Article

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Just Accepted

Discovery of Novel Dual Inhibitors of Wild-type and the Most Prevalent Drug-Resistant Mutant, S31N, of M2 Proton Channel from Influenza A Virus

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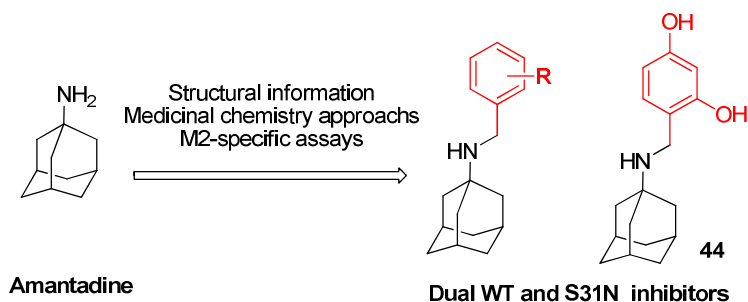
Abstract: Anti-influenza drugs, amantadine and rimantadine, targeting the M2 channel from influenza A virus are no longer effective because of widespread drug resistance. S31N is the predominant and amantadine-resistant M2 mutant, present in almost all of the circulating influenza A strains as well as in the pandemic 2009 H1N1 and the highly pathogenic H5N1 flu strains. Thus, there is an urgent need to develop second-generation

1

M2 inhibitors targeting S31N mutant. However, S31N mutant presents a huge challenge to drug discovery, and it has been considered undruggable for several decades. Using structural information, classical medicinal chemistry approaches and M2-specific biological testing, we discovered benzyl-substituted amantadine derivatives with activity against both S31N and WT, among which 4-(adamantan-1-ylaminomethyl)-benzene-1,3-diol is the most potent dual inhibitor. These inhibitors demonstrate that S31N is a druggable target and provide a new starting point to design novel M2 inhibitors that address the problem of drug-resistant influenza A infections.
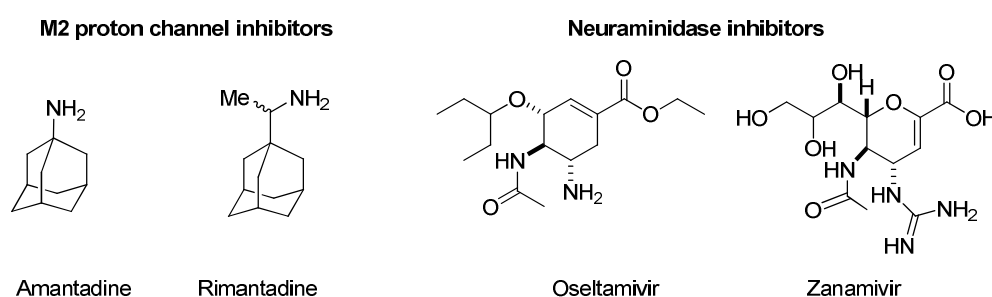
Figure 1. Structures of four approved antiviral drugs for influenza A

INTRODUCTION

Seasonal influenza infections as well as the emergence of life-threatening strains of influenza are a major worldwide health concern. Every year, influenza epidemics cause numerous deaths and millions of hospitalizations.[1,2] Besides the yearly epidemic outbreaks, influenza viruses are even more threatening pathogens due to their potency to cause pandemics, as occurred in 2009 with the emergence and worldwide spread of pandemic H1N1. Available prophylactic vaccines are not completely effective against emerging flu strains and thus effective anti-viral therapy is an essential component in the fight against influenza A infections. Currently, there are only four antivirals approved for use against influenza virus infections in humans, two targeting the M2 proton channel, amantadine (oral) and rimantadine (oral) and two targeting the neuraminidase, oseltamivir (oral), and zanimivir (inhaled) (Fig.**1**). Resistance to both classes is a problem: resistance to the

2

adamantane class of drugs is now so pervasive that the Centers for Disease Control and Prevention (CDC) has advised against its continued use[2,3] and widespread resistance to oseltamivir has been increasingly reported since 2007-2008.[4-8] Even more concerning are the reports of the emergence of flu strains with dual resistance to both adamantane and neuraminidase classes of antivirals.[9]

Resistance to M2 proton channel drugs is associated with mutations in the transmembrane domain of the M2 protein. The homotetrameric structure of the M2 channel places constraints on the types of drug-resistant mutations that can be accommodated. A single mutation to the pore-lining residues of the protein results in four changes to the residues lining the pore, where the drug binds. While many mutations can be tolerated with retention of function *in vitro*,[10-12] only a few mutants appear to be tolerated in highly transmissible viruses seen in the wild. V27A, L26F, and S31N have consistently comprised more than 99% of transmissible M2 mutants; among which S31N is now predominant in 98-100% of the transmissible amantadine-resistant H1N1, H5N1, and H3N2 strains isolated from humans, birds and swine in the last decade.[1] As a result, there is an urgent need to develop second-generation novel M2 inhibitors targeting the most prevalent S31N mutant. However, this mutation presents significant challenges to drug discovery, because the side chain of Asn31 partially fills the amantadine-binding site and also changes its physical properties markedly.

M2 inhibitors generally consist of a hydrophobic portion (adamantane in the drugs amantadine and rimantadine) connected to a polar group, which is generally a positively charged substituent such as an amine in amantadine or an aminoethyl group in rimantadine (Fig. **1**). A variety of scaffolds can be substituted for the hydrophobic adamantane group, including fused and spiro-fused multi-cyclic alkanes, acyclic branched alkanes, and silanes.[13, 14] These compounds showed excellent activity for WT, and some were highly active against V27A and L26F.[14, 15] However, none showed significantly better activity vs. S31N than had already been seen for amantadine. The polar group has also been the subject of extensive structure activity relations (SAR). Again, a

3

variety of substituents were tolerated when the target was WT, but only the simple primary amine found in amantadine gave significant activity against S31N. Thus, after several decades of medicinal chemistry investigations, no progress had been made towards inhibiting this predominant mutant form, suggesting that this variant might be essentially *undruggable*.[16-20]

Our journey to S31N inhibitors discovery started with modification of amantadine (**1**) whose potency against S31N drops approximately 12-fold from 16 μM against WT[21] to 200 μM[22] in a TEVC (two electrode voltage clamp) electrophysiological assay. Amantadine (**1**) is, however, more active than rimantadine (**2**) against S31N (199.9 μM vs > 2 mM)[23] (Fig. **2A**). We envisioned that the S31N inhibitory activity could be restored if we captured additional interactions within the channel. Recent crystallographic and NMR studies have provided major advances in our understanding of the mode of inhibition of M2 by amantadine and related drugs (Fig. **2B** and **2C**).[15,21,24-33]
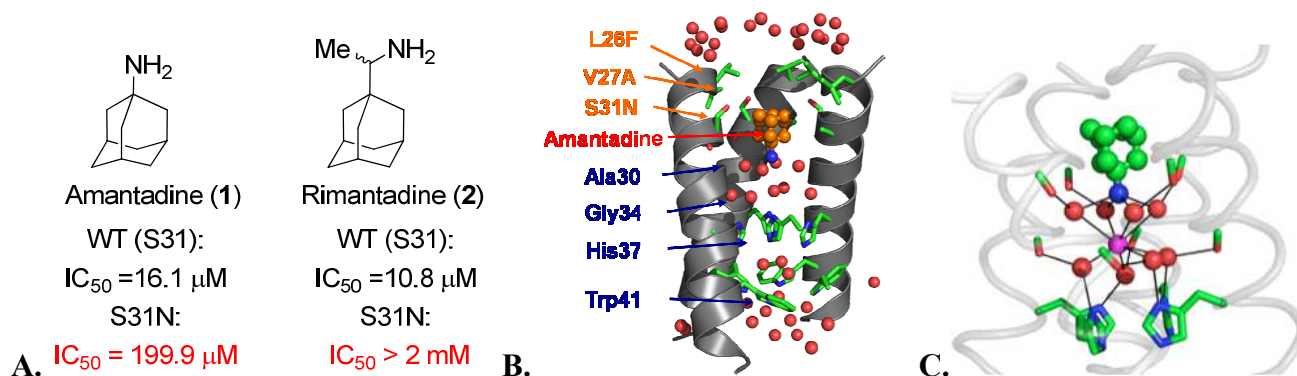
Figure 2. (**A**) Activity of amantadine and rimantadine against WT (S31) and S31N mutant in the TEVC assay. (**B**) The M2 channel-lining residues found in transmissible drug-resistant mutants are shown in orange letters. Amantadine (orange bound drug) causes a break in the aqueous path (water molecules, red balls). One helix has been removed for clarity. (**C**) Snapshot from a simulation of amantadine with WT[15]. Water molecules (red) associate with carbonyl groups (green/red sticks) in a square planar array. This array of water molecules can stabilize the bound ammonium group of amantadine (green and blue bound drug) or a centrally located water molecule (magenta).
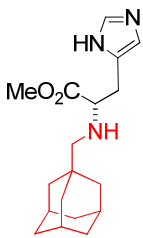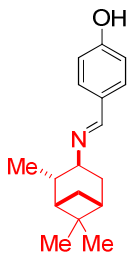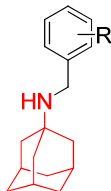
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The location of the pharmacologically relevant drug binding site in M2 was extensively debated until recently. Our earlier crystallographic structures suggested a pore-binding model,[24] while Chou and co-workers proposed a more peripheral site on the surface of the protein based on NMR studies.[25] However, last year, Chou and co-workers solved a structure, showing that drug binds to the site we had originally identified, ending the controversy, and validating our earlier crystallographic structures.[31] Specifically, the drug binds in a pocket within the channel lumen, and is surrounded by the apolar side chain of Val27, Ala30, the C-beta of Ser31, and the C-alpha of Gly34. The ammonium group projects downward towards the invariant His37 residue, but does not actually interact with this residue. Instead it is stabilized by water molecules, which are in turn stabilized by interactions with backbone carbonyl groups lining the pore.[15] The mutation S31N disrupts the size and polarity of the pore in precisely the region that accommodates the adamantane group of amantadine, explaining the decrease in potency of amantadine from 16 μM against WT to 200 μM in a TEVC electrophysiological assay. Moreover, NMR studies showed that S31N was more dynamic and possibly also more hydrated in the pore than the corresponding WT protein.[34] This led us to consider strategies in which we either increased the polarity or dimensions of the adamantane core to capture more interactions with the backbone of the channel (Fig. **3**, approach **I**). We synthesized over 300 amantadine analogues and other diverse cores, some of which have been published.[14,15,22,35-38] While we succeeded in inhibiting V27A and L26F, none of these compounds inhibited S31N.[15]
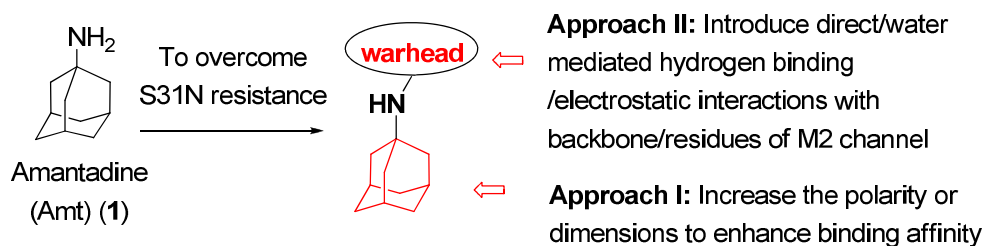
Figure 3. Two strategies to overcome drug-resistance

We turned our attention to the strategy of introducing additional groups (warhead groups) to the amine, in order to enhance affinity for S31N as well as WT by introducing direct/water mediated hydrogen binding /electrostatic interactions with backbone or the groups lining the pore of the M2 channel (Fig. **3,** approach **II)**. Examination of the structure of M2's channel showed there was sufficient space in the channel to accommodate additional substituents to mediate these interactions. However, this line of modification of amantadine (**1**) was not obvious; primary amines tended to be more potent than secondary amines and many early substitutions of the primary amines decreased activity towards the WT channel.[14] Nevertheless, two reports had shown that aromatic groups could be appended to the amines of 1-adamantanemethylamine (a rimantadine (**2**) derivative)[39] and pinanamine (**4**)[40] and showed activity against M2 channels. We therefore synthesized the most potent inhibitors in these two series, compounds **3**[39] and **4**[40], and found that they were active against WT (21% and 91% inhibition for **3** and **4**, respectively) (Table 1). However, they showed no activity against S31N (0% inhibition for both) in our electrophysiological TEVC assay. Since amantadine (**1**) is more potent against S31N (35% inhibition) than rimantadine (**2**) (13% inhibition) and pinanamine (0% inhibition[23]) in a TEVC assay, we envisioned that installation of aromatic group to the amine group of amantadine (**5-49**) might improve activity against S31N as well as WT. This led to the discovery of benzyl-substituted amantadine derivatives as dual WT and S31N inhibitors, which is described in the present manuscript. These compounds serve as starting points for design of novel compounds that address the problem of drug-resistant influenza A infections. At the same time of writing this manuscript, a pinanamine bearing imidazole derivative was reported to show weak inhibitory activity against the S31N M2 channel.[41]

6

Table 1. M2 inhibitors with an appended aromatic group[1]

Structure			
	3	**4**	**5-49**
	Rimantadine-based	Pinanamine-based	Amantadine-based
			(**this work**)
S31N % inhibition (TEVC)	0%	0%	See the text
WT % inhibition (TEVC)	$27 \pm 2\%$	$91 \pm 2\%$	

[1] The activity of the inhibitors was measured using the TEVC technique with full length AM2 protein in the *Xenopus* oocytes membrane; average of three measurements was reported (see Experimental Section in the Supporting Information).

A significant obstacle to developing inhibitors of M2 is to devise assays that are specific to inhibition of M2. In establishing an assay for S31N, we found it important to verify that the assay identified true M2 inhibitors, as we found both vesicle proton flux assays and viral inhibition assays are complicated by the fact that many amantadine-like compounds are proton carriers. Proton carriers such as chloroquine are good inhibitors of virus *in vitro,* but lack efficacy in animal models and human trials.[42-44] However, by conducting electrophysiological assays in parallel with antiviral assays it is possible to eliminate many false positives from compounds that acted as proton carriers and delay acidification of the endosome. We report here the first examples of compounds with specificity for both S31N and WT. Such compounds will be particularly important as mechanistic probes as well as serving as starting points for design of novel compounds that address the problem of drug-resistant influenza A infections.

7

Chemistry

The synthetic routes of the compounds **5, 8**, **9, 11, 12, 15, 17-23, 29-49** are shown in Scheme 1. A variety of benzyl groups were installed to N-terminal of **1** either through reductive amination (procedure **a** or **b**)[45-47] or amide bond formation followed by lithium aluminium hydride (LAH) reduction (procedure **c**).[48-50] Subsequent reactions included deprotection, oxidation of a thioether,[51] tetrazole formation,[52] or N-methylation, yielding compounds **6**, **10**, **13**, **16**, and **27**, respectively. Due to a low yield in the reductive animation with 2,4-di-hydroxybenzaldehyde, compound **44** was synthesized by reductive amination with 2-hydroxy-4-methoxy-benzaldehyde followed by demethylation with BBr$_3$.[53] Compounds **14**, **24**, **25**, **31**, **33** were prepared via similar routes (Scheme 2). The syntheses of the imine analogue, **26**[40] and the ether analogue, **23**, are shown in Scheme 3.
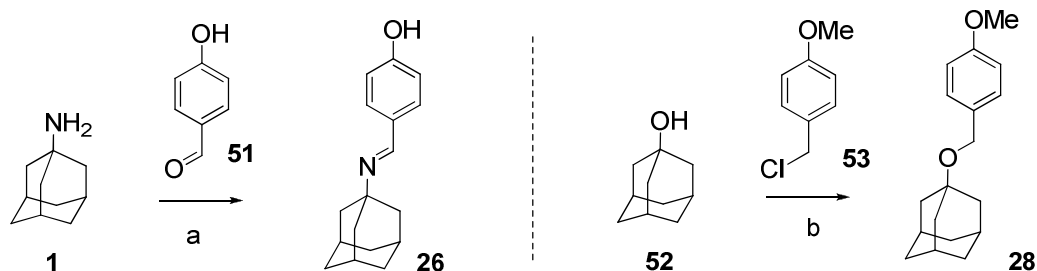
Scheme 1
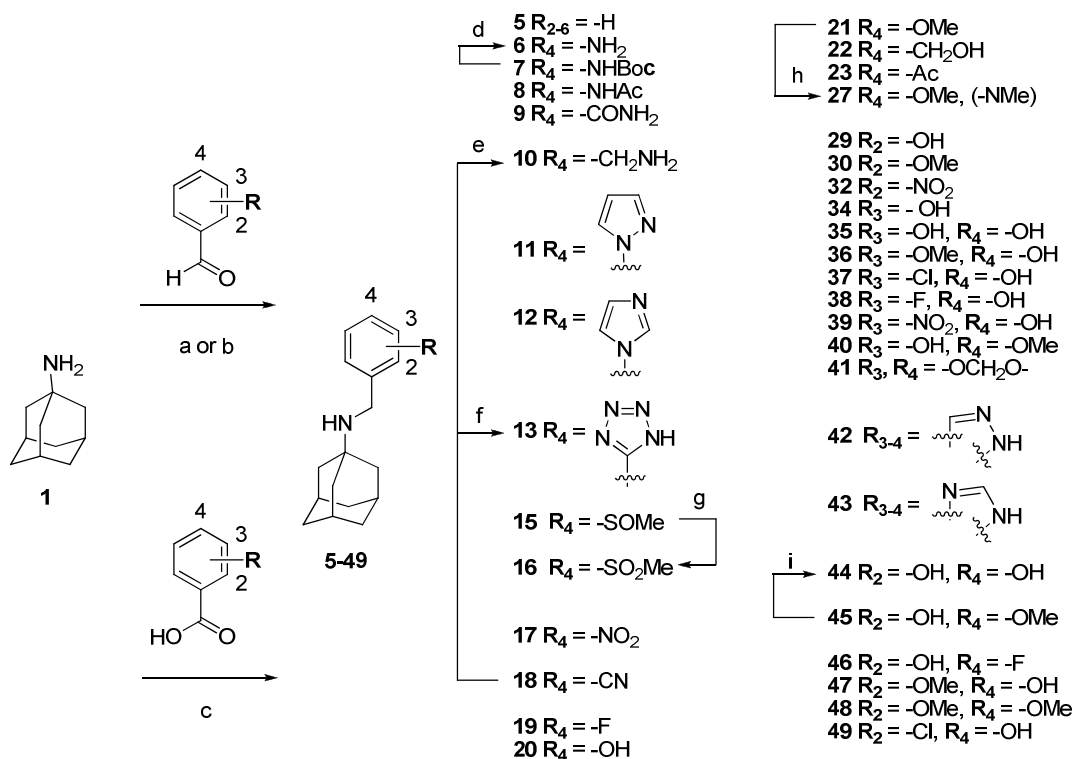
Reagents and conditions: (a) NaBH$_3$CN, MeOH, RT, overnight. (b) i. Ti(OiPr)$_4$; ii. NaBH$_4$, MeOH. (c) i. EDCI, HOAT, DIPEA, CH$_2$Cl$_2$, RT; ii. LAH, THF, Reflux. (d) HCl/dioxane, rt. (e) LAH, THF, Reflux. (f) NaN$_3$, CH$_3$CN. (g) m-CPBA (1.2 equiv), CH$_2$Cl$_2$, RT. (h) NaH, MeI (1.1 equiv), 0 °C, DMF. (i) BBr$_3$ (1.2 equiv), CH$_2$Cl$_2$, -78 to 0 °C.

Scheme 2
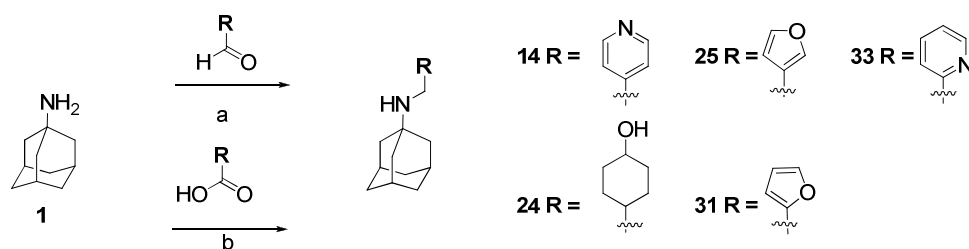
Reagents and conditions: (a) NaBH$_3$CN, MeOH, RT. (b) i. EDCI, HOAT, DIPEA, DMF, RT; ii. LAH, THF, Reflux.

Scheme 3

Reagents and conditions: (a) piperidine (cat.), benzene, Dean-Stark. (b) NaH, PMBCl (1.1 equiv), RT, DMF.

Structure-Activity Relationship (SAR) Study

The activity of the inhibitors was measured using the TEVC technique with full length M2 protein expressed in *Xenopus* oocytes.[21,54-56] All inhibitors were initially tested at 100 μM; those that inhibited the AM2 channel activity more than amantadine (Amt) (**1**) (by 35% inhibition) were chosen for measurement of their IC$_{50}$. Inhibitors of M2 enter the channel from the outside of the virus through a narrow opening near Val27. Thus, M2-blockers show slow on/off inhibition, with association occurring on the min. to hour time scale (at 1 μM) and dissociation hour to day time scale. Due to the limited stability of oocytes at low pH, we measure the percent inhibition after only 2 min, before equilibrium is reached. Due to the *kinetics of inhibition*, this provides an apparent kinetic IC$_{50}$ up to 100-fold higher than the true K$_{diss}$ measured with much longer times.[57] Nevertheless, the relative potency of the compounds tracks well with their relative potencies measured in other methods. Thus, the low μM IC$_{50}$ values of compounds seen in our primary electrophysiologic assays are

9

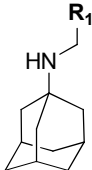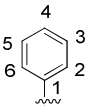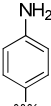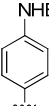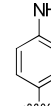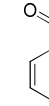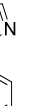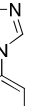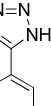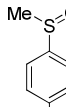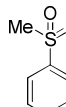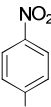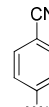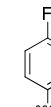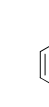numerically larger than their antiviral potency in plaque assays. While the TEVC assay underestimates the true

potencies of the compounds, it has the advantage of being remarkably reproducible, allowing us to reliably

interpret even small degrees of inhibition. All measurements were made in triplicate, with a typical standard

error being between 1% and 3%. Moreover, we observed less than 5% error when making repeat measurements

over time and with different batches of oocytes.

Since the reported nonamantadine-based aryl-containing M2 inhibitors (Table 1)[39,40] showed essentially no

inhibition against S31N, we started to explore amantadine-based derivatives with an aromatic group appended

to the primary amine group, which led to the discovery of a new series of substituted N-benzyl amantadines as

promising M2 inhibitors against S31N as well as WT. Beginning with the unsubsituted N-benzyl analogue of

amantadine we examined the effects of substitution at various positions in the ring. Because unsubstituted

amantadine is already a relatively hydrophobic drug, we focused the present studies primarily on polar

substituents to capture additional hydrogen bonding or electrostatic interactions as well as to maintain desirable

drug-like properties. Although the unsubstituted derivative **5** was inactive towards S31N (1% inhibition), it did

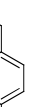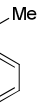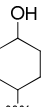show significant inhibition against the WT (77% inhibition) at 100 μM drug concentration. We therefore

focused on phenyl substituents with hydrogen-bonding donor/acceptor at 4-position (Table 2), and were

gratified to observe that the S31N inhibitory activity did increase gradually for 4-substituted series.

Introduction of a 4-amino substituent (compound **6**) increased S31N activity from 1% to 10% inhibition, but

decreased activity against WT from 77% to 31% inhibition. Interestingly, a Boc derivative of the aniline of **6**

improved S31N activity to 20% inhibition (compound **7**), and acetylation of **6,** further boosted S31N activity to

32% inhibition (compound **8**), which is similar to the activity of **1** (35% inhibition). However, the closely

related benzamide **9** and benzylamine **10** lost considerable potency against both S31N and WT.

Next, heteroaryl substituents at 4-position were examined. Introduction of a pyrazole **11**, or imidazole **12** gave compounds with some activity against S31N (26% and 25% inhibition, respectively), but still less potent than unsubstituted amantadine (**1**). A tetrazole derivative was even less active than the pyrazole and imidazole derivatives (8% inhibition) therefore, we examined other substituents at the 4-position. Replacement of aniline of **6** with its bioisomer, 4-pyridine **14** slightly increased S31N and WT activity. Other substituents at the 4-position such as sulfone **15**, sulfoxide **16**, nitro **17**, cyano **18,** and fluorine **19** showed some improvement in S31N activity compared to the parent compound **5**, but did not reach the same activity as amantadine (**1**).

The 4-phenolic derivative, 4-(adamantan-1-ylaminomethyl)-phenol (**20**) was the first compound that showed slightly higher inhibitory activity against S31N (IC_{50} = 166 μM) than amantadine (**1**) (IC_{50} = 199.9 μM). Methylation of the 4-hydroxyl group of **20**, reduced S31N inhibitory activity from 42% to 31% inhibition (compound **21**), which indicated the importance of the 4-hydroxy group. Methyl ketone **23** and methyl ether **21** showed similar S31N activity (31% inhibition). One carbon homologated derivative of **20** (compound **22**), markedly decreased S31N inhibitory activity from 42% to 17% inhibition. Interestingly, 4-cyclohexanol **24**, a saturated version of **20**, dramatically dropped S31N activity from 42% to 19% inhibition, which demonstrated that the aromaticity is crucial for S31N activity. We also found that the 4-hydroxy of **20** could not be replaced by its bioisomer, 3-furan **25**, as this modification led to a marked loss of S31N inhibitory activity from 42% to 8%, respectively.
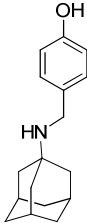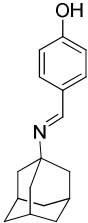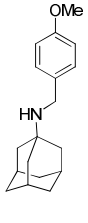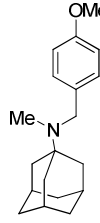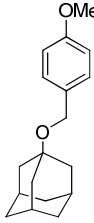
Table 2. SAR of 4-substituted adamantan-1-yl-benzyl-amines [1]

R₁=	-H Amt	(phenyl)	NH₂	NHBoc	NHAc	O=C-NH₂	CH₂NH₂	pyrazole	imidazole	tetrazole	pyridine
/ ID #	**1**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
S31N % inh/ IC$_{50}$	35 ± 2/ 199.9 μM	1 ± 1	10 ± 1	20 ± 2	32 ± 2	3 ± 1	8 ± 1	26 ± 3	25 ± 2	8 ± 1	15 ± 1
WT % inh/ IC$_{50}$	91 ± 3/ 16.1 μM	77 ± 1	31 ± 2	13 ± 2	3 ± 2	7 ± 2	3 ± 2	4 ± 3	4 ± 2	6 ± 1	6 ± 1

R₁=	Me-S=O (sulfoxide)	Me-SO₂ (sulfone)	NO₂	CN	F	OH	OMe	HO-CH₂	C(=O)Me	OH (cyclohexyl)	furan
/ ID #	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
S31N % inh/ IC$_{50}$	12 ± 1	21 ± 2	14 ± 1	8 ± 2	8 ± 2	42 ± 2/ 166 μM	31 ± 2	17 ± 2	31 ± 2	19 ± 2	8 ± 2
WT % inh/ IC$_{50}$	2 ± 2	45 ± 2	49 ± 2	36 ± 2	77 ± 1	67 ± 2	43 ± 1	26 ± 2	2 ± 2	16 ± 1	73 ± 2

[1] The activity of the inhibitors was measured using the TEVC technique with full length AM2 protein in the *Xenopus* oocytes membrane; average of three measurements was reported (see Experimental Section in the Supporting Information).
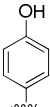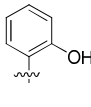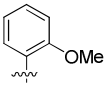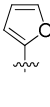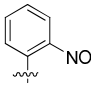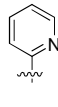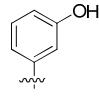
We also explored the SAR of the linkers (Table 3). A secondary amine is preferred over an imine analogue for S31N activity (42% and 21% inhibition for compounds **20** and **26**, respectively). Secondary amine linker also showed better S31N activity than either a tertiary amine or an ether linkage (31%, 20%, and 13% inhibition for compounds **21**, **27,** and **28**, respectively). Accordingly, in the following SAR studies, we kept the secondary amine linker constant.
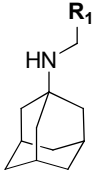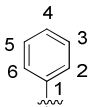
Table 3. The SAR of the linkers

Structure					
	20	**26**	**21**	**27**	**28**
S31N % inh	42 ± 2%	21 ± 1%	31 ± 2%	20 ± 3%	13 ± 1%
WT % inh	67 ± 2%	81 ± 2%	43 ± 1%	34 ± 3%	51 ± 2%
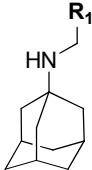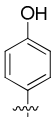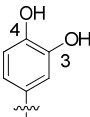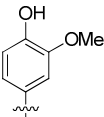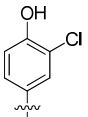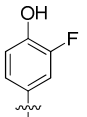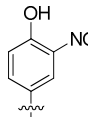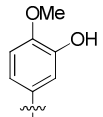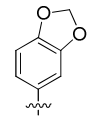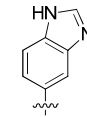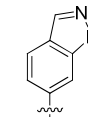
We further examined SAR of 2- and 3-substituted benzyl amine analogues (Table 4). Two phenol regioisomers of **20**, 3-hydroxy **34** and 2-hydroxy **29**, displayed much lower S31N inhibitory activity (6% and 18% inhibition, respectively), but improved WT inhibitory activity (88% and 81% inhibition, respectively). As was the case for 4-methyl ether **21** (Table 2), 2-methyl ether **30** also decreased rather than enhanced the S31N inhibitory activity. Similarly, replacement of the 2-hydroxy in **29** with a 2-furan **31**, 2-nitro-phenyl **32,** or 2-pyridyl **33** group led to the further decrease in activity.

Table 4. SAR of 2 and 3-substituted adamantan-1-yl-benzyl-amines

R$_1$=	-H Amt	(phenyl, positions 4,5,6,3,1,2)	OH (4-position)	OH (2-position)	OMe (2-position)	O (furan)	NO$_2$ (2-position)	N (pyridine)	OH (3-position)
/ ID #	1	5	20	29	30	31	32	33	34
S31N % inh/ IC$_{50}$	35 ± 2/ 199.9 μM	1 ± 1	42 ± 2/ 166 μM	18 ± 2	14 ± 1	6 ± 2	1 ± 1	0%	6 ± 2
WT % inh/ IC$_{50}$	91 ± 3/ 16.1 μM	77 ± 1	67 ± 2	81 ± 2	22 ± 2	66 ± 3	48 ± 1	12 ± 1	88 ± 1

Attention was turned to effects of 3-substituents in the context of the 4-hydroxy **20**, which had significant activity against S31N and WT (Table 5). Both electron withdrawing groups (3-Cl, 3-F, 3-NO$_2$) (compounds **37**, **38,** and **39**, respectively) and electron donating groups (3-OH, 3-OMe) (compounds **35** and **36**, respectively) reduced potency, with 3-fluoro derivative **38** showing the smallest decrease in potency (31% inhibition). 3-Methoxy analogue **36** is less effective than 3-hydroxy derivative **35**, which may be due to steric hindrance. This was supported by a more potent inhibitor **41**, in which the methylene at 3-position was constrained into a 5-membered ring. Methylation of the 4-hydroxyl group of **35** (compound **40**) maintained both WT and S31N activity and displayed slightly decreased activity than the more constrained **41**. We also found that 4-hydroxy cannot be replaced with two cyclic bioisomers, **42** and **43**, which displayed much weaker activity against S31N (10% and 12% inhibition, respectively).

Table 5. SAR of 3,4-disubstituted adamantan-1-yl-benzyl-amines
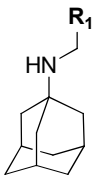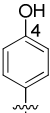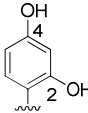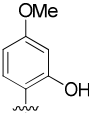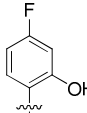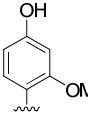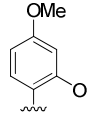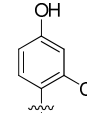
$R_1=$ / ID #	H Amt / 1	OH / 20	OH OH / 35	OH OMe / 36	OH Cl / 37	OH F / 38	OH NO / 39	OMe OH / 40	O-O / 41	HN-N / 42	N-NH / 43
S31N % inh/ IC$_{50}$	35 ± 2/ $199.9\,\mu M$	42 ± 2/ $166\,\mu M$	23 ± 1	3 ± 1	16 ± 1	31 ± 3	13 ± 2	20 ± 1	29 ± 1	10 ± 1	12 ± 1
WT % inh/ IC$_{50}$	91 ± 3/ $16.1\,\mu M$	67 ± 2	44 ± 3	76 ± 2	78 ± 1	65 ± 1	66 ± 1	46 ± 3	54 ± 2	26 ± 1	79 ± 1

Because 2- and 4-hydroxyl and methoxy analogues provided better inhibition compared to the 3-hydroxy analogue (Table 4), we further examined whether their beneficial effects were additive. Toward this end, we synthesized 2,4-disubstituted benzyl amine derivatives (Table 6). Pleasingly, 2,4-dihydroxy substituted analog, **44,** markedly enhanced S31N inhibitory activity (67% inhibition at 100 μM, IC$_{50}$ = 35.2 μM). Compound **44** was approximately 6-fold more potent against S31N than amantadine (**1**) (IC$_{50}$ = 199.9 μM), and only 2-fold less potent than amantadine (**1**) for WT (IC$_{50}$ = 16 μM) in the TEVC assay. Compound **44** was also active against the WT with an IC$_{50}$ of 59 μM, approximately 3.7-fold less potent than amantadine (**1**). The antiviral activity of **44** was also profiled in viral plaque reduction assays (Fig. **4**). The plaque assays showed that the compounds were more active than in the electrophysiological TEVC assays, which underestimated the affinities due to the brief period of inhibition. Compound **44** had an EC$_{50}$ of 3.2 μM against S31N and was 7-fold more potent than amantadine (**1**) (EC$_{50}$ = 22.5 μM) (Fig. **3A**). In the plaque assay, compound **44** was also active against the WT virus with complete inhibition of plaque formation at l μM (Fig. **3B**).

15

We also found that methylation of the 4-hydroxy **45** maintained the potency for both WT and S31N with IC$_{50}$

values of 79 μM and 41.3 μM, respectively. The importance of the 2-hydroxy was observed in other 4-

substituted analogues. For example, installation of 2-hydroxy to 4-fluoro analogue **19** markedly improved S31N

inhibitory activity from 8% to 37% inhibition (compound **46**). However, methylation of 2-hydroxy in **44** and

45 (compounds **47** and **48**, respectively) dramatically reduced both S31N and WT activities, which might be

due to the steric hindrance at this position. 2-Chloro analogue **49** also showed much lower S31N activity than

44, which may be attributed to the fact that chlorine is a weaker hydrogen acceptor than a hydroxyl group.

These data might also indicate that a hydrogen bond donating hydroxyl is essential at this position of the ring.

Table 6. SAR of 2,4-disubstituted adamantan-1-yl-benzyl-amine of selective compounds

	R1=	-H Amt	OH 4	2 OH	OH 4 2 OH	OMe OH	F OH	OH OMe	OMe OMe	OH Cl
	/ID #	1	20	29	44	45	46	47	48	49
S31N	% inh	35 ± 2	42 ± 2	18 ± 2	**67 ± 1**	**66 ± 1**	37 ± 2	12 ± 2	11 ± 1	14 ± 1
	IC$_{50}$ (μM)	199.9 μM	166 μM		**35.2 μM**	**43.1 μM**	NA	NA	NA	NA
WT	% inh	91 ± 3	67 ± 2	81 ± 2	**64 ± 1**	**56 ± 1**	87 ± 2	0	16 ± 2	78 ± 1
	IC$_{50}$ (μM)	16.1 μM	NA		**59 μM**	**79 μM**	NA	NA	NA	NA

Figure 4. (A) Plaque reduction assay with A/WSN/33-S31N influenza virus. (**B**) Plaque reduction assay with A/WSN/33-WT virus. The inhibitory effect of compound **44** and amantadine on influenza A virus replication was evaluated by plaque formation in MDCK cells as described in the Experimental Section.

Conclusions:

Using a combination of structural information of M2 proton channel and medicinal chemistry approaches, we report the discovery of promising dual M2 inhibitors for S31N and WT M2 channels, demonstrating that M2 S31N is a druggable target. Based on this work, several more potent series of S31N inhibitors have been discovered, one series has already been published[58] and others will be the subject of future reports. Such compounds will be also important as mechanistic probes to address the problem of drug-resistant influenza A infections.

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ACKNOWLEDGEMENT

SUPPORTING INFORMATION

Details of the Two-Electrode Voltage Clamp (TEVC) Assay, Plaque Reduction Assay, and Synthesis of

Compounds, **5-49,** are available free of charge via the Internet at http://pubs.acs.org.

LIST OF ABBREVIATIONS

Amantadine (Amt), Serine 31 (S31), Serine 31 Arganine (S31N), Wild type (WT), Compound (Cmpd), Two-Electrode Voltage Clamp (TEVC), % inhibition (% inh), The half maximal inhibitory concentration (IC_{50}), Madin-Darby Canine Kidney Cells (MDCK), Lithium aluminium hydride (LAH), 1-Hydroxy-7-azabenzotriazole (HOAT), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), Nuclear Magnetic Resonance (NMR), *N*,*N*-Diisopropylethylamine (DIPEA), Dimethylformamide (DMF), Room temperature (RT), *meta*-Chloroperoxybenzoic acid (*m*-MCPA).

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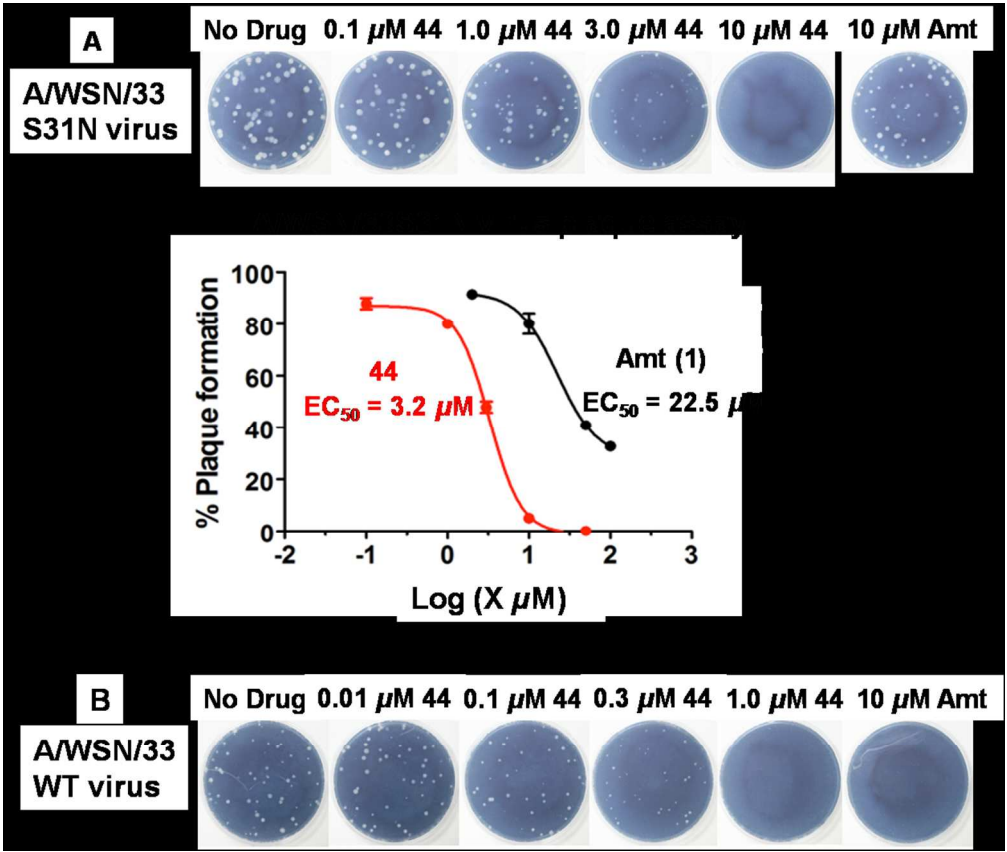
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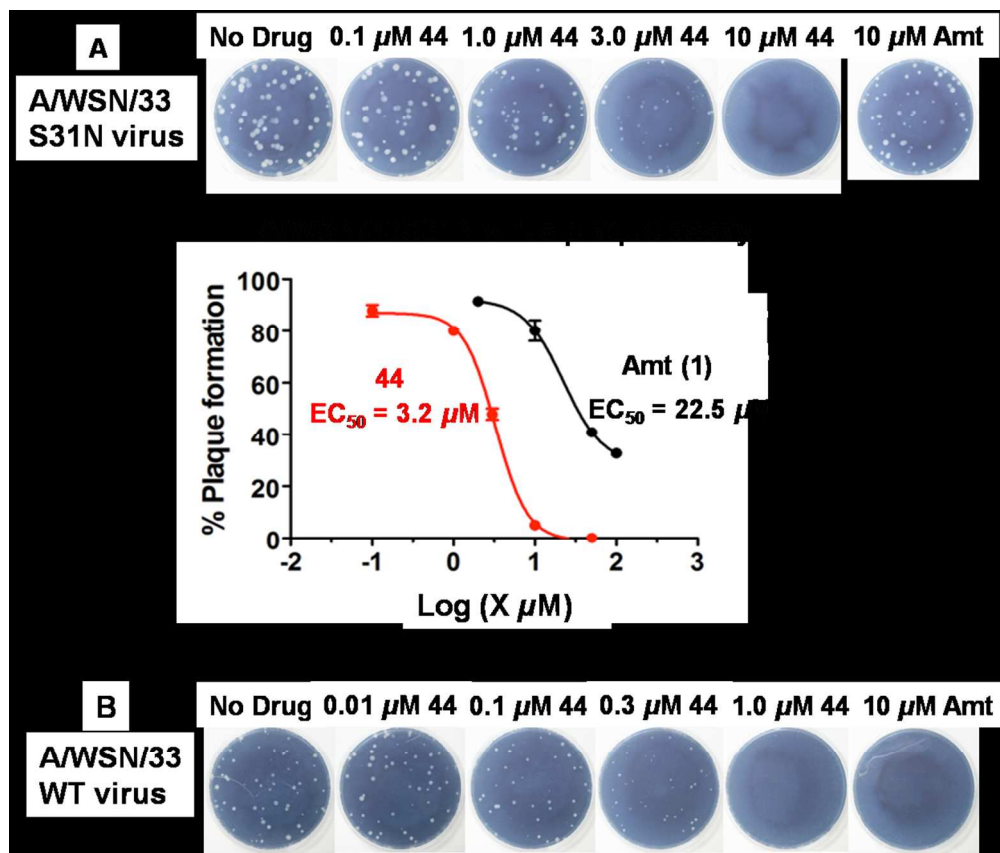
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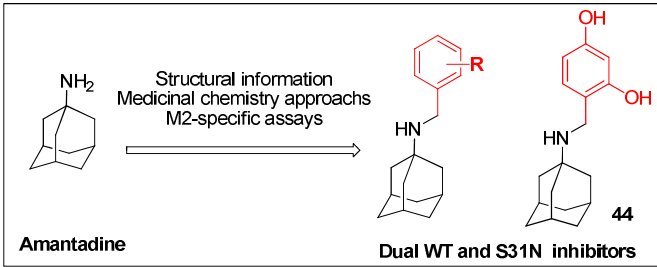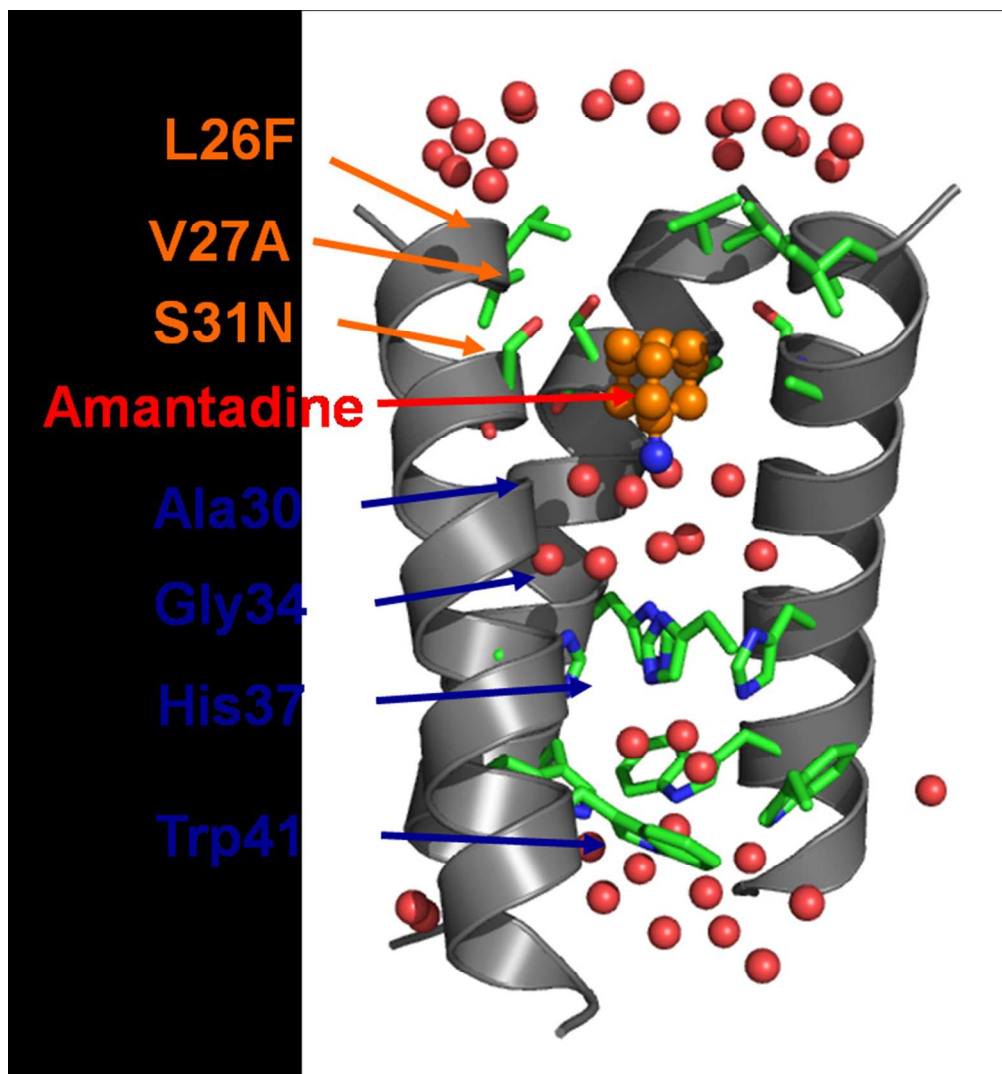
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