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Profiling the *in vitro* drug-resistance mechanism of influenza A viruses towards the AM2-S31N proton channel blockers

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Abstract

The majority of human influenza A viruses currently in circulation carry the amantadine-resistant AM2-S31N channel mutation. We previously discovered a series of AM2-S31N inhibitors with potent antiviral activity against both oseltamivir-sensitive and -resistant influenza A viruses. To understand the drug-resistance mechanism of AM2-S31N inhibitors, we performed serial viral passage experiments using the influenza virus A/California/07/2009 (H1N1) to select drug-resistant AM2 mutations against two representative AM2-S31N channel blockers (**1** and **2**). Unlike amantadine, which gives rise to resistance after a single passage, compounds **1** and **2** selected for partially resistant viruses at passages 05 and 04 with a V27I and L26I mutation, respectively. This appears to suggest compounds **1** and **2** have a higher genetic barrier to resistance than amantadine at least in cell culture. Passage with a higher drug concentration of compound **2** selected higher level resistant viruses with a double mutant L26I + A30T. The mechanism of resistance and replication fitness for mutant viruses were evaluated by electrophysiology, reverse genetics, growth kinetics, and competition assays. AM2-S31N/V27I and AM2-S31N/L26I channels achieved similar specific proton conductance as AM2-S31N, but the AM2-S31N/L26I/A30T triple mutant had drastically reduced specific proton conductance. Viral replication fitness of AM2-S31N/V27I and AM2-S31N/L26I double mutant viruses were similar to AM2-S31N containing viruses in cell culture. However, AM2-S31N/L26I/A30T viruses displayed attenuated growth as well as inability to compete with AM2-S31N viruses. The results herein offer insight regarding the resistance mechanism of AM2-S31N inhibitors, and may help guide the design of the next-generation of AM2-S31N inhibitors with a higher genetic barrier to drug resistance.

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Keywords

AM2-S31N; isoxazole; resistance; proton channel; influenza

1. INTRODUCTION

Influenza A viruses are a diverse group of respiratory pathogens that cause acute infections in birds and mammals including humans (Monto and Webster, 2013). In 2009, the emergence of a novel pandemic H1N1 influenza virus (A/H1N1/pdm09) led to a dramatic spike in hospitalizations and deaths in the United States, particularly among young adults (Shrestha et al., 2011). For emerging influenza strains such as A/H1N1/pdm09, antiviral therapies are the primary treatment option during the initial phases of a pandemic. At present, the two classes of FDA-approved antivirals are the AM2 channel inhibitors, amantadine and rimantadine (Wang et al., 2015), and neuraminidase (NA) inhibitors, oseltamivir, zanamivir, and peramivir (De Clercq, 2006; Moscona, 2005). The majority of H1N1 strains currently in circulation are resistant to amantadine and rimantadine because of the S31N mutation in the AM2 channel (Kolocouris et al., 2014; Krumbholz et al., 2009). As such, CDC discourages the use of amantadine and rimantadine for the prophylaxis and the treatment of influenza virus infection. Fortunately unlike the 2008–2009 oseltamivir-resistant seasonal H1N1 virus (Zaraket et al., 2010), the A/H1N1/pdm09 remained largely oseltamivir susceptible, although sporadic cases of oseltamivir-resistance are continuously reported (Hurt et al., 2009; Hurt AC, Hardie K, Wilson NJ, Deng YM, Osbourn M, Gehrig N, Kelso, 2011; Lackenby et al., 2011). However, even in susceptible strains, the overall effectiveness of oseltamivir has been controversial (Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, Spencer EA, Onakpoya IJ, Mahtani KR, Nunan D, Howick J, Heneghan, 2015). Therefore, there is an urgent need to develop additional antivirals to lift our dependency on a single class of influenza antivirals.

Influenza AM2 proton channel, the drug target of amantadine and rimantadine, plays a vital role in the viral replication cycle (Wang et al., 2015). AM2 channels consist of four identical 97-amino acid long subunits that oligomerize to form a homotetramer within the viral envelope (Sakaguchi et al., 1997). The conserved H37XXXW41 motif is essential for the channel conductance (Hu et al., 2011; Ma and Wang, 2018) and proton selectivity (Balannik et al., 2010; Ma et al., 2009). AM2 is activated by the low pH within early endosomes, whereby protonation of histidine 37 and opening of the tryptophan 41 gate facilitates proton flux into intracellular virions (Acharya et al., 2010; Cady et al., 2009). This process is essential for facilitating nuclear import of the viral ribonucleoprotein (vRNP) complex during viral replication. AM2 proton conductance plays a secondary role by equilibrating the pH in the Golgi complex for proper folding of viral hemagglutinin in the later stages of viral replication. The dual function of AM2 in viral replication at early and late stages of replication renders AM2 a compelling antiviral drug target.

Although relatively conserved, AM2 channels evolve mutations in the pore-lining residues to obstruct drug binding. Amantadine resistance in transmissible viruses can be caused by conservative (V27A) or nonconservative (L26F and S31N) mutations in the AM2 channel.

However, among transmissible viruses, AM2-S31N channel is the most prominent (Dong et al., 2015; Durrant et al., 2015). Amantadine resistance caused by mutations in other residues (such as 30 or 34) have been reported within individual viral particles, but have not represented a sizable population in annual surveillance reports (Dong et al., 2015). In the case of the AM2-S31N mutation, the mechanism for resistance is due to a bulkier, polar asparagine which destabilizes amantadine binding through steric interference as well as by reducing the hydrophobic interaction with the adamantane cage. Structure-guided design of AM2-S31N inhibitors has led to a series of biologically active small molecule antivirals such as the isoxazole-substituted amantadine analogs **1** and **2** (Duque et al., 2011; Li et al., 2017, 2016; Wang et al., 2013; Wu et al., 2014). AM2-S31N inhibitors structurally related to compounds **1** and **2** have been shown to inhibit multiple strains of AM2-S31N influenza A viruses, including both oseltamivir-sensitive and -resistant strains, with a high selectivity index (Li et al., 2017; Wang et al., 2018). As representative examples of our rationally designed AM2-S31N inhibitors, compounds **1** and **2** were chosen for the drug resistant mechanistic studies due to their high antiviral potency and selectivity index.

Antiviral drugs require a high genetic barrier to drug resistance in order to have sustainable therapeutic application. Since AM2-S31N inhibitors demonstrate promising antiviral activity, we set out to investigate the evolutionary course by which susceptible influenza A strains become resistant. Our group and others have found that the evolution of AM2-wild-type influenza A viruses in the presence of amantadine coincide with a low genetic barrier to resistance. Given these circumstances, there are concerns that viruses might evolve resistance against AM2-S31N inhibitors under similar drug selection pressure. To address this question, we investigated the drug-resistance mechanism of two representative AM2-S31N inhibitors **1** and **2** using a combination of electrophysiology and virology techniques. We chose the AM2-S31N-containing 2009 pandemic-like influenza virus A/California/07/2009 (H1N1) as a model virus and performed serial viral passage experiments to select resistant mutants under the increasing drug selection pressure of compounds **1** and **2**. The A/California/07/2009 (H1N1) virus was chosen because of its clinical relevance, and similar viruses are still in circulation among human in recent years. Interestingly, we found that both compounds evolved conservative mutations in the AM2 channel pore after 04 to 05 passages with reduced drug sensitivities. Higher level of resistance was obtained only after exceeding drug selection pressure using compound **2** (100-fold of EC₅₀), and a triple mutant S31N/L26I/A30T was identified to account for the higher level of resistance. However, the AM2-S31N/L26I/A30T triple mutant channel had significantly reduced specific proton conductance than the AM2-S31N proton channel. The mutant virus harboring the AM2-S31N/L26I/A30T triple mutant also had attenuated replication fitness and was not able to compete against AM2-S31N containing virus. In summary, the results presented in this study appear to suggest that AM2-S31N inhibitors have a higher genetic barrier to drug resistance than amantadine at least in cell culture, motivating further drug discovery efforts to target the AM2-S31N channel.

2. MATERIALS AND METHODS

2.1. Cells and viruses

MDCK, A549, and HEK293T cells were maintained using standard cell culture procedures. MDCK cells expressing the human beta-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal1) were used for viral infections (Hatakeyama et al., 2005). Influenza A/California/07/2009 (H1N1) was amplified in ST6Gal1-expressing MDCK cells and stored with 0.5% BSA at -80°C . Recombinant A/Udorn/1972 (rH3N2) viruses were generated as previously described (Takeda et al., 2002). Briefly, HEK293T cells were transfected with eight pHH21 plasmids (NS, M, NA, NP, H3, PA, PB1, PB2) and four pcDNA plasmids (PB1, PB2, PA, NP) using TransIT reagent (Mirus Bio LLC) in reduced serum Opti-MEM (Gibco). After transfected HEK293T cells were incubated for 8–15 h at 37°C in a 5% CO_2 atmosphere, the media was exchanged with Opti-MEM supplemented with $2\mu\text{g/ml}$ N-acetyl trypsin. After an additional 6–14 h incubation, transfected HEK293T cells were re-suspended and co-cultured with freshly passaged MDCK cells and incubated for an additional 24 h. Recovered viruses were stored with 0.5% BSA at -80°C . AM2 mutations were introduced into the M pHH21 plasmid using QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA).

2.2. Plaque reduction assay

Plaque reduction assays were performed in ST6Gal1-expressing MDCK cells as previously reported (Hu et al., 2017). Briefly, confluent cells were washed with PBS containing magnesium and calcium and infected with virus diluted in Dulbecco's modified Eagle's medium (DMEM) with 0.5% BSA for a final concentration of approximately 100 PFU per well. Viral infection was synchronized for 30 min at 4°C and then incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Viruses were then aspirated, washed, and incubated in a DMEM media overlay containing $2\mu\text{g/ml}$ N-acetyl trypsin and 1.2% avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) at 37°C in a 5% CO_2 atmosphere. Cells were stained 46 hpi with 0.2% crystal violet dye solution (0.2% crystal violet; 20% methanol). EC_{50} values were determined by the average plaque area per well using ImageJ software.

2.3. Serial passage

To select for drug resistant AM2 channels, serial passage experiments were performed using A/California/07/2009 (H1N1) virus and ST6Gal1-expressing MDCK cells as previously described (Ma et al., 2016). Briefly, for each passage cells were grown to a confluency of 80–95%, washed twice with PBS, then infected with A/California/07/2009 at an MOI of 0.001 in the presence or absence of compounds. Viruses were harvested by centrifugation when more than 80% detachment of cells were observed, typically around 48 hpi. Selected passages were sequenced to determine AM2 channel mutation. The percent total population of AM2 mutants were determined by averaging the peak area from the sequencing trace.

2.4. Two-electrode voltage clamp assay

AM2 gene of A/California/07/2009 (Genbank access # CY121681) was ordered from Genscript (Piscataway, NJ). The AM2 gene was subcloned into pGEM3 vector with EcoR I and Hind III sites. Selected mutations were introduced into the A/California/07/2009 AM2

pGEM3 vector using the QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). mRNA synthesis, culture, and microinjection of oocytes and electrophysiological TEVC recordings were carried out as previously described (Ma et al., 2009; Ma and Wang, 2018).

The specific conductance measurements were carried out as previously described (Balannik et al., 2010). Briefly, the membrane currents for individual oocytes expressing A/California/07/2009 AM2 WT or mutant AM2 channels were first recorded with TEVC electrophysiology technique. Oocytes were then washed with ND96 solution and fixed in 2% paraformaldehyde in ND96 (pH 8.5) for 30 min. Fixed oocytes were incubated in ND96 containing 1% BSA for 1 h, followed by a 1 h incubation with 14C2 (anti-AM2) monoclonal antibody at 1:500 in ND96 solution containing 1% BSA. The oocytes were washed with an ice-cold ND96 solution three times for 10 min and then incubated with Alexa Fluor 546 labeled goat anti-mouse IgG (Molecular Probes, Inc., Medford, OR) at 1:100 in ND96 containing 1% BSA for 1 h. Upon the antibody removal, the oocytes were again washed with an ice-cold ND96 solution five times for 10 min. Fluorescence images were acquired using a ZOE fluorescent Cell Imager (Bio-Rad). About one-half of the surface of each oocyte was imaged. Fluorescence intensity was quantified using Photoshop by measuring the grey scale value. Uninjected oocytes were subjected to the same conditions as described and served as a control for the auto fluorescence from the yolk (Beumer et al 1995). For each oocyte, the whole-cell current measured by TEVC was plotted against the protein expression level detected by immunofluorescence, and the slope of the obtained linear regression curve represents the relative specific activity of the channel.

For the K_d measurements, a washout protocol was applied at the end of the application of the compounds. During the washout period, in order to prevent prolonged acidification of oocytes, we applied pH 5.5 pulse, instead of continuous application of pH 5.5 barth solution. The inhibition and washout curves were fitted into the association then dissociation equation in GraphPad Prism 5. The equation is

$$\begin{aligned}
 K_{ob} &= [compound] * K_{on} + K_{off} \\
 K_d &= \frac{K_{off}}{K_{on}} \\
 Eq &= B_{max} * \frac{[compound]}{[compound] + K_d} \\
 Association &= Eq * (1 - \exp(-1 * K_{ob} * t)) \\
 Y@t_0 &= Eq * (1 - \exp(-1 * K_{ob} * t_0)) \\
 Dissociation &= Y@t_0 * \exp(-1 * K_{off} * (X - t_0)) \\
 Y &= IF(X < t_0, Association, Dissociation) + NS
 \end{aligned}$$

where Y is the percent of inhibition at time t.

2.5. Plaque purification

To obtain plaque-purified viruses, ST6Gal1-expressing MDCK cells were infected with the passaged viruses as in plaque reduction assays. After infection, an agarose media overlay (DMEM; 2µg/ml N-acetyl trypsin; 0.5% agarose) was added. After 42 hpi neutral red solution was added to the media overlay and incubated for an additional 4 h in order to visualize individual plaques. At this time, a sterile Pasteur pipet was used to pick a single plaque colony. The agarose from a single plaque was placed in DMEM with 0.5% BSA and incubated overnight at 4°C with gentle rocking to allow virus particles to diffuse into the media. Purified viruses were then amplified in ST6Gal1-expressing MDCK cells.

2.6. AM2 sequence analysis

AM2 sequences from human, avian, and swine viruses were downloaded from the NCBI Influenza Virus Research online database (www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) as of 2017. Sequence analysis was done using MEGA7 software (www.megasoftware.net). Residues 22-37 were aligned using the MUSCLE tool.

2.7. AM2 gene sequencing

The AM2 gene for A/California/07/2009 (H1N1) and recombinant A/Udorn/1972 (H3N2) was sequenced using methods previously reported (Ma et al., 2016). Briefly, the viral RNA was extracted using QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany) and was reverse-transcribed using strain-specific M segment primers. PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Purified PCR products were sequenced by Eton Bioscience, Inc.

2.8. In vitro replication assays

For multicycle growth curves, ST6Gal1-expressing MDCK cells were infected with either plaque-purified A/California/07/2009 (H1N1) or recombinant A/Udorn/1972 (rH3N2) at an MOI of 0.003. Viruses were harvested at 8, 14, 20, 26, 32, 44 h and titrated by plaque reduction assay to determine viral titers at each time point.

For viral competition assays, A/California/07/2009 (H1N1) or recombinant A/Udorn/1972 (rH3N2) containing the AM2-S31N were co-infected with either AM2-S31N/V27I, AM2-S31N/L26I, or AM2-S31N/L26I/A30T viruses at an MOI ratio of 1:100 (2.0×10^{-5} : 2.0×10^{-3}) in ST6Gal1-expressing MDCK cells. The virus co-culture was then passed three times and the AM2 gene was sequenced. The percent total population of AM2 mutants was determined by averaging the peak area from the sequencing chromatogram. For viral competition assays against AM2-S31N/L26I/A30T viruses, the average area of the two peaks (corresponding to the L26I and A30T mutations) were used to generate the error bars.

3. RESULTS

3.1. AM2-S31N inhibitors 1 and 2 have potent channel blockage and antiviral activity

Compounds **1** and **2** are hydroxyl adamantane AM2-S31N inhibitors containing aryl substitutions at the 5-position of the isoxazole group (Fig. 1A and B). We previously tested the antiviral activity of AM2-S31N inhibitors **1** and **2** against the AM2-S31N-containing

2009 pandemic-like A/California/07/09 H1N1 (A/H1N1/pdm09) virus in plaque assay (Li et al., 2017; Wang et al., 2018). It was found that compounds **1** and **2** inhibited viral replication with an EC₅₀ value of $1.2 \pm 0.2 \mu\text{M}$ and $0.3 \pm 0.04 \mu\text{M}$, respectively. The CC₅₀ in MDCK and A549 cells were reported to be $59.3 \pm 2.6 \mu\text{M}$ and $101.9 \pm 4.5 \mu\text{M}$ for compound **1**, and $146.6 \pm 62.8 \mu\text{M}$ and $> 300 \mu\text{M}$ for compound **2**, respectively (Li et al., 2017; Wang et al., 2018) (Fig. 1C and D). To determine the AM2-S31N proton channel blockage by compounds **1** and **2**, the AM2 channel from A/California/07/2009 (H1N1) virus was expressed in *Xenopus* oocytes for TECV measurements. Compound **1** and **2** showed $75.5 \pm 1.8\%$ and $84.3 \pm 0.8\%$ blockage at $100 \mu\text{M}$ drug concentration at the 2-minute time point after drug treatment (Fig. 1E and F). This is in agreement with previously reported TECV experiments using AM2-S31N under the A/Udorn/1972 background (Li et al., 2017; Wang et al., 2018). Overall, compounds **1** and **2** were found to have potent antiviral activity with low cellular cytotoxicity, with a mechanism of action dependent upon AM2-S31N channel blockage.

3.2. *In vitro* serial viral passage experiments select both conservative and non-conservative drug-resistant mutations against AM2-S31N inhibitors **1** and **2**

Serial passage experiments are routinely used to assess the genetic barriers to drug resistance for many viruses. Therefore, we performed serial viral passage experiments with the A/California/07/2009 (H1N1) virus under persistent drug selection pressure to identify resistant mutants against compounds **1** and **2**. The genetic barrier to drug resistance for amantadine against AM2-WT-containing influenza strains has been extensively studied (Astrahan and Arkin, 2011; Ma et al., 2016). Amantadine has been found to have a lower genetic barrier to drug resistance than oseltamivir, which correlates with the frequency of amantadine- and oseltamivir-resistant genotypes found in clinically isolated populations. For the serial viral passage experiments, ST6-Gal1-MDCK cells were infected with the A/California/07/2009 (H1N1) virus at MOI of 0.001 in the presence of either compound **1** or **2**. For each compound, the AM2-S31N EC₅₀ value was chosen as the initial drug concentration, and the dosage was doubled following each passage. At select passages, a dose-response curve was used to monitor drug sensitivity. The results are shown in Fig. 2 and Table 1. For the first three passages, no significant changes in drug sensitivity was observed for either compound **1** or **2**. In comparison, amantadine evolved resistance after a single passage of the AM2-N31S A/WSN/33 (H1N1) virus (Ma et al., 2016). A noticeable change in the EC₅₀ value was observed after passage 5 for compound **1** (1P05) and after passage 4 for compound **2** (2P04) (Table 1). The EC₅₀ value of compound **1** against the 1P05 virus was $7.3 \pm 0.8 \mu\text{M}$, or approximately 6-fold higher than that of the 1P00 virus. The EC₅₀ value of compound **2** against 2P04 virus was $3.7 \pm 0.4 \mu\text{M}$, approximately 12-fold increase compared to that of the 2P00 virus. Nevertheless, potent inhibition of viral replication was observed for both 1P05 and 2P04 viruses with high drug concentrations of compounds **1** and **2**, respectively.

The partial loss in drug susceptibility of 1P05 and 2P04 viruses was found to due to conservative mutations in the AM2 channel transmembrane region. When the M segment was sequenced and analyzed, we found the emergence of the conservative mutation V27I in 1P05 viruses and L26I in 2P04 viruses (Fig. 2). In order to estimate the population

distribution, the area under each chromatogram peak corresponding to a nucleic acid base change was measured (Takeda et al., 2002). For 1P05 and 2P04 viruses, more than 90% of the viral population contained the drug-selected mutation (Fig. 2). After reaching 1P07 for compound **1**, the drug concentration was maintained at 50 μ M from passages 1P07 to 1P10 to limit cytotoxic effects. No additional mutation was found in the AM2-S31N/V27I gene segment. The EC₅₀ of compound **1** against 1P10 viruses and plaque-purified 1P10 viruses were 8.3 ± 2.1 μ M (Table 1) and 4.4 ± 0.8 μ M (Table 2), respectively. After drug withdrawal at 1P10 for three additional passages, the 1P13 viral population retained the AM2-S31N/V27I mutation. Since compound **2** had a higher CC₅₀ compared to **1** (Fig. 1C and D) the drug dose was increased from 2P07 to 2P10. Higher level of resistance to compound **2** appeared at passages 08 and onwards. The emergence of a triple mutation AM2-S31N/L26I/A30T was observed in 2P08, and its population continued to increase at 2P09 and 2P10 (Fig. 2). Following drug withdrawal, the A30T mutant disappeared, and only AM2-S31N/L26I double mutant was found at 2P13. Taken together, these results demonstrate that the AM2-S31N inhibitors have a higher genetic barrier to drug resistance than amantadine at least in cell culture.

3.3. Drug-resistant mutants are located at the N-terminal drug-binding site of AM2-S31N

Both the conservative (V27I and L26I) and non-conservative (A30T) mutations were located around the N-terminal drug binding site of AM2-S31N. Previous solution NMR structure of AM2-S31N proton channel in complex with an isoxazole inhibitor WJ332 (PDB: 2LY0) showed that the channel blocker fits in the N-terminal channel pore and forms both hydrophobic and hydrogen bonds with the AM2-S31N channel (Fig. 3A). In the docking models of compounds **1** and **2** in the AM2-S31N channel (Figs. 3B and 3C), both compounds bind in a similar manner as compound WJ332. Interestingly, the hydroxyl group at the adamantane ring in compounds **1** and **2** engages in a hydrogen bond with the main-chain A30 carbonyl. In the docking model of compound **1** in complex with AM2-S31N (Fig. 3B), the distal 2-thiomethylphenyl ring forms hydrophobic interactions with the V27 side chain methyls. When valine was mutated to isoleucine (V27I), it is likely that the channel pore became constricted and could not accommodate compound **1** as efficiently as the AM2-S31N channel. In the docking model of compound **2** in complex with AM2-S31N (Fig. 3C), a mutation of the channel facing residue alanine 30 to threonine not only changes the channel pore size, but also the polarity of the channel, which may explain the complete resistance of the AM2-S31N/L26I/A30T to compounds **1** and **2**. The other conservative mutation L26I occurs at a helix-interface facing residue, therefore it might have an indirect effect on the channel pore drug-binding site. Collectively, the evolved conservative and non-conservative mutations are all located at the N-terminal drug-binding site of AM2-S31N and they have either a direct or indirect effect on the channel pore which leads to either reduced or completely loss of drug sensitivity.

3.4. Confirmation of drug resistance in TEVC and plaque assays

To confirm whether the observed drug resistance in plaque assay was solely due to the corresponding AM2 mutations, we introduced each mutation into the AM2-S31N gene of the A/California/07/2009 (H1N1) virus and recorded the drug sensitivity in TEVC assays

(Figs. 4 and 5). Both compounds **1** and **2** partially inhibited AM2-S31N/V27I and AM2-S31N/L26I double mutant channels, but with reduced potency compared with AM2-S31N when tested at 100 μ M after 2 minutes at pH 5.5. Neither compound had inhibitory activity against the AM2-S31N/L26I/A30T triple mutant (Fig. 4).

To quantitatively compare the binding affinity of compounds **1** and **2** towards AM2-S31N channel versus the evolved AM2-S31N/V27I, AM2-S31N/L26I, and AM2-S31N/L26I/A30T mutant channels, we next determined K_d values using electrophysiology (Fig. 5). To measure the K_{off} values, we applied a prolonged washout protocol at the end of compound treatment. In order to minimize the acidification of oocytes during the prolonged washout period, we applied pH 5.5 pulses instead of continuous application of pH 5.5 Barth solution. We then fit the inhibition and washout curves with association and dissociation equations using GraphPad Prism 5. The introduction of V27I mutation into AM2-S31N protein increased K_d value of compound **1** from $12.8 \pm 2.1 \mu\text{M}$ to $193.5 \pm 39.9 \mu\text{M}$; similarly, the introduction of L26I mutation into AM2-S31N protein increased K_d value of compound **2** from $7.3 \pm 0.5 \mu\text{M}$ to $28.0 \pm 5.0 \mu\text{M}$. Overall, the fold-change in binding affinity K_d values were comparable to the fold-change in antiviral EC_{50} values. Neither compound **1** nor **2** showed inhibition against AM2-S31N/L26I/A30T triple mutant channel. In summary, both the AM2-S31N/V27I and AM2-S31N/L26I mutations we identified in serial viral passage experiments indeed displayed reduced drug sensitivity in its channel function, but retained a partial degree of channel blockage. Overall, the electrophysiological assay results suggest that the observed partial phenotypic resistance in plaque assay was due to the mutations in the AM2 gene.

3.5. AM2-S31N/L26I/A30T triple mutant channel had reduced specific proton conductance than AM2-S31N, AM2-S31N/V27I, and AM2-S31N/L26I mutant channels

Next, we set out to determine the consequences of the identified mutations on channel function. As such, we measured the specific proton conductance using macroscopic whole cell current relative to the amount of AM2 channels present. The surface protein expression level was quantified by immunofluorescence via an AM2 N-terminal specific antibody (14C2) and the channel specific activity was calculated by normalizing the whole current with the surface AM2 protein level via a linear regression function (Fig. 6A). The calculated specific activities of AM2-S31N/V27I and AM2-S31N/L26I channels were about 77.6% and 93.7% of the AM2-S31N channel, respectively (Figs. 6B–D and F). In contrast, the specific activity of AM2-S31N/L26I/A30T triple mutant channel was only about 12.5% of the AM2-S31N channel (Figs. 6B, E and F). In order to determine whether proton selectivity is affected by the identified AM2 mutations, we measured current-voltage relationship for each mutant channel. We did not observe significant reversal potential changes in AM2-S31N/V27I, AM2-S31N/L26I, or AM2-S31N/L26I/A30T (data not shown), indicating that these mutant AM2 channels have very similar proton selectivity as the AM2-S31N channel from the A/California/07/2009 (H1N1) virus. Overall, these results suggest that the triple mutant AM2-S31N/L26I/A30T identified in the drug passage has attenuated channel activity, while the double mutants AM2-S31N/V27I and AM2-S31N/L26I had similar channel activity as the AM2-S31N channel.

3.6. AM2-S31N/L26I/A30T triple mutant-containing virus had compromised replication fitness compared with AM2-S31N, AM2-S31N/V27I, or AM2-S31N/L26I-containing viruses

To investigate the impact of the evolved AM2 mutations on viral replication, we performed multicycle growth curve measurements for plaque-purified viruses harboring either AM2-S31N/V27I, AM2-S31N/L26I, or AM2-S31N/L26I/A30T mutant channels. The growth curve for the AM2-S31N-containing A/California/07/2009 (H1N1) virus was included as a control for comparison. For the growth curve, the ST6-Gal1 expressing MDCK cells were infected with plaque-purified viruses at an MOI of 0.003. Viral titers were quantified at 8, 14, 20, 26, 32, and 44 hpi (Fig. 7A) by plaque assay. Plaque-purified virus harboring the AM2-S31N/V27I mutant had comparable growth to the AM2-S31N-containing A/California/07/2009 (H1N1) virus, while the plaque-purified virus harboring the AM2-S31N/L26I mutant had partially reduced growth (Fig. 7A). Plaque-purified virus harboring the AM2-S31N/L26I/A30T triple mutant had significantly reduced growth at 8, 14, 20, 26, and 32 hpi compared to both AM2-S31N, -S31N/V27I, and -S31N/L26I-containing viruses. In order to rule out the possibility that secondary mutations located in viral proteins other than AM2 might influence virus growth, we repeated the growth kinetics using recombinant A/Udorn/1972 (rH3N2) viruses containing AM2-S31N, AM2-S31N/V27I, AM2-S31N/L26I, and AM2-S31N/L26I/A30T mutant channels (Fig. 7B). The drug sensitivity of the recombinant viruses was first tested in plaque assay. The EC₅₀ values of compounds **1** and **2** against AM2-S31N, AM2-S31N/V27I, and AM2-S31N/L26I rH3N2 viruses are shown in Table 2. Overall, the EC₅₀ values of compounds **1** and **2** for rH3N2 viruses are comparable with plaque-purified viruses. Similarly, compound **2** showed no inhibition against AM2-S31N/L26I/A30T rH3N2 virus up to 30 μ M. For the growth curve, AM2-S31N/V27I and AM2-S31N/L26I rH3N2 viruses had no difference in the growth rate compared to the AM2-S31N rH3N2 virus. In contrast, the growth kinetics for the AM2-S31N/L26I/A30T rH3N2 virus was significantly attenuated, having approximately 2-log units lower viral titers from 8 to 32 hpi. From these results, it is likely that the AM2-S31N/L26I/A30T triple mutant has an effect on the replication of influenza viruses.

To further compare the replication fitness, we performed viral competition growth assays. In this experiment, ST6-Gal1 expressing MDCK cells were co-infected with A/California/07/2009 (H1N1) virus and plaque-purified viruses at an MOI ratio of 1:100. This greatly biased the infection to favor growth of the mutant viruses over the AM2-S31N containing A/California/07/2009 (H1N1) virus. It was found that AM2-S31N/V27I mutation remained dominant after three passages, and no increase in the AM2-S31N population was detected (Fig. 7C). Similarly, the population of the AM2-S31N/L26I did not change (Fig. 7E). In contrast, the population of the AM2-S31N/L26I/A30T triple mutant began to decline after passage 01, meanwhile the relative population of AM2-S31N viruses increased (Fig. 7G). After the second passage, the population of AM2-S31N viruses eclipsed the AM2-S31N/L26I/A30T triple mutant. By the third passage, there was no detectable AM2-S31N/L26I/A30T triple mutant in the population. We next performed the same viral growth competition experiments using used rH3N2 strains. Similar to plaque-purified viruses, AM2-S31N/V27I and AM2-S31N/L26I rH3N2 viruses remained the dominant population after three passages (Figs. 7D and F), while the AM2-S31N/L26I/A30T triple mutant rH3N2 virus was eclipsed by AM2-S31N rH3N2 after 2 passages (Fig. 7H). At passage 03, the AM2-S31N/L26I/A30T

triple mutant rH3N2 virus was approximately 11% of the total viral population based on the sequence analysis (Fig. 7H). Overall, the triple mutant-harboring virus appears to be driven to extinction in the presence of AM2-S31N viruses, despite an initial bias in its favor.

3.7. Frequency of AM2-S31N, AM2-S31N/V27I, AM2-S31N/L26I, and AM2-S31N/L26I/A30T mutants among circulating influenza A viruses

In order to determine the frequency of these evolved AM2 mutations in influenza strains isolated from human, swine, and avian hosts, sequences were obtained from the NCBI Genbank and aligned for analysis. Out of 29,312 AM2 protein sequences from human influenza viruses, nearly 80% contain the AM2-S31N mutation, while less than 0.5% contained the AM2-S31N/L26I double mutation and only 0.2% had the AM2-S31N/V27I double mutation (Fig. 8). For 18,686 AM2 protein sequences from avian influenza viruses, over 14% contained the AM2-S31N mutant, only 0.09% had the AM2-S31N/V27I double mutant, and around 2% had the AM2-S31N/L26I mutant. For 8,749 AM2 sequences from swine influenza viruses, over 76% contained the AM2-S31N mutant, around 1% contained the AM2-S31N/V27I double mutant, and around 3% contained the AM2-S31N/L26I double mutant. Out of the two double mutants, AM2-S31N/L26I double mutant is more frequently found in both avian and swine hosts, while the AM2-S31N/V27I double mutant is slightly higher for human isolated strains. Neither the AM2-L26I/A30T double mutant nor the AM2-S31N/L26I/A30T triple mutant was found in human, avian, or swine influenza strains in these analyses (Fig. 8). Overall, the absence of AM2-S31N/L26I/A30T triple mutant among circulating influenza A viruses suggest that this triple mutant might have deleterious effect on the fitness of viral replication.

4. DISCUSSION

Influenza viruses are a highly diverse group of respiratory pathogens that evolve as quasispecies (Lauring and Andino, 2010). The influenza virus RNA polymerase, unlike the eukaryotic DNA polymerase, lacks proof-reading function, therefore allowing mutations to continuously emerge during replication cycles. This gives Influenza viruses an intrinsic ability to rapidly adapt to a dynamic and hostile environment and, in the presence of antivirals, select for resistant variants in the population. Fortunately, the window of feasible mutations are limited to those that maintain an adequate level of replication and transmissibility. In the presence of amantadine, a large number of drug-resistant AM2 mutants were identified from cell culture experiments (Brown et al., 2010; Grambas and Hay, 1992; Hay et al., 1985), among which a subset of these was also found in mice and human that were treated with amantadine after viral infection (Abed et al., 2005; Shiraishi et al., 2003). Interestingly, the majority of selected drug-resistant mutant viruses reverted to the amantadine-sensitive wild-type (WT) AM2 after drug removal (Grambas and Hay, 1992; Suzuki et al., 2003), suggesting that the mutants might have compromised fitness of replication (Balannik et al., 2010; Stouffer et al., 2008). Indeed, electrophysiological assays showed that majority of the resulting AM2 mutants have attenuated proton conductance rates (specific conductance) (Balannik et al., 2010), which might render them less fit. As such, only a limited number of AM2 mutants were identified in transmissible viruses such as V27A, L26F, and S31N (Dong et al., 2015). All three mutants have similar proton

conductance rate to that of the AM2-WT (Balannik et al., 2010). The challenge of resisting AM2 channel inhibitors while maintaining fitness of replication may be due to the AM2 proton channel structure; the formation of a homotetramer with a narrow pore implies that a single point mutation gives rise to four amino acid changes in the channel simultaneously. Any mutation that prevents tetramer formation or results in the occlusion of channel pore will lead to the loss of function of AM2.

AM2-S31N inhibitors are analogs of amantadine that are chemically substituted at the ammonium group (Wang et al., 2015). Analogs containing an isoxazole substitution were found to stabilize the AM2-S31N by forming optimal hydrogen bond networks with Asp31 and structural water molecules within the channel pore (Wang et al., 2013). Whereas amantadine orients its amine towards the C-terminal end facing histidine 37, AM2-S31N inhibitors bind S31N with the amine facing up (N-terminal lumen) (Fig. 3A). This drug orientation positions the substituted group near the pore-facing residue 27 and helix-interfacing residue 26, stabilizing an otherwise dynamic channel. Based on the solution NMR structure of WJ332 in complex with AM2-S31N (Fig. 3A), a series of amantadine derivatives containing various aryl groups at the 5-position of the isoxazole were designed to increase the hydrophobic interactions at position 26 and 27 to improve drug binding (Li et al., 2017, 2016; Wang et al., 2018). Additionally, introduction of a hydroxyl at the adamantane cage was found to maximize the solubility and specificity for some of the more hydrophobic or branched aryl-substituted isoxazole derivatives without destabilizing the drug-channel interaction. This approach gave rise to compounds **1** and **2**, which were chosen to study the evolution and mechanism of resistance. These compounds selected for partially drug-sensitive viruses containing conservative mutations either at position 26 or 27. Complete resistance was identified with a third mutation at the pore-facing residue 30, but only when the drug selection pressure was significantly increased. All the identified AM2 mutants were located at the drug-binding site of AM2-S31N and it is likely they have either a direct or indirect impact on the drug binding. These results suggest AM2-S31N inhibitors have a higher genetic barrier to drug resistance than amantadine at least in cell culture.

We examined the binding kinetics for drug-resistant mutations selected by compounds **1** and **2** during viral passages in order to obtain a more detailed measurement of drug binding. Compound **2** was more potent in antiviral assays compared with compound **1**, and was reflected by a lower K_d value from the electrophysiological assay. Likewise, the fold-change in K_d for drug selected AM2 mutations were roughly proportional to the fold-change observed in antiviral activity measurements. The overall trend in the dissociation constants for mutant channels match the corresponding antiviral activity measurements. Overall, the electrophysiological assay results, together with the growth kinetics, and competition growth experiments, collectively suggest that the conservative mutants AM2-S31N/V27I and AM2-S31N/L26I are functionally equivalent to AM2-S31N *in vitro* while conferring partial resistance to compounds **1** and **2**. In contrast, the non-conservative mutant channel AM2-S31N/L26I/A30T has attenuated channel function and could not support viral replication *in vitro*, and it shows higher level of resistance to both compounds **1** and **2**.

Based on our findings, future efforts for AM2-S31N drug discovery should focus on compounds that not only inhibit the AM2-S31N channel, but also the AM2-S31N/V27I and

AM2-S31N/L26I double mutants in order to further increase the genetic barrier to drug resistance. Small populations of viruses containing the AM2-S31N/V27I and AM2-S31N/L26I double mutants were found in circulating human, avian, and swine strains, and our competition assays showed that these mutant viruses can sustain their population in the presence of AM2-S31N viruses in cell culture. Compounds **1** and **2** demonstrated partial activity against AM2-S31N/V27I and AM2-S31N/L26I both in antiviral and electrophysiological assays. Therefore, AM2-S31N inhibitors appear to be an ideal starting point for optimizing inhibitors against these double mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- AM2-S31N inhibitors have a higher in vitro genetic barrier to drug resistance than amantadine
- AM2-S31N/L26I and AM2-S31N/V27I confer partial drug resistance against AM2-S31N inhibitors
- AM2-S31N/L26I and AM2-S31N/V27I-containing viruses have similar fitness of replication as the AM2-S31N-containing virus
- AM2-S31N/L26I/A30T confers complete drug resistance to AM2-S31N inhibitors
- AM2-S31N/L26I/A30T-containing virus has drastically reduced fitness of replication compared to AM2-S31N-containing virus

Fig. 1. Compounds 1 and 2 have antiviral activity against A/California/07/2009 (H1N1) viruses by targeting the AM2-S31N channel

Compounds 1 and 2 are hydroxyl amantadine analogs that differ by a chemical substitution at the 5-position of the isoxazole group. (A) The 2-methylthiophenyl substituted isoxazole inhibitor 1 and (B) cyclopentyl substituted isoxazole inhibitor 2 are shown. The indicated EC_{50} and CC_{50} values have been previously reported (Li et al., 2017; Wang et al., 2018). EC_{50} values were obtained using plaque assays in ST6Gal1-expressing MDCK cells infected with influenza virus A/California/07/2009 (H1N1) in the presence of (C) compound 1 and (D) compound 2. CC_{50} values were obtained by measuring the cytopathic effect by neutral red uptake in MDCK cells treated with (C) compound 1 and (D) compound 2 for 48 h (Li et al., 2017; Wang et al., 2018). Electrophysiological measurements using AM2-S31N with the A/California/07/2009 (H1N1) background treated at the indicated concentration with (E) compound 1 and (F) compound 2 are shown.

Fig. 2. AM2 channel sequences from serial passage experiments under selection pressure from compounds 1 and 2

The influenza A/California/07/2009 (H1N1) virus was passaged at an MOI of 0.001 in ST6Gal1-expressing MDCK cells. Viruses were harvested after 2 days. Viral RNA at the indicated passages were isolated and reverse transcribed using M segment primers. M segment cDNA was then sequenced. Traces are shown for nucleic acid sequences corresponding to residues 26-31. Arrows indicate mutations not present in AM2-S31N. The relative populations of mutant AM2 viruses were determined by measuring the peak area from the sequencing traces. Mutations selected by (A) compound 1 and (B) compound 2 are shown at the indicated passage number. (A) concentration of compound 1 was increased up to 50 μM. (B) Concentration of compound 2 was increased until drug withdrawal. Compounds were not present after passage 10.

Fig. 3. Solution NMR structure and docking models of AM2-S31N inhibitors in the transmembrane domain of AM2-S31N

(A) Solution NMR structure of WJ332 in complex with AM2-S31N (PDB: 2LY0) (Wang et al., 2013) (B) Docking model of compound **1** in AM2-S31N. (C) Docking model of compound **2** in AM2-S31N. The transparency of the front helix was set as 0.5 for clarity. Docking was performed using Schrödinger Glide standard precision.

Fig. 4. Compounds 1 and 2 have antiviral activity and channel blockage against AM2-S31N/V27I and AM2-S31N/L26I double mutant channels

(A) TEVC recordings of compound 1 and 2 for AM2-S31N and AM2-S31N mutant channels were performed as previously reported (Ma et al., 2009; Ma and Wang, 2018). Compounds 1 and 2 partially inhibit the AM2-S31N/V27I and AM2-S31N/L26I mutant channels. The TEVC recording trace for AM2-S31N/L26I/A30T was scaled for clarity. The AM2-S31N/L26I/A30T was not inhibited by either compound 1 or 2. (B) Plaque assays with compounds 1 and 2 at the indicated concentrations are shown using plaque-purified A/California/07/2009 (H1N1) viruses isolated from 1P10 and 2P10 viruses.

Fig. 5. Drug binding kinetics for compound 1 and 2 against double mutant AM2-S31N/V27I and AM2-S31N/L26I channels were determined using a combined application and washout procedure

Compound **1** on (A) AM2-S31N and (B) AM2-S31N/V27I, and compound **2** on (C) AM2-S31N and (D) AM2-S31N/L26I are shown with the concentration of compound applied. Washout curves are shown on the left side of each figure. Blue bars represent the application period of pH 5.5 bath solution, whereas red bars indicate application period of pH 5.5 bath solution with either compound **1** or **2** included. K_d values are indicated on each graph, and were obtained by fitting the association then dissociation equation in GraphPad Prism 5.

Fig. 6. Mutations selected by AM2-S31N inhibitors affect the specific conductance of the channel (A) Immunofluorescent signal from anti-AM2 antibody was measured in fixed oocytes to determine the expression level of AM2 on the oocyte plasma membrane. The slope of the regression line was determined by plotting the whole cell current versus channel expression, which was then used to determine specific proton conductance. Specific proton conductance for mutant channels are shown for (B) AM2-S31N, (C) AM2-S31N/V27I, (D) AM2-S31N/L26I, and (E) AM2-S31N/L26I/A30T. (F) Bar graph representation for the percent specific proton conductance of each mutant channel is shown.

Fig. 7. *In vitro* viral replication and fitness assays for AM2-S31N mutant channels

Growth kinetics were performed on (A) plaque-purified A/California/07/2009 (H1N1) and (B) recombinant A/Udorn/1972 (rH3N2) viruses at MOI of 0.003 in ST6Gal1-expressing MDCK cells. Viruses were harvested at 8, 14, 20, 26, 32 and 44 hpi and viral titers were determined using plaque assays. Each data point in (A) and (B) represents two biological replicates. For competition assays, an MOI ratio of 1:100 for AM2-S31N and mutant AM2 viruses was used in ST6Gal1-expressing MDCK cells. The relative population of AM2 variants were quantified by the area of each sequencing trace. Plaque-purified H1N1 virus competition for (C) AM2-S31N/V27I, (E) AM2-S31N/L26I and (G) AM2-S31N/L26I/A30T and rH3N2 virus competition for (D) AM2-S31N/V27I, (F) AM2-S31N/L26I and (H) AM2-S31N/L26I/A30T are shown. Error bars in (G,H) were obtained from the average area for both L26I and A30T sequencing peaks.

Fig. 8. Analysis of the frequency of the mutations selected for by AM2-S31N inhibitors 1 and 2 in the NCBI influenza database

Total AM2 protein sequences were retrieved for human, avian, and swine hosts and aligned using MEGA7 for analysis. The fraction of mutant AM2 to total AM2 are indicated above each column. AM2-S31N (grey bar), AM2-S31N/V27I (green bar), AM2-S31N/L26I (orange bar), and AM2-S31N/L26I/A30T (red bar) mutations for human, avian, and swine hosts are shown.

TABLE 1

Serial passage experiments of compounds (1) and (2) against influenza A virus

H1N1 A/California/07/2009 ^a	(1)			(2)			Mock
	Selection Pressure ^b	EC ₅₀ ^{c,d}	AM2 Mutation	Selection Pressure ^b	EC ₅₀ ^{c,d}	AM2 Mutation	
Passage 00		1.2 ± 0.2	S31N		0.3 ± 0.04	S31N	S31N
Passage 01	1.2	2.9 ± 0.4	ND	0.25	N.D.	ND	ND
Passage 02	2.5	3.3 ± 0.2	ND	0.5	0.6 ± 0.1	ND	ND
Passage 03	5	2.3 ± 0.2	S31N	1	1.7 ± 0.2	ND	ND
Passage 04	10	3.4 ± 0.5	ND	2	3.7 ± 0.5	S31N/L26I	ND
Passage 05	20	7.3 ± 0.8	S31N/V27I	4	4.2 ± 0.4	ND	S31N
Passage 06	40	6.1 ± 1.2	ND	8	3.7 ± 0.5	ND	ND
Passage 07	50	ND	ND	16	3.2 ± 0.6	S31N/L26I + (A30+T30)	ND
Passage 08	50	8.2 ± 2.9	S31N/V27I	32	> 10	S31N/L26I + (A30+T30)	ND
Passage 09	50	8.1 ± 1.5	ND	64	> 10	ND	ND
Passage 10	50	8.3 ± 2.1	S31N/V27I	128	> 10	S31N/L26I/A30T	S31N
Passage 11	0	ND	ND	0	ND	ND	Ended Passage
Passage 12	0	ND	ND	0	ND	ND	
Passage 13	0	ND	S31N/V27I	0	5.4 ± 0.4	S31N/L26I	

^aViruses infected at MOI of 0.001 in MDCK cells expressing ST6GalI^bViruses propagated for up to 2 days in the presence of compound (1) or (2) at the indicated concentrations (μM)^cEC₅₀ (μM) = mean ± S.E.^dDrug sensitivity was determined by plaque assays and quantified by plaque area using ImageJ

ND: not determined

TABLE 2

Drug sensitivities against purified and recombinant strains

Influenza virus strain	AM2 Mutation	EC ₅₀ ^{a,b}	
		(1)	(2)
A/California/07/2009 (H1N1)			
	S31N	1.2 ± 0.2	0.3 ± 0.04
Plaque-purified A/California/07/2009 (H1N1)			
	S31N/V27I	4.4 ± 0.8	ND
	S31N/L26I	ND	2.4 ± 0.5
	S31N/L26I/A30T	ND	>30
Recombinant A/Udorn/1972 (rH3N2)			
	S31N	0.9 ± 0.06	0.3 ± 0.04
	S31N/V27I	5.6 ± 0.8	ND
	S31N/L26I	2.04 ± 0.4	1.2 ± 0.2
	S31N/L26I/A30T	ND	>30

^aEC₅₀ (μM) = mean ± S.E.^bDrug sensitivity determined by plaque assay and quantified by plaque area using ImageJ