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Discovery of potent antivirals against amantadine-resistant influenza A viruses by targeting the M2-S31N proton channel

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Abstract

Despite the existence of flu vaccines and small molecule antiviral drugs, influenza virus infection remains a public health concern that warrants immediate attention. As resistance to the only orally bioavailable drug, oseltamivir, has been continuously reported, there is a clear need to develop the next-generation of anti-influenza drugs. We chose the influenza A virus M2-S31N mutant proton channel as the drug target to address this need as it is one of the most conserved viral proteins and persist in more than 95% of currently circulating influenza A viruses. In this study, we report the development of a late-stage diversification strategy for the expeditious synthesis of M2-S31N inhibitors. The channel blockage and antiviral activity of the synthesized compounds were tested in two-electrode voltage clamp assays and antiviral assays, respectively. Several M2-S31N inhibitors were identified to have potent M2-S31N channel blockage and micromolar antiviral efficacy against several M2-S31N containing influenza A viruses.

Graphical abstract

Keywords

Influenza A virus; M2 proton channel; M2-S31N inhibitor

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Supporting information

Synthesis procedures, ¹HNMR, ¹³CNMR, and MS of intermediates and final products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

INTRODUCTION

Each year an estimated 10–15% of the population is infected by influenza viruses in the United States,¹ which accounts for 36,000 deaths and 200,000 hospitalizations.^{2, 3} In addition, emerging or re-emerging influenza strains such as H5N1 and H7N9 have the potential of leading to influenza pandemics, which are often associated with higher mortality rates.⁴ As the standard of care, vaccines are the most effective way to prevent influenza infection; however, their efficacy is limited to an immune-competent population.⁵ Moreover, vaccines have to be reproduced every year to match the antigens from viruses in the coming year, and the production process normally takes at least six months.^{6, 7} Therefore, effective vaccines are unlikely to be available at the first wave of an influenza outbreak. In such a scenario, small molecule antivirals that target the most conserved viral proteins, such as the A/M2 proton channel, are highly desired.^{8, 9}

The influenza virus is a negative-strand RNA virus belonging to the orthomyxoviridae family. Each influenza virion contains eight segments of RNA. Due to the lack of proofreading function of the influenza virus polymerase and the segmented nature of its RNAs, the influenza virus undergoes both antigenic drift and antigenic shift during its replication, which results in a large diversity of influenza types and subtypes. Although this is beneficial for viral adaption and evolution, it creates a grand challenge in designing antiviral drugs. Ideally, an antiviral drug should target the most conserved viral proteins or host factors such that its efficacy does not depend on strain variation. The M2-S31N proton channel is such a drug target that meets this criterion. It is one of the most conserved viral proteins among influenza A viruses. Genetic sequencing revealed that more than 95% of current circulating influenza A viruses carry this mutation, rendering M2-S31N a high-profile drug target.¹⁰

M2 is a proton-selective homotetrameric channel associated with the viral membrane.^{11, 12} The function of M2 is to facilitate viral uncoating during the early stage of viral replication. Additionally, in certain influenza strains, M2 also helps equilibrate the pH across the Golgi apparatus, thereby preventing premature conformational change of hemagglutinin.¹³ M2 is absolutely required during viral replication: deleting the M2 gene or blocking the proton conductance of M2 using small molecules leads to inhibition of viral replication. Thus M2 is a validated drug target. The adamantanes, such as amantadine and rimantadine, are wild-type (WT) M2 channel blockers. However, their use has been limited by prevalent drug resistance. It is important to stress that although a large number of M2 mutants were selected from cell culture, animals, and human patients upon drug treatment, only a limited number of M2 mutants evolved the fitness of transmission among humans, namely V27A, S31N, and L26F.^{14, 15} Among these, S31N is the most prevalent mutant and is persist in the majority of current circulating influenza A viruses, including the pandemic A/California/07/2009 (H1N1) strain and the recently emerged A/Switzerland/9715293/2013 (H3N2) strain in North America.¹⁶

Propelled by mechanistic studies of the proton conductance and drug inhibition mechanisms by X-ray crystallography,^{17–23} NMR,^{19–21} and molecular dynamics simulations,^{24, 25} recent years have witnessed steady progress toward designing inhibitors targeting M2-S31N,¹⁰

which was previously considered undruggable despite decades of traditional medicinal chemistry efforts. The pharmacophore of M2-S31N channel blockers consists of a hydrophobic template such as adamantane, a positively charged ammonium linker, and an aromatic head group with a hydrophobic substitution (Figure 1).^{21, 26, 27} It has been found that the adamantane template and the ammonium linker are essential for M2-S31N inhibition. Substituting adamantane with other hydrophobic cage structures leads to a decrease of M2-S31N channel inhibition. In contrast, diverse aromatic head groups with either cyclic or acyclic hydrophobic substitutions are tolerated (Figure 1B). In this study, in order to provide additional structure diverse lead compounds for in vivo animal studies, we examined the structure–activity relationships of M2-S31N inhibitors by diversifying the aromatic head groups and the hydrophobic substitutions (Figure 1D). We devised an expeditious synthesis strategy that enables us to diversify the hydrophobic substitutions at the last step, which greatly facilitates the lead optimization. Synthesized compounds were tested in both electrophysiological assays and antiviral assays. This effort led to several lead compounds with potent channel blockage and single micromolar antiviral efficacy as well as high selectivity.

RESULTS AND DISCUSSION

CHEMISTRY

In the present study, the goal is to optimize the channel blockage potency of M2-S31N inhibitors by diversifying the aromatic head groups and the hydrophobic substitutions. Toward this goal, we devised a late-stage functionalization strategy that allows us to conveniently introduce diverse hydrophobic substitutions at the last step. Specifically, the Buchwald–Hartwig C–N cross-coupling reaction was employed to install various hydrophobic substitutions on the aromatic head groups (pyrimidine, pyridine, and pyrazine). The precursors (**9**, **12**, **15**, and **19**) for the cross-couplings were prepared using previously optimized reductive amination reaction conditions.²⁶ The yield ranged from 79% to 84%. Compound **10d** was synthesized directly from 2-phenylpyrimidine-5-carbaldehyde by reductive amination. The optimized conditions for Buchwald–Hartwig cross-coupling reactions for pyrimidine were found to be Pd₂(dba)₃ (5% mol), BINAP (15% mol), and NaOtBu (1.4 equiv.) under microwave heating at 100 °C in toluene for 30 minutes. The yields ranged from 72 to 85%. When applying this condition to the synthesis of pyridine (2-substituted or 3-substituted) and pyrazine analogs, it also gave similar satisfactory yields. The yields for 2-substituted pyridine analogs, 3-substituted pyridine analogs, and pyrazine analogs ranged from 75 to 82%, 75 to 81%, and 71 to 78%, respectively. Overall, this synthesis route proved to be robust and amenable for parallel synthesis.

CHANNEL BLOCKAGE PROFILING BY TWO-ELECTRODE VOLTAGE CLAMP ASSAYS

Synthesized compounds were tested for their channel blockage against the M2-S31N proton channel using the two-electrode voltage clamp (TEVC) assay. TEVC is one of the gold standard assays for studying ion channel current conductance and drug inhibition.²⁵ In this assay, the M2-S31N channel was expressed in the oocyte cell membrane and was activated by acidic pH (pH = 5.5). All compounds were tested at 100 μM, and the channel conductance current was recorded at the two-minute time point after applying compounds.

The activity of the compound was expressed as a percentage of current inhibition. For the pyrimidine series of compounds, the intermediate compound **9**, which lacks a hydrophobic substitution, had minimal M2-S31N channel inhibition ($8.8 \pm 1.9\%$) at $100 \mu\text{M}$ (Table 1). Similar results were found for other precursors, such as compounds **12**, **15**, and **19**, and all of them had weak M2-S31N channel inhibition (Table 1). When a pyrrolidine substitution was introduced to the pyrimidine aromatic head group, compound **10a** showed increased M2-S31N channel inhibition ($47.8 \pm 2.8\%$). The activity was further improved for compounds with piperidine (**10b** and **10c**), azepane (**10f**), and azocane (**10h** and **10i**) substitutions. Compound **10d** with a benzene substitution was less active than the piperidine analog (**10c**) ($57.7 \pm 1.0\%$ versus $67.2 \pm 1.5\%$). Compound **10e** with a hydrophilic substitution, morpholine, was also much less active than the piperidine analog (**10c**) ($26.4 \pm 3.0\%$ versus $67.2 \pm 1.5\%$). A hydroxyl substitution at the 3-position of adamantane was generally tolerated for the pyrimidine series of compounds (**10b** versus **10c** and **10h** versus **10i**) except the azepane analogs (**10f** versus **10g**). Overall, these results indicate that a hydrophobic substitution on the aromatic head group is required for M2-S31N inhibition. The optimal hydrophobic substitutions are piperidine, azepane, and azocane. Similar SAR results were also found for the pyridine and pyrazine series of analogs. For example, for the 2-substituted pyridine series, compounds with azepane (**13c**) and azocane (**13d** and **13e**) substitutions were more potent than the piperidine analogs (**13a** and **13b**). For the 3-substituted pyridine series, compounds with azepane (**16c** and **16d**) and azocane (**16e** and **16f**) substitutions were similarly more potent than the piperidine analogs (**16a** and **16b**). The most potent compound from the pyridine series was **16d**, which showed $85.8 \pm 0.9\%$ inhibition at $100 \mu\text{M}$. For the pyrazine series, compounds with azepane substitutions (**20c** and **20d**) outperformed piperidine analogs (**20a**) and azocane analogs (**20e** and **20f**). In summary, pyrimidine, 3-substituted pyridine, and pyrazine are the more favorable aromatic head groups than 2-substituted pyridine, and azepane and azocane are the more favorable hydrophobic substitutions than pyrrolidine, piperidine, and morpholine (Figure 2).

ANTIVIRAL ACTIVITY OF POTENT LEAD COMPOUNDS

As M2 is essential for viral replication, M2 channel blockers are expected to have potent antiviral activity. To test this hypothesis, we evaluated several compounds (**10b**, **10c**, **10f**, **16d**, **16f**, and **20c**) for their antiviral activity and cytotoxicity. Compounds **10f**, **16d**, and **20c** all had more than 80% M2-S31N channel inhibition at $100 \mu\text{M}$. Compounds **10c** and **16f**, which had less than 70% M2-S31N channel inhibition at $100 \mu\text{M}$, were also chosen in order to determine a correlation between the channel blockage activity and the antiviral activity. The antiviral activity was tested against the amantadine-resistant A/WSN/33 (H1N1) virus, which contains the M2-S31N mutant, using the cytopathic effect (CPE) assay. All compounds were found to display single to submicromolar EC_{50} values against A/WSN/33 (H1N1), which are comparable to the antiviral efficacy of amantadine in inhibiting the WT A/Udorn/72 (H3N2) virus.²⁸ Moreover, there is a positive correlation between M2-S31N channel blockage activity and antiviral activity: compounds **10b**, **10f**, **16d**, and **20c**, which had more potent channel blockage activity in electrophysiological assays than compounds **10c** and **16f**, were also more active in the antiviral CPE assays than compounds **10c** and **16f**. These results are consistent with previous findings.²¹

As selectivity index is critical for lead optimization, the cytotoxicity of the compounds was tested in both MDCK cells and the human epithelial cells A549 (Table 2). Compound **10b** had moderate cytotoxicity, with CC₅₀ values of 33.9 μ M and 72.1 μ M against MDCK and A549 cells, respectively. The selectivity index greatly improved upon introduction of a hydroxyl group at the 3-position of adamantane, and the CC₅₀ values for compound **10c** were over 300 μ M for both MDCK and A549 cells. Although the pyrimidine analog **10f** had the most potent antiviral efficacy, with an EC₅₀ value of 0.7 μ M, it was moderately cytotoxic with CC₅₀ values of 38.1 μ M and 29.3 μ M against MDCK and A549 cells, respectively. The 3-substituted pyrimidine analogs (**16d** and **16f**) were found to have both potent antiviral activity and favorable selectivity indexes, which warrants further development. Interestingly, the pyrazine analog **20c** was more cytotoxic in MDCK cells than in A549 cells.

ANTIVIRAL ACTIVITY OF POTENT LEAD COMPOUNDS AGAINST CLINICALLY RELEVANT HUMAN INFLUENZA A STRAINS

To further prove the antiviral efficacy of M2-S31N inhibitors against M2-S31N-containing influenza A viruses, we tested two inhibitors, the pyrimidine **10c** and 3-substituted pyridine **16d**, against two clinically relevant human influenza A strains, A/Switzerland/9715293/2013 (H1N1) and A/California/07/2009 (H1N1). Both of these strains are the predominant strains circulating among humans in North America in recent years, and both of them are amantadine-resistant and contain the M2-S31N mutant.¹⁶ Thus it is expected that compounds **10c** and **16d** should have potent antiviral activity against these two strains. The antiviral EC₅₀ values of the pyrimidine compound **10c** in inhibiting A/Switzerland/9715293/2013 (H1N1) and A/California/07/2009 (H1N1) are 3.4 ± 0.8 μ M and 4.7 ± 0.8 μ M, respectively (Figure 3), which were similar to its efficacy in inhibiting A/WSN/33 (H1N1) (Table 2). Similarly, the antiviral EC₅₀ values of the pyridine compound **16d** in inhibiting A/Switzerland/9715293/2013 (H1N1) and A/California/07/2009 (H1N1) are 3.2 ± 0.7 μ M and 8.7 ± 3.7 μ M, respectively (Figure 3). In summary, compounds **10c** and **16d** were found to have consistently low micromolar antiviral efficacy against several M2-S31N-containing influenza A viruses.

CONCLUSIONS

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 the 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.^{29–31} 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. The reason why we chose M2-S31N mutant as the drug target is because it is one of the most conserved viral proteins among circulating influenza A viruses. Although it is generally assumed that M2 is not a desired antiviral drug because of the failed example of amantadine: resistance easily emerges with amantadine treatment in the case of WT viruses. However, our recent studies demonstrated that unlike amantadine, M2-S31N channel blockers have a higher genetic barrier of drug resistance that is comparable to oseltamivir.³² Therefore, M2-S31N remains a high-profile antiviral drug target to combat drug resistance. In this study, we

developed a late-stage diversification synthesis strategy that enabled us to expeditiously synthesize a focused library of M2-S31N inhibitors. All synthesized compounds were tested in electrophysiological assays, and potent lead compounds were further tested in antiviral assays. It was found that a hydrophobic substitution, preferentially piperidine and azepane, is required at the para-position of the aromatic head group for potent M2-S31N channel blockage. A number of heterocycles such as pyridine, pyrimidine, and pyrazine are tolerated. Hydroxyl group substitution on the adamantane ring appears to lower the cytotoxicity, possibly due to reduced hydrophobicity. Potent channel blockers were also consistently found to have greater antiviral efficacy. The most potent compounds **10c** and **16d** have single-digit micromolar efficacy against all three M2-S31N-containing influenza A viruses, A/WSN/22 (H1N1), A/Switzerland/9715293/2013 (H1N1), and A/California/07/2009 (H1N1). The potent antiviral efficacy, coupled with the favorable selectivity indexes, warrant further evaluation of these lead compounds in in vivo animal studies. They can be used either as monotherapy or in combination with oseltamivir to confine the outbreak of next influenza pandemic.³³

EXPERIMENTAL SECTION

Chemistry

All chemicals are commercially available and were used without further purification. Detailed synthesis procedures can be found in the Supporting Information for reactions described in Scheme 1. All final compounds were purified by flash column chromatography, and the purity of all compounds tested was 95% as determined by LC-MS. Compound characterizations (¹HNMR, ¹³CNMR, and MS) can be found in the Supporting Information.

Two-electrode voltage clamp (TEVC) assay

The compounds were tested in a two-electrode voltage clamp assay using *Xenopus laevis* frog oocytes microinjected with RNA expressing the S31N mutant of the A/M2 protein, as previously reported.³⁴ The potency of the inhibitors was expressed as percentage inhibition of A/M2 current observed after 2 minutes of incubation with 100 μM of compounds at pH 5.5. All measurements were repeated three times with different oocytes.

Plaque reduction assay

The plaque reduction assay was performed as previously reported,²¹ except MDCK cells expressing ST6Gal I were used instead of regular MDCK cells.³⁵ Briefly, confluent monolayer of ST6Gal MDCK cells were incubated with ~100 pfu virus samples in DMEM with 0.5% BSA for 1 hour at 4°C, then 37°C for 1 hour. The inoculums were removed, and the cells were washed with phosphate buffered saline (PBS). The cells were then overlaid with DMEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) and NAT (2.0 μg/mL). To examine the effect of the compounds on plaque formation, the overlay media was supplemented compounds at testing concentration. At 2 days after infection, the monolayers were fixed and stained with crystal violet dye solution (0.2% crystal violet, 20% methanol). The viruses used for this assay were A/WSN/33 (H1N1), A/Switzerland/9715293/2013 (H1N1), and A/California/07/2009 (H1N1), all of which contain the M2-S31N mutant. Influenza A viruses, A/Switzerland/9715293/2013

X-247 (H3N2), FR-1366, and A/California/07/2009 (H1N1)pdm09, FR-201, were obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA.

Cytotoxicity assay and cytopathic effect assay

Evaluation of the cytotoxicity of compounds and the efficacy of compounds against influenza induced cytopathic effect was carried out using neutral red uptake assay.³⁶ Briefly, 80,000 cells/mL MDCK cells in DMEM medium supplemented with 10% FBS and 100 U/mL Penicillin-Streptomycin were dispensed into 96-well cell culture plates at 100 μ L/well. Twenty-four hours later, the growth medium was removed and washed with 100 μ L PBS buffer; then for cytotoxicity assay, 200 μ L fresh DMEM (No FBS) medium contains serial diluted compounds was added to each well; for cytopathic effect assay, 200 μ L fresh DMEM medium contains 100 pfu of A/WSN/33 influenza virus, 2 μ g/mL NAT, and serial diluted compounds was added to each well. After incubating for 48 hours at 37 °C with 5% CO₂ in a CO₂ incubator, the medium was removed and replaced with 100 μ L DMEM medium contains 40 μ g/mL neutral red for 4 hours 37 °C. The amount of uptaken neutral red was determined at absorbance 540 nm using a Multiskan FC Microplate Photometer (Fisher Scientific). The EC₅₀ and CC₅₀ values were calculated from best-fit dose response curves with variable slope in Prism 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

WT	wild type
SAR	structure–activity relationship
DMEM	Dulbecco's modified eagle medium
MDCK	Madin–Darby Canine Kidney
TEVC	two-electrode voltage clamps

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Figure 1. M2-S31N inhibitors. (A) Examples of previously discovered M2-S31N inhibitors. (B) M2-S31N inhibitor pharmacophore. (C) Representation of compound **1** binding to the M2-S31N channel (PDB: 2LY0). (D) Synthesis of potent M2-S31N inhibitors by microwave-assisted Buchwald–Hartwig cross-coupling. N.T. = not tested.

Figure 2.
SAR summary of M2-S31N inhibitors explored in this study.

Figure 3.
Antiviral efficacy of potent M2-S31N inhibitors in inhibiting clinically relevant human influenza A viruses. The antiviral efficacy was determined using plaque reduction assays.

A. Synthesis of pyrimidine analogs **B. Synthesis of 2-substituted pyridine analogs** **C. Synthesis of 3-substituted pyridine analogs** **D. Synthesis of pyrazine analogs** **Scheme 1.**

Synthesis routes to M2-S31N inhibitors bearing various aromatic head groups and hydrophobic substitutions.

Table 1

Structure-channel blockage activity relationships of M2-S31N inhibitors

R	A	Compound ID	Electrophysiological TEVC assay* (% S31N channel inhibition)	Electrophysiological TEVC assay* (% WT channel inhibition)
	H	9	8.8 ± 1.9	N.T.
	OH	10a	47.8 ± 2.8	N.T.
	H	10b	74.4 ± 3.3	12.0 ± 0.8
	OH	10c	67.2 ± 1.5	2.0 ± 2.0
	OH	10d	57.7 ± 1.0	N.T.
	OH	10e	26.4 ± 3.0	N.T.
	H	10f	83.8 ± 2.8	0.7 ± 0.7
	OH	10g	36.4 ± 3.0	N.T.
	H	10h	79.0 ± 1.4	3.5 ± 0.7
	OH	10i	80.3 ± 2.9	N.T.
	H	12	19.5 ± 0.5	N.T.
	H	13a	67.8 ± 3.3	3.0 ± 3.0
	OH	13b	47.5 ± 2.7	N.T.
	H	13c	69.5 ± 2.1	0.9 ± 0.9

H	13d	77.6 ± 0.5	18.6 ^{**}
	OH	13e	62.9 ± 2.3	N.T.
H	15	31.9 ± 1.4	N.T.
	H	16a	40.3 ± 1.8	10.0 ^{**}
OH	16b	45.2 ± 2.0	N.T.
	H	16c	74.4 ± 2.1	N.T.
OH	16d	85.8 ± 0.9	1.6 ^{**}
	H	16e	80.9 ± 2.9	N.T.
OH	16f	56.8 ± 3.7	N.T.
	H	19	13.2 ± 0.7	N.T.
H	20a	64.4 ± 3.0	24.1 ± 2.7
	H	20b	55.9 ± 0.5	N.T.
H	20c	84.0 ± 1.6	12.3 ^{**}
	OH	20d	81.6 ± 1.3	N.T.
H	20e	74.9 ± 2.7	N.T.
	OH	20f	20.1 ± 2.8	N.T.

* Values represent the mean of three independent measurements. The data are presented as percent inhibition at 100 μ M at the two-minute time point.

** Measurements were only taken once. N.T. = not tested.

Table 2

Antiviral activity and cytotoxicity of M2-S31N inhibitors

Compounds	% M2-S31N channel inhibition at 100 μ M	CPE EC ₅₀ (μ M) A/WSN/33 (H1N1)	Plaque reduction assay EC ₅₀ (μ M) A/WSN/33 (H1N1)	Cytotoxicity CC ₅₀ (μ M) MDCK cells	Cytotoxicity CC ₅₀ (μ M) A549 cells
10b	74.4 $\pm$ 3.3	1.5 $\pm$ 0.6	1.7 $\pm$ 0.5	33.9	72.1
10c	67.2 $\pm$ 1.5	4.3 $\pm$ 0.3	4.5 $\pm$ 1.9	>300	>300
10f	83.8 $\pm$ 2.8	0.7 $\pm$ 0.1	N.T.	38.1	29.3
16d	85.8 $\pm$ 0.9	2.7 $\pm$ 0.1	3.6 $\pm$ 1.3	>100	>200

Compounds	% M2-S31N channel inhibition at 100 μ M	CPE EC ₅₀ (μ M) A/WSN/33 (H1N1)	Plaque reduction assay EC ₅₀ (μ M) A/WSN/33 (H1N1)	Cytotoxicity CC ₅₀ (μ M) MDCK cells**	Cytotoxicity CC ₅₀ (μ M) A549 cells**
16f	56.8 $\pm$ 3.7	7.9	N.T.*	>150	>150
20c	84.0 $\pm$ 1.6	1.9	N.T.*	45.7	>100

* N.T. = not tested.

** CC₅₀ values were determined by the MTT assay.