Amantadine Variant - Aryl Conjugates that Inhibit Multiple Amantadine Resistant M2 Mutant Influenza A Viruses

Christina Tzitzoglaki,[†x] Anja Hoffmann,[§x] Andreea L. Turcu,[≠y] Christos Liolios, [†] Patrick Schmerer,[§] Chunlong Ma,[‡] Jun Wang, [‡] Santiago Vázquez,[≠] Michaela Schmidtke,[§, *] Antonios Kolocouris[†,*]

[†] Laboratory of Medicinal Chemistry, Section of Pharmaceutical Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimioupolis-Zografou, Athens 15771, Greece

[§] Jena University Hospital, Department of Medical Microbiology, Section Experimental Virology, CMB Building, R. 443, Hans Knoell Str. 2, D-07745 Jena, Germany

[≠] Laboratori de Química Farmacèutica (Unitat Associada al CSIC), Facultat de Farmàcia i Ciències de l'Alimentació, and Institute of Biomedicine (IBUB), Universitat de Barcelona, Av. Joan XXIII, 27-31, Barcelona, E-08028, Spain

[‡] Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, Arizona 85721, United States

Keywords: amantadine - aryl conjugate, antiviral activity, CPE, in vitro, ion channel blocker, electrophysiology, influenza A M2 protein, mutation A30T, G34E, L26F, S31N, V27A, synthesis, TEVC, WSN/33,

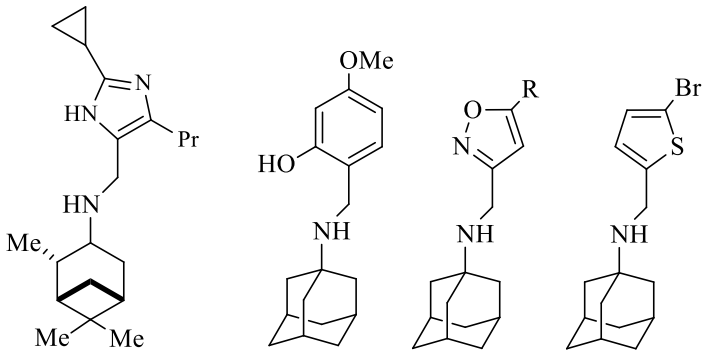
15.11.2021

Abstract

One challenge facing anti-influenza drug development is the heterogeneity of the circulating influenza A viruses, which comprise several strains with variable susceptibility to antiviral drugs. Viruses bearing the S31N mutant of the M2, such as the pandemic 2009 H1N1 and seasonal H3N2, as well as other mutants (L26F, V27A, A30T, G34E) are resistant to amantadine class of drugs. Here, we synthesized and tested many of the second generation amantadine - aryl conjugates, against the WT M2 and all the M2 amantadine resistant strains, i.e. L26F, V27A, S31N, A30T, G34E generated from WSN/33 (S31N) virus. We identified many compounds that are dual *in vitro* M2 WT and L26F virus inhibitors. Furthermore, few of them (**21**, **32**, **33**), having a rimantadine or diamantadine or 4-(1-adamantyl)aniline instead of amantadine in the conjugate, were *in vitro* inhibitors against M2 WT, L26F and S31N while one of them inhibited also the A30T virus. The electrophysiology (EP) experiments showed that these compounds blocked significantly M2 WT, L26F or even M2 V27A channels but not the M2 S31N. The observation that adamantane variants and derivatives inhibit multiple M2 mutant virus replication in cell culture, without blocking M2 channel-mediated proton current in EP is not uncommon, underlying a mechanism of antiviral activity that has not been identified.

Influenza vaccines remain the cornerstone in prophylaxis of influenza infection. [1] Due to antigenic shift and antigenic drift of influenza viruses, influenza vaccines should be reformulated each year; plus, there is generally a six-month delay in vaccine production. Additionally, the vaccines are much less active than small molecule antivirals in treating influenza-infected patients, [2] and these factors make an urgent task the development of new antivirals.

Amantadine and rimantadine were used against M2 wild-type (WT) viruses bearing an M2 with serine-31. Nevertheless, mutations in the transmembrane domain of the M2 protein were associated with resistance to M2 WT. The homotetrameric structure of the M2 channel places constraints on the types of drug-resistant mutations that can be accommodated. [3] Amino acid substitution L26F, V27A, A30T, G34E and S31N were shown to confer cross-resistance to amantadine and rimantadine demonstrating their impact for inhibitor binding to influenza A virus (IAV). [4,5,6,7,8] The vast majority, 95% of resistant viruses, bear the S31N substitution in M2, 1% have V27A and L26F, and the rarest are A30T and G34E. [9,10] The M2 S31N mutant is a natural mutation and one of the most conserved viral proteins among currently circulating influenza A virus that maintain nearly identical channel function as the M2 WT but is resistant to amantadine. On the other hand, the substitution V27A often emerged under drug selection pressure. [5,6] The other mutations confer amantadine resistance but this is not a result of the amantadine drug selection pressure. [5] Thus, the presence of L26F, V27A, and particularly S31N in IAV circulating worldwide pushed the search for novel ion channel blockers with stronger, preferably resistance-overcoming activity.



1 **2** **3:** R = Me **6**
4: R = c-Pr
5: R = Ph

Figure 1. Few amantadine or cage amine-aryl head conjugates which block both M2 WT, S31N channels and inhibit the M2 WT and S31N viruses.

Hu et al. discovered that when pinanamine, which is active against M2 WT, [11,12,13] is linked with 4-imidazole or *p*-hydroxyphenyl to the amine group, through a methylene bridge, as in **1** (Figure 1), this compound blocks M2 WT and S31N channels. [12,13] DeGrado and Wang performed extensive structure-activity relationship (SAR) investigations, [14–20] Wang or our lab [21] also kinetic studies, [22,23] of molecules including an amphiphilic or lipophilic aryl derivative linked with amantadine or 3-hydroxy-amantadine through a methylene bridge, eg. compounds **2-6** in Figure 1, using electrophysiological (EP) testing, anti-viral assaying, NMR spectroscopy and molecular dynamics (MD) simulations. DeGrado and Wang identified conjugates, eg. compounds **2**, **6** (Figure 1), acting as dual blockers of M2 WT and S31N-mediated proton currents or only against M2 S31N, eg. compound **5**. Furthermore, in previous work, we showed that the addition of the small CMe_2, as well as the longer linkers in compounds **21**, **33, 34** (Figure 2), abolishes channel blockage against M2 S31N, but not against M2 WT, i.e. the selectivity is tighter in M2 S31N due to the incomplete blocking that cause low k_{on} rates. [21] Using MD simulations and binding free energy calculations combined with EP results, we also showed [21] that conjugates **21**, **33, 34**, with

the elongated linker can also fit between the V27 to G34 region of M2TM S31N, however, this linker caused less tight binding and incomplete block.

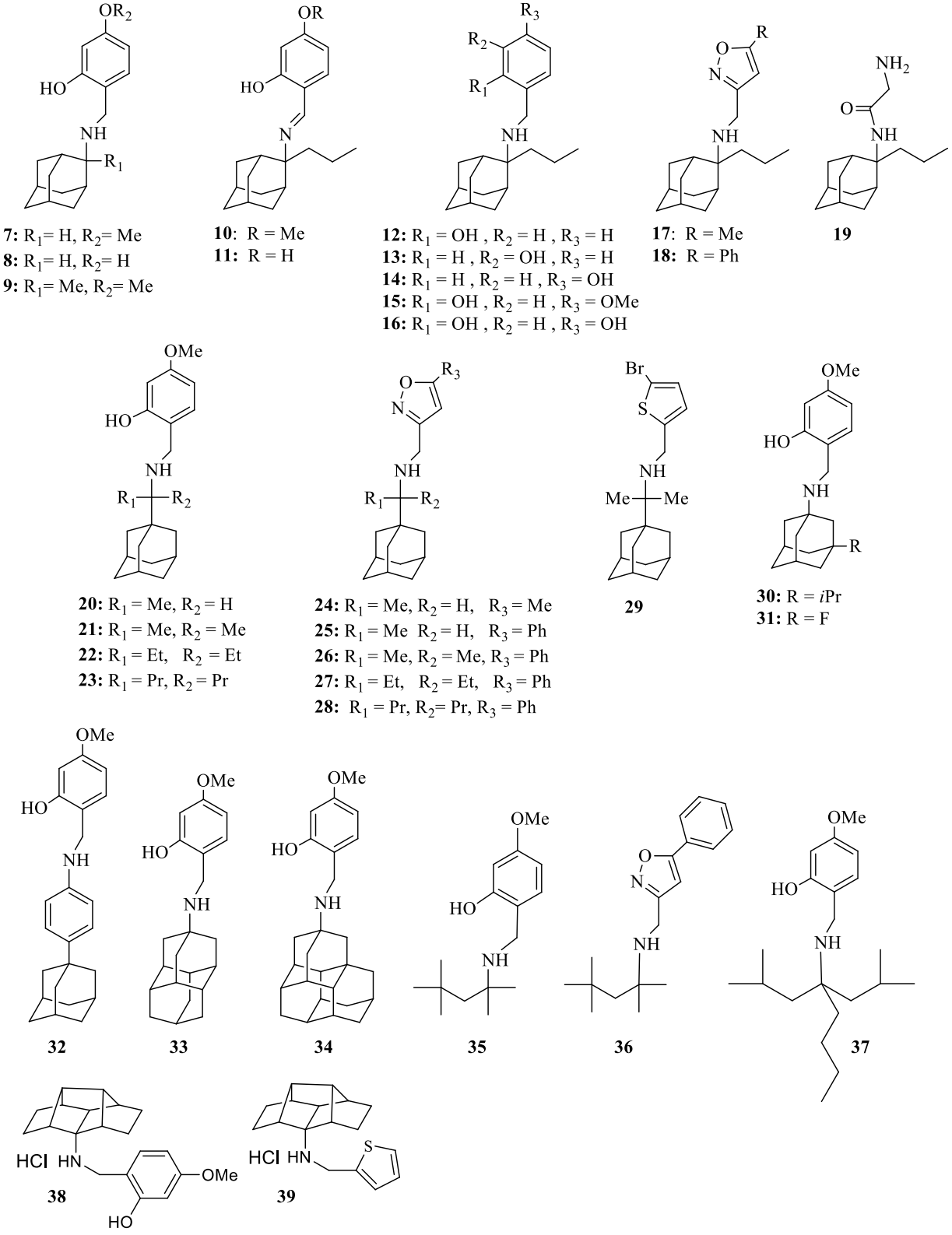


Figure 2. Chemical structures of synthetic aminoadamantane-polar head conjugates **7-39.**

As a continuation of these efforts, we explored additional SAR with variation of the adamantyl blocking group. We installed the hydroxylphenyl or substituted isoxazolyl or thiophenyl aryl groups, that were proved as successful when combined with amantadine (compounds **2**, **5, 6**, Figure 1) for blocking M2 WT and M2 S31N channel proton current and viruses carrying them, [14–16] to amantadine variants or other cage amines through a methylene bridge resulted in the aryl head conjugates **7-39** (Figure 2). In these conjugates the lipophilic amine is, instead of amantadine, as in **2**, **5**, and **6**, (a) a 2-alkyl-2-aminoadamantane, in compounds **7-19**; (b) a 3-substituted, with *i*-propyl or fluorine group, amantadine in compounds **30**, **31** (c) a rimantadine analogue with CR_2 instead of CHMe or an aminoadamantane with a longer linker between adamantyl and the amino group in compounds **20-29**, **32**; (d) the diamantylamine or triamantylamine, in compounds **33** or **34,** respectively; (e) primary *tert*-alkyl amine analogues, in compounds **35-37**; (f) an amine with a cage alkyl other than adamantyl in compounds **38** and **39.**

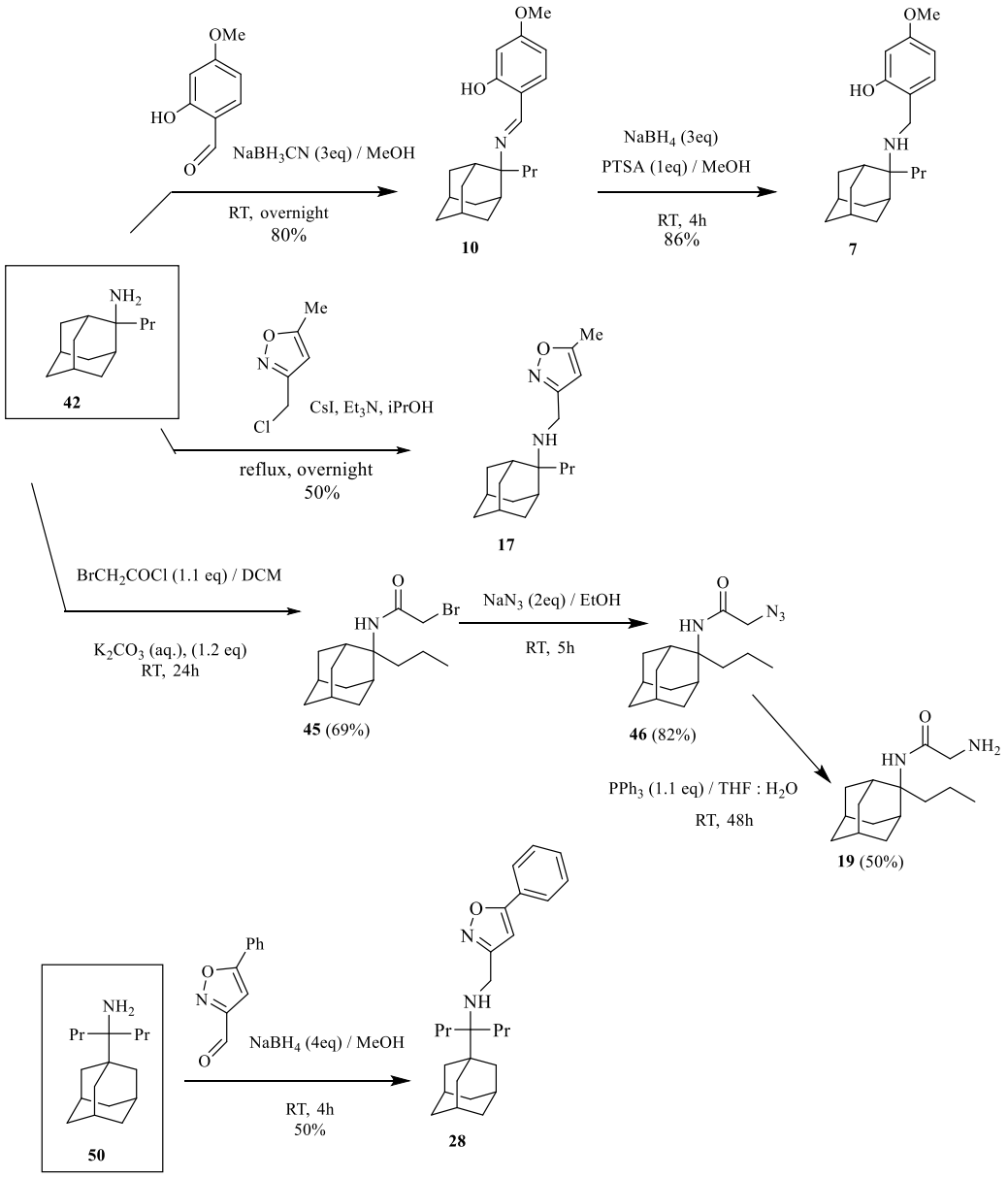
Furthermore, compounds **2**, **5**, **6** or other potent amantadine-aryl conjugtes against M2 S31N were never tested against the mutant M2 L26F, V27A channels and the corresponding viruses. These viruses carrying mutant M2 channels although nowdays less prevalent that M2 S31N viruses can provide escape muntants that may cause epidemics or pandemics. Thus, compounds **1**, **2**, **5**, **7-39** were tested against the amantadine resistant viruses L26F, V27A, A30T, G34E and S31N aiming at finding resistant breaking inhibitors.

Synthesis of lipophilic amine - aryl conjugates **2-39**

In order to install the aryl moiety on the amino group of lipophilic cages we followed a reductive alkylation reaction. First, the reaction of the primary amine with 2-hydroxy-4-methoxybenzaldehyde using $NaCNBH_3$ in methanol for 15 min, as described earlier, [15] afforded in our hands the corresponding imine (see Method A in the Supporting Information for a general description). Elongation of the reduction for few hours led to cleavage of the adduct affording the starting amine from the reaction mixture, see for example compounds **10** and **11**. These imines were also formed by refluxing the starting amine with the suitable aldehydes in benzene using a Dean-Stark adaptor. We found that a mixture of isolated imine with PTSA and $NaBH_4$ in methanol afforded the derired amine in good yields (Schemes 1, 1S). The yield was increased in presence of a Lewis acid, possibly acting by increasing reactivity of the carbonyl group. Using this protocol we prepared the *N*-(2-hydroxy-4-methoxybenzyl)methyl derivatives **7**, **9**, **15**, **20-23**, **30-39** (see also Schemes S1-S3). We also re-synthesize compounds **1**, **2** and **5** for comparison purposes.

Second, we applied also a reductive amination with $NaBH_4$ in methanol and $Ti(OiPr)_4$ as Lewis acid (see Method B in the Supporting Information for a general description) as previously applied [14] (Scheme S2). This procedure afforded *N*-(hydroxyphenyl)methyl derivatives **12-14** or the isoxazolylmethyl derivatives **18**, **25-29**. Additionally, we reacted some amines with CsI (Method C) and 3-chloromethyl-5-methylisoxazole in isopropanol to afford compounds **17** and **24** (Schemes 1).

The demetylation of the methoxy group from the 2-hydroxy-4-methoxy-benzylmoiety in compounds **7**, **10** and **15**, through treatment with excess of BBr3 afforeded derivatives **8**, **11** and **16**, respectively (Scheme S3). Finally, from the amine **42** we prepared the *N*-bromoacetyl derivative **45**, followed by the synthesis of the *N*-azidoacetyl derivative (**46**) that under Staundinger reaction condition the afforded compound **19** (Scheme S1).



Scheme 1. Synthesis of compound **19** and example of the application of reductive alkylation procedure for the preparation of compounds **7**, **17** or **28** from amines **42** or **50**, respectively.

Biological testing

CPE assay

We used the cytopathic effect (CPE) inhibition assay [24,25] to compare the antiviral activity of the compounds in Table 2 against WSN/33 (M2 consisting N31) virus variants generated by reverse genetics. In particular, we tested the inhibitory activity against the M2 sensitive to amantadine virus (WSN/33-M2-N31S) or the amantadine resistance-conferring mutant M2 L26F, V27A, A30T, S31N, G34E channels, corresponding to viruses WSN/33-M2-L26F/N31S, WSN/33-M2-V27A/N31S, WSN/33-M2-A30T/N31S, WSN/33, WSN/33-M2-G34E/N31S, respectively, in MDCK cells. The activity of the amantadine-base compounds **2**, **5** against M2 WT and M2 S31N viruses previously evaluated was confirmed; [14,15] compound **2** is active against both M2 WT and S31N viruses [15] while **5** is very potent only against M2 S31N virus. [14] The 2-alkyl-2-adamantanamine derivatives **8**, **10**, **14-16**, the rimantadine

derivatives **20-29**, the 4-(1-adamantyl)aniline and diamantylamine derivatives **32** and **33**, respectively, have at least low micromolar potency against the M2 WT virus with several compounds being submicromolar inhibitors.

When the 2-alkyl-2-adamantyl adduct in **7**, **9** and **15** is compared with 1-adamantyl in **2**, the increased in size adamantyl adduct is consistent with boosting L26F activity (as previously discussed in EP experimental results) leading to dual M2 WT, M2 L26F inhibitory activity. The 2-propyl-2-adamantanamine derivatives **14-16** having low micromolar potency against M2 WT and M2 L26F viruses. Similarly, the rimantadine-CR_2 derivatives **21-23** and **28** with both small and larger R groups at showed low micromolar potency against M2 WT and M2 L26F viruses. [14,16] When the aryl groups tested are compared in the compounds linked with the rimantadine-CR_2 analogues showed potency against only M2 WT and M2 L26F viruses.

Beyond **14-16**, **21-23** and **28** which are dual inhibitors of M2 WT and M2 L26F viruses, compound **21**, which is an efficient blocker of both M2 WT and L26F-channel mediated proton current (Table 1), yet it inhibited efficienly *in vitro* M2 WT, M2 S31N, M2 L26F, G34E viruses. Compared to **21**, compound **20** differs only by one methyl but it is less potent. Compound **32** only blocked efficiently M2 WT channel, yet it inhibits *in vitro* M2 WT, M2 S31N, M2 L26F, G34E viruses. Additionally, compound **33** blocked M2 WT and, slightly, L26F and V27A, but inhibits *in vitro* M2 WT, M2 S31N, M2 L26F viruses. Compounds **12**, **13**, **17**, **18**, **35**, **36**, **38**, **39** are not active at all. Compounds **31** and **37** have poor activity against M2 WT virus. As shown in Table 1, several compounds showed cytotoxicity at concetrations smaller that 100 μM.

Overall, compounds **21**, **32** and **33** stand out as the best anti-influenza agent, with IC_{50} ranging from submicromolar to medium micromolar for WT and for viruses carrying different mutant M2 channels (**21, 33** inhibit M2 WT, L26F, S31N and **32** also A30T).

Table 1. *In vitro* potency (IC_{50}, μM) of aminoadamantane – aryl conjugates **1, 2, 5, 7-39** tested against initial cell infection for WSN/33 viruses corresponding to M2 WT and M2 mutant sequences which are resistant to amantadine. [a]

	CC_{50} (μM)	**IC_{50} ± SD (μM)** [b]					
Cmp		M2 WT	M2 S31N	M2 L26F	M2 V27A	M2 A30T	M2 G34E
1	112.1	**6.9 ± 0.7** [c]	**10.8 ± 2** [c]	n.t. [e]	n.t. [e]	n.t. [e]	n.t. [e]
2 [15]	49.86 ± 12.28	**1.13 ± 0.03**	**8.70 ± 4.38**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
5 [14]	67.80 ± 0.62	91.35 ± 42.80	**1.18 ± 0.17**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
7	68.10 ± 3.65	**12.47 ± 6.70**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
8	>100	**1.08 ± 0.33**	36.98 ± 8.52	48.86 ± 13.71	n.a. [d]	n.a. [d]	37.75 ± 12.57
9	>100	**4.88 ± 2.76**	37.73 ± 13.38	30.25 ± 24.51	63.51 ± 30.59	50.15 ± 12.80	49.18 ± 9.05
10	69.40 ± 12.02	**1.72 ± 0.78**	n.a. [d]	**23.03 ± 10.29**	n.a. [d]	n.a. [d]	n.a. [d]
11	>100	7.50 ± 1.64	n.a. [d]	28.73 ± 7.58	n.a. [d]	n.a. [d]	n.a. [d]
12	>100	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.t. [e]
13	>100	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
14	30.22 ± 3.05	**1.17 ± 0.08**	n.a. [d]	**3.67 ± 1.73**	n.a. [d]	n.a. [d]	n.a. [d]
15	>100	**1.06 ± 0.59**	n.a. [d]	**4.20 ± 0.60**	n.a. [d]	n.a. [d]	**17.89 ± 6.56**
16	>100	**0.66 ± 0.37**	n.a. [d]	**9.29 ± 2.74**	n.a. [d]	56.58 ± 24.98	n.a. [d]
17	>100	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.t [e]	n.t. [e]
18	35.36 ± 3.23	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.t [e]	n.t. [e]
19	>100	**5.98 ± 0.49**	n.a. [d]	30.82 ± 12.17	n.a. [d]	n.a. [d]	n.a. [d]
20	>100	**0.27 ± 0.08**	41.74 ± 1.76	**17.55 ± 9.26**	78.16 ± 15.42	34.90 ± 1.32	50.65 ± 2.08

21 [21]	>100	**0.13 ± 0.02**	**19.86 ± 6.92**	**1.80 ± 0.29**	n.a. [d]	43.98 ± 8.50	**19.22 ± 9.36**
22	>100	**0.19 ± 0.08**	n.a. [d]	**1.44 ± 0.44**	n.a. [d]	n.a. [d]	n.a. [d]
23	27.34 ± 3.37	**0.37 ± 0.10**	n.a. [d]	**0.60 ± 0.23**	n.a. [d]	n.a. [d]	n.a. [d]
24	>100	**1.00 ± 0.65**	86.37 ± 8.47	91.30 ± 7.25	n.a. [d]	n.a. [d]	n.a. [d]
25	21.70 ± 8.61	**1.14 ± 0.46**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
26	67.60 ± 1.45	**0.19 ± 0.09**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
27	>100	**0.89 ± 0.64**	56.97 ± 31.59	**14.27 ± 3.04**	n.a. [d]	n.a. [d]	n.a. [d]
28	>100	**0.69 ± 0.13**	70.15 ± 7.86	**1.52 ± 0.49**	n.a. [d]	n.a. [d]	n.a. [d]
29	85.99 ± 3.60	**0.29 ± 0.17**	n.a. [d]	**9.06 ± 2.53**	n.a. [d]	n.a. [d]	n.a. [d]
30	57.24 ± 6.07	**13.21 ± 5.97**	n.a. [d]	n.a. [d]	n.a. [d]	19.10 ± 7.17	**23.44 ± 4.14**
31	>100	63.43 ±17.64	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
32 [21]	>100	**0.66 ± 0.21**	**17.43 ± 11.94**	**21.57 ± 10.65**	55.02 ± 21.57	**22.53 ± 13.06**	47.99 ± 14.34
33 [21]	>100	**0.29 ± 0.11**	**21.24 ± 8.92**	**17.31 ± 12.09**	47.47 ± 15.20	38.40 ± 6.22	55.79 ± 11.58
34	27.14 ± 9.43	**8.10 ± 2.72**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
35	>100	26.35 ±10.42	n.a. [d]	40.33 ± 17.18	n.a. [d]	38.02 ± 3.68	37.01 ± 7.23
36	>100	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
37	65.30 ± 5.80	58.94 ± 10.98	60.36 ± 2.76	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
38	75.36 ± 15.53	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
39 [26]	>100	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
Oseltamivir	>100	**0.03 ± 0.02**	**0.01 ± 0.00**	**0.04 ± 0.02**	**0.03 ± 0.01**	**0.01 ± 0.00**	**0.04 ± 0.03**

[a] definition of the viruses used: M2 WT correspond to A/WSN/33 M2 N31S virus; M2 31 correspond to WSN/33 virus; M2 L26F correspond to A/WSN/33 M2 L26F,N31S virus; M2 V27A correspond to A/WSN/33 M2 V27A,N31S virus; M2 A30T corresponds to A/WSN/33 M2 A30T, S31N virus; M2 G34E corresponds to /WSN/33 M2 G34E, S31N virus; [b] measured in triplicate; [c] determined using A/HK/68 virus, see ref. [12]; [d] n.a., not active; [e] n.t. not tested; in bold are shown interesting potencies, eg. IC_{50}'s < 25 μM.

Finally, we also tested few selected compounds against the Calif/07 and Jena/8178 strains, both carrying the S31N mutant M2 channel (Table 2). The results showed that compounds **20**, **21**, **24**, **32** and **33** inhibited significantly these viruses. We also tested 4-(1-adamantyl)aniline (**62**) (Scheme S1) which had some activity against Calif/07.

Table 2. *In vitro* efficacy (IC_{50}, μM) of Scheme 1 compounds against influenza virus A(H1N1)pdm09 viruses. [a]

Compound	**IC_{50} (μM)**	
	Jena/8178	**Calif/07**
7	74.33 ±0.00	54.59 ±0.00
8	40.96 ±8.41	62.64 ±26.04
20	**18.19 ±6.79**	**13.23 ±2.95**
21	**24.23 ±7.63**	**13.99 ±3.76**
24	**13.23 ±3.98**	**8.16 ±5.39**
27	58.77 ±21.70	53.83 ±38.83
32	**12.96 ±5.22**	**15.71 ±5.06**
33	**18.43 ±7.25**	**21.88 ±5.88**
37	n.a. [b]	73.58 ±6.85
oseltamivir	**0.14 ±0.13**	**0.29 ±0.27**
amantadine	n.a. [b]	n.a. [b]
4-(1-adamantyl)aniline, 62	37.82 ±1.54	19.88 ±7.35

[a] measured in triplicate; [b] n.a. = non active

Electrophysiology

We explored if the M2 channel mediated proton current is the target for inhibition of infuenza A viruses by testing, representatively, the compounds **21**, **32** and **33** having promising multiple strain antiviral activity. We also include compound **2** which was active against M2 WT and M2 S31N viruses.

We measured if these compounds blocked full length M2 WT, M2 S31N, M2 L26F, M2 V27A, M2 A30T, M2 G34E channel-proton mediated current using EP (Table 3). The blocking effect against full length-M2 protein was determined with a two-electrode voltage clamp (TEVC) assay at 2 min.

Table 3. %-Block of full-length Udorn after 2 min wash-in M2 current by selected compounds[a,b].

Compound	WT	L26F	V27A	A30T	S31N	G34E
2	**64 ± 1**	0	0	0	**59 ± 1**	0
21	**80.0 ± 1.7**	**89.9 ± 0.3**	20.5 ± 1.3	1.3 ± 1.3	4.8 ± 0.3	3.2 ± 3.2
32	**81.0 ± 2.1**	24.0 ± 4.3	21.2 ± 4.4	0	10.9 ± 5.0	0
33	**81.0 ± 1.6**	**37.0 ± 4.6**	**48.8 ± 1.2**	0	6.8 ± 0	11.2 ± 0.4

[a] For each compound, percent block of pH-dependent M2 current at listed concentrations (+/- s.e.m.). [b] Three replicates were used for measurements at 100 µM.

Compound **2** blocked efficiently M2 WT and M2 S31N channels. [14,16,21] Compound **21** was an efficient blocker of both M2 WT (80.0%) and L26F (89.9%) and weakly the M2 V27A channel (20.5%). Compound **32** only blocked efficiently the M2 WT (81%) and weakly the M2 L26F(24%) and V27A (21%) channels. Compound **33** was a triple M2 channel blocker; it blocked M2 WT (81%) and moderately the M2 L26F (37%), M2 V27A (48.8%) chanels. [27–29] These compounds can block M2 L26F or M2 V27A due to the extra alkyl adduct in rimantadine analogue **50** (Scheme 1) or 4-(1-adamantyl)aniline (**62**, Scheme S1), which are the amantadine analogues in conjugates **21** and **32**, or the girth and height of diamantamine **64** in compound **33** that can fit the extra space that L26F or V27A generate in the mutant M2 channels, as we previously showed by X-ray crystallography of M2(22-46) WT and V27A in complex with a spiro-adamantyl amine. [30,31] We also showed previously with MD simulations that a destabilization occurs when an amantadine-aryl conjugate is inside the pore of the M2 G34E or A30T channel since the adamantyl group faces the four polar hydroxyl groups of T30 or the four likely charged glutamate groups of E34 pointing inside the pore. [32]

It has been shown previously that amantadine-aryl conjugates or amantadine analogues with bulky alkyl adducts are slow M2 blockers. [23,21,27] Although their %-blockage against M2 channel at 2 min can be low, eg. 33% for compound **10g** against M2 S31N in ref. [23] or 21% for compound **52** in Scheme 1 against M2 WT (which is compound **5** in ref. [27]), respectively, compounds **10g** in ref. [23] and **52** [27] were active *in vitro* influenza A inhibitors, acting through M2 proton-mediated current blocking (other examples are also available in ref. [28]). In such cases the reported percentage channel blockage at the 2 min time point actually underestimated the true potency of these compounds. To validate this hypothesis in a previous work, [27] we observed that for the active *in vitro* rimantadine analogue **52** (with bulky alkyl adduct at methyle carbon bridge) the %-blocking effect was 27% at 2 min but 61% at 10 min. [27]

Compared to the EP experiments results, compounds **21**, **32, 33** inhibited the replication of M2 WT, S31N, L26F and blocked in EP the M2 WT, L26F channels but did not block the M2 S31N channel. This is also observed for **32** which inhibited the A30T *in vitro* but did not block the M2 A30T chanel. The observation that adamantane variants and amantadine analogue-aryl conjugates or pinanamine-aryl

conjugates inhibited multiple M2 mutant virus replication in cell culture, without blocking M2 channel-mediated proton current in EP in not uncommon, underlying an additional mechanism of antiviral activity that we and others also previously observed and suggested to be hemagglutinin fusion step of virus entrance. [13,21,26,33,34] A striking observation is that **33** showed a weak inhibition of M2 V27A virus *in vitro* while in EP blocked the M2 V27A channel.

The limitations of influenza vaccines together with the heterogeneous makeup of influenza viruses call for broadspectrum small molecule antivirals. We tested here several aminoadamantane - aryl conjugates aiming to find compounds with resistance-overcoming activity. This work adds to the SAR of this kind of compounds. The *in vitro* antiviral assays combined with EP experiments against M2 WT and mutant channels showed compounds **21, 33** and **32** inhibit a multiple M2 mutant influenza A viruses.

Experimental Methods

Chemistry

Representative synthetic procedures for few compounds are listed below. The details for the synthesis of the remaining compounds can be found in the Supporting Information.

Compound 7 (Procedure A). Reaction of 2-aminoadamantane 40 (88 mg, 0.583 mmol) and 2-hydroxy-4-methoxybenzaldehyde (74 mg, 0.486mmol) in MeOH (2 mL) followed by addition of $NaCNBH_3$ (110 mg, 1.75 mmol) to afford imine **43**; yield 88 mg (56 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 1.61 (d, J = 8 Hz, 2H, 4eq, 9eq-adamantyl H), 1.78 (br s, 4H, 1,3,8eq,10eq-adamantyl H), 1.89-1.95 (m, 8H, 5,6,7,8ax,10ax,4ax,9ax-adamantyl H), 3.50 (s, 2H, 2-adamantyl H) 3.80 (s, 3H, OCH_3), 6.30 (dd, J = 8, 2.4 Hz, 1H, phenyl H), 6.36 (d, J = 2 Hz, 1H, phenyl H), 7.05 (d, J = 8 Hz, 1H, phenyl H), 8.14 (s, 1H, CH=N).

Reaction of imine **43** (88 mg, 0.327 mmol) with PTSA (56 mg, 0.327 mmol) and $NaBH_4$ (50 mg, 1.31 mmol) in MeOH (2 mL) afforded amine **7**; yield 80 mg (85 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 1.57 (d, J = 8 Hz, 2H, 4eq,9eq-adamantyl H), 1.74 (br s, 4H, 1,3,8eq,10eq-adamantyl H), 1.86 (br s, 4H, 5,7,6-adamantyl H), 1.95 (m, 4H, 8ax,10ax,4ax,9ax-adamantyl H), 2.82 (s, 2H, 2 adamantyl H) 3.76 (s, 3H, OCH_3), 3.92 (s, 2H, CH_2N), 5.29 (br s, OH), 6.31 (dd, J = 8, 2.4 Hz, 1H, phenyl H), 6.43 (d, J = 2 Hz, 1H, phenyl H), 6.89 (d, J = 8 Hz, 1H, phenyl H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 27.5 (5-adamantyl C), 27.7 (7-adamantyl C), 31.6 (4,9-adamantyl C), 31.7 (8,10 adamantane-C), 37.6 (1,3-adamantane-C), 37.8 (6-adamantyl C), 49.6 (CH_2N), 55.4 (OCH_3) 61.2 (2-adamantyl C), 102.1 (3-phenyl CH), 104.5 (5-phenyl CH), 115.6 (1-phenyl C), 128.7 (6-phenyl CH), 159.7 (2-phenyl COH), 160.5 (4-phenyl $\underline{C}OCH_3$); HRMS (m/z): [M + H^+] calcd for $C_{18}H_{25}NO_2$ 287.1885, experimental 287.1890.

Compound 17 (Procedure C). To the mixture of 2-propyl-2-aminoadamantane **42** (110 mg, 0.569 mmol) and 3-chloromethyl-5-methylisoxazole (50 mg, 0.379 mmol) in isopropanol (2 mL) was added CsI (15 mg, 0.0569 mmol) and Et_3N (115 mg, 1.14 mmol) to afford amine **17;** yield 35 mg (32%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.93 (t, J = 7 Hz, 3H, $CH_2CH_2C\underline{H}_3$), 1.20-1.26 (m, 2H, $CH_2C\underline{H}_2CH_3$), 1.46 (d, J = 12 Hz, 2H, 4eq,9eq-adamantyl H), 1.61 (d, J = 12 Hz, 2H, 8eq,10eq- adamantyl H), 1.65-1.72 (m, 6H, $C\underline{H}_2CH_2CH_3$, 6,1,3-adamantyl H), 1.80 (br s, 2H, 5,7-adamantyl H), 1.93 (d, J = 12 Hz, 2H, 8ax,10ax-adamantyl H), 2.20 (d, J = 12 Hz, 2H, 4ax,9ax-adamantyl H), 2.4 (s, 3H, CH_3), 3.6 (s, 2H, CH_2N), 6.0 (s, 1H, isoxazyl CH=); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 12.3 ($CH_2CH_2\underline{C}H_3$), 14.9 (CH_3), 15.0 ($CH_2\underline{C}H_2CH_3$), 27.8 (5,7-adamantyl C), 32.6 (4,9-adamantyl C), 33.5 (8,10-adamantyl C), 34.0 (1,3-adamantyl C), 36.0 ($\underline{C}H_2CH_2CH_3$) 39.0 (6-adamantyl C), 57.9 (2-adamantyl C), 101.5 (isoxazolyl CH=),165 (isoxazolyl C=N), 170 (isoxazolyl CO); HRMS (m/z): [M + H^+] calcd for $C_{18}H_{28}N_2O$ 288.2202, experimental 288.2212.

Compound 28 (Procedure B). To the mixture of 4-(1-adamantyl)-4-heptananamine **50** (20 mg, 0.080 mmol) and 5-phenylisoxazole-3-carboxaldehyde (14 mg, 0.080 mmol) in $Ti(iPrO)_4$ (0.3 mL, 1.20 mmol) was added $NaBH_4$ (12 mg, 0.320 mmol) and MeOH (2 mL) to afford amine **28;** yield 20 mg (50%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.94 (m, 6H, 2x$C\underline{H}_3$), 1.34-1.56 (m, 8H, 2x$C\underline{H}_2C\underline{H}_2CH_3$), 1.62-1.75 (m, 12H, 2,4,6,8,9,10-adamantyl H), 1.98 (br s, 3H, 3,5,7-adamantyl H), 3.97 (s, 2H, $C\underline{H}_2NH$), 6.59 (s, 1H, isoxazolyl CH=C), 7.43-7.46 (m, 3H, phenyl), 7.74-7.78 (m, 2H, phenyl); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 14.3 (2xCH_3), 18.7 (2x$CH_2\underline{C}H_2CH_3$), 29.3 (3,5,7-adamantyl C), 35.9 (2x$\underline{C}H_2CH_2CH_3$), 36.0 (4,6,10-adamantyl C), 36.9 (2,8,9-adamantyl C), 55.9 (CH_2NH), 69.1 (CNH), 97.9 (isoxazolyl CH=), 125.9 (CH, C_6H_5), 129.0 (CH, C_6H_5), 130.4 (CH, C_6H_5), 150.0 (isoxazolyl C=N), 169.4 (isoxazolyl CO); HRMS (*m*/*z*): [M + H^+] calcd for $C_{27}H_{38}N_2O$ 406.2984, experimental 406.2965.

Compound 19. To a vigorously stirred mixture of 2-propyl-2-aminoadamantane **42** (100 mg, 0.517 mmol) in dichloromethane (8 mL) and K_2CO_3 (82 mg, 0.595 mmol) in H_2O (5 mL), was added, dropwise, a solution of bromoacetyl chloride (90 mg, 0.569mmol) in dichloromethane (5 mL). The reaction mixture was stirred at rt for 24 h. The resulting mixture was extracted with dichloromethane and the organic phase was washed with $NaHCO_3$ 10% (1x15 ml), HCl 3% (1x15mL), water and brine and evaporated under vacuum to afford the bromacetamide **45**; yield 110 mg (69 %) ; ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 14.7 ($CH_2CH_2\underline{C}H_3$), 16.1 ($CH_2\underline{C}H_2CH_3$), 27.2 (5,7-adamantyl C), 30.2 ($\underline{C}H_2CH_2CH_3$), 33.0 (4,9-adamantyl C), 33.2 (1,3-adamantyl C), 33.3 (8,10-adamantyl C), 34.7 (6-adamantyl C), 38.6 (CH_2Br), 61.5 (2-adamantyl C), 163.6 (C=O).

A mixture of the bromacetamide **45** (105 mg, 0.334 mmol) and NaN_3 (43 mg, 0.668 mmol) in ethanol (3 mL) was stirred for 5 h at rt. After evaporation of ethanol, water (10 mL) was added and the aqueous phase was extracted twice with an equal volume of diethyl ether. The combined organic phase was washed with water and brine, dried (Na_2SO_4) and evaporated to afford the oily azide **46**; yield 75 mg (82 %); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 14.6 ($CH_2CH_2\underline{C}H_3$), 16.0 ($CH_2\underline{C}H_2CH_3$), 27.2 (5,7-adamantyl C), 32.9 (4,9-adamantyl C), 33.2 (1,3-adamantyl C), 33.3 (8,10-adamantyl C), 34.9 ($\underline{C}H_2CH_2CH_3$), 38.6 (6-adamantyl C), 53.3 (CH_2N_3), 61.3 (2-adamantyl C), 165.2 (C=O).

A mixture of the azide **46** (75 mg, 0.271 mmol) and PPh_3 (78 mg, 0.298 mmol) in THF (3 mL) and water (5 mL) was stirred for 72 h at rt. The solvent was evaporated under vacuum and the residue was dissolved in Et_2O. The organic solution was washed with water and extracted with HCl 4% (2x10mL). The aqueous layer was made alkaline with solid Na_2CO_3 and the oily product formed was extracted with ether. The combined ether extracts were washed with water and brine and dried (Na_2SO_4). After evaporation of the solvent, amino acetamide **19** was afforded: yield 34 mg (50 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm) 0.89 (t, *J* = 7 Hz, 3H, $CH_2CH_2C\underline{H}_3$, 1.23-1.24 (m, 4H, 2x$CH_2C\underline{H}_2CH_3$), 1.58-1.61 (m, 2H, 4eq,9eq-adamantyl H), 1.65 (d, *J* = 12 Hz, 2H, 1,3-adamantyl H), 1.80 (br s, 2H, 8eq,10eq-adamantyl H), 1.95-2.01 (m, 6H, 5,7,6,8ax,10ax-adamantyl H), 2.25 (d, *J* = 12 Hz, 2H, 4ax,9ax-H), 3.28 (s, 2H, $COCH_2N$); ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm) 14.7 ($CH_2CH_2\underline{C}H_3$), 16.1 ($CH_2\underline{C}H_2CH_3$), 27.4 (5,7-adamantyl C), 33.0 (4,9-adamantyl C), 33.2 (8,10-adamantyl C), 35.1 ($\underline{C}H_2CH_2CH_3$), 37.1 (1,3-adamantyl C), 38.7 (6-adamantyl C), 45.4 (CH_2N), 59.8 (2-adamantyl C), 171.2 (C=O); HRMS (*m*/*z*): [M + H^+] calcd for $C_{15}H_{26}N_2O$ 250.2045, experimental 250.2052.

Two-Electrode Voltage Clamp (TEVC) Assay

The inhibitors were tested via a TEVC assay using *X. laevis* frog oocytes microinjected with RNA expressing the M2 protein as in a previous paper.[36] The blocking effect of the a derivatives against M2 was investigated with EP experiments using $M2_{Udorn/72}$. Because WSN/33-M2-N31S, WSN/33-M2-N31S-V27A, WSN/33-M2-N31S-L26F was used to compare antiviral potencies using a whole cell assay,

$M2_{V27A}$ $M2_{L26F}$ $M2_{S31N}$ were generated and studied in parallel. The potency of the inhibitors was expressed as the inhibition percentage of the A/M2 current observed after 2 min of incubation with 100 μM of compound.

Cells and viruses

Madin-Darby canine kidney (MDCK) cells (Cat.no. RIE 328, Friedrich-Loeffler Institute, Riems, Germany) were propagated as monolayer in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 2 mM L-glutamine. Amantadine-sensitive Udorn/72, amantadine-resistant WSN/33-M2-WT (with am N31 in M2) and its variant with N31S amino acid substitution in the M2 ion channel were used in this study. Briefly for the generation of WSN/33-M2-N31S [35] the plasmid pHW187-M2-N31S was altered by site-directed mutagenesis PCR and afterwards used as part of a plasmid set for the recovery of A/WSN/33 virus. [36] WSN/33-variants were propagated on MDCK cells in serum-free EMEM supplemented with 2 mM L-glutamine, 2 μg/mL trypsin, and 0.1% sodium bicarbonate (test medium). Virus containing supernatant was harvested after about 48 h of incubation at 37 °C when cytopathic effect became microscopically visible. Aliquots were stored at -80 °C until use. The M2 gene identity of all recombinant viruses was verified by sequencing.

CPE assay

Cytotoxicity and CPE inhibition studies were performed on two-day-old confluent monolayers of MDCK cells grown in 96-well plates as published. [25] Cytotoxicity was analyzed 72 h after compound addition. In CPE inhibition assay, 50 μl of a serial half-log dilution of compound in test medium (maximum concentration 100 μM) and a constant multiplicity of infection of test virus in a volume of 50 μL of the test medium were added to cells. Then, plates were incubated at 37 °C with 5% CO_2 for 48 h. Crystal violet staining and determination of the 50% cytotoxic (CC_{50}) and 50% inhibitory concentration (IC_{50}) was performed as described before. [25,26] At least three independent assays were conducted.

Supporting Information

Additional synthetic schemes, experimental details, discussion on the results, and also Figure S1 describing the orientation of 2 inside M2TM WT and M2TM S31N.

Abbreviations

cytopathic effect, CPE; electrophysiology, EP; molecular dynamics, MD; transmembrane, TM; DMPC, dimirystoyl-*sn*-glycero-3-phosphoethanolamine; protein data bank, PDB; wild type, WT; influenza A virus, IAV; structure-activity relationship, SAR

Acknowledgements

A.K. thanks Chiesi Hellas for supporting this research, Dr Natalie A. Fokina and Professor Peter R. Schreiner for providing samples of diamantane and triamantane and Assist. Professor I. Papanastasiou for helping on synthesis of 2-(1-adamantyl)-propan-2-amine. A.K. and S.V. thank Fundació La Marató de TV3 (201832) for finantial support. A.L.T thanks the Spanish *Ministerio de Educación, Cultura y Deporte* for a PhD fellowship (FPU program).

Author Information

[*]Corresponding author: A. K., e-mail: ankol@pharm.uoa.gr; M.S., michaela.schmidtke@med.uni-jena.de; [x] C.T. and A.H. equally/shared first authorship.

A.K. designed this research project and with M.S. supervised the research. C.T. did the ligand synthesis while A.L.T. prepared compounds **38-39** in S.V. lab. A.H. and P.S. generated the mutant viruses with reverse genetics and did the antiviral testing in the M.S. group. C.M. performed the EP measurements in J. W. lab. A.K. wrote the manuscript and A.L.T., S.V. and M.S. revised it.

References

(1) Grohskopf, L. A.; Shay, D. K.; Shimabukuro, T. T.; Sokolow, L. Z.; Keitel, W. A.; Bresee, J. S.; Cox, N. J. Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices - United States, 2013-2014. *Mmwr Recomm. Reports* **2013**, *62*, 1–43.

(2) Jin, H.; Chen, Z. Production of Live Attenuated Influenza Vaccines against Seasonal and Potential Pandemic Influenza Viruses. *Curr. Opin. Virol.* **2014**, *6*, 34–39.

(3) Astrahan, P.; Arkin, I. T. Resistance Characteristics of Influenza to Amino-Adamantyls. *Biochim. Biophys. Acta - Biomembr.* **2011**, *1808*, 547–553.

(4) Abed, Y.; Goyette, N.; Boivin, G. Generation and Characterization of Recombinant Influenza A (H1N1) Viruses Harboring Amantadine Resistance Mutations Generation and Characterization of Recombinant Influenza A (H1N1) Viruses Harboring Amantadine Resistance Mutations. **2005**, *49*, 556–559.

(5) Furuse, Y.; Suzuki, A.; Oshitani, H. Large-Scale Sequence Analysis of M Gene of Influenza A Viruses from Different Species: Mechanisms for Emergence and Spread of Amantadine Resistance. *Antimicrob. Agents Chemother.* **2009**, *53*, 4457–4463.

(6) Furuse, Y.; Suzuki, A.; Kamigaki, T.; Oshitani, H. Evolution of the M Gene of the Influenza A Virus in Different Host Species: Large-Scale Sequence Analysis. *Virol. J.* **2009**, *6*, 67.

(7) Bright, R. A.; Medina, M. J.; Xu, X.; Perez-Oronoz, G.; Wallis, T. R.; Davis, X. M.; Povinelli, L.; Cox, N. J.; Klimov, A. I. Incidence of Adamantane Resistance among Influenza A (H3N2) Viruses Isolated Worldwide from 1994 to 2005: A Cause for Concern. *Lancet* **2005**, *366*, 1175–1181.

(8) Lan, Y.; Zhang, Y.; Dong, L.; Wang, D.; Huang, W.; Xin, L.; Yang, L.; Zhao, X.; Li, Z.; Wang, W.; Li, X.; Xu, C.; Yang, L.; Guo, J.; Wang, M.; Peng, Y.; Gao, Y.; Guo, Y.; Wen, L.; Jiang, T.; Shu, Y. A Comprehensive Surveillance of Adamantane Resistance among Human Influenza A Virus Isolated from Mainland China between 1956 and 2009. *Antivir. Ther.* **2010**, *15*, 853–859.

(9) 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*, 1–20.

(10) Garcia, V.; Aris-Brosou, S. Comparative Dynamics and Distribution of Influenza Drug Resistance Acquisition to Protein M2 and Neuraminidase Inhibitors. *Mol. Biol. Evol.* **2014**, *31*, 355–363.

(11) Zhao, X.; Li, C.; Zeng, S.; Hu, W. Discovery of Highly Potent Agents against Influenza A Virus. *Eur. J. Med. Chem.* **2011**, *46*, 52–57.

(12) Dong, J.; Chen, S.; Li, R.; Cui, W.; Jiang, H.; Ling, Y.; Yang, Z.; Hu, W. Imidazole-Based Pinanamine Derivatives: Discovery of Dual Inhibitors of the Wild-Type and Drug-Resistant Mutant of the Influenza A Virus. *Eur. J. Med. Chem.* **2016**, *108*, 605–615.

(13) Zhao, X.; Li, R.; Zhou, Y.; Xiao, M.; Ma, C.; Yang, Z.; Zeng, S.; Du, Q.; Yang, C.; Jiang, H.; Hu, Y.; Wang, K.; Mok, C. K. P.; Sun, P.; Dong, J.; Cui, W.; Wang, J.; Tu, Y.; Yang, Z.; Hu, W.

Discovery of Highly Potent Pinanamine-Based Inhibitors against Amantadine- and Oseltamivir-Resistant Influenza A Viruses. *J. Med. Chem.* **2018**.

(14) Wang, J. J.; Wu, Y.; Ma, C.; Fiorin, G.; Pinto, L. H.; Lamb, R. A.; Klein, M. L.; Degrado, W. F. Structure and Inhibition of the Drug-Resistant S31N Mutant of the M2 Ion Channel of Influenza A Virus. *Proc Natl Acad Sci U S A* **2013**, *110*, 1315–1320.

(15) Wang, J.; Ma, C.; Wang, J.; Jo, H.; Canturk, B.; Fiorin, G.; Pinto, L. H.; Lamb, R. A.; Klein, M. L.; DeGrado, W. F. Discovery of Novel Dual Inhibitors of the Wild-Type and the Most Prevalent Drug-Resistant Mutant, S31N, of the M2 Proton Channel from Influenza A Virus. *J. Med. Chem.* **2013**, *56*, 2804–2812.

(16) Wu, Y.; Canturk, B.; Jo, H.; Ma, C.; Gianti, E.; Klein, M. L.; Pinto, L. H.; Lamb, R. A.; Fiorin, G.; Wang, J.; Degrado, W. F. Flipping in the Pore: Discovery of Dual Inhibitors That Bind in Different Orientations to the Wild-Type versus the Amantadine-Resistant S31N Mutant of the Influenza a Virus M2 Proton Channel. *J. Am. Chem. Soc.* **2014**, *136*, 17987–17995.

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

(18) 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.

(19) 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.

(20) Williams, J. K.; Tietze, D.; Wang, J.; Wu, Y.; Degrado, W. F.; Hong, M. Drug-Induced Conformational and Dynamical Changes of the S31N Mutant of the Influenza M2 Proton Channel Investigated by Solid-State NMR. *J. Am. Chem. Soc.* **2013**, *135*, 9885–9897.

(21) Tzitzoglaki, C.; McGuire, K.; Lagarias, P.; Konstantinidi, A.; Hoffmann, A.; Fokina, N. A.; Ma, C.; Papanastasiou, I. P.; Schreiner, P. R.; Vázquez, S.; Schmidtke, M.; Wang, J.; Busath, D. D.; Kolocouris, A. Chemical Probes for Blocking of Influenza A M2 Wild-Type and S31N Channels. *ACS Chem. Biol.* **2020**, *15*, 2331–2337.

(22) Hu, Y.; Hau, R. K.; Wang, Y.; Tuohy, P.; Zhang, Y.; Xu, S.; Ma, C.; Wang, J. Structure-Property Relationship Studies of Influenza A Virus AM2-S31N Proton Channel Blockers. *ACS Med. Chem. Lett.* **2018**, *9*, 1111–1116.

(23) Wang, Y.; Hu, Y.; Xu, S.; Zhang, Y.; Musharrafieh, R.; Hau, R. K.; Ma, C.; Wang, J. 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. *J. Med. Chem.* **2018**, *61*, 1074–1085.

(24) Rey-Carrizo, M.; Barniol-Xicota, M.; Ma, C.; Frigolé-Vivas, M.; Torres, E.; Naesens, L.; Llabrés, S.; Juárez-Jiménez, J.; Luque, F. J.; Degrado, W. F.; Lamb, R. A.; Pinto, L. H.; Vázquez, S. Easily Accessible Polycyclic Amines That Inhibit the Wild-Type and Amantadine-Resistant Mutants of the M2 Channel of Influenza A Virus. *J. Med. Chem.* **2014**, *57*, 5738–5747.

(25) Schmidtke, M.; Schnittler, U.; Jahn, B.; Dahse, H.-M.; Stelzner, A. A Rapid Assay for Evaluation of Antiviral Activity against Coxsackie Virus B3, Influenza Virus A, and Herpes Simplex Virus Type 1. *J. Virol. Methods* **2001**, *95*, 133–143.

(26) Torres, E.; Duque, M. D.; Vanderlinden, E.; Ma, C.; Pinto, L. H.; Camps, P.; Froeyen, M.; Vázquez, S.; Naesens, L. Role of the Viral Hemagglutinin in the Anti-Influenza Virus Activity of Newly Synthesized Polycyclic Amine Compounds. *Antiviral Res.* **2013**, *99*, 281–291.

(27) Drakopoulos, A.; Tzitzoglaki, C.; McGuire, K.; Hoffmann, A.; Konstantinidi, A.; Kolokouris, D.; Ma, C.; Freudenberger, K.; Hutterer, J.; Gauglitz, G.; Wang, J.; Schmidtke, M.; Busath, D. D.; Kolocouris, A. Unraveling the Binding, Proton Blockage, and Inhibition of Influenza M2 WT and S31N by Rimantadine Variants. *ACS Med. Chem. Lett.* **2018**, *9*, 198–203.

(28) Barniol-Xicota, M.; Gazzarrini, S.; Torres, E.; Hu, Y.; Wang, J.; Naesens, L.; Moroni, A.; Vázquez, S. Slow but Steady Wins the Race: Dissimilarities among New Dual Inhibitors of the

Wild-Type and the V27A Mutant M2 Channels of Influenza A Virus. *J. Med. Chem.* **2017**, *60*, 3727–3738.
(29) 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.
(30) Thomaston, J. L.; Polizzi, N. F.; Konstantinidi, A.; Wang, J.; Kolocouris, A.; Degrado, W. F. Inhibitors of the M2 Proton Channel Engage and Disrupt Transmembrane Networks of Hydrogen-Bonded Waters. *J. Am. Chem. Soc.* **2018**, 15219–15226.
(31) Thomaston, J. L.; Konstantinidi, A.; Liu, L.; Lambrinidis, G.; Tan, J.; Caffrey, M.; Wang, J.; Degrado, W. F.; Kolocouris, A. X-Ray Crystal Structures of the Influenza M2 Proton Channel Drug-Resistant V27A Mutant Bound to a Spiro-Adamantyl Amine Inhibitor Reveal the Mechanism of Adamantane Resistance. *Biochemistry* **2020**, *59*, 627–634.
(32) Musharrafieh, R.; Lagarias, P.; Ma, C.; Hau, R.; Romano, A.; Lambrinidis, G.; Kolocouris, A.; Wang, J. Investigation of the Drug Resistance Mechanism of M2-S31N Channel Blockers through Biomolecular Simulations and Viral Passage Experiments. *ACS Pharmacol. Transl. Sci.* **2020**, *3*, 666–675.
(33) Kolocouris, A.; Tzitzoglaki, C.; Johnson, F. B.; Zell, R.; Wright, A. K.; Cross, T. A.; Tietjen, I.; Fedida, D.; Busath, D. D. Aminoadamantanes with Persistent in Vitro Efficacy against H1N1 (2009) Influenza A. *J. Med. Chem.* **2014**, *57*, 4629–4639.
(34) Torres, E.; Fernández, R.; Miquet, S.; Font-Bardia, M.; Vanderlinden, E.; Naesens, L.; Vázquez, S. Synthesis and Anti-Influenza a Virus Activity of 2,2-Dialkylamantadines and Related Compounds. *ACS Med. Chem. Lett.* **2012**, *3*, 1065–1069.
(35) Schade, D.; Kotthaus, J.; Riebling, L.; Kotthaus, J.; Müller-Fielitz, H.; Raasch, W.; Hoffmann, A.; Schmidtke, M.; Clement, B. Zanamivir Amidoxime- and N-Hydroxyguanidine-Based Prodrug Approaches to Tackle Poor Oral Bioavailability. *J. Pharm. Sci.* **2015**, *104*, 3208–3219.
(36) Hoffmann, E.; Neumann, G.; Kawaoka, Y.; Hobom, G.; Webster, R. G. A DNA Transfection System for Generation of Influenza A Virus from Eight Plasmids. *Proc. Natl. Acad. Sci.* **2000**, *97*, 6108–6113.

TOC GRAPHIC