant 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 2007-8 influenza season. In fact, A-322278 appears to be somewhat less effective in protecting mice challenged with the H274Y NA mutant compared to the WT virus. As we have previously reported similar LD50 values for the two viruses in mice (i.e. 10³ PFUs) (2), it is possible that such difference could be attributable to low level cross resistance between the two drugs not measurable in in vitro assays. Clinical trials of A-322278 are warranted in the context of an eventual pandemic and considering the limited alternatives to oseltamivir.
References


Table 1. Effect of treatment with neuraminidase inhibitors on mice infected with the recombinant wild-type (A/H1N1) influenza virus.

<table>
<thead>
<tr>
<th>Regimens</th>
<th>Mean % of weight loss on day 5</th>
<th>Weight loss P value compared to untreated group</th>
<th>Mortality rate</th>
<th>Mean day of death (MDD)</th>
<th>Lung viral titers on day 4 (PFU/lung) ± SD</th>
<th>Lung viral titers P value compared to untreated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>17.4</td>
<td>NA</td>
<td>100%</td>
<td>5.8</td>
<td>1.9 x 10^6 ± 0.1 x 10^6</td>
<td>NA</td>
</tr>
<tr>
<td>1 mg/kg Oseltamivir 4h PRE</td>
<td>2.3</td>
<td>&lt;0.01</td>
<td>0%</td>
<td>NA</td>
<td>3.9 x 10^4 ± 1 x 10^4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 mg/kg A-322278 4h PRE</td>
<td>1.4</td>
<td>&lt;0.01</td>
<td>0%</td>
<td>NA</td>
<td>6 x 10^4 ± 2 x 10^4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 mg/kg Oseltamivir 48h POST</td>
<td>3.9</td>
<td>&lt;0.01</td>
<td>11%</td>
<td>5</td>
<td>5.2 x 10^4 ± 0.9 x 10^4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 mg/kg A-322278 48h POST</td>
<td>9.1</td>
<td>&lt;0.05</td>
<td>11%</td>
<td>5</td>
<td>3.2 x 10^4 ± 1.3 x 10^4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 mg/kg Oseltamivir 48h POST</td>
<td>1.2</td>
<td>&lt;0.001</td>
<td>0%</td>
<td>NA</td>
<td>5.2 x 10^3 ± 0.1 x 10^3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10 mg/kg A-322278 48h POST</td>
<td>9.5</td>
<td>&lt;0.05</td>
<td>0%</td>
<td>NA</td>
<td>3.0 x 10^4 ± 1.7 x 10^4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

PRE, preinfection; POST, postinfection; NA, not applicable.

* p<0.05 vs 10 mg/kg A-322278 48 h POST.

Note: Lung viral titers were done in triplicate.
Table 2. Effect of treatment with neuraminidase inhibitors on mice infected with the recombinant mutant H274Y (A/H1N1) influenza virus.

<table>
<thead>
<tr>
<th>Regimens</th>
<th>Mean % of weight loss on day 5</th>
<th>Weight loss P value compared to untreated group</th>
<th>Mortality rate</th>
<th>Mean day of death (MDD)</th>
<th>Lung viral titers on day 4 (PFU/lung) ± SD</th>
<th>Lung viral titers P value compared to untreated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>22.2</td>
<td>NA</td>
<td>100%</td>
<td>5.1</td>
<td>$1.0 \times 10^6 \pm 0.3 \times 10^6$</td>
<td>NA</td>
</tr>
<tr>
<td>1 mg/kg Oseltamivir 4h PRE</td>
<td>23.7</td>
<td>$&gt;0.05$</td>
<td>100%</td>
<td>5</td>
<td>$1.9 \times 10^5 \pm 0.3 \times 10^5$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>1 mg/kg A-322278 4h PRE</td>
<td>10.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$&lt;0.01$</td>
<td>0%</td>
<td>NA</td>
<td>$6.9 \times 10^4b \pm 0.7 \times 10^4$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>10 mg/kg A-322278 4h PRE</td>
<td>5</td>
<td>$&lt;0.001$</td>
<td>0%</td>
<td>NA</td>
<td>$3.5 \times 10^4 \pm 0.4 \times 10^4$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>1 mg/kg Oseltamivir 48h POST</td>
<td>20.1</td>
<td>$&gt;0.05$</td>
<td>100%</td>
<td>5.2</td>
<td>$2.3 \times 10^5 \pm 0.9 \times 10^5$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>1 mg/kg A-322278 48h POST</td>
<td>19</td>
<td>$&gt;0.05$</td>
<td>75%</td>
<td>5.4</td>
<td>$8.3 \times 10^4c \pm 0.5 \times 10^4$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>10 mg/kg Oseltamivir 48h POST</td>
<td>21.5</td>
<td>$&gt;0.05$</td>
<td>75%</td>
<td>5.1</td>
<td>$1.4 \times 10^5 \pm 0.6 \times 10^5$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>10 mg/kg A-322278 48h POST</td>
<td>15.6</td>
<td>$&lt;0.05$</td>
<td>25%</td>
<td>5</td>
<td>$1.3 \times 10^4d \pm 0.3 \times 10^4$</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

PRE, preinfection; POST, postinfection; NA, not applicable.

<sup>a</sup> $p<0.001$ vs 1 mg/kg Oseltamivir 4 h PRE.

<sup>b</sup> $p<0.001$ vs 1 mg/kg Oseltamivir 4 h PRE.

<sup>c</sup> $p<0.001$ vs 1 mg/kg Oseltamivir 48 h POST.

<sup>d</sup> $p<0.001$ vs 10 mg/kg Oseltamivir 48 h POST.

Note: Lung viral titers were done in triplicate.