Amantadine Variants Activity Against Multiple Influenza A Viruses

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Abstract

Future pandemic influenza necessitates the development of new drugs against the current circulating, amantadine and rimantadine drugs resistant, influenza A M2 S31N viruses. The possibility of an antigenic shift to M2 S31 necessitates ranking the biological activities of amantadine variants. Several amantadine variants have been tested by different laboratories, but various M2 wild type influenza A strains have been used with different sensitivity against amantadine and the unambiguous comparison between potencies is not straightforward. Here, we compared the anti-influenza activities of 57 synthetic amantadine variants against influenza A WSN/33 viruses with amantadine-sensitive M2 WT, with a range of over three digits providing a reference set of potencies for structure-activity relationships, and amantadine-resistant M2 S31N proteins (and observed no potent compounds). 17 compounds were selected and tested against M2 L26F, V27A, A30T, G34E viruses. We tested few reference compounds using electrophysiology and explored point mutations which both showed that M2 is the target of potent antiviral potency against the M2 WT, L26F, V27A viruses. Major findings are: (a) Several amantadine variants from Kolocouris group block only M2 WT and M2 L26F-mediated proton current and the corresponding viruses replication. (b) A compound from Vazquez's group is a triple blocker of M2 WT, L26F, V27A channels and viruses replication. (c) A compound from Vazquez's group blocks only M2 L26 channel and virus replication. (d) Several compounds from Kolocouris group have potent activity against several influenza A M2 WT and three M2 S31N viruses, eg. the pandemic A/H1N1/California/07/2009 (H1N1pdm09) or A/H1N1/PuertoRico/08/1934 without blocking M2 S31N. The compounds and their cocktails while not to be more toxic than amantadine might be useful for re-purposing of amantadine class of drugs in the case (i) of the prevalence of M2 L26F and or M2 V27A strains (ii) of an antigenic shift of the virus to M2 WT and (iii) because they inhibited a broad panel of M2 WT and M2 S31N viruses including the H1N1pdm09). (d) We showed that the mechanism of antiviral activity against A/California/07/2009 or A/PR/08/1934 and possibly also M2 WT viruses compared to WSN/33 viruses is not due to inhibition of an early stage of virus infection or a late stage of M2 channel function during endocytosis or inhibition of HA binding to host cells or a different pH for HA fusion or a lysosomotropic effect.

Introduction

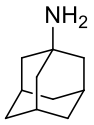
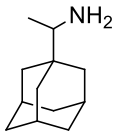
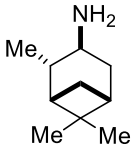
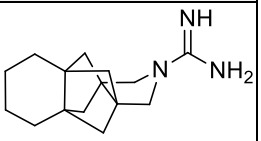
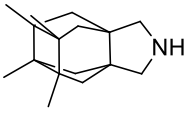
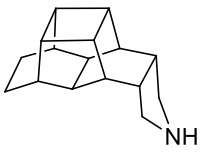
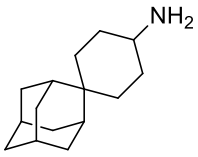
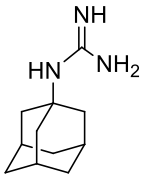
Amantadine (*Amt*, **1**) and rimantadine (*Rim*, **2**) (Table 1) are blockers of proton transport by M2 ion channel of influenza A wild type (WT) virus, [1,2,4] and were clinically approved as prophylactics and therapeutics against influenza A viruses. [3] Several groups published high resolution structures and data from mutation experiments showing that M2 protein channel is blocked by **1** and **2** via a M2 pore-binding mechanism. [4–9] Additionally, amantadine variants, especially those with hydrophobic adducts, can act as lysosomotropic drugs, [10] which accumulate in intracellular vesicles through membrane permeation by the electroneutral form and increase intravesicular pH, causing endosome and/or trans-Golgi network neutralization and inhibition of viral reproduction. [11] Noteworthy, SARS-CoV-2 is inhibited by chloroquine, probably because it acts as a lysosomotropic drug. [12]

Resistance to M2 WT proton channel drugs is associated with mutations in the TM domain of the M2 protein. [13–15] The homotetrameric structure of the M2 channel places constraints on the types of drug-resistant mutations that can be accommodated. [16,17] The amino acid substitutions L26F, V27A, A30T, G34E and S31N were shown to confer cross-resistance to *Amt* (**1**) and *Rim* (**2**) against influenza A viruses. [18–20] The vast majority, 95%, of resistant viruses bear the S31N substitution in M2, 1% have V27A, and L26F, A30T, and G34E are rare. [21,22] The substitution V27A most often emerged under drug selection pressure. [23,18] The other mutations confer *Amt* (**1**) resistance but this is not a result of the *Amt* (**1**) drug selection pressure. [23] The M2 S31N mutant is a natural mutation and one of the most conserved in viral proteins among currently circulating influenza A viruses that happens to maintain nearly identical channel function as the M2 WT but is resistant to *Amt* (**1**). The presence of L26F, V27A, and particularly S31N in influenza A viruses circulating worldwide pushes the search for novel ion channel blockers with stronger, preferably resistance-overcoming activity.

After early work on potent synthetic amantadine analogues, [24] many other amantadine variants, [25–30] polycyclic cage amines and their guanidino analogues have been synthesized, [31,32] eg.

compounds **3-8.** Compounds **3** and **8** block the M2 WT- proton mediated current. Compounds **4**, **6** [31] block both M2 WT- and M2 V27A-proton mediated current and inhibit these viruses replication. Compounds **5** [31] and **7** [33] are blockers of the M2 WT, V27A, L26F channels. Although these compounds do not block M2 S31N channel and new derivatives that block M2 S31N have been developed, [34,35] they are still valuable due to the unpredictable mutations of the virus M2 S31 channel in current epidemics.

Table 1. Chemical structures of representative amantadine variants and analogues and their potencies against M2 WT and the amantadine resistant M2 V27A, M2 L26F and M2 S31N mutant channels.

	Compound No/Structure			
	1	**2**	**3**	**4**
				
A/M2 WT IC_{50}	16.1 µM	16.1 µM	6.02 µM	3 µM
A/M2 V27A IC_{50}	>500 µM	>500 µM	n.a.	0.29 µM
A/M2 S31N IC_{50}	200 µM	200 µM	>100 µM	>100 µM
	5	**6**	**7**	**8**
				
A/M2 WT IC_{50}	18.0 µM	2.1 µM	18.7 µM	15.0 µM
A/M2 V27A IC_{50}	0.70 µM	17.2 µM	0.3 µM	n.a.
A/M2 S31N IC_{50}	>100 µM	>100 µM	>100 µM	n.a.
A/M2 L26F IC_{50}	8.6 µM	n.a.	5.6 µM	n.a.

n.a., not active

While several amantadine variants have been tested by different laboratories, various strains of M2 WT virus have been used with different sensitivity against *Amt* (**1**). [24,25,30,31] Subsequently, the unambiguous comparison between potencies is not straightforward. We provided here a data set of 57 amantadine variants with in vitro antiviral potencies (Figure 1) against the WSN/33-M2-N31S virus strain, which has as natural strain the *Amt* (**1**)-resistant influenza virus A/WSN/33 (M2 N31). In the A/WSN/33 M2-N31S strain the M2 key amino acid residues 26, 27, 30, 31 and 34 of A/WSN/33 M2-S31 are identical with the Udorn strain with M2 WT protein. From now on we will use the term M2 WT for the M2 N31S channel of the A/WSN/33 M2-N31S strain. We selected and tested 17 representative amantadine variants against the *Amt* (**1**)-resistant influenza viruses with M2 L26F, M2 V27A, M2 A30T, M2 G34E, i.e. the A/WSN/33 M2-N31S L26F, A/WSN/33 M2-N31S V27A, A/WSN/33 M2-N31S A30T, A/WSN/33 M2-N31S G34E viruses. [17] To render the in vitro inhibitory activities against influenza A comparable we confirmed that the compounds act at the same protein target. Thus, for a subset of compounds we carried out electrophysiology (EP) experiments and showed that only the active compounds block the M2 channel-mediated proton current. Since these compounds may be useful as future antivirals, we also studied the mutations that few representative compounds caused to M2TM WT pore under conditions of drug pressure.

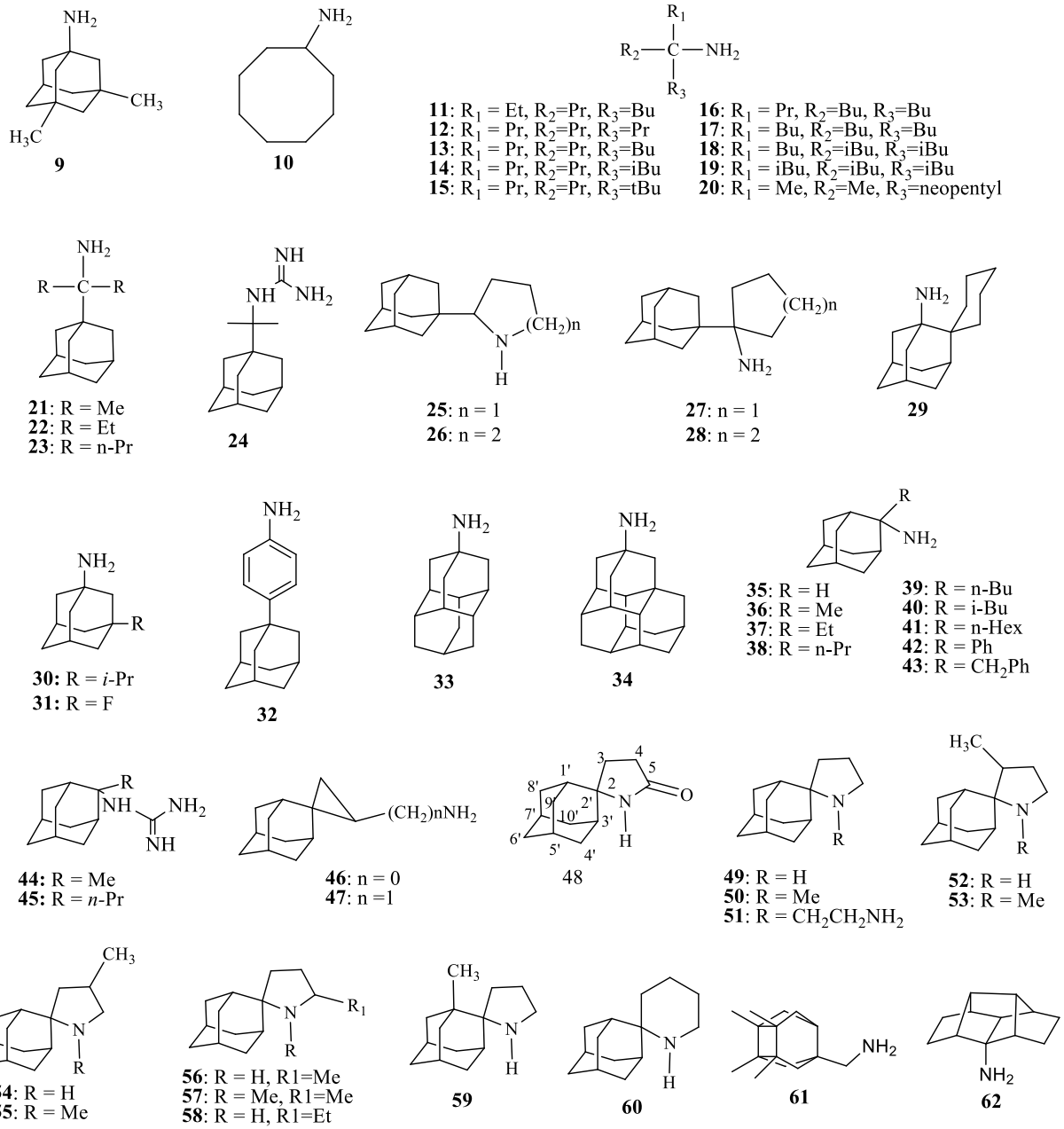


Figure 1. Chemical structures of the 57 amantadine variants tested against influenza A M2 WT and M2 S31N (WSN/33 N31S and WSN/33, respectively).

Amt (**1**) or *Rim* (**2**) are inactive against influenza A/H1N1/Calif/07/2009 (A/Calif/07/09 or H1N1pdm09), A/H1N1/PuertoRico/8/1934 (A/PR/8/34 or APR8), A/H1N1/WS/33 having M2 channel with asparagine at position-31 of M2 and active against A/H3N2/Victoria/3/75 and A2/H2N2/Taiwan/1/64 having M2 channel with serine-31 but also against the A/H1N1/PR/8/1934

strain which has the M2 A30T S31N protein. Strikingly, few amantadine variants inhibited the A/H1N1/PR/8/1934 (A30T/S31N) [37,38] and the pandemic strain A/California/07/2009 (S31N) [28] influenza A strains, without blocking efficiently the M2 S31N channel according to isothermal titration calorimetry, EP, solid state NMR chemical shifts, and molecular dynamics simulations, [39,40] which also showed that M2 N31 M2 mutation abolishes the lipophilic pocket enclosed by V27 side chains causing a propensity for *Amt* (**1**) variants to change orientation pointing ammonium group N-ward in the M2 S31N pore compared to their tight C-ward orientation in the M2 WT pore. [8,9]

To explore this biological activity for a larger set of amantadine variants, we tested many of the compounds against the A/H1N1/California/07/2009, the A/H1N1/PuertoRico/8/1934, and the A/H1N1/WS/33 viruses, all having M2 S31N proteins. Compounds were also tested against the influenza A M2 WT viruses A/H3N2/Victoria/3/75 (A/Victoria/3/75) and A2/H2N2/Taiwan/1/64.

Results and Discussion

Compounds set

Memantine (**9**) and cyclooctanamine (**10**), are *tert*-alkyl amines analogues of amantadine **11-20**. Compounds **21-28** include a linear alkyl or heterocyclic or carbocyclic substitution and are *Rim* (**2**) analogues. Compounds **29**, and **30 or 31** have a spirocyclohexyl at C-2 position and a F or iPr group at C-3 position of adamantane, respectively. Compounds **32**, and **33, 34** include a phenyl as linker between amino group and 1-adamantyl group, and diamantyl, triamantyl, respectively. Compounds **35-60** have an amino group at C-2 position and also include additional carbon atoms in the form of a linear alkyl or saturated 3-, 5-, 6-membered heterocycle. Compounds **35-45** include an alkyl at C-2 adamantane carbon, **46**, **47** contain a spirocyclopropyl, **48-59** contain a spiropyrrolidine while **60** a spiropiperidine ring. Cage amines **61, 62** were tested as polycyclic analogues. Guanidine derivatives **24, 44, 45** were also included after the observation that **4** and **8** are potent anti-influenza A compounds. We re-synthesized many of the amantadine variants

previously reported **8**, **21-23**, **25-29, 32-43**, **46-50**, **52-57**, **59-62** (see Supporting Information). Several new compounds were synthesized and tested, ie. **11-20, 30, 31, 44, 45, 52-55, 51, 56, 58** (Schemes S1-S6).

Biological activity

Antiviral activity against WSN/33 and WSN/33-M2-revertant viruses

Compounds **1**, **2-**(*R*), **2-**(*S*), **2-**(*rac*), **9-62** were tested in CPE inhibitory assays [41] in MDCK cells against two influenza virus strains, having M2 WT and M2 N31 viruses (A/WSN/33 M2 N31S and A/WSN/33 respectively); A/WSN/33 M2 N31S was produced by reverse genetics from A/WSN/33 strain. The IC_{50} values are shown in Table 2. None of the tested compounds showed potency against the M2 N31 (A/WSN/33) strain. Most of the compounds were potent against M2 WT virus, with **21**, **24**, **33** endowed with nanomolar inhibitory potency, i.e. IC_{50} values 0.03, 0.04, 0.07 μM, respectively, very similar to that of *Rim* (**2**) (IC_{50} = 0.02-0.04 μM) and 9-fold more potent than *Amt* (**1**) (IC_{50} = 0.38 μM). Of note, the presence of the amino group is crucial for the activity since lactam **28** have I_{C50} value 100 μM. Compounds **25**, **36-38**, **45-47**, **49** and **60** have IC_{50} values between 0.1-0.3 μM. Compounds **22, 23, 26, 34, 35, 44, 50, 52, 54-56** have activities in the range of IC_{50} values 0.5-1 μM and compounds **57, 32, 52** have IC_{50} values 1-3 μM. Compounds **10**, **20**, **27**, **51**, **58**, **59** have IC_{50} values 4-7 μM, **30**, **31** have IC_{50} values 14.7, 16.2 μM, and **40, 43** have IC_{50} values 22.6, 31.3 μM, respectively. Compounds **41**, **42** are not active. Compared to *Amt*-(**1**), the more elongated molecules **21** and **33** have the highest potency, similar to *Rim* (**2**). [39] The enantiomers of *Rim* (**2**) have equal potency to **2-**(*rac*). The effect of moving the amino group from 1- at 2-position does not affect potency (compared **1** with **35**) but results in higher toxicity in cells (compared **1** with **35, 36**). The installation of an alkyl group from Me to Pr at the 2-position of **35** does not reduce potency (compare **1** or **35** *vs* **36**-**38**, Table 2) However, further increase of the alkyl size is detrimental for potency (see compounds **39**-**43**). In particular, compared to **36** longer alkyls (n-butyl or n-hexyl in compounds **39** or **41**, respectively) or branching (*i*-butyl in compound **40**) reduce potency, eg. from 10-fold and 100-fold for *n*-Bu and *i*-Bu, respectively, while potency

disappears in n-hexyl derivative **41**. While 2-phenyl substitution, as in **42**, leads to an inactive compound, the benzyl substituent, as in **43**, leads to a 46-fold less potent compound, compared to **1** and **35**. The spirocyclopropanamines **46**, **47**, and the spiropirrolidine **49** and the spiropiperidine **60** have potencies similar to that of *Amt* (**1**), in qualitative agreement with previous results with different influenza A strains. [25,36] The effect of the N-methylation of pyrrolidine ring in compounds **50, 53, 55, 57** is the 4-fold reduction in potency compared to **49, 52, 54, 56**, respectively. The guanidine derivatives **24**, **44**, **45** have similar activities compared to the parent amines **21, 36, 37**, in agreement with previous observations. [38]

It is striking that small changes in structure resulted in remarkable changes in potencies or large changes that did not affect the potency. Such cases are presented when the potency is reduced from ca. 4 μM for the *n*-Bu adduct in **39** to 41 μM for the *i*-Bu adduct in **40**; the 30-fold reduction in potency from 0.03 μM in **21** to ca. 1 μM to **22**, **23**; the 120-fold reduction in potency or potency abolishment in rimantadine analogues **27** or **28**. However, pyrrolidine analogue of rimantadine **25** and piperidine analogue **26** have IC_{50} values 0.19 and 0.90 μM, respectively. The most serious effect of C-alkylation to potency was observed in pyrrolidine **59** which reduces potency 10-fold compared to **49** and to memantine (**9**) which is inactive. The second amino group in spiropyrrolidine **51** reduces the potency by 10-fold compared to compound **51**. Noteworthy, compared to *Amt* (**1**) (IC_{50} = 0.33 μM) the increased in girth of the cage alkyl in diamantane **34** results only in 2-fold potency reduction (IC_{50} = 0.72 μM) while the 3-isopropyl-1-amantadine **31** is mediocre in potency (IC_{50} c.a. 17 μM). The effect of a single bridgehead fluorination to *Amt* (**1**) is similar and **30** has a potency ca. 14 μM. The acyclic analogues of amantadine **11-19** were inactive except neo-pentylamine (**20**) with a potency c.a. 5 μM in agreement with previous results from EP studies. [30] Cyclooctylamine has a potency c.a. 7 μM.

Table 2. Antiviral activity and cell cytotoxicity against influenza M2 WT [a,b] virus.

Compound	IC_{50} (μM) [c]	CC_{50} (μM) [c]
1	0.38 ± 0.17	>100
2-(*rac*)	0.04 ± 0.01 [d,f,42]	>100
2-(*R*)	0.04 ± 0.01	>100
2-(*S*)	0.02 ± 0.01	>100
9	not active	101.92±1.64
10	6.85±3.76	>100
11-19	not active	>100
20	5.36 ± 2.01	>100
21	0.03 ± 0.02 [f,39]	>100
22	1.01 ± 0.13 [f,39]	>100
23	1.06 ± 0.23 [f,39]	71.28±11.46
24	0.04 ± 0.01	>100
25	0.19 ± 0.09	>100
26	0.80 ± 0.37	>100
27	4.34 ± 2.94 [f,43]	95.16±4.19
28	not active [f,43]	>100
29	not active	61.64
30	14.12 ± 3.72	> 100
31	16.19 ± 5.36	> 100
32	2.51 ± 1.24	> 100
33	0.07 ± 0.02	> 100
34	0.72 ± 0.33	> 100
35	0.50 ± 0.38	70.50±25.31
36	0.33 ± 0.10 [f,43]	25.63±7.65
37	0.29 ± 0.23 [f,43]	>100
38	0.34 ± 0.10 [f,43]	>100
39	3.83 ± 1.71	>100
40	31.33 ± 11.88 [d]	>100
41	>31.6	31.64±23.27
42	>100	>100
43	22.59 ± 8.58	>100
44	0.69 ± 0.26	>100
45	0.29± 0.14	>100
46	0.16 ± 0.09	>100
47	0.12 ± 0.06	>100
48	~100	>100
49	0.34 ± 0.08 [f,43]	>100
50	0.90 ± 0.29 [f,43]	>100
51	4.33 ± 2.26	>100
52	0.68 ± 0.32	>100
53	2.98 ± 1.46	>100

54	0.85± 0.41	>100
55	0.68 ± 0.32	>100
56	0.96 ± 0.64	>100
57	1.92 ± 1.07	>100
58	7.01 ± 1.84	>100
59	6.12 ± 2.59	>100
60	0.34 ± 0.19	>100
61	1.75	>100
62	not active	>100

[a] Influenza M2 WT correspond to WSN/33 M2 N31S virus; [b]all compounds were inactive against M2 N31 virus, i.e. the WSN/33 strain. [c] mean and standard deviations of the 50% inhibitory concentration (IC_{50}) and the 50% cytotoxic concentration (CC_{50}) of at least three independent assays determined in Madin-Darby canine kidney cells; [d] compound 40 was considered as less potent inside the series; [e] n.t., not tested, n.a., not active; the lowest IC_{50} value is grey shaded and written with bold text and the highest measured IC_{50} value is grey shaded and written with plain text; [f] data published in indicated reference.

We selected a few compounds with representative antiviral activity against the M2 WT virus, for testing their potency against other *Amt* (**1**) resistant viruses, ie., those carrying the M2 L26F, V27A, A30T and G34E (Table 3). This compounds set include the mid-nanomolar aminoadamantane derivatives **21**, **24**, **33**, the compounds **22**, **23**, **34**-**37**, **44**, **45**, **49**, **61** with IC_{50} values in the range 0.3 µM to 2 µM, the compounds **20** and **32** with IC_{50} values 5.36 and 2.51 µM, respectively, the compounds **30** and **31** with IC_{50} values of c.a. 14.12 and 16.19 µM, respectively, and the not active, acyclic, primary *tert*-alkyl amine **18** as well as 29, **62**. We observed that compounds **21**, **24** and **33** also have low micromolar potencies against the L26F strain and, additionally, diamantane **33** also inhibits V27A, with IC_{50} of 15.5 µM. Compounds **23**, **37** and **45** also showed low micromolar potency against the L26F mutant, in the range 0.5-2 µM and **22** and **49** have IC_{50} values 5.95 and 8.75 µM, respectively. Neopentylamine **18**, and the aminoadamantane derivatives **20**, **30**-**32** and **34** are generally not active or have weak potency and **35**, **36** and **44** have IC_{50} values in the range of 16-23 µM). Except of the weak activity of compound 32 against M2 A30T virus none of the compounds is active against the resistant to *Amt* (**1**) strains, M2 A30T and G34E.

Table 3. Antiviral activity against influenza A M2 L26F, [a] V27A, [b] A30T, [c] G34E [d] viruses. [a]

Compound	IC_{50} (μM) [e]			
	M2 L26F	M2 V27A	M2 A30T	M2 G34E
18	n.a [f]	n.a	n.a	n.a
20	n.a	n.a	n.a	n.a
21	0.79 ± 0.37	n.a	n.a	n.a
22	5.95 ± 3.35	n.a	n.a	n.a
23	0.55 ± 0.23	n.a	n.a	n.a
24	1.01 ± 0.50	n.a	n.a	n.a
29	n.a	n.a	n.a	n.a
30	69.45 ± 28.73	n.a.	n.a	n.a.
31	n.a	n.a	n.a	n.a
32	45.30 ± 10.76	n.a.	40.64±18.78	n.a
33	0.74 ±0.26	15.46 ± 6.97	n.a	n.a
34	n.a.	n.a.	n.a.	n.a.
35	20.97 ± 5.00	n.a	n.a	n.a
36	16.10 ± 8.72	n.a	n.a	n.a
37	1.99 ± 0.74	n.a	n.a	n.a
44	23.13 ± 6.34	n.a	n.a	n.a
45	1.79 ±1.05	n.a	n.a	n.a
49	8.75 ± 4.72	n.a	n.a	n.a
61	3.84 ± 1.99	n.a	n.a	n.a
62	n.a	n.a	n.a	n.a

[a] Influenza M2 L26F correspond to A/WSN/33 M2 N31S L26F; [b] influenza A M2 V27A correspond to A/WSN/33 M2 N31S V27A; [c] influenza A A30T correspond to A/WSN/33 M2 N31S A30T; [d] influenza A M2 G34E correspond to A/WSN/33 M2 N31S G34E; [e] mean and standard deviations of the 50% inhibitory concentration (IC_{50}) of at least three independent assays determined in Madin-Darby canine kidney cells; [f] n.a., not active.

Functional inhibition of M2 channels by selected amantadine variants

We verified that M2 channel is the protein target for inhibition of influenza A viruses by measuring the blocking effect of few compounds against full length M2 using EP (Table 4, see also Supporting Information [36]). We measured the blocking effect against full length M2 protein with a two-electrode voltage clamp (TEVC) assay at 2 min. We tested compounds **21**, **24**, and **33**, that have high in vitro antiviral potencies against M2 WT and M2 L26F as well as **29, 61** and **62**. EP testing confirmed that the compounds **21**, **24**, **61** and likely **33** are acting by blocking M2 WT channel; compound **33** seems to

be a slow blocker as we showed previously for compound **23**. [39] Compounds **21**, **24**, **29**, and **61** blocked efficiently M2 L26F channel. While **21**, **24** and **61** blocked both M2 WT and M2 L26F channels, compound **29** is selective only for M2 L26F. Noteworthy, compound **61** is a triple inhibitor of the M2 WT, L26F and V27A mutant channels.

The observation that results showed that several of the compounds in Figure 1, such as **18, 25, 27, 37-43, 49-55, 58, 58** were endowed with antiviral potency against the *Amt* (**1**) or *Rim* (**2**) resistant M2 S31N A/H1N1/Calif/07/2009 virus [28] can be attributed to an alternative antiviral mechanism. Their activity may be due to increasing the pH in endosomes. This lysomotropic effect of amantadine variants has been suggested as the mechanism [44] of severe acute respiratory syndrome-coronavirus-2 (SARS-Cov-2) by amantadine decrease the viral load in positive patients suggesting that it can be prescribed as a prophylactic that prevents symptomatology caused by SARS-CoV-2 coronavirus. [45]

Table 4. Inhibitory effect of selected compounds on M2 WT, L26F, V27A, S31N proton channel function. [a]

	M2 WT	M2 L26F	M2 V27A	M2 A30T	M2 S31N	M2 G34E
21	80.3±0	93.3±0.7	0	n.t. [b]	0	n.t.
24	79.4±0.9	91.7±0.6	16.5±0	n.t.	0	n.t.
29	0	65.5 ± 0.5	1.6 ± 1.6	n.t.	0	n.t.
33	30.8±2.1	12.2±1.1	6.5±0.0	n.t.	6.9±0	n.t.
61	84.2 ± 0.5	79.2 ± 1.6	91.0 ± 1.0	n.t.	2.0 ± 1.0	n.t.
62	4.8 ± 2.8	2.9 ± 0.1	0	n.t.	0	n.t.

[a] Inhibition by 100 μM of compound for 2 min (% ± SE). Three replicates were used for measurements; n.t., not tested.

M2 mutants selection by drug resistance to M2 WT

Resistance of M2 WT to *Amt* (**1**) develops rapidly *in vitro,* [5,46,47] in mice, [48] and in the clinical setting [49] through a small set of mutations, primarily L26F, V27A, V27T, A30T, S31N, and G34E. These are residues whose side chains are near the 4-fold symmetric amantadine binding site. We examined in more detail the resistance development

pathways of H3N2 virus with M2 protein WT to a few compounds (**21**, **27**, **36**, **38**). We carried out passaging experiments with plaque sequencing analysis, that is, amino-acid translation of the M-segment in the presence of the compounds **21**, **27**, **36**, **38** (Table 5).

Table 5. Mutations developing in influenza WT M2 WT [a] after passaging amantadine variants **21**, **27**, **36** or **38**.

H3N2 [a]	**Compound** *[b]*	**21**	**27**	**36**	**38**
P *[c]*	**Plaque** *[f]*	**1 µg/ml** *[d]*	**1 µg/ml**	**1 µg/ml**	**5 µg/ml**
2	**1**	V27A [e]	V27A	WT	A30T
	2	WT	V27A	WT	A30T
	3	-	V27A	WT	A30T
5	**1**	A30T*	V27A	A30T	A30T
	2	V27A	V27A	A30V	A30T
	3	V27A	V27A	A30T	A30T
		10 µg/ml	-	**2 µg/ml**	**5 µg/ml**
10	**1**	V27A	-	A30T	A30T
	2	V27A	-	A30T	A30T
	3	V27A	-	A30T	A30T

[a] Parent strain is A/Hong Kong/1/1968 (H3N2 M2 WT); *[b]* compound number from Figure 1; [c] passage number; [d] MDCK cells were bathed in media containing the concentrations specified; [e] sequences of resistant progeny of WT induced by compounds in the top row; *[f]* Three separate plaques were sampled and sequenced at passages 2, 5, and 10.

The development of viral resistance falls into three clusters. The first cluster include compound **38**, to which the WT virus rapidly develops resistance through mutation at A30, particularly to threonine, suggesting that this drug block the M2 WT, but do not block M2 A30T. The second cluster include compounds that do not block V27A, ie. **21**, **27**, **36**. These resistant strains suggest that different strains have different drug sensitivities. It may, therefore, be possible to design a cocktail of compounds to prevent resistance development for influenza A M2-blockers. These compounds cause the mutation A30T since A30 is in contact with the adduct or the mutation V27A since adamantyl cage is in contact with V27. [8,9] Some of these derivatives or cocktails may be valuable based on the possibility of the virus to mutate from S31N back to S31.

Antiviral activity against other amantadine sensitive-M2 WT and amantadine resistant-M2 S31N viruses

To investigate further the observation that amantadine variants, such as **38**, **49**, have antiviral potency against A/H1N1/Calif/07/2009 [45] or A/H1N1/PuertoRico/8/1934 [38,50] without blocking the M2 S31N channel, we tested compounds against the M2 S31N viruses A/H1N1/Calif/07/2009, A/H1N1/PuertoRico/8/1934, A/H1N1/WS/33 (Table 6). Also few compounds were tested against the M2 WT viruses A/H3N2/Victoria/3/75 and A2/H2N2/Taiwan/1/64. *Rim* (**2**) is inactive against A/H1N1/Calif/07/2009, A/H1N1/PR/8/1934, A/H1N1/WS/33 having M2 channel with asparagine-31 but active against A/H3N2/Victoria/3/75 and A2/H2N2/Taiwan/1/64 having M2 channel with serine-31 but also against A/H1N1/PR/8/1934 with M2 A30T S31N. [28,38]

We found several low micromolar inhibitors (**18, 25, 27, 37-43, 49-55, 58, 58**) against A/H1N1/Calif/07/2009 with **27** having a submicromolar activity. Also we indentified several low micromolar against A/H1N1/PuertoRico/8/1934, which is M2 A30T/S31N virus, (**2, 15, 28, 35-40, 42. 43, 51, 53, 59**) with **15**, **28, 38, 39, 40, 42, 51, 53, 59** having a submicromolar activity. We observed also micromolar activities for compounds **36, 37, 40** against A/H1N1/WS/33. Compounds **2, 17, 18, 28, 35-40, 42, 43, 51, 53, 54, 56, 57** have low micromolar activity against A/H3N2/Victoria/3/75 with M2 WT channel while all compounds tested, ie. **2, 28**, **35-40, 42, 43, 51, 59** have low micromolar potency against M2 WT A2/H2N2/Taiwan/1/64. Thus, several compounds blocked replication of M2 S31N viruses without blocking M2 S31N-mediated proton current for the 2-min drug treating of the M2 channel (Table 4).

Table 6. Antiviral activity and cell cytotoxicity against influenza A M2 WT and S31N viruses.

Compound	IC_{50} (μM) [a]				
	A/Calif/07/2009 (H1N1) M2 S31N	A/Victoria/3/75 (H3N2) M2 WT	A2/Taiwan/1/64 (H2N2) M2 WT	A/PR/8/34 (H1N1) M2 A30T/S31N	A/WS/33 (H1N1) M2 N31
2	106 ± 41	0.53 ± 0.07	1.64 ± 0.29	3.26 ± 0.5	314 ± 135
9	70.8 ± 10.5	72.24 ± 10.58	n.t. [b]	n.t.	n.t.
10	363 ± 3.76	10.16 ± 2.58	n.t.	n.t.	n.t.
11	61.2 ± 6.6	39 ± 4.5	n.t.	n.t.	n.t.
12	41.9 ± 11.8	91 ± 1.5	n.t.	n.t.	n.t.
14	241 ± 121	105.4 ± 35	n.t.	n.t.	n.t.
15	43.2 ± 1.11	33.9 ± 7.32	n.t.	0.79 ± 0.80	37.4 ± 11.8
16	293 ± 273	21.71 ± 2.19	n.t.	n.t.	n.t.
17	340 ± 231	11.89 ± 2.31	n.t.	n.t.	n.t.
18	11.7 ± 1	3.76 ± 0.37	n.t.	n.t.	n.t.
19	398 ± 176	43.6 ± 6.07	n.t.	n.t.	n.t.
25	15.4 ± 2.4	n.t.	n.t.	n.t.	n.t.
26	7.03 ± 1.20	n.t.	n.t.	n.t.	n.t.
27	0.79 ± 0.14	n.t.	n.t.	n.t.	n.t.
28	3.62 ± 0.49	2.31 ± 0.37	4.22 ± 0.98	0.3 ± 0.5	53.7 ± 11.5
35	151 ± 33 [28]	3.32 ± 0.89	0.81 ± 0.27	3.76 ± 1.02	112 ± 14.8
36	53.9 ± 12.6 [28]	2.02 ± 0.43	0.54 ± 0.54	0.44 ± 0.44	18.7 ± 4.49
37	19.0 ± 3 [28]	2.02 ± 0.43	0.81 ± 0.27	1.81 ± 0.94	22.5 ± 2.89
38	4.71 ± 0.92 [28]	23.4 ± 7.6	0.54 ± 0.54	0.5 ± 0.2	37.6 ± 8.4
39	7.42 ± 0.91 [28]	4.02 ± 1.19	1.46 ± 0.25	0.3 ± 0.5	34.6 ± 4.1
40	5.75 ± 0.37 [28]	13.26 ± 2.02	0.36 ± 0.12	0.3 ± 0.5	20.5 ± 3.7
42	17.4 ± 1.41 [28]	5.18 ± 1.05	0.24 ± 0.24	0.3 ± 0.5	86.0 ± 19.6
43	9.72 ± 1.38 [28]	17 ± 3.1	0.24 ± 0.32	1.2 ± 1.1	26.9 ± 14.5
46	399 ± 297	n.t.	n.t.	n.t.	n.t.
47	30.8 ± 49	n.t.	n.t.	n.t.	n.t.
49	8.1 ± 2.1	n.t.	n.t.	n.t.	n.t.
50	7.92 ± 1.51	n.t.	n.t.	n.t.	n.t.
51	2.66 ± 0.33	0.56 ± 0.21	0.48 ± 0.24	0.3 ± 0.5	34.2 ± 8.4
52	7.2 ± 2	55.3 ± 0.5	n.t.	n.t.	9.60 ± 1.40
53	10.2 ± 1.2	5.67 ± 1.80	n.t.	0.79 ± 0.80	-
54	9.5 ± 1.6	19.2 ± 3.5	n.t.	n.t.	n.t.
55	7.0 ± 0.8	58.3 ± 11.5	n.t.	n.t.	n.t.
56	34.2 ± 4.4	3.2 ± 2	n.t.	n.t.	n.t.
57	7.7 ± 2	16.2 ± 7.5	n.t.	n.t.	n.t.
58	8.7 ± 1.7	38.5 ± 1	n.t.	n.t.	n.t.
59	34.1 ± 1.2	34.1 ± 7.9	1.21 ± 0.25	0.7 ± 0.5	14.9 ± 1.6
60	19.8 ± 17.1	n.t.	n.t.	n.t.	n.t.

[a] EC_{50} ± its standard error from mini-plaque testing for dose-response or single-dose screens, using cultured MDCK cells, based on least-squares fitting of single-site binding curves. No microscopic evidence of cytotoxicity to MDCK cells was detected after 18 hour exposure at 50 μM except with compound **41**, where a 5 μM dose was used instead; [b] n.t.; not tested.

Development of resistance to amantadine-sensitive A/Victoria/3/75 (H3N2, M2 WT) and amantadine-resistant A/Calif/07/2009 (H1N1, M2 S31N) to amantadine variants

Resistance testing with semi-weekly passages in MDCK cell cultures was performed for amantadine **1** against an amantadine-sensitive H3N2 virus; and for compound **38** and a cocktail of **26**, **27**, and **60** against amantadine-resistant H1N1 (2009) (Table 7).

The cocktail components were selected to represent a diverse, random set of ring adducts at both C1 and C2. In the *Amt* (**1**) - H3N2 system, drug resistance appeared after one passage in the presence of drug, with no detectable activity of *Amt* (**1**) against the progeny from passage 1 or passage 2 at 50 µM, but normal *Amt* (**1**) activity against the original virus *post hoc* (EC_{50} 3.0 ± 0.5 µM N=9). In contrast, in the **38**-H1N1 system, virus progeny produced in the presence of drug at passages 1-5 maintained full drug sensitivity (EC_{50} 2.1-5.4 µM). Resistance to **38** developed between passage 6 and passage 12, becoming significant after passage 10. Likewise, the cocktail of **26**, **27**, and **60** remained effective through 6 passages, but the H1N1 virus developed resistance by passage 10.

Table 7. Resistance testing for amantadine **1** against an amantadine-sensitive H3N2 virus and potent amantadine variants against amantadine-resistant H1N1 (2009) (see Table 6).

Passage #	**1** (5 µM) A/Victoria/3/75 (H3N2, M2 WT) EC_{50} ± S.E. (µM)	**38 (**5 µM) A/Calif/07/2009 (H1N1, M2 S31N) EC_{50} ± S.E. (µM)	**26, 27, 60 (**5 µM) A/Calif/07/2009 (H1N1, M2 S31N) EC_{50} ± S.E. (µM)
0	2.77 ± 0.29	4.71 ± 0.92	0.99x ± 0.15
1	Inactive	5.4 ± 1.4	-
2	Inactive	3.7 ± 0.5	-
5	N.D.	2.1 ± 1.6	-
6	N.D.	-	1.20x ± 0.07
8	N.D.	18.5 ± 1.0	-
10	N.D.	76 ± 9	7.9x ± 0.8
12	N.D.	149 ± 115	n.t.
Drug **29**		10.2 ±1.7	

n.t.; not tested.

The passage-12 **38**-resistant mutant was subsequently tested and found to be sensitive to **28** (ie. the EC_{50} modestly reduced at 10.2 μM compared to 3.6 μM against H1N1 (2009)). Without any drug in the medium, the development of viral resistance to compound **38** was negligible, ie. the EC_{50} retested at passage 0 was 4.7 ± 0.7 μM, at passage 10: 3.0 ± 0.3, and at passage 30: 7.7 ± 0.6 μM.

The compound that was very successful against H1N1 (2009), **38**, induced resistance in the WT within 2 passages. As was published before, no changes from the parent A/California/07/2009 were observed for the amino-acid translation of the M-segment of the passage-12 **38**-resistant strain for residues sequenced, 10-73. Hence, resistance did not develop by selection of additional amantadine-resistance mutations in M2. The lack of sequence changes for M2(S31N)-bearing virus in the presence of compound **38** mentioned above indicates that M2(S31N)-bearing virus has a different escape route than M2(WT)-bearing virus. In the latter case changes inside the M2 pore confer resistance while in the former no mutations were observed in the M2 channel amantadine binding-site and therefore some other change in the virus is implicated.

Mechanism of action of amantadine variants against amantadine-resistant S31N viruses investigated

Pre-exposure of virus to drug before cells infection

We also performed experiments with A/Calif/07/09 inhibition to show that this virus inhibition does not take place at late stages of virus endocytosis M2 protein function compared to other viruses. We showed that **38** is capable of blocking virus infection when drug exposure occurs prior to virus access to cells, presumably virus blocking occurs on the viral envelope-associated M2 protein.

EP experiments for 30-minute exposure of M2 S31N with (and without) compound **38**

To provide additional evidence that amantadine variants did not block M2 S31N at latter stage of infection we performed a 30-minute exposure EP experiment with and without compound **38** (Figure 2). The results revealed no blocking of M2 S31N by 38 during the 30 min exposure.

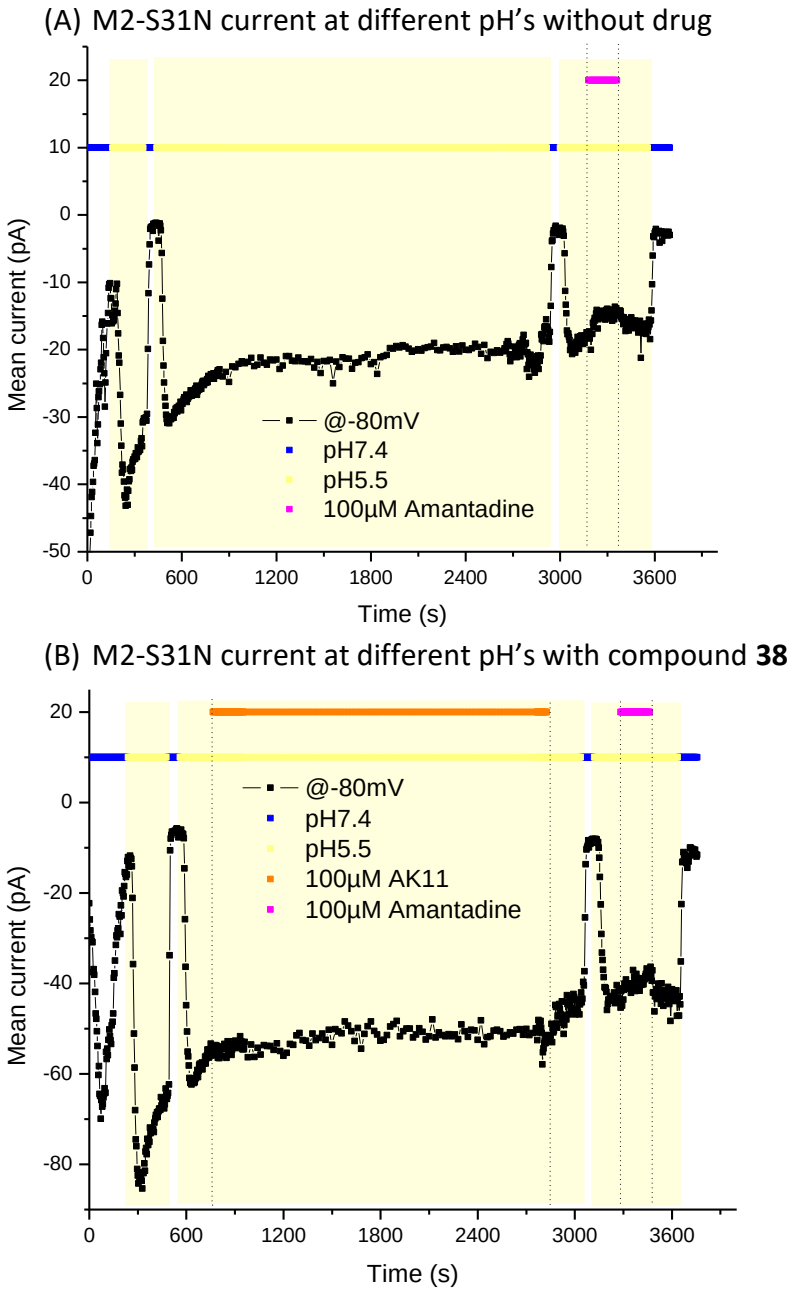


Figure 2. Panels (A), (B) show the 30-minute exposure of M2 S31N without or with **38**, respectively.

Since the sequences of WSN/33/H1N1 and A/California/07/2009/H1N1 differ just on residue 43 of M2 (43T and 43L respectively, see Table S1) [50] another target seems to interfere with amantadine variants blocking A/California/07/2009/H1N1 and other S31N viruses (see Tables 6 S2).

Binding of haemagglutinin to the virus

In recent papers, we suggested that haemagglutinin (HA) can be involved for the antiviral activity of amantadine variants to viruses (including some M2 S31N viruses) and find mutations of HA in aminoacids that may influence either HA binding to cell receptors or possibly the efficiency of the conformational changes that may take place at lower pH. [28,37,38,51]

We performed additional experiments to attempt identifying the stage at which virus propagation is blocked. HA-inhibition assays were used to investigate whether amantadine variants inhibited receptor binding (see Supporting Information). A haemolysis assay was used to see whether the compounds inhibited the acid induced fusion activity of the virus in a similar manner to the antivirals Arbidol (Umifenovir) [52] or tertiary butylhydroquinone (TBHQ). [53] These drugs inhibit between the viral envelope and the cell membrane of the target cell by preventing contact between the virus and target host cells which prevents viral entry to the target cell, and therefore protects it from infection. With APR8 virus and compound **38**, which had been shown to be very efficient at inhibiting propagation of this virus (Table 6), there was no detectable inhibition of haemolytic activity compared with control. Therefore, the block in viral propagation seems that it does not occur at the virus-endosomal membrane fusion stage.

Endosome neutralizers

To explore further the possibility of a mechanism of antiviral activity of amantadine variants against some M2 S31N viruses [28,37,38,51] with implication of HA, we thought that amantadine variants may increase the endosomal pH and thus indirectly inhibit the HA conformational change which is required for fusion. We selected virus mutants resistant to the amantadine variants, and

they had an increased fusion pH and mutations in HA. Also, when we compared a few H1N1 strains (including H1N1pdm09), their sensitivity to the amantadine variants correlated with their fusion pH. These results suggested that HA can represent a sensitivity factor. [11,37,51] However, the intriguing observation that X-31 (A/Aichi/2/1968) and APR8 had the same fusion pH (5.0; which is low compared to other strains), and yet X-31 is totally insensitive to amantadine variants, while APR8 has very robust sensitivity, seems to be contradictory. Perhaps H1 HA is more sensitive to this pH effect compared to H3 HA. [11,37,51]

In this work we also showed that Bovine papillomavirus (BPV) infection of Bovine embryonic kidney cells is not inhibited by compounds **38** (which is submicromolar inhibitor of A/PR/8/34 having M2 S31N/A30T) and **27** (which is submicromolar inhibitor of A/Calif/07/09 having M2 S31N) in the 1-20 μM range (see Supporting Information) even though it is inhibited by modest concentrations of endosome neutralizers (lysosomotropic efect), chlorpromazine, ammonium, chloroquine, and bafilamycin A1. This argues that **38** and **27** are not good endosome neutralizers lacking a chloroquine effect. [54] Additionally it has been found that chloroquine effect alone (endosomal or perhaps viral alkalinization) works in cell culture but not in humans. [55]

Neurotoxicity study

Compounds tested here might be useful for re-purposing of amantadine class for compounds not only because of antigenic shift of the virus to M2 WT but also because the compounds tested here and cocktails inhibit a broad panel of viruses. There was no evidence of cytotoxicity (inhibition of cell growth or cell morphological changes) for any of the compounds at dosage ≤50 μM. Toxicity of compounds **38** and **49** were assessed in CD-1 mice and compared to amantadine **1** (Table 8).

Intraperitoneal doses of all three compounds were non-lethal at 30 mg/kg. Compound **38** was lethal to all 3 mice tested at 100 mg/kg and **1** and **49** were lethal at 300 mg/kg. Some signs of neural effects were seen with **49** at 30 mg/kg, but not with **1** and **38**.

Table 8. Toxicity from I.P. injections of CD-1 mice.

Dose (mg/kg)	Amantadine **1**	Compound **18**	Compound **31**
30	0 (2:2) [a]	0 (1:2)	0 (2:1) [b]
100	0 (2:1) [c]	3 (2:1)	2 (3:1) [d]
300	3 (2:1)	N.D.	3 (2:1)

[a]Number of deaths within 48 hours (number of males injected: number of females injected). With compound 8 at 100 mg/kg, one male and one female died.[b] Slightly abnormal gate. Some quivers. Hyper-responsive to touch or noise. [c] Moderate trembling or quivers. [d] Ataxic gait. Moderately abnormal gait. Slightly or somewhat impaired mobility. Mild tremors. Motor incoordination.

Discussion

Here, we provided a determined study for 57 synthetic amantadine variants using anti-viral assay against influenza A virus with M2 WT and all amantadine resistant M2 mutant strains. We reported activities against WSN/33-M2 N31S covering a range of over three digits (0.03 to 31.33 μM). Changes in structure of adamantane, which are often subtle, result in remarkable changes in blocking efficiency for M2 channels and potency against the corresponding influenza A M2 strains. Several compounds from Kolocouris lab, eg **21, 24**, block both M2 WT and M2 L26F-mediated proton current and these viruses replication, while **62** is a triple blocker of M2 WT, M2 L26F and M2 V27A channels and these viruses replication, and **29** only blocks M2 L26F channel and virus replication. We evaluated that their mechanism of inhibition is M2 blocking by EP and identified consistent point mutations for representative compounds against M2 WT viruses. Compounds **29** and **62** developed from Vazquez may provide useful probe molecules for these M2 channels and corresponding viruses inhibition.

The difference in the anti-viral potency of the amantadine variants against A/WSN/33 virus and A/Calif/09 or A/PR/8/34 viruses with asparagine at position 31 of M2 (and possibly M2 WT viruses) that was shown and the lack of M2 S31N blocking activity is not understood. [11,38,28] Our previous results showed also that aminoadamantane-aryl group conjugates are active even against the WSN/33, [56] without blocking the M2 S31N channel nor bind HA and their mechanism of action needs further investigation. These findings suggested additional mechanisms of anti-viral activity,

plausibly a lysosomotropic effect for amantadine variants with lipophilic adducts inhibiting virus at concentrations usually higher than ca. 5 μM. [11,38,28,56] These compounds are lipophilic and can be accumulated and buffer late-stage endosomes to prevent acid-induced fusion of the virus envelope with the endosome membrane. [57] Such lysosomotropic agents, like chloroquine, are effective against many types of viruses *in vitro*, possibly including also the SARS-CoV-2. [12]

However, we showed that the mechanism of antiviral activity against A/Calif/09 or A/PR/8/34 and possibly also M2 WT viruses compared to WSN/33 viruses is not due to inhibition of an early stage of virus infection or a late stage of M2 channel function during endocytosis or inhibition of HA binding to host cells or a different pH for HA fusion or a lysosomotropic effect. If these additional mechanisms will be rationalized, this knowledge may be used to develop antivirals to block other viruses including the SARS-CoV-2. It should be emphasized that we did not explore if the drugs can inhibit another stage of entry of the virus into a new host cell, where M1 protein must be dissociated from the RNPs, allowing them to enter the nucleus. This event allows the import of incoming RNPs into the nucleus. [58] Thus, intraviral buffering inhibiting M1-RNP disruption could be an adamantane mechanism-of-action.

We provided a neurotoxicity study in mice for selected compounds showing that variants other than amantadine showed similar toxicity profile. The compounds and their cocktails while not to be more toxic than amantadine might be useful for re-purposing of amantadine class of drugs because: (a) they inhibited a broad panel of M2 WT (and can be useful in the case of an antigenic shift of the virus to M2 WT) and M2 S31N viruses including the H1N1pdm09, and (b) they can inhibit M2 L26F or even M2 V27A strains that in the future can be prevalent virus strains.

Conclusion

The results for amantadine variants against influenza A presented here provide a reference study. We reported activities for 57 compounds against WSN/33-M2 N31S covering a range of over three digits (0.03 to 31.33 μM). Several compounds block in TEVC experiments both M2 WT and M2 L26F-mediated proton current and the corresponding viruses replication. We identified one triple blocker of M2 WT, M2 L26F and M2 V27A channels and viruses replication a compound that only blocks M2 L26F channel and virus replication. With WT viruses, which more readily developed resistance to these compounds, the pattern of resistant M2 sequence changes suggests that mutants have specific drug sensitivities, which could lead the way to the development of impenetrable cocktails. We paid an effort to explore features that may be responsible for the fact that some amantadine variant inhibited A/Calif/09 or A/PR/8/34 without blocking M2 S31N channel-mediated proton current compared to WSN/33 viruses. We found that this is not due to inhibition of an early stage of virus infection or a late stage of M2 channel function during endocytosis or inhibition of HA binding to host cells or a different pH for HA fusion or a lysosomotropic effect. However, these compounds and their cocktails while not to be more toxic than amantadine might be useful in the case a re-purposing of amantadine class of drugs will be needed.

Supporting Information

Synthetic chemistry methods and experimental methods for chemical synthesis and experimental methods for biological evaluation and neurotoxicity data, sequences alignments of M2 proteins from different viruses and SI references (six Schemes and two Tables).

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AK designed this research project which includes the graduate project thesis of MS. MS, IS, CT, CL in AK group synthesized compounds; MS and CT contributed equally. ALT in SV group re-synthesized compounds **29, 61, 62**. EP experiments were performed by CM in JW lab (see Table 4) and by DF group (see Figure 2). Antiviral testing against WSN viruses was performed by AH, PS, KD in MS group and against other viruses by BJ. RZ performed the point mutations experiments. IA, FS performed the neurotoxicity experiments. SW did the HA binding experiments. AK wrote the manuscript and MS, ALT, SV revised it.

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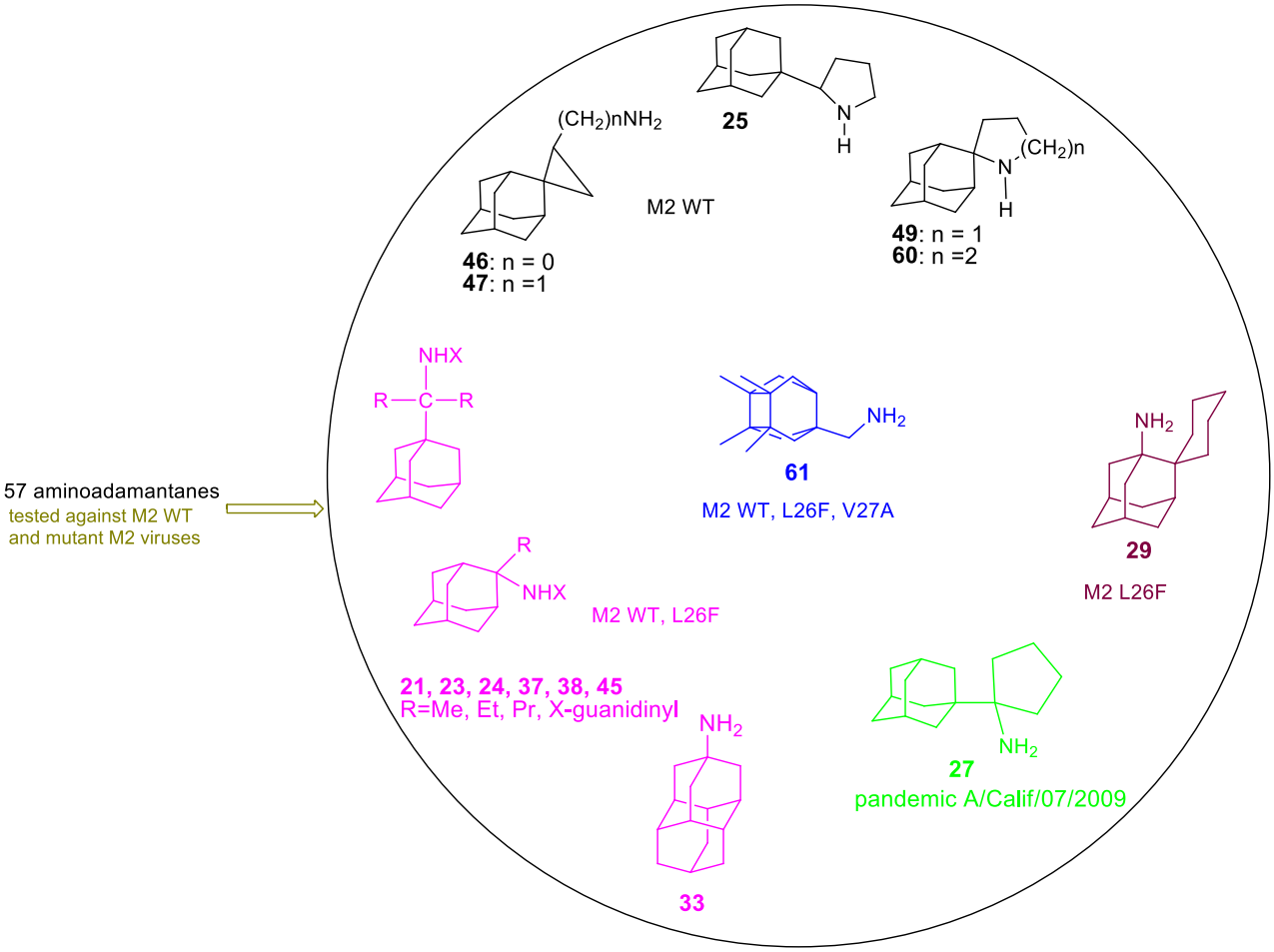
TOC Graphic


57 aminoadamantanes
tested against M2 WT
and mutant M2 viruses
(CH2)nNH2
46: n = 0
47: n =1
25
M2 WT
(CH2)n
49: n = 1
60: n =2
NHX
R—C—R
21, 23, 24, 37, 38, 45
R=Me, Et, Pr, X-guanidinyl
NH2
61
M2 WT, L26F, V27A
R
NHX
M2 WT, L26F
NH2
29
M2 L26F
NH2
33
NH2
27
pandemic A/Calif/07/2009