Published in final edited form as:

J Med Chem. 2018 February 08; 61(3): 1074–1085. doi:10.1021/acs.jmedchem.7b01536.

In 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

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Abstract

Influenza viruses are respiratory pathogens that are responsible for both seasonal influenza epidemics and occasional influenza pandemics. The narrow therapeutic window of oseltamivir, coupled with the emergence of drug resistance, calls for the next-generation of antivirals. With our continuous interest in developing AM2-S31N inhibitors as oral influenza antivirals, we report here the progress of optimizing the in vitro pharmacokinetic (PK) properties of AM2-S31N inhibitors. Several AM2-S31N inhibitors, including compound **10b**, were discovered to have potent channel blockage, single to submicromolar antiviral activity, and favorable in vitro PK properties. The antiviral efficacy of compound **10b** was also synergistic with oseltamivir carboxylate. Interestingly, binding kinetic studies (K_d , K_{on} and K_{off}) revealed several AM2-S31N inhibitors that have similar K_d values but significantly different K_{on} and K_{off} values. Overall, this study identified a potent lead compound (**10b**) with improved in vitro PK properties that is suitable for the in vivo mouse model studies.

Graphical Abstract

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting information: Molecular Formula Strings.

Keywords

Influenza A virus; AM2 proton channel; AM2-S31N inhibitor; antiviral

INTRODUCTION

Influenza viruses are respiratory pathogens that are responsible for both seasonal influenza epidemics and sporadic influenza pandemics.¹ Influenza A and B viruses co-circulate among humans in each influenza season; however, the strains and ratios change over time and it is often difficult to predict which strains will be circulating in the next influenza season and when the next influenza pandemic will emerge.² In addition, the mortality rates for different subtypes of influenza viruses vary significantly. For example, the mortality rates for human infections with avian H5N1 and H7N9 viruses are 52.7% and 38.6%, respectively (WHO), comparing to the 0.1%–2.5% mortality rate associated with past H1N1 influenza pandemic viruses.^{3, 4} Moreover, people at the high-risk groups of influenza virus-related complications are more vulnerable for influenza virus infection than immune-competent adults.⁵ Therefore, effective antiviral drugs are undoubtedly needed for the prevention and treatment of influenza virus infection.⁶

There are two classes of FDA-approved influenza antivirals: M2 channel blockers (amantadine and rimantadine),⁷ and neuraminidase inhibitors (oseltamivir, zanamivir, and peramivir).⁸ Amantadine and rimantadine are no longer recommended by CDC for the prevention and treatment of influenza virus infection due to the prevalent drug resistance.⁹ Oseltamivir is currently the only FDA-approved oral influenza drug. Zanamivir is administered through intranasal spray; therefore, its use is limited in infants and critically ill patients. Recently approved peramivir is an intravenous drug and its use is restricted in clinical settings. Although majority of current circulating influenza viruses remain sensitive to oseltamivir, oseltamivir-resistant viruses have been continuously reported,^{10, 11} and more alarmingly the 2007–2008 seasonal influenza H1N1 strain circulating in North America and Japan was completely resistant to oseltamivir due to the H275Y mutation in the neuraminidase gene.^{12, 13} In this regard, the next generation of oral influenza drugs with novel mechanism of action is clearly needed.

More than 95% of current circulating influenza A viruses carry the AM2-S31N mutant in their AM2 genes,¹⁴ which confer to amantadine and rimantadine resistance. Given the high prevalence of the AM2-S31N mutant, we hypothesize that AM2-S31N inhibitors will have potent antiviral activity against both oseltamivir-sensitive and –resistant influenza A strains. Indeed, we have shown that our rationally designed AM2-S31N inhibitors inhibit multiple

human influenza A viruses with submicromolar efficacy, including oseltamivir-resistant strains.^{15–18} Encouraged by these preliminary results, in this study we report our progress in optimizing the in vitro PK properties of AM2-S31N inhibitors with the aim of prioritizing lead compounds for the next step in vivo mice studies. Through iterative cycles of hypothesis-driven PK optimization, we identified one compound, **10b**, with optimal in vitro PK properties. Interestingly, unlike most other previously reported AM2-S31N inhibitors which have fast K_{on} and slow K_{off} values, compound **10b** is a slow binding channel blocker with slow K_{on} and K_{off} values but having a low micromolar K_d . Subsequent kinetic studies of several representative AM2-S31N inhibitors revealed that the antiviral efficacy of AM2-S31N inhibitors has a better correlation with their K_d values, rather than their percentage of channel blockage at the two-minute time point.

RESULTS AND DISCUSSION

CHEMISTRY

Compounds **8a–8f** were synthesized according to a previous optimized synthesis route (Scheme 1A).¹⁸ Briefly, condensation of methyl ketone **1** and diethyl oxalate **2** using potassium *tert*-butoxide as the base gave the β -ketone ester intermediate **3**, which was cyclized to isoxazole ester **4** by heating at 50 °C in methanol with hydroxylamine hydrochloride. The ester **4** was reduced to alcohol **5** by sodium borohydride and was then converted to bromide **6** by triphenyl phosphine and carbon tetrabromide in dichloromethane. Last, alkylation of bromide **6** with the amantadine analog **7** gave the isoxazole-containing AM2-S31N inhibitor **8**. The overall yields range from 15% to 36%. Compounds **10a–10g** were synthesized by direct alkylation (Scheme 1B). The yields range from 62% to 85%. The synthesis of compounds **11**, **14**, **15**, and **16** was reported before.^{16, 18, 19}

HYPOTHESIS-DRIVEN OPTIMIZATION OF THE IN VITRO PK PROPERTIES OF AM2-S31N INHIBITORS

The lead optimization was guided by the solution NMR structure of compound **11** in complex with AM2-S31N (19–49) (PDB: 2LY0) (Figure 1).¹⁹ Compound **11** binds to the central cavity of the AM2-S31N channel with its aryl group projecting toward the N-terminal channel lumen. The distal thiophene substitution from compound **11** forms hydrophobic interactions with V27 side chain methyls, the isoxazole and the amine form bidentate hydrogen bonds with the N31 side chain amide, and the adamantane cage fits in the cavity created by the G34 from AM2-S31N.

As a first step to optimize the in vitro PK properties of the AM2-S31N inhibitors, we profiled the mouse liver microsomal stability of compound **11**, which is one of the most potent AM2-S31N inhibitors we have discovered so far (Table 1).¹⁹ It was found that compound **11** had an ultra-short half-life of 1.0 minute in mouse liver microsomes, which occluded it from being tested in in vivo mouse studies. Inspection of the chemical structure of compound **11** revealed that there might be three possible metabolic soft-spots: the adamantane, the isoxazole, and the thiophene.²⁰ Amantadine was shown to be metabolized to hydroxyl adamantane in vivo,²¹ the isoxazole ring can be cleaved at the N–O bond,²² and the thiophene ring is known to be easily oxidized.²³ To increase the microsomal stability of

compound **11**, we first searched the FDA-approved drug database for orally bioavailable adamantane-containing drugs, hoping to gain insights how to improve the microsomal stability of adamantane. The search revealed two compounds, vildagliptin (**12**) and saxagliptin (**13**) (Figure 2A), both of which are dipeptidyl peptidase-4 (DPP-4) inhibitors and are used as anti-diabetic drugs. Vildagliptin (**12**) and saxagliptin (**13**) each contain hydroxyl adamantane instead of adamantane. The biological half-life $T_{1/2}$ of vildagliptin (**12**) and saxagliptin (**13**) were 2–3 h and 2.5 h, respectively, following a single oral dose in healthy volunteers.²⁴ The presence of hydroxyl adamantane instead of adamantane in vildagliptin (**12**) and saxagliptin (**13**) might confer to their high microsomal stability and oral bioavailability. We therefore applied the same strategy in our PK optimization and converted compound **11** to the hydroxyl adamantane-containing compound **14** (Figure 2B). Compound **14** had similar channel blockage and antiviral efficacy as compound **11** (Table 1), and had an improved selectivity index over compound **11**. Gratifyingly, the half-life of compound **14** in mouse microsomes was increased to 31.5 minutes. This result suggests that hydroxyl adamantane is indeed more metabolically stable than adamantane. Following the same rationale, we tested the mouse microsomal stability of another hydroxyl adamantane-containing AM2-S31N inhibitor, compound **15**. The mouse microsomal half-life of compound **15** was 13.4 minutes, which was a significant improvement compared to compound **11**, but still requires further optimization. Next, to remove another possible metabolic labile spot, the thiophene,²³ in compounds **11**, **14**, and **15**, we tested the microsomal stability of compound **10a**, which contains a cyclopropyl substitution instead of thiophene. The mouse microsomal half-life of compound **10a** was 12.8 minutes, which was a significant improvement compared to compound **11** ($T_{1/2}$ =1.0 minute). Substituting adamantane in **10a** with hydroxyl adamantane gave compound **10b**, which had a half-life of more than 145 minutes. Encouraged by this result, we then synthesized a few other cyclopropyl-containing analogs **10c**, **10d**, **10e**, **10f**, and **10g**. Compound **10d** did not block the AM2-S31N channel (5.3% channel blockage at 100 μ M) and therefore had no antiviral activity. All other compounds (**10c**, **10e**, **10f** and **10g**) had potent antiviral activity (EC_{50} < 3.1 μ M) and a high selectivity index. Compounds **10c**, **10e**, and **10g** were also stable in mouse microsomes with $T_{1/2}$ of 97.2, > 145, and > 145 minutes, respectively. Despite the potent antiviral activity of compounds **10b**, **10c**, **10e**, **10f**, and **10g**, they had a moderate to weak channel blockage against the AM2-S31N channel (33.8% to 67.3% at 100 μ M). It is noteworthy that the value of percentage channel blockage reported herein was the value at the two-minute time-point after applying 100 μ M of compound to the oocyte bathing medium. Therefore, this value may or may not represent the true binding potency of the compound since this experiment is under kinetic control. The apparent discrepancy between these compounds' weak to moderate percentage channel blockage at the 2-minute time point and their potent antiviral activity suggest that they might be slow-binding inhibitors. To confirm this hypothesis, we performed detailed kinetic studies of these AM2-S31N inhibitors (**10b**, **10c**, **10e**, **10f**, and **10g**), and the results are shown in the next section.

In parallel, guided by the solution NMR structure of AM2-S31N in complex with compound **11** (PDB: 2LY0) (Figure 1), we also designed and synthesized isoxazole adamantane analogs with cyclobutyl (**8a**, **8b**), cyclopentyl (**8c**, **8d**), and cyclohexyl (**8e**, **8f**) substitutions. Substituting cyclopropyl with cyclobutyl, cyclopentyl, and cyclohexyl should be

accommodated as this substitution forms hydrophobic interaction with the V27 side chain methyls (Figure 1). As expected, all compounds were found to have potent channel blockage against the AM2-S31N channel (>84% at 100 μ M) and potent antiviral activity against the AM2-S31N-containing A/California/07/2009 (H1N1) virus (EC_{50} 1 μ M). However, replacing cyclopropyl in compound **10b** with cyclobutyl (**8b**), cyclopentyl (**8d**), and cyclohexyl (**8f**) led to a gradual decrease of the mouse microsomal stability (Table 1). These results suggest cyclopropyl substitution is more metabolically stable than the corresponding cyclobutyl, cyclopentyl, and cyclohexyl substitutions.

BINDING KINETIC STUDIES OF AM2-S31N INHIBITORS

In our previous studies, we plotted the AM2-S31N inhibitors' percentage channel blockage at the two-minute time point with their antiviral activity in a plaque assay.¹⁵ It was found that compounds with a higher percentage of channel blockage normally have more potent antiviral efficacy. However, in this study we found a few exceptions such as compounds **10b**, **10c**, **10e**, **10f** and **10g**, which all had moderate to weak channel blockage ranging from 33.8% to 67.3% at the two-minute time points when tested at 100 μ M drug concentration. According to the previous correlation, the predicted antiviral EC_{50} values of these compounds should have been greater than 8 μ M.¹⁵ However, all of them had potent antiviral activity with EC_{50} values less than 3.1 μ M. One possible explanation is that these compounds might be slow-binding inhibitors and equilibrium might not be achieved at the two-minute time-point; therefore, the reported percentage channel blockage at the two-minute time point actually underestimates the true potency of these compounds. To validate this hypothesis, we chose compounds **10b**, **10c**, **10e**, **10f** and **10g** for detailed kinetic studies. Two additional compounds **10a** and **16** were included as controls for comparisons.

The K_d values of compounds **10a**, **10b**, **10c**, **10e**, **10f**, **10g**, and **16** were obtained by fitting the inhibition and washing curves (Figure 3A) with the association and dissociation equations (Figure 3B). All compounds were tested at 100 μ M. The best-fit results are shown in Table 2. As shown in Figure 3A and Table 2, despite the moderate to low channel blockage at the two-minute time point for compounds **10b**, **10c**, **10e**, and **10f** (44.8%–67.3%), they had comparable K_d values (4.5 μ M–11.5 μ M) as the potent channel blocker compound **10a** (7.7 μ M). Consistent with our hypothesis, compounds **10b**, **10c**, **10e**, and **10f** are indeed slow-binding inhibitors as shown by their slow K_{on} ($<1 \times 10^4$ $M^{-1}min^{-1}$) and slow K_{off} (<0.1 min^{-1}) values. Slow K_{off} is defined as the value being 10-fold lower than the fast K_{off} value. In contrast, compound **10a** has fast K_{on} ($>1 \times 10^4$ $M^{-1}min^{-1}$) and slow K_{off} (<0.1 min^{-1}) values. Compound **10g** has the lowest K_{on} of 1745 ± 102 $M^{-1}min^{-1}$ and the highest K_d of 22.7 ± 2.0 μ M, yet it had potent antiviral activity with an EC_{50} of 2.6 ± 0.7 μ M. In contrast, compound **16** has both fast K_{on} ($>1 \times 10^4$ $M^{-1}min^{-1}$) and fast K_{off} (>0.1 min^{-1}), with a K_d of 17.3 ± 2.8 μ M.

In summary, on the basis of channel binding kinetics, AM2-S31N inhibitors can be grouped into three categories: (1) Compounds with fast K_{on} and slow K_{off} , such as compound **10a**. This group of compounds normally has potent antiviral activity. In this case, the percentage channel blockage at the two-minute time point is an accurate predictor for the antiviral activity. (2) Compounds with slow K_{on} and slow K_{off} , such as the newly discovered slow-

binding inhibitors **10b**, **10c**, **10e**, **10f**, and **10g**. Compounds in this category may or may not have potent antiviral activity, which depends on their K_d values. Because of their slow K_{on} values, the percentage channel blockage at the two-minute time point cannot accurately predict the antiviral potency. (3) Compounds with fast K_{on} and fast K_{off} such as compound **16**. Similar to the slow-binding inhibitors, the antiviral potency of compounds in this category depends on their K_d values, not their percentage inhibition at the two-minute time point.

It is noteworthy that although K_d values are more accurate predictors for the compounds' antiviral activity, in practice it is not feasible to measure the K_d value for every compound. The reason for this is that K_d measurements require bathing AM2-S31N-expressing oocytes at pH 5.5 for an extended period of time (>30 minutes), which often results in cell death and membrane leakage. On average, we could only obtain one quality binding and washing curve out of five measurements from different oocytes. Therefore, instead of simply filtering compounds based on their percentage channel blockage at the two-minute time point in the primary electrophysiological assays, all compounds should be tested in secondary antiviral plaque assays in parallel. In this way, potent compounds that show slow-binding kinetics in inhibiting AM2-S31N proton channel will not be mistakenly filtered.

ANTIVIRAL ACTIVITY OF COMPOUND **10b** AGAINST DRUG-RESISTANT INFLUENZA A VIRUSES

The antiviral activity of compound **10b** was further tested against five additional human influenza A viruses, among which A/Denmark/528/2009 (H1N1), A/Washington/29/2009 (H1N1), A/Texas/04/2009 (H1N1), and A/North Carolina/29/2009 (H1N1) are resistant to both amantadine and oseltamivir carboxylate, while A/Switzerland/9715293/2013 (H3N2) is resistant to amantadine but sensitive to oseltamivir. These five strains, together with A/California/07/2009 (H1N1), all contain the AM2-S31N mutant. It was found that compound **10b** inhibited all six strains with EC_{50} values less than 1 μ M (Figure 4). In addition, compound **10b** also showed potent inhibition against the AM2-S31N-containing A/WSN/33 (H1N1) virus with an EC_{50} value of 0.5 ± 0.2 μ M. The cytotoxicity CC_{50} values of compound **10b** were greater than 300 μ M for both MDCK and A549 cells, which corresponds to a selectivity index of greater than 375. Overall, AM2-S31N inhibitors such as **10b** were shown to have potent antiviral activity against AM2-S31N-containing influenza A viruses with diverse genetic backgrounds.

IN VITRO PK PROFILING OF COMPOUND **10b**

Given compound **10b**'s potent antiviral activity, high selectivity index, and long half-life in mouse microsomes, we further profiled other in vitro PK properties of compound **10b**, which include human liver microsome stability, cell permeability, and cytochrome P450 inhibition (Table 3). It was found that compound **10b** has high membrane permeability in Caco-2 cells and it did not show inhibition against the five isoforms of CYP enzymes: 1A2, 2C9, 2C19, 2D6, and 3A4. Compound **10b** has a similar high stability in the human liver microsomes, with a half-life greater than 145 minutes. Compound **10b** also did not violate the Lipinski rule of five. In summary, the in vitro PK profiling predicts that compound **10b** will likely have high oral bioavailability.

COMBINATION THERAPY POTENTIAL OF COMPOUND **10b** WITH OSELTAMIVIR CARBOXYLATE

Combination therapy has been proven effective in decreasing the incidence of drug resistance as well as in lowering cytotoxicity.²⁶ As such, we are interested in exploring the combination therapy potential of AM2-S31N inhibitors such as **10b** with oseltamivir carboxylate. As AM2-S31N inhibitor **10b** targets a different viral replication stage than oseltamivir carboxylate, it is expected that a combination of compound **10b** with oseltamivir carboxylate will have a synergistic antiviral effect. To test this hypothesis, we applied the standard fractional inhibitory concentration index (FICI) method to evaluate the combination therapy effect of compound **10b** with oseltamivir carboxylate.^{27–30} A FICI value of lower or higher than 1 indicates synergy or antagonism, respectively. It was found that all five sets of combination gave FICI less than 0.5 (Table 4), suggesting strong synergism by combining **10b** with oseltamivir carboxylate. Among the five sets of results, a combination of 0.039 μ M **10b** with 1.97 nM oseltamivir (combination ratio 1:10) exerted the highest synergistic efficacy, with a FICI value of 0.28.

DOCKING MODEL OF COMPOUND **10b** IN AM2-S31N

To gain insight into how compound **10b** binds to the AM2-S31N channel, we performed molecular modeling using the Schrödinger Glide standard precision docking. The solution NMR structure of AM2-S31N (PDB: 2LY0) was used as the protein template. It was found that compound **10b** can bind to the AM2-S31N channel in two different conformations (Figures 5A and 5B). In one conformation (Figure 5A), the isoxazole nitrogen from **10b** forms a hydrogen bond with the N31 side chain amide carbonyl from one of the monomers, while the ammonium from **10b** forms another hydrogen bond with the N31 side chain amide carbonyl from the opposite monomer. In the another binding confirmation (Figure 5B), the isoxazole nitrogen and ammonium from **10b** form a bidentate hydrogen bond with the N31 side chain amide carbonyl from one monomer. In both conformations (Figures 5A and 5B) the cyclopropyl substitution from **10b** form hydrophobic interactions with the V27 side chain methyls, and the hydroxyl substitution on adamantane forms a hydrogen bond with the A30 backbone amide carbonyl.

CONCLUSIONS

Although novel drugs are urgently needed to address the unmet medical needs, drug discovery is unfortunately a lengthy and expensive venture.³¹ In the case of AM2-S31N drug discovery, no progress was made for decades until the report of the first-generation AM2-S31N inhibitors in 2013.^{19, 32} Thereafter, lead optimization led to the development of more potent and selective AM2-S31N channel blockers that inhibit multiple strains of human influenza A viruses, including viruses that are resistant to amantadine, oseltamivir, or both. However, the first generation of AM2-S31N inhibitors such as compound **11** was found to have low stability in mouse microsomes, precluding them from further development. Guided by a hypothesis-driven approach, we were able to significantly improve the microsomal stability of AM2-S31N inhibitors. The lead compound **10b** had a $T_{1/2}$ of more than 145 minutes in both mouse and human liver microsomes, and it was also highly permeable in Caco-2 cells and did not inhibit five isoforms of CYP. Interestingly, albeit the moderate

percentage channel blockage (47.9 ± 1.0 %) of compound **10b** at the two-minute time point when tested at 100 μ M, **10b** had similar antiviral activity as compound **10a** which showed 85.4 ± 2.2 % channel blockage at the two-minute time point when tested at 100 μ M. The potent antiviral activity of compound **10b** was unexpected: according to previous correlation study results, the predicted antiviral EC_{50} value of compound **10b** would be more than 10 μ M. However, in reality, compound **10b** inhibits multiple AM2-S31N-containign influenza A viruses with EC_{50} values ranging from 0.2 μ M to 0.8 μ M (Figure 4). The apparent discrepancy led us to examine the binding kinetics of these AM2-S31N inhibitors. By fitting the binding and washing curves with the association and dissociation equations, we calculated the K_{on} , K_{off} , and K_d values of compounds **10a** and **10b**, along with a few other compounds (**10c**, **10e**, **10f**, **10g**, and **16**). The results showed that the newly discovered compounds **10b**, **10c**, **10e**, **10f**, and **10g** are slow-binding inhibitors and the binding equilibrium was not achieved at the two-minute time point when tested at 100 μ M. Therefore, the reported percentage inhibition at the two-minute time point underestimates the true potency of these compounds (**10b**, **10c**, **10e**, **10f**, and **10g**). As such, although compound **10b** had significantly less percentage channel blockage than **10a** at the two-minute time point (47.9 % versus 85.4 %), they had similar K_d values (4.5 μ M versus 7.7 μ M). These results suggest that the K_d value is a more accurate predictor of the compound's antiviral activity rather than the percentage channel blockage at the two-minute time point.

Overall, the results presented herein may have important implications for other ion channel drug discovery program. As the electrophysiological assay is time-consuming, it is generally used as a primary assay to test compounds at a single drug concentration. However, as this process is under kinetic control, slow-binding inhibitors might be missed from this primary screening. To ensure slow-binding inhibitors are not being mistakenly filtered, functional assays should be performed in parallel.

In summary, this study led to the development of a promising lead compound (**10b**) with potent antiviral activity, high selectivity index, and favorable in vitro PK properties; and combination therapy of **10b** with oseltamivir carboxylate also showed strong synergism.

EXPERIMENTAL SECTION

Chemistry.

Chemicals were ordered from commercial sources and were used without further purification. Synthesis procedures for reactions described in Scheme 1 were show below. All final compounds were purified by flash column chromatography. 1H and ^{13}C NMR spectra were recorded on a Bruker-400 NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to residual solvent (CD_3OD) 3.31 ppm, (DMSO- d_6) 2.50 ppm, and ($CDCl_3$) 7.24 ppm or from internal standard tetramethylsilane (TMS) 0.00 ppm. The following abbreviations were used in reporting spectra: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; ddd, doublet of doublet of doublets. All reactions were carried out under N_2 atmosphere, unless otherwise stated. HPLC-grade solvents were used for all reactions. Flash column chromatography was performed using silica gel (230–400 mesh, Merck). Low-resolution mass spectra were obtained using an ESI

technique on a 3200 Q Trap LC/MS/MS system (Applied Biosystems). The purity was assessed by using a Shimadzu LC-MS with a Waters XTerra MS C-18 column (part #186000538), 50 × 2.1 mm, at a flow rate of 0.3 mL/min; λ = 250 and 220 nm; mobile phase A, 0.1% formic acid in H₂O, and mobile phase B', 0.1% formic in 60% isopropanol, 30% CH₃CN and 9.9% H₂O. All compounds submitted for testing in TEVC assay, plaque assay, cytotoxicity assay, and PK studies were confirmed to be > 95.0% purity by LC-MS traces.

Synthesis Procedures.

ethyl 5-cyclopentyl-1,2-oxazole-3-carboxylate (4b).—To a stirred solution of diethyl oxalate (1.46 g, 1.1 equiv) and 1-cyclopentylethanone (1.12g, 1 equiv) in toluene was added a solution of potassium tert-butoxide in THF (1.2 equiv) dropwise. The resulting solution was stirred at room temperature overnight. The reaction was quenched with 1N HCl and extracted with ethyl acetate (3×). The combined organic layers were dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product was dissolved in methanol and hydroxylamine hydrochloride (1.04g, 1.5 equiv.) was added. The solution was heated to 50 °C for 6 hours. The solvent was removed under reduced pressure and the resulting isoxazole ester was purified by flash column chromatography (10–20% EtOAc/Hexane) to give the final product as a white solid. Yield: 56%. ¹H NMR (400 MHz, CDCl₃) δ 6.37 (s, 1H), 4.40 (q, J = 7.2 Hz, 2H), 3.26–3.18 (m, 1H), 2.11–2.05 (m, 2H), 1.79–1.62 (m, 6H), 1.37 (t, J = 7.2 Hz, 3H). C₁₁H₁₅NO₃, EI-MS: m/z (M+H⁺): 210.2 (calculated), 210.0 (found).

3-(bromomethyl)-5-cyclopentyl-1,2-oxazole (6b).—The ester **4b** (0.21g, 1 equiv.) was dissolved in methanol and cooled down to 0 °C. NaBH₄ (0.15g, 4 equiv.) was added in small portions to the solution over 10 min. The mixture was warmed to room temperature and stirred for four hours. The reaction was quenched by adding diluted HCl and the organic solvent was removed under reduced pressure. The resulting aqueous layer was extracted with ethyl acetate (3×), and the organic layers were combined and dried over MgSO₄, and the solvent was removed under reduced pressure. This hydroxyl intermediate **5b** was used for the next step without further purification. Hydroxyl intermediate (0.17g, 1 equiv) was dissolved in DCM, and the resulting solution was cooled down to 0 °C. CBr₄ (0.50g, 1.5 equiv) and PPh₃ (0.39g, 1.5 equiv) were added sequentially. The solution was stirred at 0 °C for 20 minutes and gradually warmed up to room temperature. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (20% hexane/DCM) to give the desired intermediate **6b**. Yield: 62%. ¹H NMR (400 MHz, CDCl₃) δ 6.06 (s, 1H), 4.39 (s, 2H), 3.24–3.15 (m, 1H), 2.14–2.05 (m, 2H), 1.83–1.65 (m, 6H). C₉H₁₂BrNO, EI-MS: m/z (M+H⁺): 231.1 (calculated), 231.0 (found).

3-(bromomethyl)-5-cyclobutyl-1,2-oxazole (6a).—The synthesis and characterization of bromide **6a** was reported.¹⁸

3-(bromomethyl)-5-cyclohexyl-1,2-oxazole (6c).—synthesis and characterization of bromide **6c** was reported.¹⁸

General procedure of alkylations.

The bromide (1 equiv.) and amantadine or 1-amino-3-hydroxyadamantane or 5-aminoadamantan-2-one (1.5 equiv.) were dissolved in isopropanol; CsI (0.1 equiv.) and triethyl amine (2 equiv.) were then added. The reaction mixture was heated to reflux overnight. The solvent was removed under reduced pressure, and the resulting residue was extracted with ethyl acetate and water. The organic layer was separated, dried over anhydrous MgSO_4 , filtered, and concentrated under reduced pressure. The mixture was then purified by silica gel flash column chromatography (5–10% $\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$) to give the final product.

N-[(5-cyclobutyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (8a).—Compound **8a** was synthesized according to the above described alkylation procedure starting with bromide **6a**. The characterization of compound **8a** was reported before.¹⁸

3-[[[(5-cyclobutyl-1,2-oxazol-3-yl)methyl]amino]adamantan-1-ol (8b).—

Compound **8b** was synthesized according to the above described alkylation procedure starting with bromide **6a**. Yield: 78%. ^1H NMR (400 MHz, CDCl_3): δ 6.03 (s, 1H), 3.83 (s, 2H), 3.63–3.55 (m, 1H), 2.41–2.31 (m, 2H), 2.31–2.21 (m, 4H), 2.10–1.90 (m, 4H), 1.72–1.64 (m, 6H), 1.64–1.59 (m, 4H), 1.55–1.50 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 176.51, 163.24, 99.27, 69.64, 54.51, 50.00, 44.34, 41.07, 37.01, 35.09, 32.07, 30.73, 28.00, 18.74. $\text{C}_{18}\text{H}_{26}\text{N}_2\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 303.4 (calculated), 303.0 (found).

N-[(5-cyclopentyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (8c).—Compound **8c** was synthesized according to the above described alkylation procedure starting with bromide **6b**. Yield: 75%. ^1H NMR (400 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 6.85 (s, 1H), 4.22–4.15 (m, 2H), 3.20–3.08 (m, 1H), 2.17–2.10 (m, 3H), 2.08–2.02 (m, 6H), 2.02–1.95 (m, 2H), 1.75–1.61 (m, 12H). ^{13}C NMR (100 MHz, CDCl_3): δ 178.91, 157.08, 100.94, 59.01, 38.73, 37.48, 35.85, 35.38, 31.84, 29.14, 25.24. $\text{C}_{19}\text{N}_2\text{H}_{28}\text{N}_2\text{O}$, EI-MS: m/z ($\text{M}+\text{H}^+$): 301.4 (calculated), 301.0 (found).

3-[[[(5-cyclopentyl-1,2-oxazol-3-yl)methyl]amino]adamantan-1-ol (8d).—

Compound **8d** was synthesized according to the above described alkylation procedure starting with bromide **6b**. Yield: 82%. ^1H NMR (400 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 6.71 (s, 1H), 4.20–4.11 (m, 2H), 3.23–3.12 (m, 1H), 2.40–2.32 (m, 2H), 2.14–1.99 (m, 4H), 1.98–1.85 (m, 4H), 1.81–1.61 (m, 10H), 1.59–1.50 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 180.05, 155.89, 100.68, 68.50, 60.21, 42.73, 37.44, 36.66, 34.17, 31.80, 30.19, 25.20. $\text{C}_{19}\text{H}_{28}\text{N}_2\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 317.4 (calculated), 317.0 (found).

N-[(5-cyclohexyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (8e).—Compound **8e** was synthesized according to the above described alkylation procedure starting with bromide **6c**. The characterization of compound **8e** was reported before.¹⁸

3-[[[(5-cyclohexyl-1,2-oxazol-3-yl)methyl]amino]adamantan-1-ol (8f).—

Compound **8f** was synthesized according to the above described alkylation procedure starting with bromide **6c**. Yield: 85%. ^1H NMR (400 MHz, $\text{CD}_3\text{Cl}+\text{CD}_3\text{OD}$): δ 6.57 (s, 1H),

4.19–4.02 (m, 2H), 2.80–2.63 (m, 1H), 2.43–2.24 (m, 2H), 2.06–1.79 (m, 8H), 1.79–1.58 (m, 7H), 1.59–1.45 (m, 2H), 1.45–1.16 (m, 5H). ^{13}C NMR (100 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 180.06, 155.59, 100.11, 68.79, 60.48, 42.68, 36.70, 36.31, 35.30, 34.10, 30.89, 30.11, 25.55, 25.47. $\text{C}_{20}\text{H}_{30}\text{N}_2\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 331.5 (calculated), 332.0 (found).

Compounds **10a–10g** were synthesized using the general alkylation procedure described above starting from commercial available bromides.

N-[(5-cyclopropyl-1,2-oxazol-3-yl)methyl]adamantan-1-amine (10a).—The characterization of 10a was reported before.¹⁹

3-[[[(5-cyclopropyl-1,2-oxazol-3-yl)methyl]amino]adamantan-1-ol (10b).—Yield: 72%. ^1H NMR (400 MHz, CDCl_3): δ 5.92 (s, 1H), 3.83 (s, 2H), 2.29–2.23 (m, 2H), 2.02–1.91 (m, 2H), 1.68–1.63 (m, 5H), 1.61–1.57 (m, 4H), 1.53–1.48 (m, 2H), 1.05–0.98 (m, 2H), 0.95–0.89 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 174.76, 164.18, 98.63, 70.25, 54.02, 50.16, 44.26, 41.31, 36.88, 35.41, 30.98, 8.29, 8.08. $\text{C}_{17}\text{H}_{24}\text{N}_2\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 289.4 (calculated), 289.0 (found).

5-[[[(5-cyclopropyl-1,2-oxazol-3-yl)methyl]amino]adamantan-2-one (10c).—Yield: 80%. ^1H NMR (400 MHz, CDCl_3): δ 5.88 (s, 1H), 3.76 (s, 2H), 2.60–2.53 (m, 2H), 2.28–2.21 (m, 1H), 2.00–1.87 (m, 11H), 1.04–0.97 (m, 2H), 0.93–0.87 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 217.3, 175.03, 163.72, 98.46, 50.75, 46.64, 43.00, 41.69, 38.62, 37.42, 28.90, 8.67, 8.62. $\text{C}_{17}\text{H}_{22}\text{N}_2\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 287.4 (calculated), 287.0 (found).

3-[[[(5-cyclopropyl-1,3,4-oxadiazol-2-yl)methyl]amino]adamantan-1-ol (10d).—Yield: 71%. ^1H NMR (400 MHz, CDCl_3): δ 3.97 (s, 2H), 2.32–2.23 (m, 2H), 2.16–2.05 (m, 1H), 1.68–1.61 (m, 6H), 1.60–1.55 (m, 4H), 1.53–1.47 (m, 2H), 1.13–1.11 (m, 2H), 1.11–1.09 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 168.50, 164.95, 69.48, 53.78, 49.61, 43.95, 40.74, 35.99, 34.60, 30.55, 7.87, 5.70. $\text{C}_{16}\text{H}_{23}\text{N}_3\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 290.4 (calculated), 290.0 (found).

3-[[[(5-cyclopropyl-1,2,4-oxadiazol-3-yl)methyl]amino]adamantan-1-ol (10e).—Yield: 84%. ^1H NMR (400 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 4.15 (s, 2H), 2.35–2.28 (m, 2H), 2.22–2.12 (m, 1H), 1.96–1.90 (m, 2H), 1.89–1.78 (m, 4H), 1.70–1.61 (m, 4H), 1.54–1.47 (m, 2H), 1.26–1.16 (m, 4H). ^{13}C NMR (100 MHz, $\text{CDCl}_3+\text{CD}_3\text{OD}$): δ 183.64, 162.35, 68.08, 60.75, 45.82, 42.45, 36.75, 35.34, 33.85, 30.28, 10.53, 7.18. $\text{C}_{16}\text{H}_{23}\text{N}_3\text{O}_2$, EI-MS: m/z ($\text{M}+\text{H}^+$): 290.4 (calculated), 290.0 (found).

N-[(5-cyclopropyl-1,3,4-thiadiazol-2-yl)methyl]adamantan-1-amine (10f).—Yield: 78%. ^1H NMR (400 MHz, CDCl_3): δ 4.18 (s, 2H), 2.42–2.34 (m, 1H), 2.14–2.08 (m, 3H), 1.74–1.67 (m, 9H), 1.67–1.60 (m, 3H), 1.25–1.18 (m, 2H), 1.12–1.06 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 174.15, 42.42, 40.17, 36.45, 29.48, 11.65, 11.38. $\text{C}_{16}\text{H}_{23}\text{N}_3\text{S}$, EI-MS: m/z ($\text{M}+\text{H}^+$): 290.4 (calculated), 290.0 (found).

3-[[[5-cyclopropyl-1,3,4-thiadiazol-2-yl)methyl]amino}adamantan-1-ol (10g).—

Yield: 78%. ¹HNMR (400 MHz, DMSO-*d*₆): δ 4.66 (s, 2H), 2.63–2.55 (m, 1H), 2.30–2.23 (m, 2H), 1.82–1.76 (m, 6H), 1.61–1.50 (m, 4H), 1.50–1.43 (m, 2H), 1.29–1.22 (m, 2H), 1.06–1.00 (m, 2H). ¹³CNMR (100 MHz, DMSO-*d*₆): δ 175.97, 67.82, 59.95, 54.82, 46.05, 43.87, 37.93, 36.93, 34.30, 30.13, 12.04, 11.42. C₁₆H₂₃N₃OS, EI-MS: *m/z* (M+H⁺): 306.4 (calculated), 306.0 (found).

Characterization of compounds **11**, **14**, **15**, and **16** was reported before.^{16, 18, 19}

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 either the AM2-WT or the AM2-S31N mutant of the AM2 protein, as previously reported.³³ The M2 sequences used for this study were identical to the A/California/07/2009 (H1N1) M2 sequence (AM2-S31N) and the corresponding AM2-N31S mutant (AM2-WT). The M2 sequences from A/WSN/33 (H1N1) and A/California/07/2009 (H1N1) differ only by one amino acid at the transmembrane region at position 28: in A/WSN/33 (H1N1) is valine, in A/California/07/2009 (H1N1) is isoleucine. The potency of the inhibitors was expressed as percentage inhibition of AM2 current observed after two minutes of incubation with 100 μM of compounds at pH 5.5. All measurements were repeated three times with different oocytes. For kinetic studies, during the binding period, the oocyte was fluxed with pH 5.5 buffer containing 100 μM of compound until binding equilibrium was achieved. During the washing period, in order to prevent prolonged acidification of oocytes, a pH 5.5 pulse, instead of continuous application of pH 5.5 barth solution, was applied until equilibrium was achieved.

Plaque assay.

The plaque assay was performed as previously reported,^{29, 30} except MDCK cells expressing ST6Gal I were used instead of regular MDCK cells.³⁴ Briefly, a confluent monolayer of ST6Gal I MDCK cells was incubated with ~100 pfu virus/well in DMEM with 0.5% BSA for 1 hour at 4 °C, then at 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 with compounds at testing concentrations. After two days of incubation at 37 °C with 5% of CO₂ in the cell culture incubator, the overlay was removed and the cell monolayers were fixed and stained with crystal violet dye solution (0.2% crystal violet, 20% methanol). Influenza A viruses A/Switzerland/9715293/2013 X-247 (H3N2), FR-1366, A/Washington/29/2009 (H1N1), FR-460, A/North Carolina/29/2009 (H1N1), FR-488, and A/California/07/2009 (H1N1), 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. Influenza virus A/Denmark/528/2009 (H1N1) was obtained from Dr. Elena Govorkova at St. Jude Children's Research Hospital. Influenza virus A/Texas/04/2009 (H1N1) was obtained from Dr. James Noah at the Southern Research Institute.

Cytotoxicity assay.

Evaluation of the cytotoxicity of compounds and the efficacy of compounds against influenza-induced cytopathic effect was carried out using the neutral red uptake assay.²⁵ Briefly, 80,000 cells/mL of MDCK or A549 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 the cytotoxicity assay, 200 μ L fresh DMEM (no FBS) medium containing 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, then 100 μ L DMEM medium containing 40 μ g/mL neutral red was added and the plate was incubated for four hours at 37 °C. The amount of uptaken neutral red was determined at absorbance 540 nm using a Multiskan FC Microplate Photometer (Fisher Scientific). The CC₅₀ values were calculated from best-fit dose response curves with variable slope in GraphPad Prism version 5.

Combination therapy of compound **10b** with oseltamivir carboxylate.

The synergistic antiviral effect of compound **10b** and oseltamivir carboxylate was evaluated in cell cultures as described previously using CPE assay.^{29, 30} Five combinations of compound **10b** and oseltamivir carboxylate, at the fixed EC₅₀ ratios of 10:1, 5:1, 1:1, 1:5 and 1:10, were included. In each combination, a stock solution with the designated EC₅₀ ratio of compound **10b** and oseltamivir carboxylate was first made, then six 3-fold serial dilutions of the stock solution were made and tested to obtain the dose response curve, based on which EC₅₀ of individual **10b** or oseltamivir carboxylate was determined. Subsequently, fractional inhibitory concentration index (FICI) was calculated using the following formula: FICI = ((EC₅₀ of **10b** in combination)/(EC₅₀ of **10b** alone)) + ((EC₅₀ of oseltamivir carboxylate in combination)/(EC₅₀ of oseltamivir carboxylate alone)). FICI < 0.5 was interpreted as a significant synergistic antiviral effect.

Microsome stability.

Test compounds were incubated at 37°C with liver microsomes (mouse or human) (Xenotech) at 1 μ M drug concentration in the presence of a NADPH regenerating system at 0.5 mg/ml microsomal protein. Testosterone (3A4 substrate), Propafenone (2D6), and Diclofenac (2C9) were included as positive controls. Compounds were incubated with microsomes in the presence of a NADPH regenerating system (Sigma, Cat. No. N0505, Lot SLBH3107V). Aliquots at 0, 5, 10, 20, 30 and 60 minutes post incubation were collected and immediately mixed with cold acetonitrile containing internal standard (IS). Compounds incubated with microsomes without NADPH regenerating system for 60 minutes were also included. All the samples were analyzed by LC/MS/MS; disappearance of test compound were assessed based on peak area ratios of analyte/IS. Following equations were applied to calculate the microsome clearance:

$$\text{Elimination rate constant (k)} = -\text{gradient}; \text{Half life (T}_{1/2}\text{)} \left(\text{min} \right) = \frac{0.693}{k}$$

$CL_{int(mic)} = 0.693 / \text{half life} / \text{mg microsome protein per mL}$; Liver wt: 40 g/kg, 30 g/kg, 32 g/kg, 20 g/kg and 88 g/kg for rat, monkey, dog, human and mouse; Using $CL_{int(mic)}$ to calculate the whole the liver clearance: $\text{mg microsomal protein/g liver weight} : 45 \text{ mg/g}$ for 5 species; $CL_{int(liver)} = CL_{int(mic)} * \text{mg microsomal protein/g liver weight} * \text{g liver weight/kg body weight}$.

Caco-2 cell permeability assay.

Caco-2 Cells (ATCC) were cultured in MEM with 10% FBS. Cells at the passage number from 30 to 50 are seeded onto PET membranes of 96-well Insert Plates at 1×10^5 cells/ cm^2 for 21 to 28 days to reach confluent cell monolayer. The integrity of the monolayer was verified by performing Lucifer yellow rejection assay. The quality of the monolayer were verified by measuring the Unidirectional (A→B) permeability of fenoterol (low permeability marker), propranolol (high permeability marker) and Bi-directional permeability of Digoxin (a P-glycoprotein substrate marker) in duplicate wells. Assay conditions for test compounds listed as followings:

- Test concentration: 2 μM (DMSO 1%);
- Replicates: n=2;
- Directions: bi-directional transport including A→B and B→A;
- Incubation time: single time point, 2 hours;
- Transport buffer: HBSS containing 10 mM HEPES, pH7.4;
- Incubation condition: 37°C, 5% CO_2 , 95% relative humidity.

Testing compounds were diluted in transport buffer and acetonitrile (containing internal standard) and the resulting solution is designated as T0 sample. At the end of incubation, sample solutions from both donor and receiver wells were immediately mixed with cold acetonitrile containing internal standard (IS). All samples including T0 samples, donor samples and receiver samples were analyzed using LC/MS/MS. Concentrations of test compound were expressed as peak area ratio of analytes versus IS without a standard curve.

The apparent permeability coefficient P_{app} (cm/s) was calculated using the equation:

$$P_{app} = \left(dC_r / d_t \right) \times V_r / \left(A \times C_0 \right)$$

Where dC_r / d_t is the cumulative concentration of compound in the receiver chamber as a function of time ($\mu\text{M/s}$); V_r is the solution volume in the receiver chamber (0.075 mL on the apical side, 0.25 mL on the basolateral side); A is the surface area for the transport, i.e. 0.0804 cm^2 for the area of the monolayer; C_0 is the initial concentration in the donor chamber (μM).

CYP inhibition.

Compound was incubated at 10 μM with human liver microsomes with protein concentration at 0.253 mg/mL. α -Naphthoflavone, Sulfaphenazole, (+)-N-3-benzylrivanol, quinidine, and

ketoconazole were used as positive controls. The mixture of test compound or positive control were pre-warmed with human liver microsomes at 37 °C for 10 minutes. Phenacetin, diclofenac, *S*-mephenytoin, dextromethorphan, and midazolam were mixed and were used as substrates of CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively. After addition of substrate mixture and cofactor NADPH, the solution was incubated for another 10 minutes. The samples were then quenched with stop solution containing internal standard. Next, the solution was centrifuged at 4000 rpm (Centrifuge, 5810R Eppendorf) at room temperature for 20 minutes. The supernatant was diluted with purified water (v/v 2:1), which was shaken at 1000 rpm (Titer plate shaker, Thermo) for 10 minutes before LC-MS/MS injection. The formation of the metabolite acetaminophen, 4'-hydroxy diclofenac, 4'-hydroxy mephenytoin, dextropropan, and 1'-hydroxy midazolam were determined by LC-MS/MS and IC₅₀ values were calculated. SigmaPlot (V.11) was used to plot the mean CYP Activity (% VC) versus the test compound concentrations with non-linear regression analysis. IC₅₀ values were reported as "> 100 μM" when % inhibition at highest concentration (100 μM) was less than 50%.

The IC₅₀ values were extrapolated using the following equation:

$$IC_{50} = x \times (100 - \%inhibition \text{ at } x) / \%inhibition, \text{ suppose Hill slope} = 1, \text{ where } x = \text{concentration of test compounds}.$$

ACKNOWLEDGEMENTS

This research is supported by startup funding from the University of Arizona and NIH grant A1119187 to J.W. R.M. was supported by the NIH training grant T32 GM008804. We thank Dr. David Bishop for proofreading and editing the manuscript.

ABBREVIATIONS USED

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

REFERENCES

1. Webster RG; Monto AS; Braciale TJ; Lamb RA Textbook of Influenza. John Wiley & Sons, Ltd: West Sussex, 2013.
2. Zhang WQ; Webster RG Can we beat influenza? Science 2017, 357, 111–111. [PubMed: 28706015]
3. Zhu H; Lam TT; Smith DK; Guan Y Emergence and development of H7N9 influenza viruses in China. Curr Opin Virol 2016, 16, 106–113. [PubMed: 26922715]
4. Lai S; Qin Y; Cowling BJ; Ren X; Wardrop NA; Gilbert M; Tsang TK; Wu P; Feng L; Jiang H; Peng Z; Zheng J; Liao Q; Li S; Horby PW; Farrar JJ; Gao GF; Tatem AJ; Yu H Global epidemiology of avian influenza A H5N1 virus infection in humans, 1997–2015: a systematic review of individual case data. Lancet Infect Dis 2016, 16, e108–118. [PubMed: 27211899]
5. https://www.cdc.gov/flu/about/disease/high_risk.htm, accessed on 11/20/2017.

6. Shaw ML The next wave of influenza drugs. *ACS Infect Dis* 2017, 3, 691–694. [PubMed: 28892353]
7. Wang J; Li F; Ma C Recent progress in designing inhibitors that target the drug-resistant M2 proton channels from the influenza A viruses. *Biopolymers* 2015, 104, 291–309. [PubMed: 25663018]
8. Loregian A; Mercorelli B; Nannetti G; Compagnin C; Palu G Antiviral strategies against influenza virus: towards new therapeutic approaches. *Cell Mol Life Sci* 2014, 71, 3659–3683. [PubMed: 24699705]
9. Wang J; Qiu JX; Soto C; DeGrado WF Structural and dynamic mechanisms for the function and inhibition of the M2 proton channel from influenza A virus. *Curr Opin Struct Biol* 2011, 21, 68–80. [PubMed: 21247754]
10. Gubareva LV; Besselaar TG; Daniels RS; Fry A; Gregory V; Huang W; Hurt AC; Jorquera PA; Lackenby A; Leang SK; Lo J; Pereyaslov D; Rebelo-de-Andrade H; Siqueira MM; Takashita E; Odagiri T; Wang D; Zhang W; Meijer A Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2015–2016. *Antiviral Res* 2017, 146, 12–20. [PubMed: 28802866]
11. Webster RG; Govorkova EA Continuing challenges in influenza. *Ann N Y Acad Sci* 2014, 1323, 115–139. [PubMed: 24891213]
12. Hurt AC The epidemiology and spread of drug resistant human influenza viruses. *Curr Opin Virol* 2014, 8, 22–29. [PubMed: 24866471]
13. Matsuzaki Y; Mizuta K; Aoki Y; Suto A; Abiko C; Sanjoh K; Sugawara K; Takashita E; Itagaki T; Katsushima Y; Ujike 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. [PubMed: 20202225]
14. 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, e0119115. [PubMed: 25768797]
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, 1580–1590. [PubMed: 28182419]
16. 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. [PubMed: 28433777]
17. 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, 726–733. [PubMed: 27657178]
18. Li F; Ma C; DeGrado WF; 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, 1207–1216. [PubMed: 26771709]
19. Wang J; Wu Y; Ma C; Fiorin G; Wang J; Pinto LH; Lamb RA; Klein ML; DeGrado WF 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, 1315–1320. [PubMed: 23302696]
20. Kerns E; Di L Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization. Academic press, Boston, 2016.
21. Wanka L; Iqbal K; Schreiner P The lipophilic bullet hits the targets: medicinal chemistry of adamantane derivatives. *Chem Rev* 2013, 113, 3516–3604. [PubMed: 23432396]
22. Kalgutkar AS; Nguyen HT; Vaz AD; Doan A; Dalvie DK; McLeod DG; Murray JC In vitro metabolism studies on the isoxazole ring scission in the anti-inflammatory agent leflunomide to its active alpha-cyanoenol metabolite A771726: mechanistic similarities with the cytochrome P450-catalyzed dehydration of aldoximes. *Drug Metab Dispos* 2003, 31, 1240–1250. [PubMed: 12975333]
23. Gramec D; Peterlin Masic L; Sollner Dolenc M Bioactivation potential of thiophene-containing drugs. *Chem Res Toxicol* 2014, 27, 1344–1358. [PubMed: 25014778]
24. White JR Dipeptidyl peptidase-IV inhibitors: pharmacological profile and clinical use. *Clin Diabetes* 2008, 26, 53–57.

25. Repetto G; del Peso A; Zurita JL Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. *Nat Protoc* 2008, 3, 1125–1131. [PubMed: 18600217]
26. Hayden F Newer influenza antivirals, biotherapeutics and combinations. *Influenza Other Respir Viruses* 2013, 7, 63–75. [PubMed: 23279899]
27. Huggins JW; Robins RK; Canonico PG Synergistic antiviral effects of ribavirin and the C-nucleoside analogs tiazofurin and selenazofurin against togaviruses, bunyaviruses, and arenaviruses. *Antimicrob Agents Chemother* 1984, 26, 476–480. [PubMed: 6151377]
28. Meletiadis J; Pournaras S; Roilides E; Walsh TJ Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 2010, 54, 602–609. [PubMed: 19995928]
29. Hu Y; Zhang J; Musharrafieh RG; Ma C; Hau R; Wang J Discovery of dapivirine, a nonnucleoside HIV-1 reverse transcriptase inhibitor, as a broad-spectrum antiviral against both influenza A and B viruses. *Antiviral Res* 2017, 145, 103–113. [PubMed: 28778830]
30. Hu Y; Zhang J; Musharrafieh R; Hau R; Ma C; Wang J Chemical genomics approach leads to the identification of hesperadin, an aurora B kinase inhibitor, as a broad-spectrum influenza antiviral. *Int J Mol Sci* 2017, 18, E1929. [PubMed: 28885544]
31. DiMasi JA; Grabowski HG; Hansen RW Innovation in the pharmaceutical industry: New estimates of R&D costs. *J Health Econ* 2016, 47, 20–33. [PubMed: 26928437]
32. Wang J; Ma C; Wang J; Jo H; Canturk B; Fiorin G; Pinto LH; Lamb RA; Klein ML; DeGrado WF 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, 2804–2812. [PubMed: 23437766]
33. Balannik V; Wang J; Ohigashi Y; Jing X; Magavern E; Lamb RA; DeGrado WF; Pinto LH Design and pharmacological characterization of inhibitors of amantadine-resistant mutants of the M2 ion channel of influenza A virus. *Biochemistry* 2009, 48, 11872–11882. [PubMed: 19905033]
34. Hatakeyama S; Sakai-Tagawa Y; Kiso M; Goto H; Kawakami C; Mitamura K; Sugaya N; Suzuki Y; Kawaoka Y Enhanced expression of an α 2,6-linked sialic acid on MDCK cells improves isolation of human influenza viruses and evaluation of their sensitivity to a neuraminidase inhibitor. *J Clin Microbiol* 2005, 43, 4139–4146. [PubMed: 16081961]

Figure 1. Solution NMR structure of compound **11** in complex with the transmembrane domain of AM2-S31N (PDB: 2LY0).¹⁹ The transparency of the front helix was set as 0.5 for clarity.

Figure 2.

Hypothesis-driven optimization of the microsomal stability of AM2-S31N inhibitors. (A) Two FDA-approved oral drugs that contain the hydroxyl adamantane. (B) Strategy to increase the microsomal stability of AM2-S31N inhibitor **11**.

Figure 3.

Kinetic studies of AM2-S31N inhibitors. (A) Binding and washing curves were recorded for two oocytes. One of the representative recording traces is shown. During the washing period, in order to prevent prolonged acidification of oocytes, a pH 5.5 pulse, instead of continuous application of pH 5.5 barth solution, was applied. The red bar shows when pH 5.5 pulse was applied, and the blue bar shows when 100 μM of compound was applied at pH 5.5. Non-

labeled areas shows when pH 8.5 solution was applied. (B) The association and dissociation equations used to fit the curves in Figure 3A.

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Figure 4.

Plaque assays of compound **10b** in inhibiting human influenza A viruses. The EC_{50} values are the mean $\pm$ standard deviation of two independent experiments. Representative plaque assay images from the one of the two independent experiments are shown.

Figure 5. Docking models of compound **10b** in the transmembrane domain of AM2-S31N (PDB: 2LY0).¹⁹ The transparency of the front helix was set as 0.5 for clarity. Docking was performed using Schrödinger Glide standard precision.

A. Synthesis of isoxazole-containing AM2-S31N inhibitors

B. Direct alkylation

Scheme 1.

Synthesis of AM2-S31N inhibitors.

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Table 1.

Channel blockage, antiviral efficacy, cytotoxicity, and mouse microsomal stability of AM2-S31N inhibitors.

Structure/Compound ID	Electrophysiological TEVC assay ^d (% S31N channel inhibition)	Electrophysiological TEVC assay ^d (% WT channel inhibition)	EC ₅₀ (μM)A/California/07/2009 (H1N1) ^b	CC ₅₀ (μM) C ₆ NDCK cells	CC ₅₀ (μM) A549 cells	T _{1/2} (min) ^d	CL _{int} (diver)(mL/min/kg)
11	90.3 ± 3.4	11.2 ± 1.3	0.3 ± 0.1	100	177.5 ± 16.2	1.0	5290.9
14	82.2 ± 2.3	N.T.	0.1 ± 0.03	>200	>300	31.5	174.1
15	86.1 ± 1.9	N.T.	0.1 ± 0.04	>200	>200	13.4	410.4
10a	85.4 ± 2.2	19.3 ± 2.5	0.2 ± 0.01	125.3 ± 8.2	130.3 ± 15.0	12.8	430.3
10b	47.9 ± 1.0	2.3 ± 0.6	0.8 ± 0.05	>300	>300	>145	<38.0
10c	57.6 ± 1.5	6.1 ± 0.5	1.7 ± 0.5	>300	>300	97.2	56.5
10d	5.3 ± 2.9	N.T.	>30	>300	>300	N.T.	N.T.
10e	44.8 ± 0.8	N.T.	3.1 ± 0.9	>300	>300	>145	<38.0

10f	67.3 ± 3.2		10.2 ± 0.6	0.7 ± 0.1	>300	346.5 ± 19.2	N.T.	N.T.
10g	33.8 ± 0.9		6.6 ± 1.6	2.6 ± 0.7	>300	>300	<38.0	>145
8a	90.3 ± 0.6		N.T.	0.5 ± 0.1	102.3 ± 6.1	95.8 ± 3.6	N.T.	N.T.
8b	87.2 ± 0.4		N.T.	0.6 ± 0.1	>300	>300	302.8	18.1
8c	85.7 ± 1.6		8.4 ± 1.8	0.8 ± 0.1	9.0 ± 0.8	16.4 ± 2.7	N.T.	N.T.
8d	84.3 ± 2.1		0.7 ± 0.5	0.3 ± 0.1	146.6 ± 62.8	>300	781.3	7.0
8e	90.0 ± 1.7		N.T.	1.0 ± 0.1	7.5 ± 0.7	8.1 ± 0.7	N.T.	N.T.
8f	89.9 ± 1.0		N.T.	0.5 ± 0.1	92.2 ± 4.7	189.5 ± 9.9	718.3	7.6

^aPercentage channel blockage is presented as the mean ± standard deviation of three independent experiments.

^bAntiviral assay was performed in plaque assay

The EC₅₀ values are the mean ± standard deviation of two independent experiments.

^cCytotoxicity was measured using the neutral red method with a 48 h incubation time.²⁵

The CC₅₀ values are the mean ± standard deviation of three independent experiments.

$t_{1/2}$ was determined in mouse liver microsomes.
N.T. = not tested.

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Table 2.

Fitting results from kinetic studies of AM2-S31N inhibitors.^a

Compounds	10a	10b	10c	10e	10f	10g	16
% AM2-S31N channel blockage at two-minute time point at 100 μM	85.4 ± 2.2	47.9 ± 1.0	57.6 ± 1.5	44.8 ± 0.8	67.3 ± 3.2	33.8 ± 0.9	81.4 ± 0.8
Antiviral EC ₅₀ (μM)	0.2 ± 0.01	0.8 ± 0.05	1.7 ± 0.5	3.1 ± 0.9	0.7 ± 0.1	2.6 ± 0.7	6.8 ± 0.7
cLogP ^b	3.406	2.561	2.353	1.847	3.509	2.521	5.024
Time0 (min)	10	10	10	11	10	20	5
B _{max} (max inhibition at equilibrium)	91.2 ± 1.0	92.3 ± 0.7	90.7 ± 0.8	94.8 ± 1.0	85.1 ± 1.5	100	76.5 ± 3.4
K _{on} (M ⁻¹ min ⁻¹)	13651 ± 517	4754 ± 121	5578 ± 184	2378 ± 83	6567 ± 442	1745 ± 102	41484 ± 5439
K _{off} ^c (min ⁻¹)	0.088 ± 0.003	0.017 ± 0.0003	0.064 ± 0.002	0.022 ± 0.0005	0.061 ± 0.003	0.040 ± 0.001	0.621 ± 0.079
K _d (μM)	7.7 ± 0.7	4.5 ± 1.0	11.5 ± 0.6	9.3 ± 0.5	9.2 ± 1.0	22.7 ± 2.0	17.3 ± 2.8
Binding kinetics	Fast on, slow off	Slow on, slow off				Fast on, Fast off	

^aThe best fit values for the inhibition by compounds **10a**, **10b**, **10c**, **10e**, **10f**, **10g**, and **16**. Results were obtained in Prism 5.0 using the equations shown in Figure 3B. The compound concentration was 100 μM. The results are the mean ± standard deviation of two independent experiments.

cLogP was calculated using the QikProp in Schrödinger Glide.
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Table 3.

In vitro PK properties of AM2-S31N inhibitor 10b.

Compound structure	Lipinski rule of five ^a	Permeability ($\times 10^{-6}$ cm/s) ^b	Mouse liver microsome stability $T_{1/2}$ (min)	Human liver microsome stability $T_{1/2}$ (min)	CYP inhibition ^c
10b	M.W. = 289.4 HBD = 3 HBA = 4 cLogP = 2.6	Caco-2 cells: A to B: 27.57 B to A: 28.33	>145	> 145	1A2: IC ₅₀ > 100 μ M 2C9: IC ₅₀ > 100 μ M 2C19: IC ₅₀ > 100 μ M 2D6: IC ₅₀ > 100 μ M 3A4: IC ₅₀ > 100 μ M

^a Lipinski rule of five: MW<500, HBD<5, HBA<10, clogP<5, values were calculated using the QikProp in Schrödinger Glide^b Bidirectional permeability in Caco-2 cells was assessed over a 120-minute incubation at 37°C and 5% CO₂ with saturated humidity^c CYP inhibition was tested at 10 μ M, and IC₅₀ was derived from the standard curve.

Table 4.

Combination therapy of 10b with oseltamivir carboxylate.

Combination Ratio (EC ₅₀)	EC ₅₀ in Combination		EC ₅₀ alone		EC ₅₀ Equivalent ^a		FICI ^b
	10b (μM)	Oseltamivir (nM)	10b (μM)	Oseltamivir(nM)	10b	Oseltamivir	
10:1	0.13 ± 0.014	0.06 ± 0.007			0.394	0.005	0.40
5:1	0.14 ± 0.031	0.11 ± 0.016			0.424	0.009	0.43
1:1	0.092 ± 0.014	0.42 ± 0.027	0.33 ± 0.01	12.0 ± 2.5	0.279	0.035	0.31
1:5	0.078 ± 0.021	1.91 ± 0.18			0.236	0.159	0.40
1:10	0.039 ± 0.0044	1.97 ± 0.22			0.118	0.164	0.28

^aConcentration in EC₅₀ equivalent was the normalized concentration that was calculated by dividing the EC₅₀ of drug in combination with its EC₅₀ alone.

^bFICI was the sum of **10b** and Oseltamivir EC₅₀-equivalent concentrations used in each combination.