Comparative Activities of Oseltamivir and A-322278 in Immunocompetent and Immunocompromised Murine Models of Influenza Virus Infection

Michael G. Ison, Vasiliy P. Mishin, Thomas J. Braciale, Frederick G. Hayden, and Larisa V. Gubareva

1Division of Infectious Diseases and International Health, Department of Medicine, 2Bierne Carter Immunology Center, and 3Department of Pathology, University of Virginia Health Sciences Center, Charlottesville

We developed an immunocompromised murine model of influenza virus infection and demonstrated comparable efficacy of oral oseltamivir and A-322278 (both given at dosages of 10 mg/kg/day) in reducing viral replication, decreasing weight loss, and prolonging survival. Once the treatment was discontinued, severe combined immunodeficient (SCID) mice had progressive viral replication and clinical decline. Drug-resistant variants were detected in 4 (29%) of 14 and 2 (13%) of 15 mice (both BALB/c and SCID) treated with oseltamivir or A-322278, respectively; no resistant variants were detected in placebo-treated mice. Amino acid substitutions in the hemagglutinin receptor-binding site at aa 137 or 225 were detected in cloned resistant isolates. A substitution in the neuraminidase (NA) active site (Arg292Lys) was detected in the cloned virus recovered from an oseltamivir-treated mouse. This model would be useful for elucidation of the molecular mechanisms of resistance to NA inhibitors and for testing of anti-influenza therapy options that might prevent the emergence of resistant variants.

In patients with compromised immunity, influenza virus may cause severe lower respiratory tract involvement; unusual syndromes, including encephalitis, myocarditis, and hepatitis; and, in transplant recipients, graft dysfunction and rejection [1, 2]. Although several case series indicate that neuraminidase (NA) inhibitor therapy may be beneficial [3–5], there have been no controlled, prospective studies of NA inhibitors in the management of influenza in patients with substantial immune compromise.

Prolonged viral shedding frequently occurs during influenza virus infection in immunocompromised individuals and is associated with an increased risk of the emergence of antiviral resistance to both M2 ion channel inhibitors [6] and NA inhibitors [7]. Single-amino-acid substitutions in the NA active site confer resistance to NA inhibitors. Although the emergence of resistance is uncommon with oseltamivir therapy in immunocompetent adults (<1%) [8, 9], it is more common in children, particularly hospitalized ones (as many as 18%) [10, 11], and in immunocompromised patients. The emergence of drug-resistant variants has been described in immunocompromised patients treated with either zanamivir or oseltamivir [8, 12–14].

A-315675 is a novel NA inhibitor that has in vitro potency similar to that of oseltamivir (GS4071) against most influenza A viruses but is more potent against influenza B strains [15–17]. It also retains antiviral activity against several common oseltamivir-resistant variants, including H274Y (N1) and E119V (N2) [18]. This activity against oseltamivir-resistant strains might be
important in immunocompromised patients, who may die of influenza infection after resistant virus emerges while they are receiving therapy with available agents. As with oseltamivir, A-315675 is delivered orally as a prodrug (A-322278).

Since there is limited information on the activity of any of the anti-influenza agents in immunocompromised hosts, in the present study we compared the activity of oseltamivir and A-322278 in both immunocompetent (BALB/c) and immunocompromised (SCID) murine models and examined viral isolates recovered during therapy for evidence of antiviral resistance.

**METHODS**

**Compounds.** A-315675 and its isopropyl ester prodrug, A-322278, were provided by Abbott Laboratories. Oseltamivir carboxylate (GS4071) and its ethyl ester prodrug, oseltamivir phosphate (GS4104), were provided by Hoffman-La Roche, in the form of dry powders. A-315675 and oseltamivir carboxylate were used in in vitro experiments, whereas A-322278 and oseltamivir phosphate were used in vivo. Stock solutions of each drug were prepared in distilled water at a concentration of 10 mg/mL, and aliquots were frozen at −20°C. Preliminary data provided by each manufacturer indicated that 10 mg/kg/day was the appropriate dosage of both study agents in mice [19]. For oseltamivir, this dosage has recently been shown to be active in a murine model of H5N1 disease caused by a human 2004 isolate [20].

**Mice.** The murine experiments were approved by the Animal Care and Use Committee and were conducted at the Center for Comparative Medicine at the University of Virginia, Charlottesville, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Groups of ~8-week-old female SCID mice (Fox Chase SCID C.B-Igh-1b/IcrTac-PrkdcSCID, bred on a BALB/c background; Taconic) and 8-week-old female BALB/c mice (BALB/CAnNTac; Taconic) and 8-week-old female BALB/c mice were used [21, 22]. For viral challenge, the mice were lightly anesthetized with ether chloroform and 10-fold dilutions were made and were inoculated onto quadruplicate monolayers of MDCK cells, using previously published methods [25]. Supernatants from wells showing cytopathic effects on day 2 were harvested for subsequent antiviral susceptibility testing of isolates; these isolates were selected to reduce the possibility of a significant growth advantage of wild-type virus over drug-resistant mutants. On day 7, virus replication in all cell monolayers was confirmed by hemadsorption using a 0.5% suspension of fresh chicken red blood cells. Virus titers were determined by the Karber method, which is described elsewhere [26], and are presented as \( \log_{10} \) median TCID50 per gram of lung.

**NA-inhibition assay.** Evaluation of virus susceptibility to NA inhibitor was assessed in the NA enzymatic activity inhibition assay, as described elsewhere [27]. The IC50 was determined by assaying the NA activity of virus in the presence of serial half-log dilutions (from 10 μmol/L to 0.01 nmol/L) of each NA inhibitor. Previously characterized oseltamivir- and A-315675–resistant mutants of the same NA subtype (N2) were used as positive controls.

**Plaque-reduction assay.** Susceptibility testing of the virus isolates was also performed using the plaque-reduction assay, as described elsewhere [28]. The effective NA inhibitor concentration (the EC50) was defined as that causing a 50% reduction in plaque diameter, compared with the control plaques produced in the absence of the drug.

**Sequence analysis of the viral hemagglutinin (HA) and NA genes.** Viral RNA extraction from cell culture supernatants,
reverse-transcription polymerase chain reactions (PCRs), and sequence analysis of the HA and NA genes were performed as described elsewhere [8]. The sequences of primers used for PCR amplification are available on request. The sequence alignments were performed by use of the Influenza Sequence Database, using the amino acid numbering for the N2 NA antigenic subtype [29] and the H3 HA antigenic subtype [30, 31]. The HA and NA gene sequences of the mouse-adapted A/Japan/305/57 (H2N2) virus and its variants have been deposited in the GenBank database (accession numbers AY643085–AY643089).

**Statistical analysis.** A Wilcoxon rank-sum test conducted using Splus 2000 (release 3; Insightful) was used to compare the median weight change and lung titers on each day. Limited analysis was performed on experiment 1, because of the rapid death of mice in the placebo group.

**RESULTS**

**Lethal infection.** After lethal infection, placebo-treated mice rapidly lost weight after infection until death (figure 1A). Placebo-treated BALB/c mice died by day 7, and placebo-treated SCID mice died by day 9. Drug-treated BALB/c mice had transient weight loss followed by recovery, whereas drug-treated SCID mice had modest weight loss while receiving treatment, with rapid weight loss after discontinuation of drug treatment. By day 12, all SCID mice had died or had been killed because of marked weight loss. No important differences were noted between the drug groups.

Lung virus titers in oseltamivir- or A-322278–treated SCID mice were 1.4 log_{10} lower on day 2 and 0.75–1.1 log_{10} lower on day 7 than those in placebo-treated SCID mice. Smaller differences (≤1.0 log_{10}) between the placebo and drug-treated groups were found in BALB/c mice than in SCID mice during therapy (table 1). Drug-treated BALB/c mice had lower virus titers on day 12 after discontinuation of treatment, whereas SCID mice had increases in titers, to levels comparable to those found in untreated mice, after discontinuation of treatment (table 1).

**Sublethal infection.** The placebo-treated BALB/c mice had transient weight loss on days 8–10, followed by recovery, whereas placebo-treated SCID mice had progressive weight loss after day 2 (figure 1B). No placebo-treated BALB/c or SCID mice died of infection during the 14-day follow-up period, despite substantial weight loss in the SCID group. The oseltamivir-treated BALB/c mice had gradual weight gain throughout the study period, whereas A-322278–treated BALB/c mice had slight weight loss. The A-322278–treated SCID mice had mild weight loss after day 5 and progressive weight loss after day 9. The oseltamivir-treated SCID mice had stable weights. In SCID mice, both treatment groups had significantly less weight loss on day 14 than did the placebo group. The difference in day 7—but not day 14—between the 2 treatment groups was statistically significant (P = .047).

In the drug-treated BALB/c mice, lung viral titers were 1.3–1.6 log_{10} lower than those in the placebo-treated mice on day 2 (P < .01, for each treatment group) and 0.9–2.0 log_{10} lower on day 7 (P < .05, for each treatment group) (table 1). Two of 6 BALB/c mice had residual lung viral titers on day 14, irrespective of their treatment group. Drug-treated SCID mice had 1.1–1.4 log_{10} lower titers on day 2 (oseltamivir, P = .04; A-322278, P = .09) and 1.7 log_{10} lower titers on day 7 (P < .01, for each treatment group) (table 1), compared with placebo-treated SCID mice. Once therapy was discontinued in SCID mice, virus replication rebounded to the levels seen in placebo-treated mice. No statistically significant differences were observed in viral titers between the 2 study drugs in either the BALB/c or the SCID mice.

**NA inhibitor susceptibility.** The challenge virus A/Japan/305/57 (H2N2) was found to be highly susceptible to both inhibitors by enzyme-inhibition assay (table 2). There were no changes in the IC_{50} of isolates recovered from drug-treated or placebo-treated mice after 7 or 14 days of treatment (table 2).

Reduction of plaque size by 50% for the challenge virus was achieved with concentrations <1 ng/mL for both NA inhibitors, and complete inhibition of plaque formation was observed at 10 ng/mL (figure 2). Forty-six viruses recovered from either placebo-treated (n = 17) or drug-treated (n = 29) mice were tested by plaque assay in MDCK cells. None of the viruses recovered from placebo-treated mice showed changes in drug susceptibility. Two of 8 viruses from oseltamivir-treated BALB/c mice (both 7 days after discontinuation of a 7-day treatment course), 2 of 6 oseltamivir-treated SCID mice (both on study day 21, 7 days after discontinuation of therapy), and 2 of 10 A-322278–treated SCID mice (both on study day 14, 7 days after discontinuation of therapy) showed changes in drug susceptibility (figure 2E and 2F). All 6 viruses formed distinct plaques at NA inhibitor concentrations ≥100 ng/mL, although the number of the plaques was reduced in comparison with that in control wells. This indicates that the majority of the variants in the virus population retained their drug-susceptible phenotype in MDCK cells.

Sequence analysis was conducted on 3 individual plaques produced by viruses isolated from 1 oseltamivir-treated BALB/c mouse and 1 A-322278–treated SCID mouse on day 14 (7 days after discontinuation of therapy). One clone from the oseltamivir-treated mouse contained mutations conferring Arg292Lys and Thr464Ala substitutions in NA and Gly225Glu (HA1 subunit) and Val193Ala (HA2 subunit) substitutions in HA. This cloned virus was highly resistant to oseltamivir (IC_{50} >3000 nmol/L) and somewhat less susceptible to A-315675 (IC_{50} 3.0–5.0 nmol/L) in NA-inhibition assays, most likely because of the substitution at the conserved residue at aa 292. The 3 virus clones from the A-322278–treated mouse had no NA mutations detected, but all had a single Arg137Gly substitution in HA (HA1
Figure 1. Weight changes in mice, by treatment group. Graphs represent the percentage weight gain or loss from baseline by treatment group during lethal (experiment 1 [A]) and sublethal (pooled data from experiments 2 and 3 [B]) infection. In the sublethal infection, the extent of weight loss at day 14 was significantly greater in the placebo-treated SCID mice than in those treated with oseltamivir ($P = 0.03$) or A-322278 ($P = 0.04$).
Table 1. Lung viral titers by study day.

<table>
<thead>
<tr>
<th>Experiment, mouse group</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 12 or 14&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Lethal infection (2 mice/group)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCID placebo</td>
<td>6, 5.75</td>
<td>6.25, 5.25</td>
<td>5.75</td>
</tr>
<tr>
<td>SCID A-322278</td>
<td>4.5, 4.5</td>
<td>4.5, 4.75</td>
<td>6, 6</td>
</tr>
<tr>
<td>SCID oseltamivir</td>
<td>4.5, 4.5</td>
<td>4.75, 5.25</td>
<td>5.75, 5</td>
</tr>
<tr>
<td>BALB/c placebo</td>
<td>5.5, 5.75</td>
<td>5.5, 5</td>
<td>NA</td>
</tr>
<tr>
<td>BALB/c A-322278</td>
<td>4.75, 5</td>
<td>4, 4.25</td>
<td>0, 0</td>
</tr>
<tr>
<td>BALB/c oseltamivir</td>
<td>4.75, 4.75</td>
<td>5, 4.25</td>
<td>2, 2.25</td>
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<tr>
<td>Sublethal infection (6 mice/group)</td>
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<td></td>
</tr>
<tr>
<td>SCID placebo</td>
<td>5.00 ± 1.07</td>
<td>6.00 ± 0.77</td>
<td>6.08 ± 0.26</td>
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<tr>
<td>SCID A-322278</td>
<td>3.88 ± 0.97</td>
<td>4.29 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.79 ± 0.71</td>
</tr>
<tr>
<td>SCID oseltamivir</td>
<td>3.63 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.25 ± 0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.25 ± 0.50</td>
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<tr>
<td>BALB/c placebo</td>
<td>5.54 ± 0.62</td>
<td>4.63 ± 0.26</td>
<td>0.75 ± 1.20</td>
</tr>
<tr>
<td>BALB/c A-322278</td>
<td>3.92 ± 1.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.63 ± 1.72&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.50 ± 0.79</td>
</tr>
<tr>
<td>BALB/c oseltamivir</td>
<td>4.21 ± 0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.71 ± 0.71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.71 ± 1.10</td>
</tr>
</tbody>
</table>

NOTE. Data are individual viral titers for the lethal infection experiment and mean ± SD viral titers for the sublethal infection experiments. NA, not applicable.
<sup>a</sup> Day-12 results are given for the lethal infection experiment, and day-14 results are given for the sublethal infection experiments.
<sup>b</sup> P = .01, vs. placebo.
<sup>c</sup> P = .02, vs. placebo.

Table 2. Drug-susceptibility testing of isolates in the NA-inhibition assay.

<table>
<thead>
<tr>
<th>Mouse group or virus isolate</th>
<th>Mouse isolates tested, no.</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, range, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A-315675</td>
</tr>
<tr>
<td>Mice</td>
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<td></td>
</tr>
<tr>
<td>BALB/c placebo</td>
<td>7</td>
<td>0.4–0.6</td>
</tr>
<tr>
<td>BALB/c A-322278</td>
<td>7</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>BALB/c oseltamivir</td>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>SCID placebo</td>
<td>6</td>
<td>0.3–0.4</td>
</tr>
<tr>
<td>SCID A-322278</td>
<td>11</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>SCID oseltamivir</td>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>Viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/Japan/305/57; egg-grown challenge virus</td>
<td>0.4–1.3</td>
<td>0.4–0.9</td>
</tr>
<tr>
<td>Oseltamivir-resistant mutant, Arg292Lys</td>
<td>5–5.8</td>
<td>&gt;3000</td>
</tr>
<tr>
<td>Oseltamivir-resistant mutant, Glu119Val</td>
<td>1.3–1.8</td>
<td>80–100</td>
</tr>
<tr>
<td>A-315675–resistant mutant, Glu119Asp</td>
<td>30–50</td>
<td>1.0–1.8</td>
</tr>
</tbody>
</table>

NOTE. ND, not determined.
Figure 2. Susceptibility testing in the plaque-reduction assay. Confluent MDCK cell monolayers were infected with the challenge mouse-adapted A/Japan/305/57 (H2N2) virus (A and B) and with the virus isolates recovered from the virus-infected SCID mice treated with either PBS (C and D) or oseltamivir (E and F). The plaque-reduction assay was performed in the presence of either A-315675 (A, C, and E) or oseltamivir (B, D, and F) at concentrations of 0, 0.1, 1, 10, 100, and 1000 ng/mL.

We have previously confirmed similar findings in studies using NA inhibitors in immunocompetent [19, 32, 33] and immunocompromised [34] mice and represent, to our knowledge, the first report of the activity of A-322278 in both immunocompetent and immunocompromised mice. Since A-322278 has antiviral activity against some oseltamivir-resistant variants and has efficacy comparable to that of oseltamivir, A-322278 might be useful for salvage therapy in patients who develop resistance while receiving oseltamivir therapy. This specific question should be addressed in future studies.

Prolonged replication of influenza virus in drug recipients appears to be a risk factor for the emergence of resistant variants. Oseltamivir treatment has been associated with the emergence of resistance in as many as 18% of hospitalized, immunocom-
petent children, and several cases have been documented in immunocompromised patients [10, 13]. In the present study, resistant variants were detected by plaque-reduction assay in 21% (6 of 29) of the virus isolates recovered from the drug-treated mice. Interestingly, the NA inhibitor–resistant viruses were not detected by initial screening using an NA-inhibition assay, probably because of the predominance of the wild-type variant in the virus population. It is possible that a higher frequency of resistance emergence would be observed with greater selective antiviral pressure (e.g., higher drug doses or, perhaps, more-prolonged administration of subtherapeutic doses), but we did not address this hypothesis in the present study. Virus propagation in cell culture in the absence of the drug may alter the ratio between the drug-resistant and wild-type variants, because of a difference in fitness. Therefore, in future studies, it would be prudent to conduct biological cloning of the virus present in the lung homogenates before propagation in cell culture.

The virus clone recovered from the A-322278–treated mouse was drug susceptible in the NA-inhibition assay and drug resistant in the MDCK cell plaque assay. The detected substitution at aa 225 in the HA receptor–binding site was most likely responsible for the virus drug resistance in cell culture. This substitution has the potential to reduce HA binding to the cell receptors, thus reducing the need for NA activity and reducing the virus susceptibility to NA inhibitors. The virus recovered from the oseltamivir-treated mouse contained a substitution in the HA receptor–binding site at aa 137. Although the effect of HA substitutions in the emergence of drug resistance in cell culture has been recognized, their role in in vivo drug resistance has not been convincingly demonstrated. Interestingly, the substitutions at aa 137 and 225 have also been identified in HA from viruses recovered from experimentally infected humans treated with oseltamivir [8].

No substitutions were detected in the NA active site of the virus clone recovered from the A-322278–treated mouse; if they were only minor species, they may have emerged without our detecting them. The virus clone derived from the oseltamivir-treated mouse had a substitution in the NA active site (aa 292). Interestingly, this mutation, along with the HA mutation, resulted in drastically reduced susceptibility to oseltamivir but not to A-315675. Because the substitution at aa 292 is the most commonly detected change in the N2-containing viruses recovered from oseltamivir-treated patients [9], the availability of drugs like A-322278 could provide a valuable therapeutic option.

Overall, the present study demonstrates that this immunocompromised murine model replicates the prolonged viral shedding, response to antiviral therapy, and emergence of resistant variants seen in influenza virus infections in immunocompromised humans. This model can be used to address questions concerning the role of the host immune response, the molecular mechanisms of drug resistance operating in vivo, and therapeutic options in the control of influenza virus infections. Additional studies are needed, using the model, to test the response of different influenza virus strains to available and experimental anti-influenza therapy options, including antiviral combinations. Further development of A-322278 is warranted on the basis of the available data on this compound, including the results of this study. This novel compound might be useful in managing patients infected with resistant variants that retain susceptibility to A-322278.

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References