Structure–Property Relationship Studies of Influenza A Virus AM2-S31N Proton Channel Blockers

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Supporting Information

ABSTRACT: Majority of current circulating influenza A viruses carry the S31N mutation in their M2 genes, rendering AM2-S31N as a high profile antiviral drug target. With our continuous interest in developing AM2-S31N channel blockers as novel antivirals targeting both oseltamivir-sensitive and -resistant influenza A viruses, we report herein the structure–property relationship studies of AM2-S31N inhibitors. The goal was to identify lead compounds with improved microsomal stability and membrane permeability. Two lead compounds, **10d** and **10e**, were found to have high mouse and human liver microsomal stability ($T_{1/2} > 145$ min) and membrane permeability (>200 nm/s). Both compounds also inhibit both currently circulating oseltamivir-sensitive and -resistant human influenza A viruses (H1N1 and H3N2) with EC_{50} values ranging from 0.4 to 2.8 μ M and a selectivity index of >100 . We also showed for the first time that AM2-S31N channel blockers such as **10e** inhibited influenza virus replication at both low and high multiply of infection (10^2 – 10^6 pfu/mL) and the inhibition was not cell-type dependent. Overall, these studies have identified two promising lead candidates for further development as antiviral drugs against drug-resistant influenza A viruses.

KEYWORDS: Influenza A virus, AM2 proton channel, AM2-S31N inhibitor, microsomal stability, antiviral

Influenza viruses are negative sense, segmented RNA viruses that are the causative agents for annual influenza epidemic and sporadic influenza pandemics.¹ Despite the availability of small molecule antivirals and influenza vaccines, there is an influenza season every year. More concerning is the emerging of influenza pandemic outbreaks that normally occur every 10 to 20 years.² Part of explanation for the reoccurring influenza virus infection might be because influenza virus not only infects human but also many animals such as swine, migrating birds, chicken, horse, sea lions, etc. As such, there are multiple sources where human can contract the virus. When healthy immunocompetent adults are infected with seasonal influenza viruses, the symptoms are normally mild, and it is rarely fatal.³ Therefore, it might be somewhat surprising to learn that influenza virus infection is currently listed among the top-ten leading causes of deaths in the United States.⁴ The number of influenza virus-related mortality actually surpasses that of breast cancer. There are several factors that might contribute to the surprising death toll of influenza virus infection: (1) influenza virus is transmissible through the airways and can be quickly spread among humans. In each seasonal influenza epidemic, an estimate of 10–20% of the population are infected; (2) mortality rate of influenza virus infection among people in high-risk groups is high.⁵ They include seniors 65 years or older, people with chronic diseases such as cardiovascular diseases, diabetes, and high blood pressure, and

people with compromised immune system. In such cases, influenza virus infection normally serves as a trigger of these pre-existing conditions. Overall influenza virus infection is a persistent public health concern that cannot possibly be ignored.

Currently there are two classes of FDA-approved small molecule influenza antivirals:⁶ (1) adamantanes such as amantadine and rimantadine. They are channel blockers of the influenza virus AM2 proton channel and inhibit the early stage of viral replication by blocking the virus uncoating. (2) Neuraminidase (NA) inhibitors such as oseltamivir, zanamivir, and peramivir. They are mimics of sialic acid and inhibit the late stage of viral replication by blocking the virus egress. Resistance to both classes of drugs now necessitates the development of newer influenza antivirals.⁷ Majority of influenza A viruses ($>95\%$) are now resistant to adamantanes due to the AM2-S31N mutation in their M2 genes, and CDC no longer recommends the use of adamantanes in the prophylaxis and treatment of influenza virus infection. Resistance to oseltamivir has been continuously reported, and more alarmingly, the 2007–2009 seasonal influenza virus circulating in North American and Japan was

Received: August 2, 2018

Accepted: October 3, 2018

Published: October 3, 2018

completely resistant to oseltamivir due to the H275Y mutation in its NA gene.^{7,8} To tackle these drug-resistant viruses, several drug candidates are currently in development,^{6,9} which include both direct-acting antivirals such as the PA (polymerase acidic protein) endonuclease inhibitor baloxavir marboxil (approved in Japan and in late stage clinical trial in U.S.), polymerase inhibitor T-705 and PB2 inhibitor JNJ-63623872, as well as host-targeting antivirals such as nitazoxanide and DAS181. In addition, a large number of other drug targets are also actively pursued in the early stage of development.¹⁰

To design novel antivirals that are active against both oseltamivir-sensitive and -resistant influenza A viruses, we focus on targeting the influenza AM2-S31N proton channel.^{14–16} AM2-S31N is a high profile antiviral drug target, and more than 95% of current circulating influenza A viruses carry this mutation.¹⁷ Therefore, AM2-S31N channel blockers are expected to inhibit both oseltamivir-sensitive and -resistant influenza A viruses. As a proof-of-concept, we have shown that our rationally designed AM2-S31N inhibitors not only have potent channel blockage but also effective antiviral activity against multiple human influenza A viruses that are in circulation in recent years, including both H1N1 and H3N2 viruses that are resistant to either amantadine, oseltamivir, or both.^{14–16} Importantly, the newly developed AM2-S31N inhibitors showed a higher genetic barrier to drug resistance than amantadine, and drug resistance only emerged under high drug selection pressure after several passages.^{18,19} To further advance these promising lead compounds to in vivo mice model studies and prove their in vivo antiviral efficacy, we report herein our progress in profiling the metabolism stability of previously reported AM2-S31N inhibitors. One candidate, compound **4**, was found to have good metabolic stability. Subsequent structure–activity and –property studies led to the discovery of two lead compounds, **10d** and **10e**, that have a long half-life in mouse and human liver microsomes ($T_{1/2} > 145$ min) as well as a high membrane permeability ($P_e > 200$ nm/s). Both compounds **10d** and **10e** showed improved selectivity index compared to that of compound **4**. Importantly, compounds **10d** and **10e** retain potent antiviral activity against both oseltamivir-sensitive and -resistant human H1N1 and H3N2 influenza A viruses ($EC_{50} = 0.4–2.8$ μ M), rendering them as promising candidates for the next step in vivo metabolic and efficacy studies in mice.

We have discovered several classes of adamantyl containing AM2-S31N inhibitors based on structure-guided design and medicinal chemistry optimization.^{11,12,14–16,20} Representative examples of the most potent AM2-S31N inhibitors were shown in Table 1. They had single to submicromolar efficacy in inhibiting AM2-S31N-containing human influenza A viruses, including current circulating H1N1 and H3N2 strains. As a first step to profile the in vitro absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties, we first tested their microsomal stability in mouse liver microsomes. Surprisingly, all compounds except compound **4** had low microsomal stability ($T_{1/2} < 30$ min) (Table 1), precluding them from further progression. Encouragingly, compound **4** showed good microsomal stability with a $T_{1/2} > 145$ min, and the predicted liver clearance is less than 38.0 mL/min/kg. Therefore, compound **4** was selected as a lead compound for further optimization.

Given the promising results of compound **4**, a library of thiophene containing AM2-S31N inhibitors were synthesized and tested for channel blockage, antiviral activity, cellular cytotoxicity, and microsomal stability. Specifically, two series of new adamantane analogs, the monoaryl adamantanes (**10a–10i**)

Table 1. Microsomal Stability Profiling of Previously Reported AM2-S31N Inhibitors

Compound structure and ID	MLM 0.5	
	$T_{1/2}$ (min)	$CL_{int(liver)}$ (mL/min/kg)
1 (ref ¹¹)	19.4	282.7
2 (ref ¹¹)	0.8	6762.7
3 (ref ¹¹)	22.1	248.3
4 (ref ¹²)	> 145	< 38.0
5 (ref ¹³)	1.0	5290.9
6 (ref ¹⁴)	4.4	566

and the bis-aryl adamantanes (**12a–12i**), were designed and synthesized (Figure 1).

The design was based on the AM2-S31N inhibitor pharmacophore (Figure 1A), which was derived from previous structure–activity relationship studies.¹⁶ Both series of adamantane analogs (**10a–10i** and **12a–12i**) meet the requirements of the AM2-S31N pharmacophore. Therefore, it is likely

Figure 1. Design and synthesis of AM2-S31N inhibitors. (A) Pharmacophore of AM2-S31N inhibitors. Adamantane cage fits in the hydrophobic cavity formed by Gly34, the ammonium linker forms a hydrogen bond with the Asn31 side chain carbonyl, and the substitution on the aryl group forms hydrophobic interactions with the Val27 side chain. (B) Solution NMR structure of monoaryl AM2-S31N inhibitor **4** in complex with AM2-S31N (19–49) (PDB: 2MUV).¹² (C) Solution NMR structure of bis-aryl AM2-S31N inhibitor **5** in complex with AM2-S31N (19–49) (PDB: 2LY0).¹³ (D) Synthesis of monoaryl adamantanes by reductive amination. (E) Synthesis of biaryl adamantanes by Suzuki–Miyaura cross-coupling reaction.

Table 2. Channel Blockage, Antiviral Activity, Cytotoxicity, and Mouse Liver Microsomal Stability of AM2-S31N Inhibitors

Structure/Compound ID	TEVC assay ^a (% S31N channel inhibition)	TEVC assay ^a (% WT channel inhibition)	% plaque formation at 10 μ M ^b	EC ₅₀ (μ M) A/California/07/2009 (H1N1) ^b	CC ₅₀ (μ M) MDCK cells ^c	T _{1/2} (min)	CL _{int} (liver) (ml/min/kg)
4	76.3 $\pm$ 2.5	77.2 $\pm$ 4.3	3.2 $\pm$ 0.5	2.9 $\pm$ 0.8	123.2 $\pm$ 8.6	>145	<38.0
10a	78.5 $\pm$ 2.4	91 $\pm$ 1.6	1.8 $\pm$ 0.3	1.0 $\pm$ 0.1	70.2 $\pm$ 3.6	24.5	223.6
10b	70.6 $\pm$ 2.2	56.1 $\pm$ 0.8	0	2.2 $\pm$ 1.9	82.1 $\pm$ 5.2	32.1	170.7
10c	72.6 $\pm$ 1.4	86.5 $\pm$ 0.3	0	0.6 $\pm$ 0.1	43.1 $\pm$ 2.8	9.1	603.6
10d	64.7 $\pm$ 3.5	11.4 $\pm$ 1.1	3.9 $\pm$ 1.2	1.1 $\pm$ 0.2	238.1 $\pm$ 2.1	>145	<38.0
10e	75.6 $\pm$ 1.2	3.2 $\pm$ 1.5	2.5 $\pm$ 1.9	0.9 $\pm$ 0.1	317.4 $\pm$ 12.0	>145	<38.0
10f	56.6 $\pm$ 1.3	6.5 $\pm$ 1.4	31.4 $\pm$ 0.5	3.1 $\pm$ 0.3	380.7 $\pm$ 24.9	100.8	54.5
10g	48.3 $\pm$ 0.5	1.9 $\pm$ 0.9	31.3 $\pm$ 0.5	5.1 $\pm$ 0.6	363.7 $\pm$ 18.3	N.T.	N.T.
10h	36.0 $\pm$ 0.4	3.9 $\pm$ 0.0	108.0 $\pm$ 1.8	N.T.	N.T.	N.T.	N.T.
10i	33.5 $\pm$ 1.2	2.7 $\pm$ 0.9	102.1 $\pm$ 0.4	N.T.	N.T.	N.T.	N.T.
12a	86.1 $\pm$ 1.2	17.9 $\pm$ 3.2	58.5 $\pm$ 3.2	12.16 $\pm$ 1.11	19.5 $\pm$ 2.0	2.8	1939.0
12b	84.6 $\pm$ 1.2	0.3 $\pm$ 0.2	27.9 $\pm$ 0.7	2.1 $\pm$ 0.2	80.5 $\pm$ 5.5	N.T.	N.T.
12c	37.6 $\pm$ 0.6	0	67.5 $\pm$ 1.9	N.T.	N.T.	N.T.	N.T.
12d	74.5 $\pm$ 1.1	0	44.1 $\pm$ 1.1	N.T.	N.T.	N.T.	N.T.
12e	73.4 $\pm$ 1.8	0	47.7 $\pm$ 1.2	N.T.	N.T.	N.T.	N.T.
12f	78.7 $\pm$ 1.3	0	20.4 $\pm$ 1.4	2.5 $\pm$ 0.8	65.2 $\pm$ 5.7	N.T.	N.T.
12g	4.4 $\pm$ 2.4	0	74.0 $\pm$ 0.5	N.T.	N.T.	N.T.	N.T.
12h	68.3 $\pm$ 1.2	0	40.5 $\pm$ 1.0	N.T.	N.T.	N.T.	N.T.
12i	64.3 $\pm$ 2.4	0	52.0 $\pm$ 0.7	N.T.	N.T.	N.T.	N.T.
5	90.3 $\pm$ 3.4	11.2 $\pm$ 1.3	0	0.3 $\pm$ 0.1	100	1.0	5290.9

^aValues represent the mean of three independent measurements $\pm$ standard deviation. Compounds were tested at 100 μ M concentration.

^bAntiviral activity was tested with the A/California/07/2009 (H1N1) virus, which contains the AM2-S31N mutant, in plaque assay. Values represent the mean of two independent measurements $\pm$ standard deviation. ^cCytotoxicity was tested by incubating MDCK cells with compounds for 48 h, and the cells were stained with neutral red.²⁴ N.T. = not tested.

that they will have potent channel blockage and antiviral activity. Representative examples of monoaryl and bis-aryl AM2-S31N inhibitors are compounds **4** and **5**, respectively, and their binding modes to the AM2-S31N channel were determined by solution NMR as shown in Figure 1B,C, respectively.^{12,13} The monoaryl adamantanes were synthesized using our previously optimized reduction amination condition (Figure 1D),²¹ and the yields range from 62% to 85%. The bis-aryl adamantanes were synthesized using the expeditious microwave-mediated Suzuki–Miyaura cross-coupling reaction (Figure 1E), and the yields range from 75% to 92%.

The challenge of structure–property relationship studies is that the ADMET properties need to be optimized without negatively affecting the channel blockage and antiviral efficacy. In the first step, we focus on optimizing the mouse liver microsomal stability because mouse is a commonly used animal model for the evaluation of the in vivo antiviral efficacy of influenza antivirals.^{22,23} Changing the bromide in compound **4** with iodide resulted in compound **10a**, which had improved antiviral activity (EC_{50} = 1.0 $\pm$ 0.1 μ M vs 2.9 $\pm$ 0.8 μ M) but drastically reduced microsomal stability ($T_{1/2}$ = 24.5 min vs >145 min) (Table 2). The selenium analog **10b** had similar antiviral activity as the thiophene **4**, but it was less metabolically stable ($T_{1/2}$ = 32.1

Table 3. PK Predictions, Membrane Permeability, Physiochemical Properties, and Mouse Microsomal Stability of Lead Compounds 10d and 10e

Compound structure	Predicted values by Glide QikProp ^a	Egg-PAMPA Permeability (nm/s)	Lipinski rule of five ^a	Mouse liver microsome stability $T_{1/2}$ (min)	Human liver microsome stability $T_{1/2}$ (min)
10d	Caco-2: 892 nm/s MDCK: 2163 nm/s (<25 poor, >500 great) % human oral absorption in GI: 100 ($<25\%$ is poor)	252.814	M.W. = 342.3 HBD = 2 HBA = 3 cLogP = 3.69	>145	>145
10e	Caco-2: 892 nm/s MDCK: 2374 nm/sec (<25 poor, >500 great) % human oral absorption in GI: 100 ($<25\%$ is poor)	204.422	M.W. = 389.3 HBD = 2 HBA = 3 cLogP = 3.80	>145	>145

^aMembrane permeability and cLogP were calculated by the Schrödinger Glide QikProp program.

Table 4. Antiviral Activity of Compounds 10d and 10e in Inhibiting Oseltamivir-Sensitive and -Resistant Influenza A Viruses

Compound structures	A/California/07/2009 (H1N1) EC ₅₀	A/Switzerland/9715293/2013 (H3N2) EC ₅₀	A/Wisconsin/67/2005 (H3N2) EC ₅₀	A/Washington/29/2009 (H1N1) EC ₅₀	A/Texas/04/2009 (H1N1) EC ₅₀	A/North Carolina/29/2009 (H1N1) EC ₅₀	A/Denmark/528/2009 (H1N1) EC ₅₀	CC ₅₀ MDCK cells
	Amantadine-resistant (AM2-S31N) Oseltamivir-sensitive			Amantadine-resistant (AM2-S31N) Oseltamivir-resistant (H275Y)
10d	1.1 ± 0.2 μM	0.5 ± 0.1 μM	1.4 ± 0.1 μM	0.7 ± 0.1 μM	0.6 ± 0.1 μM	2.8 ± 0.2 μM	2.0 ± 0.3 μM	238.1 ± 2.1 μM
10e	0.9 ± 0.1 μM	0.7 ± 0.1 μM	0.8 ± 0.1 μM	0.4 ± 0.1 μM	0.6 ± 0.1 μM	1.1 ± 0.2 μM	0.7 ± 0.1 μM	317.4 ± 12.0 μM
Oseltamivir Carboxylate	2.4 ± 0.3 nM	12.3 ± 5.2 nM	2.5 ± 0.5 nM	> 200 nM	> 200 nM	>200 nM	> 200 nM	> 20 μM

min vs >145 min). Replacing the adamantane in **4** with ring-expanded adamantane gave compound **10c**, which had increased antiviral activity ($EC_{50} = 0.6 \pm 0.1 \mu M$ vs $2.9 \pm 0.8 \mu M$) but drastically reduced microsomal stability ($T_{1/2} = 9.1$ min vs >145 min). In contrast, replacing adamantane in compound **4** with hydroxyl-adamantane yielded compound **10d** that not only had improved antiviral activity ($EC_{50} = 1.1 \pm 0.2 \mu M$ vs $2.9 \pm 0.8 \mu M$) but also high microsomal stability ($T_{1/2} > 145$ min). Encouraged by this result, we then synthesized the iodide analog **10e**. It was found that the iodide analog **10e** retained the potent antiviral activity and high microsomal stability ($EC_{50} = 0.9 \pm 0.1 \mu M$, $T_{1/2} > 145$ min). Both compounds **10d** and **10e** also had an improved selectivity index than compound **4**. Two additional bromothio-phenyl analogs **10f** and **10g** had reduced antiviral activity compared to compound **10d**, and compound **10f** was also less metabolically stable than **10d** ($T_{1/2} = 100.8$ min vs >145 min). The bromothiazole analogs **10h** and **10i** had drastically reduced channel blockage and antiviral activity, showing less than 40% channel inhibition at $100 \mu M$. For the bis-aryl adamantane analogs **12a–12i**, only compounds **12b** and **12f** had comparable antiviral activity to that of compound **10d**. The antiviral EC_{50} values of **12b** and **12f** were 2.1 ± 0.2 and $2.5 \pm 0.8 \mu M$, respectively. However, both **12b** and **12f** had reduced selectivity index compared to that of compound **10d**; therefore, they were not further pursued. All other bis-aryl compounds (**12a**, **12c**, **12d**, **12e**, **12g**, **12h**, and **12i**) had significantly reduced antiviral activity as shown by the values of percentage plaque formation at $10 \mu M$. All compounds (**10a–10i** and **12a–12i**) were also tested against the wild-type (WT) AM2 channel, and compounds **4**,

10a, and **10c** were found to have potent channel blockage against AM2-WT ($>77\%$ inhibition at $100 \mu M$). In summary, through the structure–property relationship studies, we identified two lead compounds **10d** and **10e** that had potent channel blockage, antiviral activity, a high selectivity index, and optimal microsomal stability.

The membrane permeability of the two lead compounds **10d** and **10e** was further profiled (Table 3). Compounds **10d** and **10e** were predicted by the Schrödinger Glide QikProp program to have high membrane permeability and oral absorption (Table 3). Next, to experimentally determine their membrane permeability, compounds **10d** and **10e** were tested in the parallel artificial membrane permeability assay (PAMPA), which is commonly used as an in vitro model of passive, transcellular permeation. Both compounds **10d** and **10e** showed high membrane permeability with P_e greater than 200 nm/s in the Egg-PAMPA assay, indicating they can passively diffuse through the transcellular membrane. Both compounds **10d** and **10e** also did not violate the Lipinski rule of five. The microsomal stability of compounds **10d** and **10e** was further confirmed in human liver microsomal stability test, and both compounds **10d** and **10e** were found to have a long half-life with $T_{1/2} > 145$ min. Taken together, compounds **10d** and **10e** appear to be promising leads for further development.

One of the major therapeutic advantages of AM2-S31N inhibitors is that they have no cross-resistance with neuraminidase inhibitors such as oseltamivir.¹⁸ Therefore, they are expected to have potent antiviral activity against both oseltamivir-sensitive and -resistant influenza A viruses. To

prove this hypothesis, we tested the antiviral activity of compounds **10d** and **10e** against several human influenza A viruses, including both H1N1 and H3N2 strains (Table 4). All viruses contain the AM2-S31N mutation in their M2 genes and are resistant to amantadine. In addition, the four oseltamivir-resistant strains encode the H275Y mutant in their neuraminidase gene, which confer to their resistance to oseltamivir. It was found that compounds **10d** and **10e** inhibited all seven influenza A viruses with EC₅₀ values ranging from 0.4 to 2.8 μ M.

The antiviral activity of compound **10e** was further validated when MDCK cells were infected with the A/California/07/2009 (H1N1) or the A/Wisconsin/67/2005 (H3N2) virus at high MOIs, a condition that mimics the late stage of treatment when the virus was already amplified in the host. It was found that compound **10e** significantly suppressed the viral replication when MOI was as high as 10⁶ pfu/mL for both influenza strains (Figure 2). In contrast, oseltamivir carboxylate was only effective

Figure 2. Antiviral activity of compound **10e** in inhibiting A/California/07/2009 (H1N1) (A) and A/Wisconsin/67/2005 (H3N2) (B) viruses at different MOIs (10¹–10⁶ pfu/mL) using plaque assay. The numbers shown are viral titers at initial infection. The images shown were representative results from two repeats.

at low MOIs (10¹ to 10³ pfu/mL) and was not effective at high MOIs (10⁴–10⁶ pfu/mL). Overall, compound **10e** showed potent inhibition against human H1N1 and H3N2 influenza A strains at both low and high MOIs.

To test whether the antiviral efficacy of compound **10e** is cell-type dependent, we performed viral titer reduction assay using the A/California/07/2009 (H1N1) virus and the A549 cells. In this assay, confluent A549 cells were infected with the A/California/07/2009 (H1N1) virus at MOI of 0.001, the cell culture supernatant was collected at 24, 48, and 72 h postinfection, and the viral titer was quantified by plaque assay. Compared with the DMSO control, treatment with 10 μ M of compound **10e** significantly reduced the viral titers of 1.8, 2.1, and 2.9 log₁₀ units at 24, 48, and 72 h, respectively (Figure 3). In comparison, oseltamivir carboxylate only delayed viral replication and showed no effect at 72 hpi under similar drug selection pressure (10-fold of EC₅₀ value).

The binding of compound **10e** in the AM2-S31N channel was modeled by the Schrödinger Glide standard precision docking program. In the energy-minimized docking pose (Figure 4), compound **10e** fitted inside the channel with the thiophene group facing toward the N-terminal of the channel. The hydroxyl group from **10e** forms a hydrogen bond with the backbone amide carbonyl from one of the helices, and the ammonium from **10e**

Figure 3. Antiviral activity of compound **10e** in inhibiting A/California/07/2009 (H1N1) virus replication in A549 cells. The results shown were from two repeats.

Figure 4. Docking model of compound **10e** in the transmembrane domain of AM2-S31N (PDB: 2LY0).¹³ The transparency of the front helix was set as 0.7 for clarity.

forms another hydrogen bond with the side chain amide carbonyl from the neighboring helix. The iodothiophene substitution forms hydrophobic interactions with the V27 side chain methyls. Overall, the docking pose of compound **10e** is similar to that of compound **4** (PDB: 2MUV) (Figure 1b).

Drug discovery is a lengthy and expansive endeavor,²⁵ and lead compounds can fail in any step during the early preclinical development phase or the later human clinical trials. Therefore, it is essential to provide additional backup compounds with favorable PK properties for the following in vivo animal and human studies. Herein, we report our progress of optimizing the mouse microsomal stability and cell membrane permeability of thiophene-containing AM2-S31N inhibitors. Starting from a promising lead compound **4**, we were able to identify two compounds **10d** and **10e** with improved antiviral efficacy and selectivity index. The optimized lead compounds **10d** and **10e** retained high mouse liver microsomal stability ($T_{1/2}$ > 145 min) and had favorable membrane permeability in the PAMPA assay (P_e > 200 nm/s) and a high selectivity index (SI > 100). As a demonstration of the therapeutic value of AM2-S31N inhibitors, compounds **10d** and **10e** were found to have potent antiviral potency against several oseltamivir-sensitive and -resistant human influenza A viruses. Compound **10e** was also able to inhibit A/California/07/2009 (H1N1) and A/Wisconsin/67/2005 (H3N2) at MOIs ranging from 10² to 10⁶ pfu/mL, and the antiviral activity of compound **10e** was further confirmed in human A549 cell line. Taken together, the potent antiviral efficacy, a high selectivity index, a long half-life in mouse liver microsomes, and a high membrane permeability of the identified lead compounds **10d** and **10e** warrant their further development as orally bioavailable influenza antivirals. Indeed, several FDA-approved oral drugs such as rivaroxaban, chlorothal, brotizolam, and lornoxicam similarly contain halogen-deactivated thiophene,²⁶ which reassures continuous development of this series of compounds.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.8b00336.

Synthesis procedures; characterization of compounds; antiviral and cytotoxicity assay; electrophysiological assay; mouse microsomal stability assay; membrane permeability assay (PDF)

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Funding

This research is supported by the startup funding from the University of Arizona and the NIH grant AI119187 to J.W.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

WT, wild type; DMEM, Dulbecco's modified Eagle's medium; MDCK, Madin–Darby Canine Kidney; TEVC, two-electrode voltage clamp

■ REFERENCES

- (1) Webster, R. G.; Monto, A. S.; Braciale, T. J.; Lamb, R. A. *Textbook of Influenza*; Wiley: 2013.
- (2) <https://www.cdc.gov/flu/pandemic-resources/basics/past-pandemics.html> (Accessed on 09/28/2018).
- (3) Clinical Signs and Symptoms of Influenza. <https://www.cdc.gov/flu/professionals/acip/clinical.htm> (accessed on 09/28/2018).
- (4) Leading Causes of Death. <https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm> (accessed on 09/28/2018).
- (5) People at High Risk of Developing Serious Flu–Related Complications, https://www.cdc.gov/flu/about/disease/high_risk.htm (accessed on 09/28/2018).
- (6) Shaw, M. L. The Next Wave of Influenza Drugs. *ACS Infect. Dis.* **2017**, *3*, 691–694.
- (7) Hurt, A. C. The epidemiology and spread of drug resistant human influenza viruses. *Curr. Opin. Virol.* **2014**, *8*, 22–29.
- (8) Matsuzaki, Y.; Mizuta, K.; Aoki, Y.; Suto, A.; Abiko, C.; Sanjoh, K.; Sugawara, K.; Takashita, E.; Itagaki, T.; Katsushima, Y.; Ujiike, M.; Obuchi, M.; Odagiri, T.; Tashiro, M. A two-year survey of the oseltamivir-resistant influenza A(H1N1) virus in Yamagata, Japan and the clinical effectiveness of oseltamivir and zanamivir. *Virol. J.* **2010**, *7*, 53.
- (9) Koszalka, P.; Tilmanis, D.; Hurt, A. C. Influenza antivirals currently in late-phase clinical trial. *Influenza Other Respir. Viruses* **2017**, *11* (3), 240–246.
- (10) Lorigan, A.; Mercorelli, B.; Nannetti, G.; Compagnin, C.; Palu, G. Antiviral strategies against influenza virus: towards new therapeutic approaches. *Cell. Mol. Life Sci.* **2014**, *71* (19), 3659–3683.
- (11) Li, F.; Ma, C.; Hu, Y.; Wang, Y.; Wang, J. Discovery of Potent Antivirals against Amantadine-Resistant Influenza A Viruses by

Targeting the M2-S31N Proton Channel. *ACS Infect. Dis.* **2016**, *2* (10), 726–733.

(12) Wu, Y.; Canturk, B.; Jo, H.; Ma, C.; Gianti, E.; Klein, M. L.; Pinto, L. H.; Lamb, R. A.; Fiorin, G.; Wang, J.; DeGrado, W. F. Flipping in the pore: discovery of dual inhibitors that bind in different orientations to the wild-type versus the amantadine-resistant S31N mutant of the influenza A virus M2 proton channel. *J. Am. Chem. Soc.* **2014**, *136* (S2), 17987–17995.

(13) Wang, J.; Wu, Y.; Ma, C.; Fiorin, G.; Wang, J.; Pinto, L. H.; Lamb, R. A.; Klein, M. L.; DeGrado, W. F. Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (4), 1315–1320.

(14) Hu, Y.; Wang, Y.; Li, F.; Ma, C.; Wang, J. Design and expeditious synthesis of organosilanes as potent antivirals targeting multidrug-resistant influenza A viruses. *Eur. J. Med. Chem.* **2017**, *135*, 70–76.

(15) Li, F.; Hu, Y.; Wang, Y.; Ma, C.; Wang, J. Expeditious Lead Optimization of Isoxazole-Containing Influenza A Virus M2-S31N Inhibitors Using the Suzuki-Miyaura Cross-Coupling Reaction. *J. Med. Chem.* **2017**, *60* (4), 1580–1590.

(16) Li, F.; Ma, C.; DeGrado, W. F.; Wang, J. Discovery of Highly Potent Inhibitors Targeting the Predominant Drug-Resistant S31N Mutant of the Influenza A Virus M2 Proton Channel. *J. Med. Chem.* **2016**, *59* (3), 1207–1216.

(17) Dong, G.; Peng, C.; Luo, J.; Wang, C.; Han, L.; Wu, B.; Ji, G.; He, H. Adamantane-resistant influenza A viruses in the world (1902–2013): frequency and distribution of M2 gene mutations. *PLoS One* **2015**, *10* (3), e0119115.

(18) Ma, C.; Zhang, J.; Wang, J. Pharmacological Characterization of the Spectrum of Antiviral Activity and Genetic Barrier to Drug Resistance of M2-S31N Channel Blockers. *Mol. Pharmacol.* **2016**, *90* (3), 188–198.

(19) Musharrafieh, R.; Ma, C. L.; Wang, J. Profiling the in vitro drug-resistance mechanism of influenza A viruses towards the AM2-S31N proton channel blockers. *Antiviral Res.* **2018**, *153*, 10–22.

(20) Wang, Y.; Hu, Y.; Xu, S.; Zhang, Y.; Musharrafieh, R.; Hau, R. K.; Ma, C.; Wang, J. Vitro Pharmacokinetic Optimizations of AM2-S31N Channel Blockers Led to the Discovery of Slow-Binding Inhibitors with Potent Antiviral Activity against Drug-Resistant Influenza A Viruses. *J. Med. Chem.* **2018**, *61* (3), 1074–1085.

(21) Wang, J.; Ma, C.; Wang, J.; Jo, H.; Canturk, B.; Fiorin, G.; Pinto, L. H.; Lamb, R. A.; Klein, M. L.; DeGrado, W. F. Discovery of novel dual inhibitors of the wild-type and the most prevalent drug-resistant mutant, S31N, of the M2 proton channel from influenza A virus. *J. Med. Chem.* **2013**, *56* (7), 2804–2812.

(22) Hu, Y.; Musharrafieh, R.; Ma, C.; Zhang, J.; Smee, D. F.; DeGrado, W. F.; Wang, J. An M2-V27A channel blocker demonstrates potent in vitro and in vivo antiviral activities against amantadine-sensitive and -resistant influenza A viruses. *Antiviral Res.* **2017**, *140*, 45–54.

(23) Smee, D.; Barnard, D., Methods for Evaluation of Antiviral Efficacy Against Influenza Virus Infections in Animal Models. In *Antiviral Methods and Protocols*; Gong, E. Y., Ed.; Humana Press: 2013; Vol. 1030, pp 407–425.

(24) Repetto, G.; del Peso, A.; Zurita, J. L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* **2008**, *3* (7), 1125–1131.

(25) DiMasi, J. A.; Grabowski, H. G.; Hansen, R. W. Innovation in the pharmaceutical industry: New estimates of R&D costs. *J. Health Econ.* **2016**, *47*, 20–33.

(26) Scott, K. A.; Njardarson, J. T. Analysis of US FDA-Approved Drugs Containing Sulfur Atoms. *Top. Curr. Chem. (Cham)* **2018**, *376* (1), 5.