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Discovery of M2 channel blockers targeting the drug-resistant double mutants M2-S31N/L26I and M2-S31N/V27A from the influenza A viruses

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Abstract

Influenza virus infections are a persistent threat to human health due to seasonal outbreaks and sporadic pandemics. Amantadine and rimantadine are FDA-approved influenza antiviral drugs and work by inhibiting the viral M2 proton channel. However, the therapeutic potential for the antiviral amantadine/rimantadine was curtailed by the emergence of drug-resistant mutations in its target protein M2. In this study, we identified four amantadine-resistant M2 mutants among avian and human influenza A H5N1 strains circulating between 2002 to 2019: the single S31N and V27A mutants, and the S31N/L26I and S31N/V27A double mutants. Herein, utilizing two-electrode voltage clamp (TEVC) assays, we screened a panel of structurally diverse M2 inhibitors against these single and double mutant channels. Three compounds **6**, **7**, and **15** were found to significantly block all three M2 mutants: M2-S31N, M2-S31N/L26I, and M2-S31N/V27A. Using recombinant viruses generated from reverse genetics, we further showed that these compounds also inhibited the replication of recombinant viruses harboring either the single S31N or double S31N/L26I and S31N/V27A mutants. This work represents the first example in developing antivirals by targeting the drug-resistant double mutants of M2 proton channels.

Graphical Abstract

Keywords

Influenza virus; M2; amantadine; antiviral; drug resistance; proton channel

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1. Introduction

Influenza viruses are zoonotic pathogens that commonly infect humans, birds and swine. In humans, influenza particles circulate via aerosols to infect the upper respiratory tract, triggering pneumonia, opportunistic infections, and could potentially trigger cardiac arrest. Severity of infection is dependent on factors that include transmissibility of strain, site of infection within the host (upper or lower respiratory tract), host immune evasion, and replication fitness. Since the discovery of the highly pathogenic avian influenza (HPAI) strain H5N1 in 1997 in Hong Kong, there has been a concern over a new severe H5N1 flu pandemic because: 1) H5N1 infection leads to high case fatality rate (>50%) (de Jong et al., 2006; Hatta et al., 2001; Lai et al., 2016); 2) H5N1 has the potential for human-to-human transmissibility (Herfst et al., 2012; Wang et al., 2008; Zhang et al., 2013); and 3) H5N1 is not recognizable by the immune system for most of the human population. Vaccines against HPAI H5N1 have been licensed by the FDA, but the high mutation rate associated with viral replication cannot guarantee its effectiveness in a future outbreak. Establishing antiviral agents before a pandemic will dramatically reduce the loss of human life. Moreover, antivirals may be used as a viable prophylactic strategy as well. The FDA has approved three classes of drugs for influenza viruses: the neuraminidase (NA) inhibitors oseltamivir, zanamivir and peramivir; M2 channel blockers amantadine and rimantadine; and the recently released endonuclease inhibitor baloxavir marboxil. Because all three classes of influenza drugs are susceptible to drug resistance (Hurt, 2014; Jones et al., 2018; Wang et al., 2015), it is necessary to respond with appropriate therapeutic intervention based on the drug-resistance profile of circulating strains (Ferguson et al., 2005; Hu et al., 2017a; Li et al., 2015; Wang et al., 2015; Zhang et al., 2017).

The M2 channel has been regarded as a viable target for antivirals, since it retains an essential function in viral replication and is relatively conserved among different strains (Wang et al., 2015; Wang et al., 2011c). The 97-amino acid channel forms a transmembrane tetramer within the viral envelope. Upon pH activation in early endosomes, M2 conducts protons to the C-terminal end of the channel toward the interior of the virus, facilitating the dissociation of viral ribonucleoprotein complexes from the matrix protein M1 for nuclear import. The channel has an ectodomain (residues 1-22), transmembrane domain (23-46), C-terminal amphipathic helix (47-61), and unstructured C-terminal tail (residues 62-97). The proton conductance mechanism relies on the conserved H37XXXW41 sequence which is responsible for selectively gating H⁺ ions (Hu et al., 2010; Thomaston et al., 2018). Channel blockers interfere with the proton conductance mechanism by binding to the transmembrane pore (Cady et al., 2011; Jing et al., 2008; Thomaston et al., 2018; Wang et al., 2011c; Wu et al., 2014), thereby inhibiting viral replication.

Amantadine was the first M2 blocker discovered during early antiviral screening campaigns. Amantadine contains a hydrocarbon cage and a primary amine that favorably binds to the pore of the channel through hydrophobic and electrostatic interactions atop the H37 residue. However, as with most antivirals, the M2 channel of influenza viruses can develop several mutations to prevent drug-mediated proton blockage. The pace to which amantadine resistance emerges upon its widespread use has led to restrictions of amantadine as an antiviral. Nevertheless, recent efforts in developing second-generation M2 channel blockers

by targeting amantadine-resistant M2 mutants have led to the discovery of several classes of inhibitors with both *in vitro* (Hu et al., 2018; Hu et al., 2017b; Li et al., 2017; Li et al., 2016a; Li et al., 2016b; Wang et al., 2011b; Wang et al., 2018; Wu et al., 2014) and *in vivo* (Hu et al., 2017c) antiviral efficacy. This progress invigorates the interest in developing antivirals by targeting the viral ion channels (Wang, 2016). In clinically isolated strains of influenza viruses, three major amantadine-resistant mutations in the M2 channels have been identified: L26F, V27A, and S31N (Dong et al., 2015). Several advancements in the structural characterization of M2 helped the development of not only mono-specific V27A and S31N inhibitors, but also dual inhibitors that target either both WT and V27A (Wang et al., 2011a) or both WT and S31N channels simultaneously (Wang et al., 2013a; Wu et al., 2014). However, no M2 channel blockers have been developed against drug-resistant double mutants such as the M2-S31N/L26I and the M2-S31N/V27A, which are the predominant mutations found in H5N1 viruses.

H5N1 from 2002-2012 have been found to be resistant to M2 and NA inhibitors (Govorkova et al., 2013). In the case of human and avian origin H5N1 isolated between 1996 to 2005 in Vietnam, Cambodia, Malaysia and Thailand, more than 90% contain the double mutant M2-S31N/L26I channel (Cheung et al., 2006). Influenza strains with double M2 mutations have been identified in seasonally circulating strains as well, with the most commonly identified double mutant is the S31N/V27A (Dong et al., 2015). M2-S31N/V27A has been identified in H1, H3, H5, and H7 strains from human, avian and swine reservoirs (Dong et al., 2015). Specifically, the M2-S31N/V27A mutation was observed in an immunocompetent child infected with the 2009 pandemic H1N1 without the treatment of adamantane (Anton et al., 2010). Alarming, in recombinant H1N1, the double mutant M2-S31N/V27A led to the highest mortality rate *in vivo* compared to WT or any of the single amantadine-resistant mutants, and the M2-S31N/V27A double mutant showed similar replication rates in cell culture as the WT (Abed et al., 2005). Altogether, the prevalence of the M2 double mutants, especially M2-S31N/L26I and M2-S31N/V27A, among H5N1 viruses offer an opportunity for therapeutic intervention. As of date, no antiviral has been developed to target these amantadine-resistant double M2 mutants (S31N/L26I and S31N/V27A). As such, in this work, we aimed to identify potent channel blockers against M2-S31N/L26I and M2-S31N/V27A double mutants. This effort led to the discovery of three compounds **6**, **7**, and **15** that significantly block all three M2 mutants: M2-S31N, M2-S31N/L26I, and M2-S31N/V27A. We further demonstrated their antiviral efficacy in cell culture using recombinant viruses generated from reverse genetics. In summary, this study represents the first example in developing antivirals against influenza viruses by targeting the predominant double M2 mutants.

2. Materials and methods

2.1 M2-S31N inhibitors

The M2 inhibitors tested in this study were from our previously published studies. Specifically, compounds **1-5** were reported in (Li et al., 2016b); compounds **6**, **8**, and **16** were reported in (Li et al., 2016a); compounds **7**, **11**, **12**, **13**, **14**, and **15** were reported in (Li

et al.,2017; compound **9** was reported in (Wang et al., 2013b); and compound **10** was reported in (Hu et al., 2017b).

2.2 Cell lines and viruses

Madin-Darby Canine Kidney (MDCK) and HEK293T cells were maintained in complete media (high glucose DMEM supplemented with L-glutamine, 10% fetal bovine serum and 100IU/ml penicillin) under 5% CO₂ at 37°C and passaged using standard cell culture procedures. Recombinant influenza A virus strain A/Udorn/1972 (rH3N2) was propagated in MDCK cells and stored with 0.5% BSA at –80°C.

2.3 Recombinant viruses

Recombinant viruses were constructed using influenza A/Udorn/1972 (rH3N2) background as previously described (Neumann et al., 1999). Eight pHH21 plasmids corresponding to each gene segment and four pcDNA plasmids encoding polymerase complex subunits were used to transfect 80-90% confluent HEK293T cells. HEK293T cells were co-cultured in MDCK cells approximately 24 h after transfection. Recombinant viruses were harvested and amplified in MDCK cells, and viral titers were determined by plaque assay. Mutations were introduced into the M segment pHH12 plasmid using QuikChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). For each recombinant virus, the M segment sequence was confirmed for each recombinant virus through Eton Bioscience, Inc.

2.4 Plaque reduction and growth curve assays

Plaque reduction assay was performed as previously described (Musharrafieh et al.,2018). EC₅₀ value was determined using ImageJ software to quantify the percent plaque size area and plotted using best-fit dose response curves. Growth curves were performed in MDCK cells using recombinant A/Udorn/72 at an MOI of 0.003. Infectious media was harvested at 6 time points and titers were quantified using endpoint dilution in plaque assays.

2.5 Electrophysiological TEVC assay

TEVC assays were performed using *Xenopus laevis* oocytes. Briefly, M2 from the influenza virus A/Udorn/1972 (H3N2) strain was inserted into pGEM3 vector followed by in vitro transcription. Synthesized M2 mRNA was microinjected into oocytes and incubated for one to four at 18°C for M2 expression (Musharrafieh et al., 2018). Single and double mutations in the M2 channel were introduced as described above.

2.6 M2 Sequence Analysis

M2 sequences from human and avian H5N1 viruses were obtained on mid 2019 from the NCBI Influenza Virus Research online database (www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database). Sequence analysis was done using MEGA6 software (www.megasoftware.net). Residues 26-31 were aligned using the MUSCLE tool for analysis.

3. Results and Discussion

3.1. Prevalence of amantadine-resistant M2 mutants among avian and human H5N1 viruses

During the first outbreak of avian H5N1 in 1997, a small cluster of human infections were identified. However, around 2003, international spillover of circulating avian H5N1 to humans were reported (Lai et al., 2016). Therefore, to determine the prevalence of amantadine-resistant M2 channels for avian and human H5N1 isolates, we reanalyzed sequences collected between 2002 to present. M2 sequences from human and avian H5N1 viruses were obtained mid 2019 from the NCBI Influenza Virus Research online database and analyzed. Strains resistant to amantadine in avian isolates between 2002-2019 are predominantly S31N (28.6% of amantadine-resistant strains) or S31N/L26I (60.9% of amantadine-resistant strains) (Fig 1). Other drug-resistant mutations such as the V27A or S31N/V27A were isolated in much fewer numbers. From 2002-2007, the predominant amantadine-resistant M2 contained the S31N/L26I mutation as shown in Fig. 1. For the human H5N1 viruses, the highest number of isolates were collected during 2005-2007, whereby the V27A mutation was the most frequent mutant, followed by the double mutant S31N/L26I. From 2002-2019, the double mutant S31N/L26I remained the predominant amantadine-resistant mutant, accounting up to 50.6% of total drug-resistant isolates. There were no amantadine-resistant strains found in strains collected in the past six years (2013-2019), but this period had a smaller sample size (18). Altogether, there is a significant percentage of amantadine-resistant M2 double mutants in both human and avian H5N1 strains, particularly between 2002-2007 for human isolates and 2002-2004 for avian isolates.

Within both human and avian H5N1 strains, we did not observe any M2-L26I/V27A double mutants, nor an appreciable amount of M2-S31N/L26I/V27A triple mutants (data not shown). This may suggest that only a limited arrangement of multiple amantadine-resistant substitutions within M2 are allowed.

3.2. Identification of potent M2 channel blockers for S31N/L26I and S31N/V27A mutants

To date, there has been no report on the inhibition of these double mutants by M2 inhibitors. With our continuous interest in developing M2 channel blockers, we aimed to expand the spectrum of activity of S31N inhibitors to S31N/V27A and S31N/L26I as well. Amantadine derivatives such as **WJ332** were developed to inhibit the single M2-S31N mutant with better potency than amantadine on WT channels (Wang et al., 2013b; Williams et al., 2013).

WJ332 contains an isoxazole with amantadine and aryl substituents (Fig. 2A). A closer examination of the molecular interactions of drug binding shows that both the isoxazole and aryl group are in close proximity with residues 26 and 27 (Fig. 2A) (Wang et al., 2013b; Williams et al., 2013). Therefore, we hypothesize that varying the isoxazole and aryl substitutions might lead to inhibitors targeting the M2 double mutants S31N/L26I and S31N/V27A.

With this strategy, we selected a library of M2-S31N inhibitors with diverse aryl or biaryl chemical modifications as shown in Fig. 2B. All compounds (**1-16**) selected had potent inhibition against the M2-S31N mutant as tested before. Using two-electrode voltage clamp

(TEVC) assay, we tested this series of M2-S31N inhibitors to profile their channel blockage efficacy against both S31N/L26I and S31N/V27A M2 double mutant channels expressed in oocytes. Specifically, M2 gene from the influenza virus A/Udorn/1972 (H3N2) strain with single or double amantadine-resistant mutations were inserted into pGEM3 vector followed by *in vitro* transcription. Synthesized M2 mRNA was microinjected into oocytes for expression. Each compound was tested at 100 μ M for 2-min application, and the results are presented in Fig. 3. Except for compounds **5** and **16**, all compounds that inhibited the M2-S31N channel conductance had nearly identical inhibition for the M2-S31N/L26I double mutant channel as well. In contrast, most of the compounds tested had reduced effect on the conductance of M2-S31N/V27A double mutant. Nevertheless, three compounds, **6**, **7** and **15** demonstrated 30-40% inhibition against the M2-S31N/V27A double mutant channel.

3.3. Compounds **6**, **7**, and **15** demonstrate antiviral activity in plaque assay.

H5N1 viruses are currently restricted to biosafety level 3 (BSL-3) containment labs, and clinical isolates of H5N1 viruses harboring the M2-S31N/L26I or the M2-S31N/V27A double mutants are not readily accessible. As such, in order to determine whether the M2 double mutant inhibitors selected from the electrophysiological assay will also inhibit viral replication, we used reverse genetics to generate recombinant viruses that encode either the single- or double-mutant M2 variants. Recombinant viruses were constructed using the background sequence from the influenza A/Udorn/1972 (rH3N2) virus.

All three rH3N2 viruses, rH3N2 M2-S31N, rH3N2 M2-S31N/L26I, and rH3N2 M2-S31N/V27A, had similar growth curves, indicating no effect caused by the single or double mutation (Fig. 4A). The antiviral efficacy of compounds **6**, **7** and **15** against the recombinant rH3N2 viruses was tested in plaque assay (Fig. 4B, table 1). The EC₅₀ values of compounds **6**, **7** and **15** against rH3N2 M2-S31N/L26I virus were 0.5 ± 0.2 , 1.2 ± 0.04 , 2.0 ± 0.9 μ M, respectively, slightly higher than that of the H1N1 M2-S31N virus. For the rH3N2 M2-S31N/V27A virus, the EC₅₀ values were approximately ten-fold higher than that of rH3N2 M2-S31N/L26I. Overall, these results are consistent with the electrophysiology assay results, and these compounds might represent a starting point for further chemical optimization of inhibitors against the amantadine-resistant single and double mutants of M2 channels from influenza A viruses.

4. Conclusions

The inhibition of amantadine-resistant single and double M2 mutants from the influenza viruses was investigated. M2-S31N remains the dominant amantadine-resistant strain in avian, swine and human influenza viruses, and is believed to have emerged prior to amantadine use in the early 19th century. Therefore, the focus on targeting the single-mutant S31N should continue. However, the rise of double mutants such as S31N/V27A and S31N/L26I with similar fitness levels as WT may allow for sustained presence in circulating seasonal and pandemic strains.

In this study, we identified two amantadine-resistant M2 double mutants among avian and human H5N1 viruses: the predominant M2-S31N/L26I mutant and the less frequent M2-S31N/V27A mutant. It was found that M2-S31N inhibitors in general also inhibit M2-S31N/

L26I channel in both electrophysiological assay and cellular antiviral assay, suggesting an additional L26I did not significantly alter the M2 structure and drug sensitivity. From the **WJ332**/M2-S31N complex structure (Fig. 2A), side chain of L26 faces towards exterior of the M2 four-helix bundle, therefore its mutation might have minimal or no effect on the drug binding inside the channel. In contrast, M2-S31N/V27A double mutant channel appeared to be significant different from the M2-S31N channel, as majority of the M2-S31N inhibitors tested were less active against the M2-S31N/V27A mutant, except three compounds **6**, **7** and **15**. The side chain of V27 faces the interior of the channel and forms a hydrophobic interaction with the thiophene ring from **WJ332** (Fig. 2A), and V27A mutation might have a direct impact on drug binding.

Although M2-S31N/V27A occurs at a low frequency among H5N1 viruses, its importance should not be ignored. This is because the M2-S31N/V27A mutant virus had similar fitness of replication as the WT, but is more virulent. It is possible that this double mutant might become the predominant mutant among not only HPAI H5N1 viruses, but also seasonal H1N1 and H3N2 viruses, given the fact that influenza viruses exist in quasispecies and the dynamics might change overtime. The three compounds we identified herein **6**, **7**, and **15** are promising candidates for further optimization into potent channel blockers against not only M2-S31N but also M2-S31N/L26I and M2-S31N/V27A.

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Highlights

- M2-S31N/L26I and M2-S31N/V27A are prevalent among H5N1 viruses
- Recombinant viruses harboring these M2 double mutants replicate as efficient as WT in cell culture
- Three compounds 6, 7, and 15 were identified to effectively block these double M2 mutants in addition to M2-S31N
- The three compounds 6, 7, and 15 inhibited the replication of recombinant viruses harboring these M2 double mutants

Fig. 1.

Analysis of the frequency of amantadine-resistant M2 from clinically isolated H5N1 strains between 2002 and 2019. Strains were obtained from NCBI influenza database and aligned using MEGA7. (Top) Out of a total of 2805 avian strains, 684 contained amantadine-resistant mutations. (Bottom) Out of 264 strains that have been isolated from humans, 148 were amantadine-resistant.

Fig. 2. Solution NMR structure of **WJ332** in complex with M2-S31N (A) and the chemical structures of adamantane derivatives developed against M2-S31N mutant. Compounds **1-16** contain either an adamantane or hydroxyl adamantane with aryl or biaryl substituents.

Fig. 3. Functional inhibition of amantadine-resistant S31N, S31N/L26I, and S31N/V27A mutations by compounds **1-16**. TEVC recordings of each mutant were performed for each compound at 100 μ M as previously described (Ma et al., 2016). The results are mean $\pm$ standard deviation from three repeats.

Fig. 4.

Replication rates and neutralization assay against amantadine-resistant mutants. (A) Growth curves of S31N, S31N/V27A and S31N/L26I rH3N2 were performed in MDCK cells. At each time point, a small volume was removed and titrated to determine PFU/ml. Values represent mean $\pm$ standard deviation from two biological replicates. (B) Compound **6** was tested at different concentrations in plaque assay using rH3N2 viruses. EC₅₀ values for S31N/L26I and S31N/V27A were 1.5 ± 0.7 and 11.6 ± 2.5 μ M, respectively.

Table 1.

The antiviral efficacy of lead compounds against double mutant recombinant viruses

Compound	CC_{50}^a (μ M)	EC_{50}^b (μ M)		
		H1N1	rH3N2	
		S31N	S31N/L26I	S31N/V27A
Amantadine	>300	>300	ND	ND
6	30.6 ± 5.0	0.5 ± 0.2	1.5 ± 0.7	11.6 ± 2.5
7	>200	0.1 ± 0.04	0.5 ± 0.2	26.9 ± 10.6
15	59.3 ± 2.6	1.2 ± 0.2	2.0 ± 0.9	23.7 ± 5.1

ND: Not determined

^aCC₅₀ values were obtained using neutral red cytopathic effect assay in MDCK cells^bEC₅₀ values were obtained using plaque reduction assays in MDCK cells Values are expressed as mean $\pm$ standard deviation from duplicate experiments