Pharmacological Characterization of the Spectrum of Antiviral Activity and Genetic Barrier to Drug Resistance of M2-S31N Channel Blockers

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ABSTRACT

Adamantanes (amantadine and rimantadine) are one of the two classes of Food and Drug Administration–approved antiviral drugs used for the prevention and treatment of influenza A virus infections. They inhibit viral replication by blocking the wild-type (WT) M2 proton channel, thus preventing viral uncoating. However, their use was discontinued due to widespread drug resistance. Among a handful of drug-resistant mutants, M2-S31N is the predominant mutation and persists in more than 95% of currently circulating influenza A strains. We recently designed two classes of M2-S31N inhibitors, S31N-specific inhibitors and S31N/WT dual inhibitors, which are represented by N-[(5-cyclopropyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (WJ379) and N-[(5-bromothiophen-2-yl)methyl]adamantan-1-amine (BC035), respectively. However, their antiviral activities against currently circulating influenza A viruses and their genetic barrier to drug resistance are unknown. In this report, we evaluated the therapeutic potential of these two classes of M2-S31N inhibitors (WJ379 and BC035) by profiling their antiviral efficacy against multidrug-resistant influenza A viruses, in vitro drug resistance barrier, and synergistic effect with oseltamivir. We found that M2-S31N inhibitors were active against several influenza A viruses that are resistant to one or both classes of Food and Drug Administration–approved anti-influenza drugs. In addition, M2-S31N inhibitors display a higher in vitro genetic barrier to drug resistance than amantadine. The antiviral effect of WJ379 was also synergistic with oseltamivir carboxylate. Overall, these results reaffirm that M2-S31N inhibitors are promising antiviral drug candidates that warrant further development.

Introduction

Influenza viruses are the causative agents that lead to annual seasonal influenza epidemics as well as sporadic, more devastating influenza pandemics (Rappuoli and Dormitzer, 2012; Monto and Webster, 2013). On average, influenza virus infection leads to 36,000 deaths in the United States and approximately 250,000–500,000 deaths globally each year (Thompson et al., 2003, 2004). However, current countermeasures against influenza infection are curtailed by the emergence of drug-resistant influenza A strains. Resistance to the only orally bioavailable neuraminidase inhibitor, oseltamivir, has been continuously reported and was prevalent in the 2008–2009 influenza season (Cheng et al., 2009; Samson et al., 2013). The use of M2 channel blockers (amantadine and rimantadine) is no longer recommended by the Centers for Disease Control and Prevention due to prevalent drug resistance (Bright et al., 2006; Fiore et al., 2008). Thus, novel antivirals are clearly needed. Toward this goal, we chose M2-S31N as the drug target to develop the next generation of antiviral drugs. The M2-S31N mutant is an ideal antiviral drug target because: 1) M2-S31N persists in more than 95% of currently circulating influenza A viruses among humans, including oseltamivir-sensitive and oseltamivir-resistant strains (Dong et al., 2015), thus targeting M2-S31N is likely to yield broad-spectrum antiviral drugs; and 2) M2 is a validated antiviral drug target, and there is no such homolog proton channel encoded by the human genome, thus M2 inhibitors are expected to have high selectivity. As a homotetrameric proton-selective channel, one mutation in M2 actually results in four changes at the same time, which leads to a profound impact on the structure and function of this very constricted channel. As a result, only a very limited number of M2 mutants confer both drug resistance and transmissibility among humans, of which M2-S31N is the predominant mutant. This small set of transmissible mutants suggests that M2 is a highly conserved drug target compared with other viral proteins, rendering it an ideal drug target for the development of anti-influenza drugs (Wang et al., 2015).

M2-S31N mutant was traditionally tagged as an “undruggable” target, and decades of traditional medicinal chemistry campaign failed to yield a single hit compound (Wanka et al., 2013; Wang et al., 2015). Nevertheless, guided by information...
gathered from previous structure-activity relationship studies of amantadine (Wang et al., 2015) and breakthroughs in M2 structural biology and mechanistic studies (Hong and DeGrado, 2012), steady progress has been made toward the design of M2-S31N inhibitors (Wang et al., 2013a; Wu et al., 2014; Li et al., 2016). They are broadly classified as S31N-specific inhibitors and S31N/wild-type (WT) dual inhibitors. S31N-specific inhibitors target the S31N channel only, not the WT, and one representative example is N-[(5-cyclopropyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (WJ379) (Wang et al., 2013b). S31N/WT dual inhibitors target both S31N and WT M2 channels, and one representative example is N-[(5-bromothiophene-2-yl)methyl]adamantan-1-amine (BC035) (Wu et al., 2014). Their channel blockade and antiviral effects have been confirmed in electrophysiological two-electrode voltage clamp (TEVC) assays and antiviral plaque assays, respectively. Their modes of binding were revealed by both solution and solid-state NMR studies (Cady et al., 2010, 2011; Wang et al., 2013b; Williams et al., 2013; Wu et al., 2014).

In this study, we further evaluated the therapeutic potential of both classes of M2 inhibitors, S31N-specific inhibitors and S31N/WT dual inhibitors. Specifically, from the standpoint of developing therapeutics, we are interested in testing whether the potent antiviral effect of these compounds on the model virus, A/WSN/33 (H1N1), can be extended to the currently circulating influenza A strains among humans. Furthermore, with these tool compounds in hand, we would like to address the issue of drug resistance. For example, will the S31N mutant virus become resistant to the newly developed S31N inhibitors under drug selection pressure? If yes, which mutations will be selected? How many passages does it take for the S31N mutant virus to evolve drug resistance? In addition, as we already learned the lessons of antibiotics and antivirals, resistance is unfortunately inevitable. It is not a question of yes or no but rather a question of when (Clavel and Hance, 2004; Hayden and de Jong, 2011; McKimm-Breschkin, 2013; Walsh and Wencziewicz, 2014; Blair et al., 2015). With this in mind, what one can do is to slow down the resistance evolution rather than eradicate it. Toward this goal, we would like to test the combination therapy potential of M2-S31N inhibitors with oseltamivir, as this offers a means to delay resistance evolution against both oseltamivir and M2-S31N inhibitors.

Materials and Methods

Cell Lines, Viruses, and Viral Infection. Madin-Darby canine kidney (MDCK) cells were grown at 37°C in 5% CO₂ atmosphere in Dulbecco’s modified Eagle's medium (DMEM; high glucose, with L-glutamine) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. MDCK cells overexpressing β-galactoside α-2,6-sialyltransferase I (ST6Gal I) were obtained from Dr. Yoshihiro Kawaoaka at the University of Wisconsin (Madison, WI) through material transfer agreement and were maintained in the presence of 7.5 μg/ml puromycin, except when they were used for viral infection. Influenza A virus strains A/California/07/2009 (H1N1) and A/Texas/04/2009 (H1N1) were obtained from Dr. James Noah at the Southern Research Institute (Birmingham, AL), and influenza A virus strains A/Denmark/524/2009 (H1N1) and A/Denmark/528/2009 (H1N1) were obtained from Dr. Elena Gervorkova at St. Jude Children’s Research Hospital (Memphis, TN). Virus stocks were amplified in MDCK cells in the presence of 2 μg/ml N-acetyl trypsin. Two days post infection, the culture media were harvested, and cell debris was removed by centrifugation at 3000 rpm for 30 minutes. Virus titers were determined by plaque assay using MDCK cells expressing ST6Gal I.

Compounds. WJ379 and BC035 were synthesized in house as previously described (Wang et al., 2013b; Wu et al., 2014). Amantadine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO).

Plaque Assay. Plaque assays were carried out as previously described (Jing et al., 2008; Bahnunik et al., 2009), except MDCK cells expressing ST6Gal I were used instead of regular MDCK cells. In brief, a confluent monolayer of ST6Gal I MDCK cells was incubated with ~100-pfu virus samples in DMEM with 0.5% bovine serum albumin for 1 hour at 4°C, then 37°C for 1 hour. The inocula were removed, and the cells were washed with phosphate-buffered saline (PBS). The cells were then overlaid with DMEM containing 1% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) and N-acetyl trypsin (2.0 μg/ml). To examine the effect of the compounds on plaque formation, the overlay media were supplemented with compounds at testing concentrations. At day 2 after infection, the monolayers were fixed and stained with crystal violet dye solution (0.2% crystal violet, 20% methanol).

Cytotoxicity Assay and Cytopathic Effect Assay. The cytotoxicity of compounds was determined in MDCK cells. Cells were treated with different concentrations of WJ379 or BC035 and stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours. A virus-induced cytopathic effect (CPE) assay was performed as previously described (Atkins et al., 2012; Beyleveld et al., 2013). In brief, MDCK cells were grown in DMEM supplemented with 10% FBS in 96-well plates. When 90% confluence was reached, cells were washed once with 100 μl of PBS buffer and infected with virus at multiplicity of infection (MOI) of 0.002. Then DMEM (without FBS) with indicated concentrations of compounds was added. After incubating at 37°C for 72 hours, 20 μl of 5 μg/ml MTT reagent was added to each well, and the plates were incubated at 37°C for 4 hours. Then the cells were washed once with PBS, and 100 μl of 0.1 N HCl in isopropanol was added. The absorbance was read at 560 nm with a plate reader. The assays were repeated in quadruplicate.

Serial Passage Experiments and Resistance Mutation Identification. MDCK cells were infected with A/WSN/33 (H1N1) virus or A/WSN/33 N31S (H1N1) at a MOI of 0.001 for 1 hour. Then the inoculum was removed, and MDCK cells were incubated with compounds at concentrations shown in Table 2 or in the absence of compounds to evaluate cell culture–adapted mutations. In the first passage, the applied compound concentrations were slightly below their corresponding EC₅₀ values. In the following passages, the compound concentrations were gradually increased, as shown in Table 2. In each passage, the viruses were harvested when significant cytopathic effect was observed, which usually takes 2–3 days after virus infection. The titers of harvested viruses were determined by plaque assay. At selected passages, as shown in Table 2, influenza M segments were subjected to sequencing, and S31N-resistant mutations identified from serial drug-passage experiments were confirmed in quintuplicate using the CPE assay described earlier except that neutral red was used for staining instead of MTT. In brief, MDCK cells were seeded in 96-well plates for 24 hours. Seven serial
half-logarithmic dilutions of WJ379 and oseltamivir carboxylate were prepared and added to MDCK cells infected with influenza A virus A/WSN/33 (H1N1) (MOI 0.001) in the 96-well plate. After incubation at 37°C in a humidified incubator with 5% CO₂ for 72 hours, the medium was removed, and cells were stained with 100 μM of neutral red in DMEM (40 mg/l) for 4 hours. After washing once with 150 μl of PBS, the neutral red was extracted from cells with 150 μl of neutral red destain solution (ethanol/water/glacial acetic acid: 50/49/1 (v/v/v)) to form a homogenous solution (Repetto et al., 2008). The absorbance at 540 nm was measured using a Multiskan FC Microplate Photometer (Fisher Scientific, Houston, TX). Drug-drug interaction was evaluated using the MacSynergy II software program, which automatically calculates volumes of synergy or antagonism for each three-dimensional plot of data (Prichard and Shipman, 1990; Tarbet et al., 2012). The value ranges 0–25, 25–50, 50–100, or over 100 μM²% (μM × μM × %) are considered as insignificant, minor but significant, moderate, or strong synergy or antagonism, respectively. Synergy plots were made at the 95% confidence limit.

M2 Sequence Analysis. All M2 protein full-length sequences of influenza virus A isolated from humans were obtained from the Influenza Research Database (http://www.fludb.org, accessed on January 18, 2016). A total of 18,393 M2 sequences were aligned by MEGA6 (www.megasoftware.net) using the ClustalW method. The frequencies of the appearance of substitutions of L26I and I32T were identified. As these human clinical isolates did not produce countable plaques using regular MDCK cells (data not shown), an engineered MDCK cell line overexpressing the human ST6Gal I was used for this purpose as it expresses N-acetyl sialic acid linked to galactose by an α-2,6-linkage on its cell surface (Hatakeyama et al., 2005). This cell line mimics human epithelial cells and is the only cell line that is suitable to perform plaque assays for human influenza viruses (Matrosovich et al., 2003; Hatakeyama et al., 2005). With this cell line and optimized conditions, we were able to get uniform and reproducible plaques with all four strains. Representative images of plaque assays with A/California/07/2009 (H1N1) and A/Texas/04/2009 (H1N1) (amantadine-resistant, oseltamivir-resistant) are shown in Fig. 1. The antiviral EC₅₀ values of compound WJ379 were in the submicromolar range, whereas for compound BC035, the EC₅₀ values were in the low micromolar range (Fig. 1, D and E). Of note, oseltamivir carboxylate only reduced plaque size, but not plaque number, whereas M2-S31N inhibitors completely inhibited plaque formation.

### Results

**WJ379 and BC035 Are Potent M2-S31N Channel Blockers and Antivirals against S31N Containing the A/WSN/33 (H1N1) Virus.** We previously reported on two classes of S31N inhibitors, S31N-specific inhibitors (Wang et al., 2013b; Li et al., 2016) and S31N/WT dual inhibitors (Wang et al., 2013a; Wu et al., 2014), which are represented by WJ379 and BC035, respectively. To select nontoxic drug concentrations for the following serial viral passage experiments, the cytotoxicity and selectivity indexes of these two compounds were determined. The CC₅₀ values of WJ379 and BC035 were 125 and 123 μM, respectively (Table 1). Their antiviral activities were further quantified in both plaque assays and cytopathic effect assays using the influenza A virus strain A/WSN/33 (H1N1) (Table 1). Both the plaque assay and CPE assay were standard antiviral assays used to determine the efficacy of antiviral drugs. The difference is that the plaque assay quantifies the change of viral titer upon drug treatment, whereas the CPE assay quantifies the change of cell survival upon viral infection and drug treatment (Atkins et al., 2012; Beylefeld et al., 2013). In general, there is a positive correlation between these two assays. In the plaque assays, the EC₅₀ values of WJ379 and BC035 against the A/WSN/33 (H1N1) virus were 0.49 and 2.2 μM, respectively, which were consistent with those of WJ379 and BC035 in the CPE assays (0.36 and 6.20 μM, respectively) (Table 1). The selective indexes of WJ379 and BC035 were 255 and 56, respectively. On the basis of these results, the highest drug concentrations used for the following serial viral passage experiments were set to 5 and 16 μM for WJ379 and BC035, respectively, which conferred minimal cytotoxicity.

**M2-S31N Inhibitors Are Potent Antivirals against Human Clinical Isolates of Multidrug-Resistant Influenza A Viruses.** To determine whether the potent antiviral activity of WJ379 and BC035 on A/WSN/33 (H1N1) can be extended to other S31N-containing influenza A viruses, WJ379 and BC035 were tested in plaque assays against human clinical isolates of multidrug-resistant influenza A viruses, including A/California/07/2009 (H1N1) (amantadine-resistant, oseltamivir-sensitive), A/Texas/04/2009 (H1N1) (amantadine-resistant, oseltamivir-resistant), A/Denmark/524/2009 (H1N1) (amantadine-resistant, oseltamivir-sensitive), and A/Denmark/528/2009 (H1N1) (amantadine-resistant, oseltamivir-resistant). As these human clinical isolates did not produce countable plaques using regular MDCK cells (data not shown), an engineered MDCK cell line overexpressing the human ST6Gal I was used for this purpose as it expresses N-acetyl sialic acid linked to galactose by an α-2,6-linkage on its cell surface (Hatakeyama et al., 2005). With this cell line and optimized conditions, we were able to get uniform and reproducible plaques with all four strains. Representative images of plaque assays with A/California/07/2009 (H1N1) and A/Texas/04/2009 (H1N1) are shown in Fig. 1. The antiviral EC₅₀ values of compound WJ379 were in the submicromolar range, whereas for compound BC035, the EC₅₀ values were in the low micromolar range (Fig. 1, D and E). Of note, oseltamivir carboxylate only reduced plaque size, but not plaque number, whereas M2-S31N inhibitors completely inhibited plaque formation.

**M2-S31N Inhibitors Have a Higher In Vitro Genetic Barrier to Drug Resistance than Amantadine.** To evaluate the in vitro genetic barrier to drug resistance of these two S31N inhibitors, serial viral passage experiments in MDCK cells were performed with the influenza A/WSN/33 (H1N1) virus. The drug concentrations of BC035 and WJ379 applied at passage 1 were slightly below their EC₅₀ values and were gradually increased in subsequent passages (Table 2). In the first two passages, the progeny viruses maintained full sensitivity to their respective compounds, as indicated by the EC₅₀ values (Table 2). At passage 3, the EC₅₀ values of WJ379 and BC035 were moderately increased by 4.2- and 2.8-fold, respectively. Significant resistance was observed at passage 4 in which the EC₅₀ values increased more than

<table>
<thead>
<tr>
<th>M2-S31N channel inhibition</th>
<th>85%/65%</th>
<th>76%/N.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque assay</td>
<td>0.49</td>
<td>2.2</td>
</tr>
<tr>
<td>EC₅₀ (μM)</td>
<td>0.36</td>
<td>6.20</td>
</tr>
<tr>
<td>Cellular toxicity</td>
<td>125</td>
<td>123</td>
</tr>
<tr>
<td>IC₅₀ (μM)</td>
<td>255</td>
<td>56</td>
</tr>
</tbody>
</table>

N.T., not tested; SI, selectivity index.

*W2-S31N channel inhibition was measured using two-electrode voltage clamp assay as described (Wang et al., 2013b; Wu et al., 2014). The data are expressed as percentage inhibition at 100/30 μM compound concentration.

*Reported in Wang et al. (2013b).

*Reported in Wu et al. (2014).

*EC₅₀ in plaque assays was determined with the A/WSN/93 (H1N1) virus.

*EC₅₀ in CPE assay was determined with the A/WSN/33 (H1N1) virus using MTT.

*IC₅₀ was assayed using MTT after incubating MDCK cells with increasing concentrations of compounds for 72 hours.

*SI was calculated by dividing IC₅₀ values over plaque assay EC₅₀ values.
10-fold in both cases. To test the persistence of the resulting drug-resistant mutants, the viruses were continuously pas-
saged for two more rounds in the presence of compounds, and
then five rounds in the absence of compounds. We found that
the viruses at passage 11 were resistant to both WJ379 and
BC035.

To rule out the possibility that the observed resistance was
due to cell culture–adapted mutations, the A/WSN/33 (H1N1)
virus was also passed in the absence of compound, and the
drug sensitivities of the resulting viruses against WJ379 and
BC035 were tested in plaque assays. At passage 6, the EC50
values of WJ379 and BC035 were 0.65 and 2.9 μM, respec-
tively, which were similar to their corresponding EC50 values
at passage 0, indicating that the elevated EC50 values in serial
passage experiments in the presence of WJ379 and BC035
were not due to the virus adaption in cell culture.

As a comparison, we carried out a serial viral pas-
sage experiment with the A/WSN/33 N31S (H1N1) virus
against amantadine. The A/WSN/33 N31S (H1N1) virus is
amantadine-sensitive. The EC50 value of amantadine against
the A/WSN/33 N31S virus (H1N1) was 0.26 μM. Amantadine
was applied at 0.2 μM in passage 1 (Table 2), which was close
to its EC50 value. This condition was chosen to make a side-by-
side comparison with the passage experiments of WJ379 and
BC035. In contrast to the emergence of complete resistance to
WJ379 and BC035 by the A/WSN/33 (H1N1) virus at passage
4, complete amantadine resistance against the WSN N31S
(H1N1) virus was observed as early as passage 1 (30% plaque
remaining even when treated with 30 μM amantadine). These
results indicate that M2-S31N inhibitors, such as WJ379 and
BC035, have a higher genetic barrier to resistance than
amantadine.

Sequencing M2 Genes Reveals Mutations that Confer
Resistance to M2-S31N Inhibitors. To identify how M2-
S31N evolved to become resistant to WJ379 and BC035, the
M2 genes from viruses at passages 0, 3, 4, 5, 6, and 9 were
reverse-transcribed and sequenced. The sequencing traces are
shown in Fig. 2, and the resulting M2 mutations at each
passage are shown in Table 2. For WJ379, no M2 mutation
was identified up to passage 3. At passage 4, a substantial
population of L26I mutation emerged, and its percentage
continued to rise throughout passages 5 and 6 and completely

Fig. 1. Antiviral efficacy of compounds WJ379 and BC035 on clinically isolated influenza A viruses in plaque assay. The assay was carried out with
(H1N1). (C) Plaque assays of BC035 on A/California/07/2009 (H1N1). (D) Dose-response curves of WJ379 on various influenza A strains. (E) Dose-
response curves of BC035 on various influenza A strains.
Channel blockage is expressed as percentage of current property of M2 channels as well as their drug inhibition (Pinto N31D/I32T (double mutations). The TEVC assay is one of the I32T (single mutation), M2-N31D (single mutation), and M2-L26I (single mutation), M2-N31S (single mutation), and M2-L26I (100%) mutations Have Reduced Drug Sensitivity to M2-S31N whereas another mutation, I32T, which emerged at passage 5, overtook L26 at passage 9. In addition, another mutation, N31S, was also identified at passage 6. However, this mutant reverted back to N31 (WT) at passage 9 when the drug selection pressure was removed. Nevertheless, the virus containing the single mutant L26I at passage 9 was still resistant to WJ379 (Fig. 3A). For the mutants selected by BC035, 100\% inhibition was observed even when amantadine was added at 30 \( \mu \text{M} \) (30\% of plaque remained compared with no drug control).

### TABLE 2

<table>
<thead>
<tr>
<th>Passage No.</th>
<th>WJ379 EC50</th>
<th>Mutation</th>
<th>BC035 EC50</th>
<th>Mutation</th>
<th>Ama EC50</th>
<th>Resb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{M} )</td>
<td>( \mu \text{M} )</td>
<td>( \mu \text{M} )</td>
<td>( \mu \text{M} )</td>
<td>( \mu \text{M} )</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.49</td>
<td>N31</td>
<td>2.2</td>
<td>N31</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.30</td>
<td>N.T.</td>
<td>2.0</td>
<td>N.T.</td>
<td>0.20</td>
<td>Resb</td>
</tr>
<tr>
<td>2</td>
<td>0.60</td>
<td>N.T.</td>
<td>4.0</td>
<td>N.T.</td>
<td>Not continued after passage 1 due to significant resistance</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.20</td>
<td>N.T.</td>
<td>8.0</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.50</td>
<td>L26I</td>
<td>16.0</td>
<td>N31D/I32T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>L26I</td>
<td>16.0</td>
<td>N31D/I32T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>N31D/L26I</td>
<td>16.0</td>
<td>N31D/I32T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>N.T.</td>
<td>0</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
<td>I32T (100%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
<td>22.7</td>
<td>I32T (100%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>N.T.</td>
<td>0</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>&gt;30</td>
<td>0</td>
<td>N.T.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ama, amantadine; Res, rimantadine; N.T., not tested.

*Complete resistant viruses were selected as shown by the plaque-reduction assays. No complete plaque inhibition was observed even when amantadine was added at 30 \( \mu \text{M} \) (30\% of plaque remained compared with no drug control).*

**M2 Mutants Selected from Serial Passage Experiments Have Reduced Drug Sensitivity to M2-S31N Inhibitors in TEVC Assays.** In the aforementioned serial passage experiments, we identified two amino acid substitutions, L26I and N31S, for WJ379 and two amino acid substitutions, N31D and I32T, for BC035. As we cannot distinguish whether these two substitutions occur in the same M2 protein or at two different M2 proteins, we therefore generated all possible M2 constructs and tested their drug sensitivity in TEVC assays. Specifically, in the case of WJ379, we recorded the channel blockage of WJ379 against M2-S31N/L26I (single mutation), M2-N31S/L26I (double mutations) in TEVC assays. In the case of BC035, we recorded its channel blockage against M2-S31N/I32T (single mutation), M2-N31D (single mutation), and M2-N31D/I32T (double mutations). The TEVC assay is one of the gold-standard assays to study the proton conductance property of M2 channels as well as their drug inhibition (Pinto et al., 1992; Wang et al., 1993; Pinto and Lamb, 2006). Channel blockage is expressed as percentage of current inhibition at a 2-minute time point after compound treatment. Figure 3 shows the percentage of current inhibition of the M2 mutants by 100 \( \mu \text{M} \) WJ379 or 100 \( \mu \text{M} \) BC035. For the M2 mutants selected by WJ379, M2-N31S was nearly completely resistant to WJ379, as its conductance was inhibited less than 10\% by 100 \( \mu \text{M} \) WJ379 (Dong et al., 2015). Another single mutant, M2-S31N/L26I, remained sensitive to WJ379, although it was less sensitive than the original M2-S31N. The percentage of current inhibitions for M2-S31N/L26I and M2-S31N were 64\% and 82\%, respectively, in the presence of 100 \( \mu \text{M} \) WJ379. Dose-response experiments revealed that the IC\(_{50}\) of WJ379 against the M2-S31N/L26I mutant was 72.9 \( \mu \text{M} \), in contrast to 27.9 \( \mu \text{M} \) against M2-S31N (Fig. 4A). Similar to the results of the single mutant M2-N31S, the double mutant M2-N31S/L26I was nearly completely resistant to WJ379, showing 11\% inhibition by 100 \( \mu \text{M} \) WJ379 (Fig. 3A). For the mutants selected by BC035, 100 \( \mu \text{M} \) BC035 had minimal inhibition against the M2-N31D single mutant and the M2-N31D/I32T double mutant (Fig. 3B). However, the M2-S31N/I32T single mutant remained sensitive to BC035 (Fig. 3B). The IC\(_{50}\) of BC035 against M2-S31N/I32T was 50.5 \( \mu \text{M} \), only a 2.5-fold increase compared with the IC\(_{50}\) value of BC035 against M2-S31N (Fig. 4B).

**Correlation between Electrophysiological TEVC IC\(_{50}\) Values with Antiviral EC\(_{50}\) Values Reveals the Importance of Drug Inhibition Kinetics.** In general, there is a positive correlation between an M2 channel blocker’s electrophysiological IC\(_{50}\) value and its antiviral EC\(_{50}\) value. A more potent channel blocker normally has higher antiviral activity (Wang et al., 2013b; Li et al., 2016). However, when correlating the IC\(_{50}\) and EC\(_{50}\) values of WJ379 and BC035 against M2 mutants, we found different results (Table 3). The single mutant (S31N/L26I) selected by WJ379 only conferred a 2.7-fold increase in TEVC IC\(_{50}\) values (calculated based on 2-minute time points); however, it led to more than a 60-fold increase in antiviral EC\(_{50}\) value. This discrepancy led us to further investigate the kinetics of drug binding in both S31N and S31N/L26I channels. It is of note that the percentage of channel blockage was recorded 2 minutes after compound treatment at pH 5.5. An examination of the current-inhibition traces revealed that, for S31N channel inhibition, equilibrium
Fig. 2. M2 gene sequencing traces. M2 genes at passages 0, 3, 4, 5, 6, and 9 were sequenced by Sanger sequencing. The regions (residues 18–37) that cover detected mutations are shown. Mutations are indicated by arrows. Sequencings from WJ379 serial passage experiments are shown in (A–F): WJ379, passage 0 (A); WJ379, passage 3 (B); WJ379, passage 4 (C); WJ379, passage 5 (D); WJ379, passage 6 (E); WJ379, passage 9 (F). Sequencings from BC035 serial passage experiments are shown in (G–L): BC035, passage 0 (G); BC035, passage 3 (H); BC035, passage 4 (I); BC035, passage 5 (J); BC035, passage 6 (K); BC035, passage 9 (L).
was not reached at the 2-minute time point (Fig. 5A) when tested at 100 \( \mu M \), which means the true IC\(_{50} \) is actually lower than the recorded IC\(_{50} \) if enough time is given to achieve equilibrium. In contrast, for M2-S31N/L26I inhibition by WJ379 and M2-S31N/I32T inhibition by BC035, the TEVC IC\(_{50} \) values at 2-minute time points were true IC\(_{50} \) values, as equilibriums were reached before the 2-minute time point (Fig. 5, A and B). Fitting the current-conductance trace of WJ379 in inhibiting M2-S31N/L26I using a one-phase exponential decay equation gave a time constant of 8.5 seconds, which is much faster than the 45.3-second time constant in the case of M2-S31N inhibition by WJ379. Similar results were observed for BC035: the time constants for BC035 in inhibiting M2-S31N and M2-S31N/I32T were 29.5 and 5.5 seconds, respectively. Another example of slow drug inhibition kinetics is amantadine in inhibiting the WT-M2 channel (Fig. 5C).

To get the true IC\(_{50} \) values of amantadine in inhibiting M2-WT and WJ379 in inhibiting M2-S31N due to their slow binding kinetics, we chose to record the percentage of current inhibition at 5- and 10-minute time points instead of the regular 2-minute time point. As shown in Fig. 6A, when the WT channel was treated with amantadine, the IC\(_{50} \) values were 12.5, 4.7, and 2.5 \( \mu M \) when the percentage of current inhibition data were collected at 2-, 5-, and 10-minute time points, respectively. A representative TEVC trace of amantadine in inhibiting M2-WT at 10 \( \mu M \) was shown in Fig. 6B, which clearly showed different percentage of inhibition at 2-, 5-, and 10-minute time points. Similarly, in the case of M2-S31N inhibition by WJ379 (Fig. 6C), the IC\(_{50} \) values were 27.1, 8.7, and 4.0 \( \mu M \) when recorded at 2, 5, and 10 minutes, respectively, and the representative TEVC trace of WJ379 in inhibiting M2-S31N at 10 \( \mu M \) is shown in Fig. 6D. In summary, for the M2-WT channel inhibition by amantadine and the M2-S31N channel inhibition by WJ379, the IC\(_{50} \) value in TEVC assays at 2-minute time points underestimates their true IC\(_{50} \) values. After adjusting the binding kinetics, there appears to be a good correlation between the IC\(_{50} \) and EC\(_{50} \) fold increase from passage 0 to passage 11 (Table 3): the IC\(_{50} \) increased 18.2-fold (calculated based on 10-minute time points), and the EC\(_{50} \) increased more than 60-fold. The percentage of current inhibition was typically recorded at 2-minute time points because oocyte cells used for the current recording cannot survive under pH 5.5 for an extended period of time. To get data at 5 and 10 minutes, repetitive recordings had to be performed with multiple oocytes.

In summary, although M2-S31N/L26I and M2-S31N/I32T remained sensitive to WJ379 and BC035, respectively, the fast binding kinetics rendered these two drugs much less effective. The M2-S31N Inhibitor WJ379 Shows a Synergistic Antiviral Effect with Oseltamivir. One useful strategy to reduce the pace of drug resistance development is combination therapy, which has established efficacy in the treatment of human immunodeficiency virus infection (Gulick et al., 1997, 1998; De Clercq, 2007). Unlike M2-S31N inhibitors, oseltamivir is a neuraminidase inhibitor, which interrupts the viral replication cycle by preventing virus release from infected cell surfaces. Therefore, oseltamivir is expected to show a synergistic effect with M2-S31N inhibitors in combination therapy. To test this hypothesis, we combined WJ379 and oseltamivir carboxylate using the two-drug combination protocol (Hayden, 2013; Dunning et al., 2014). The efficacy of oseltamivir carboxylate alone was first determined by CPE assay. The EC\(_{50} \) of oseltamivir carboxylate against A/WSN/33 (H1N1) in MDCK cell culture was 18.4 nM in the CPE assay. In the combination therapy, a matrix was created to combine 10-10,000 nM WJ379 with 1-1000 nM oseltamivir carboxylate. Compounds were added to confluent MDCK cells that were infected with the A/WSN/33 (H1N1) virus at MOI 0.01. Cell viability was evaluated at 72 hours after infection by neutral red staining. The synergistic effect was calculated with MacSynergy II software (Prichard and Shipman, 1990; Prichard et al., 1993; Smee et al., 2010). As shown in Fig. 7, a region of significant synergy (29.0–42.4 \( \mu M \)) was observed for WJ379 and oseltamivir carboxylate at concentrations of 320–1000 and 32–100 nM, respectively. The calculated net
effect across the entire surface was a volume of synergy of 166, indicating a strong synergy.

Discussion

Despite the existence of influenza vaccines and antivirals, influenza virus infection is still one of the leading causes of death in the United States (Thompson et al., 2003, 2004). Among the two classes of Food and Drug Administration–approved anti-influenza drugs, adamantanes are no longer recommended, which leaves oseltamivir as the only orally bioavailable drug on the market. With the continuous prescription of oseltamivir, it will only be a matter of time before predominant flu strains become resistant to it. The alarming fact is that oseltamivir-resistant strains have been continuously reported, and certain strains appear to adapt to the fitness of transmission among humans, which could lead to the next influenza pandemic (Hurt et al., 2009; Kelso and Hurt, 2012; Hurt, 2014). In addressing this unmet medical need, we revisited the M2 proton channel and aim to develop the next generation of antivirals by targeting the M2-S31N mutant. M2-S31N is the predominant mutant among currently circulating influenza A viruses, including those that are resistant to oseltamivir (Dong et al., 2015). Thus, targeting M2-S31N is expected to yield broad-spectrum antivirals that are active against both oseltamivir-sensitive and oseltamivir-resistant strains (Wang, 2015). To test this hypothesis, we chose two M2-S31N inhibitors, WJ379 and BC035, which represent S31N-specific inhibitors and S31N/WT dual inhibitors, respectively, and tested their antiviral efficacy in plaque assays. It was found that WJ379 had potent antiviral activity against all four human clinical isolates with submicromolar efficacy, including two 2009 pandemic strains that are resistant to amantadine and rimantadine (A/Texas/04/2009 and A/Denmark/528/2009). BC035 similarly inhibited both oseltamivir-sensitive and oseltamivir-resistant influenza A strains with low micromolar EC50 values. These results highlight the great therapeutic potential of the first-in-class M2-S31N inhibitors: they can serve as the second line of defense should oseltamivir fail to confine the next influenza outbreak caused by influenza A strains.

With these tool compounds in hand, we began to investigate the potential issue of drug resistance. This is critical, as addressing the efficacy is only the first step in developing antiviral drugs. Ideal antiviral drugs should also bear a high genetic barrier to drug resistance such that they can be used for a longer period of time. The genetic barrier is defined in this study as the ease of resistance generation, although it is also defined in other reports as the number of mutations needed for resistance (Vingerhoets et al., 2005; Fofana et al., 2013). The first-generation M2 channel blockers amantadine and rimantadine were rendered ineffective due to the emerging M2-S31N mutant. Related to the second generation of M2 channel blockers, which are the M2-S31N inhibitors, the concern is whether the M2-S31N channel will similarly develop resistance to S31N inhibitors. To address this, two S31N inhibitors, WJ379 and BC035, were subjected to serial passage experiments in an attempt to select resistant viruses. Not surprisingly, resistant strains were selected for both compounds at passage 4. In comparison with the genetic

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<td>TEVC IC50 (μM)</td>
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Fig. 5. Representative TEVC recording traces showing different inhibition kinetics. (A) WJ379 in inhibiting M2-S31N and M2-S31N/L26I channels. WJ379 inhibited the M2-S31N channel much slower than the M2-S31N/L26I channel. (B) BC035 in inhibiting M2-S31N and M2-S31N/I32T channels. BC035 inhibited the M2-S31N channel much slower than the M2-S31N/I32T channel. The inhibition kinetics time constant was determined by fitting the period of recording trace when the compounds are present to the one-phase exponential decay equation \[ f(t) = A \exp(-t/T1 + C) \] in Clampfit 10.3. (C) Amantadine (Ama) in inhibiting the M2-WT channel.
barriers to drug resistance for the two classes of Food and Drug Administration–approved anti-influenza drugs, M2-S31N inhibitors outperformed amantadine and are only slightly less effective than oseltamivir. In general, complete resistance to oseltamivir was generated around passage 5 (Triana-Baltzer et al., 2011; Renzette et al., 2014). Sequencing the resulting resistant viruses revealed interesting findings. For the S31N-specific inhibitor WJ379, M2-S31N simply reverted back to N31S to become resistant to WJ379. This mutation is a dominant positive mutation, as the M2-N31S/L26I double mutant was also completely resistant to WJ379. The single mutant M2-S31N/L26I alone was only partially resistant to WJ379. For the M2-S31N/WT dual inhibitor BC035, we observed similar phenomena. As BC035 is a potent inhibitor of both the S31N and the S31 (WT) channels, M2-S31N could not revert back to S31 to become resistant to it; instead, it changed to N31D. N31D is also a dominant positive mutation, and the M2-N31D/I32T was completely resistant to BC035. The single mutation M2-S31N/I32T remained sensitive to BC035, and this mutation only caused a 2-fold EC50 increase. Interestingly, both of these highly resistant mutants, N31S and N31D, appeared to be less fit than the N31 mutant in cell culture; both N31S and N31D reverted back to N31 after releasing drug selection pressure.

Fast drug-binding kinetics were observed for both WJ379 in inhibiting M2-S31N/L26I and BC035 in inhibiting M2-S31N/I32T. It is possible that M2-S31N/L26I and M2-S31N/I32T might either adapt different structures or have different dynamics than M2-S31N, which allows drugs to easily escape from the channel cavity. For example, M2-S31N/L26I and M2-S31N/I32T might have an enlarged N-termini channel entrance such that drug can easily escape from the channel. Alternatively, M2-S31N/L26I and M2-S31N/I32T might be more dynamic than M2-S31N; thus, drugs have a higher probability to escape the channels when they adapt the Openout-Closedin conformation (Khurana et al., 2009).

Although resistant mutants (L26I and I32T) were selected from serial passage experiments in cell culture under the drug selection pressure of WJ379 and BC035, respectively, both mutants appear to be rare among human influenza A viruses. We retrieved and analyzed all of the human influenza A virus M2 sequences from the Influenza Research Database (http://www.fludb.org, accessed on January 18th, 2016). L26I occurred 70 times, and I32T only occurred twice out of 18,393 M2 sequences. These results indicate that viruses carrying either of these two mutants might have a reduced fitness of transmission among humans, although more stringent experiments need to be followed to test their fitness and transmissibility.

As resistance development is, unfortunately, inevitable, one standard approach to delay resistance evolution under drug...
selective pressure is combination therapy. Combination therapy has a proven track record of reducing resistance evolution and side effects, and it is highly recommended for people with compromised immune systems who might need extended anti-

viral treatment to clear the viruses (Ison, 2013). As an example, the triple-drug combination therapy, commonly known as highly active antiretroviral therapy, was shown to be more effective over single- or double-drug combination therapy in suppressing human immunodeficiency virus drug resistance (Gulick et al., 1997, 1998; De Clercq, 2007). Therefore, exploring a double-drug combination of M2-S31N inhibitors with oseltamivir in the prevention and treatment of influenza infection is a rational approach to mitigate drug resistance. The need for combination therapy is further justified because monotherapy with oseltami-

vir was suboptimal in the treatment of H5N1-infected patients, with a mortality rate as high as 60% (Abdel-Ghafar et al., 2008; Kandun et al., 2008). It has been shown in a number of studies that a combination of 2 inhibitors (amantadine or rimantadine) with neuraminidase inhibitors (oseltamivir carboxylate or per-

amivir), ribavirin, or a combination of all three was in general more effective in vitro and in vivo than either drug alone (Kandun et al., 2008; De Clercq, 2007). Therefore, exploring a double-drug combination of M2-S31N inhibitors with oseltamivir, coupled with the strong synergistic effect of M2 inhibitors with oseltamivir, may be a more effective option for controlling influenza viruses in the future.

In summary, the potent, broad-spectrum antiviral activities of M2-S31N inhibitors against currently circulating influenza A viruses, coupled with the strong synergistic effect of M2-

inhibitors with oseltamivir, provide compelling evidence for further development of these second-generation M2 channel blockers.

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Authorship Contributions

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Conducted experiments: Ma, Zhang.

Performed data analysis: Ma, Zhang, Wang.

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