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Amantadine variant – aryl conjugates that inhibit multiple M2 mutant – amantadine resistant influenza a viruses

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ABSTRACT

Influenza A viruses can cause a serious future threat due to frequent mutations. Amantadine and rimantadine drugs inhibit influenza A M2 wild-type (WT; bearing in the protein M2 proton channel serine at position-31) viruses by binding and blocking M2 WT channel-mediated proton current. The resistant to these drugs influenza A viruses bearing the S31N mutant in the M2 proton channel can be inhibited by amantadine – aryl conjugates, in which amantadine and an aryl group are linked through a methylene, which block M2 S31N channel-mediated proton current. However, the M2 amantadine/rimantadine resistant viruses bearing one of the four mutations L26F, V27A, A30T, G34E in residues that line the M2 channel pore pose an additional concern for public health.

Here, we designed 33 compounds based on the structure of three previously published and potent amantadine-aryl conjugates against M2 S31N virus, by replacing amantadine with 16 amantadine variants. The compounds were tested against M2 WT and the five M2 amantadine resistant viruses aiming at identifying inhibitors against multiple M2 mutant – amantadine resistant viruses.

We identified 16 compounds that inhibited *in vitro* two influenza A viruses with M2 WT or L26F channels. Additionally, compounds **21** or **32** or **33**, which are conjugates of the rimantadine variant with CMe_2 (instead of CHMe in rimantadine) or the diamantylamine or the 4-(1-adamantyl)benzenamine with the 2-hydroxy-4-methoxyphenyl aryl group, were *in vitro* inhibitors against three influenza A viruses with M2 WT or L26F or S31N, while compound 21 inhibited also *in vitro* the M2 G34E virus and compound **32** inhibited also *in vitro* the M2 A30T virus. Also, using electrophysiology, we showed that compound **21** was an efficient blocker of the M2 WT and M2 L26F channels, compound **32** blocked efficiently the M2 WT channel and compound **33** blocked the M2 WT, L26F and V27A channels. The drug metabolism and pharmacokinetics studies showed that these compounds need further optimization.

1. Introduction

Amantadine and rimantadine were used for 35 years as antiviral drugs against influenza A M2 wild-type (WT) virus. These drugs are acting against M2 WT virus (bearing in the protein M2 proton channel serine at position-31) replication by binding the transmembrane (TM) pore of the M2 proton channel [1] and blocking the M2 WT-mediated proton current [2].

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Since 2005, amantadine and rimantadine have been discontinued due to the prevalence of viruses bearing the M2 S31N mutant, such as the pandemic 2009 H1N1 strain and seasonal H3N2 strains. The TM of the homotetrameric M2 protein, which is the pore of the proton channel, places constraints on the types of amantadine drug resistant mutations that can be developed [3]. The 95% of amantadine resistant viruses bear the S31N mutation in M2 and 1% have the V27A or L26F mutation with these three mutant viruses being clinical isolates [4–6]. The rarest amantadine resistant viruses are those with M2 A30T or M2 G34E [7]. It has been also shown that the frequency of emergence of resistant strains varies among different geographical areas [4,8,9]. The M2 S31N mutation is distributed worldwide, the M2 L26F primarily in Hong Kong and New Zealand, the M2 V27A mainly in China and Indonesia, the M2 A30T primarily in China, and the M2 G34E only in USA. Compounds that inhibit multiple-mutant M2 viruses are useful for a future threat due to the frequent mutations of the influenza A virus [10].

The first reported compounds that blocked M2 WT and M2 S31N channels according to electrophysiology (EP), have the pinanamine scaffold linked, through a methylene, to the *p*-hydroxyphenyl or the imidazolyl groups (e.g., see compound **1** in Fig. 1) [11,15]. The replacement of pinanamine by amantadine led to amantadine – aryl conjugates, e.g. compounds **2–6** [11–14] (Fig. 1), as second generation adamantane-based drugs. After extensive structure-activity relationships (SARs) studies [12–14,16–19] several potent inhibitors of M2 S31N virus were identified, e.g. compounds **2–6** [11–14]. In particular, compounds **2** and **6** were dual blockers of M2 WT and S31N channels by EP and inhibited *in vitro* M2 WT and M2 S31N viruses, while compound **5** blocked only the M2 S31N channel by EP and inhibited *in vitro* M2 S31N virus. These compounds blocked M2 S31N or M2 WT channels by binding the channel pore between V27 and H37 or between V27 and G34, respectively, as previously showed by NMR and MD simulations [11,13,20] and as we have confirmed by MD simulations and binding free energy calculations [19,21].

Aiming at identifying inhibitors of multiple mutant M2 – amantadine resistant viruses, we synthesized the 33 compounds **7–39** shown in Fig. 2 (compounds **32**–**34** have been published and tested previously against only M2 WT and M2 S31N viruses [19]) as analogues of amantadine – aryl conjugates **2**, **5**, **6** [11–13]. We also synthesized amantadine – aryl conjugates **2** and **5** previously reported in ref. [11] and ref. [12], respectively, for comparison. The amantadine – aryl conjugate **2** [13] or **5** [12] or **6** [14] have, as an aryl group, the 2-hydroxy-4-methoxyphenyl, the 5-phenyl-isoxazolyl and the 2-bromothiophenyl, respectively. Totally, we synthesized 35 compounds and evaluated their *in vitro* antiviral activity in this research.

We designed compounds **7**–**39** by replacing amantadine with 16 amantadine variants. The following classes of our synthesized compounds are shown in Fig. 2: (a) compounds **7**–**18**, having 2-alkyl-2-adamantylamines, conjugated with the 2-hydroxy-4-methoxyphenyl group; (b) compounds **30** and **31**, having a 3-substituted amantadine (with an *i*-propyl group or a fluorine atom at 3-position, respectively), conjugated with the 2-hydroxy-4-methoxyphenyl group; (c) compounds **20**–**29** having the rimantadine (i.e. with the CHMe bridge between 1-adamantyl and amino groups) or rimantadine variants (with a CR_2 bridge between 1-adamantyl and amino groups, R = Me, Et, Pr) conjugated with the 2-hydroxy-4-methoxyphenyl, the 5-alkyl-isoxazolyl or the 2-bromothiophenyl group; (d) compound **32**, having the 4-(1-adamantyl) benzenamine, conjugated with the 2-hydroxy-4-methoxyphenyl group; (d) compounds **33** and **34**, having the diamantylamine or the triamantylamine cores, respectively, conjugated with the 2-hydroxy-4-methoxyphenyl group or the 2-bromothiophenyl group; (e) compounds **35**–**37**, having two primary *tert*-alkyl amines, conjugated with the 2-hydroxy-4-methoxyphenyl group or 2-bromothiophenyl group; (f) compounds **38** and **39** having a polycyclic cage amine, conjugated with the 2-hydroxy-4-methoxyphenyl group or the 2-bromothiophenyl group.

The 35 synthesized compounds (**2**, **5**, **7**–**39**) were tested *in vitro* against an influenza A strain bearing the M2 WT and five viruses bearing the M2 mutant – amantadine resistant proteins, M2 S31N, M2 L26F, M2 V27A, M2 A30T and M2 G34E [22]. Between **7**–**39**, we selected few compounds and we tested them against the pandemic influenza A H1N1/Calif/07 and H1N1 Jena/8178/09 strains, both having M2 S31N proteins.

2. Results and discussion

2.1. Synthesis of amantadine variant – aryl conjugates

To link the aryl group through a methylene with the amino group of the amantadine variants we used a reductive amination reaction. First,

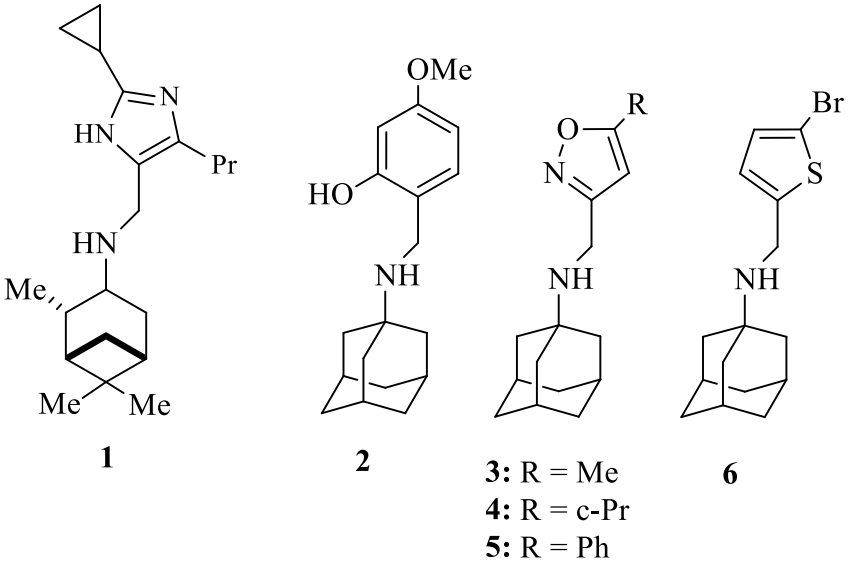


Fig. 1. Chemical structure of the pinanamine-aryl conjugate **1** [11] and the amantadine – aryl conjugates **2, 4, 5, 6** [12–14] which blocked efficiently M2 S31N channel or **2, 6** [12–14] which blocked also M2 WT channel according to EP and inhibited *in vitro* the M2 S31N or M2 S31N and M2 WT viruses, respectively; compound **3** was not an efficient blocker of neither two M2 channels [11].

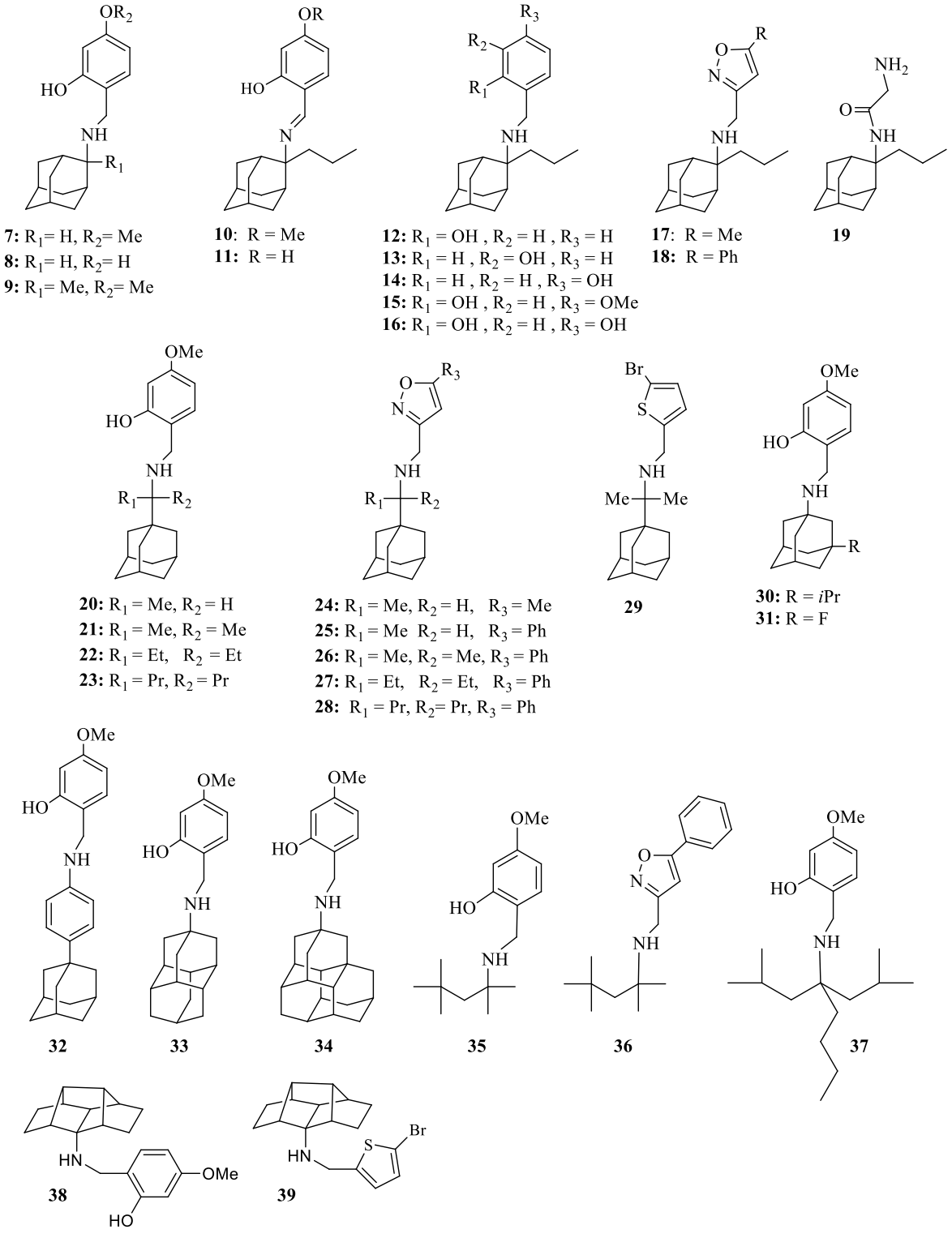


Fig. 2. Chemical structures of synthetic amantadine variant – aryl conjugates, **7**–**39.**

we applied the previously described reaction [13] of a primary amine with 2-hydroxy-4-methoxybenzaldehyde using $NaCNBH_3$ in methanol for 15 min. However, this reaction afforded in our hands the corresponding imine precursor of the desired amine (Method A in the Supporting Information). We synthesized these precursor imines also by refluxing the starting amine with the suitable aldehyde in benzene using a Dean-Stark adaptor. Elongation of the reductive amination reaction for few hours led to cleavage affording the starting amine, as we observed for compounds **10** and **11**. Strikingly, we observed that a mixture of the imine precursor with a catalytic amount of *p*-toluenesulfonic acid (PTSA) and $NaBH_4$ in methanol afforded the derired amine in good yields, as is described in Schemes 1 and S1. Using this protocol, we prepared the *N*-(2-hydroxy-4-methoxyphenyl)methyl derivatives **2**, **5**, **9**, **15**, **20**–**23**, **30**–**39**, as is described in Scheme S1.

Second, we applied a reductive amination with $NaBH_4$ in methanol and $Ti(OiPr)_4$ as Lewis acid (Method B in the Supporting Information) as

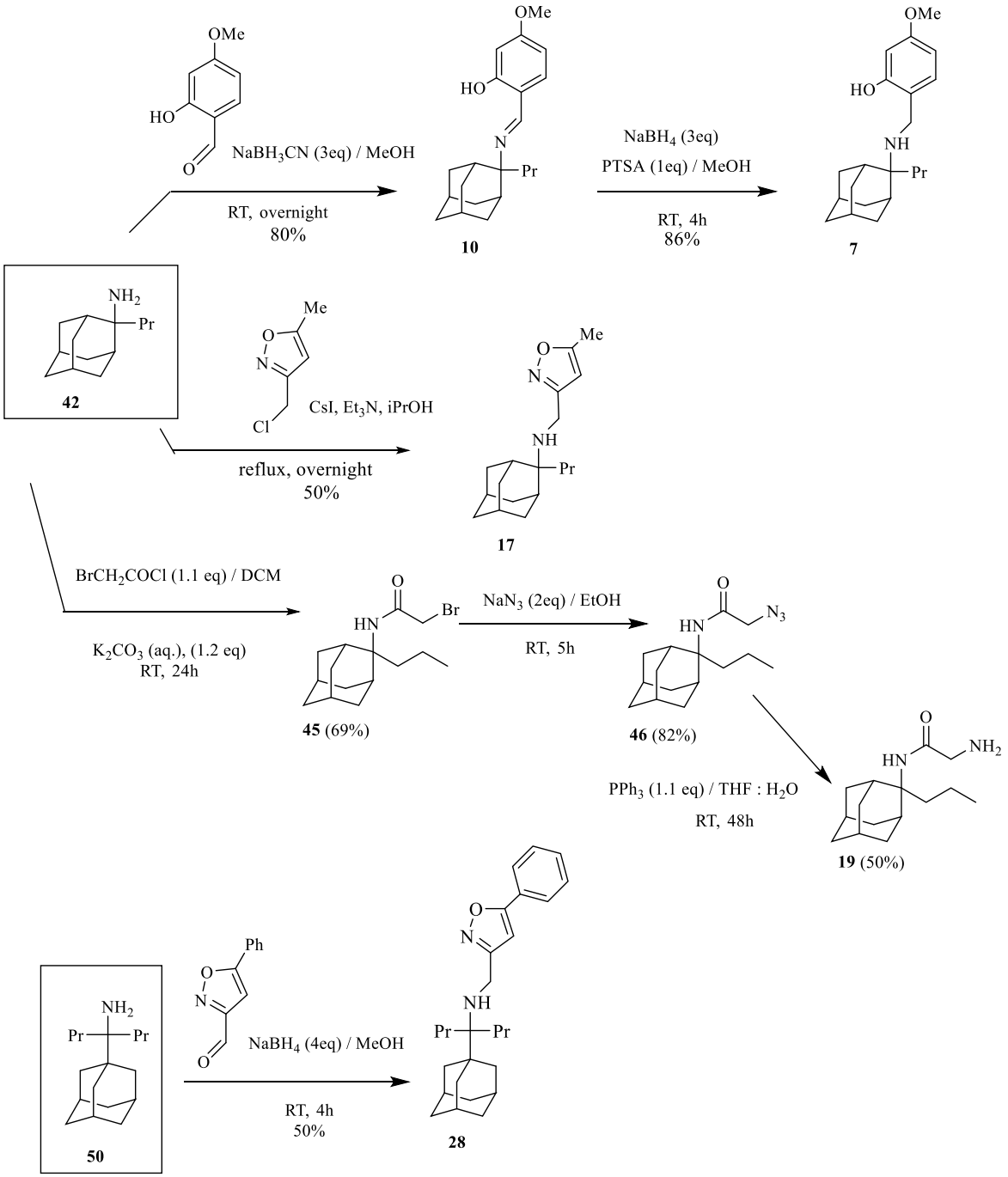
was previously applied [12] (Scheme S2). This procedure afforded the *N*-(hydroxyphenyl)methyl derivatives **12–14** or the *N*-(5-methylisoxazolyl)methyl derivatives **18** and **25–28**. Additionally, we also synthesized compounds **17** and **25** by reacting the corresponding amines with CsI (Method C) and 3-chloromethyl-5-methylisoxazole in isopropanol, as described in Scheme 1.

We carried out the demethylation of the (2-hydroxy-4-methoxyphenyl) methyl group in compounds **7**, **10** and **15**, through treatment with excess of BBr_3 to afford derivatives **8**, **11** and **16**, respectively (Scheme S3). Finally, starting from the amine **42**, we prepared the *N*-bromoacetyl derivative **45**, and the *N*-azidoacetyl derivative **46** which, under Staundinger reaction conditions, afforded compound **19** (Scheme 1).

2.2. Biological testing

2.2.1. Virus inhibition assay results

We used the cytopathic effect (CPE) inhibition assay [22] in MDCK cells to measure the inhibitory potency (EC_{50} values) of the 35 compounds **2**, **5**, **7–39** against an influenza A strain bearing the M2 WT and the amantadine resistant influenza A viruses bearing one of the mutations S31N, L26F, V27A, A30T, G34E in M2 protein. Compounds **1** (compound 48 in Ref. [23]), **2** (compound 45 in Ref. [13]), **3**, **4**, **5** (compounds with lab codes M2WJ369, M2WJ379, M2WJ352 in Ref. [12]) and **6** (compound 11 in Ref. [14]) have been previously evaluated, using the plaque reduction assay and we also measured the cytotoxicity (Table 1). We



Scheme 1. Representative examples of the chemistry applied for the preparation of compounds **7**, **17** or **28** starting from amines **42** or **50**, respectively, and for preparation of compound **19** starting from amine **42** (see also Schemes S1–S3).

resynthesized compounds **2**, **5** and compared the previously measured values of antiviral activity (EC_{50} values) against M2 WT and M2 S31N viruses (shown in Table 1) [12,13] using the plaque reduction assay with our results using the CPE inhibition assay. In Table 1 we showed in bold the most interesting EC_{50} potencies with the highest value being ~23.5 μM; we applied a grey shading in compound numbers corresponding to molecules that have been synthesized in this work; with green shading the most interesting compounds **21**, **32**, **33** are shown.

We confirmed the antiviral potency of the previously evaluated compounds **2** and **5** against M2 WT and M2 S31N viruses and observed that compound **2** is potent against both M2 WT and S31N viruses, while compound **5** is very potent only against M2 S31N virus. These compounds are devoid of any activity against all the other M2 mutant-amantadine resistant viruses.

Table 1
In vitro anti-influenza A potency (EC_{50}, μM) of amantadine variant – aryl conjugates that were tested against initial cell infection.

	CC_{50} (μM)	EC_{50} ± SD (μM) [b]					
Cmp		M2 WT [a]	M2 S31N [a]	M2 L26F [a]	M2 V27A [a]	M2 A30T [a]	M2 G34E [a]
1 [23]	112.1	**6.9 ± 0.7** [c]	**10.8 ± 2** [c]	n.t. [e]	n.t. [e]	n.t. [e]	n.t. [e]
2 [13]	49.86 ± 12.28	**1.13 ± 0.03**	**8.70 ± 4.38**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
3 [12]	n.t. [e]	n.t. [e]	104 [c]	n.t. [e]	n.t. [e]	n.t. [e]	n.t. [e]
4 [12]	n.t. [e]	n.t. [e]	**16** [c]	n.t. [e]	n.t. [e]	n.t. [e]	n.t. [e]
5 [12]	67.80 ± 0.62	91.35 ± 42.80	**1.18 ± 0.17**	n.a. [d]	n.a. [d]	n.a. [d]	n.a. [d]
6 [14]	123	**4.6** [c]	**1.8** [c]	n.t. [e]	n.t. [e]	n.t. [e]	n.t. [e]
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 [19]	>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 [19]	>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 [19]	>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 [24]	>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] See Supporting Information about cells and viruses used; [b] measured in triplicate; [c] determined using WSN/33 and A/HK/68 (M2 WT) viruses for compound **1** see ref. [23] or using WSN/33 and Udorn/72 (M2 WT) viruses, see ref. [12] for compounds **3** and **4** and see ref. [14] for compound **6**; [d] n.a., not active; [e] n.t. not tested. In bold are shown the best potencies, corresponsing to EC_{50} values lower than ~ 23.5 μM. Compounds first synthesized in this work are in grey shading; green shading is used for highlighting the most interesting compounds: **21**, **32** and **33**.

We compared the effect in potency against M2 WT and M2 mutant-amantadine resistant viruses when we replaced amantadine in amantadine – aryl conjugates conjugates **2**, **5** and with the 16 amantadine variants conjugated with 2- or 3- or 4-hydroxylphenyl or 2,4-dihydroxylphenyl or 2-hydroxy-4-methoxyphenyl or 5-methyloxazol-3-yl or 5-phenyloxazol-3-yl group.

(a) In compounds **7–19**, the 2-adamantylamine or 2-methyl- or 2-propyl-2-adamantylamine was conjugated with 2- or 3- or 4-hydroxylphenyl or 2,4-dihydroxylphenyl or 2-hydroxy-4-methoxyphenyl or 5-methyloxazolyl or 5-phenyloxazolyl group. We observed that the 2-adamantylamine derivatives **7**, **8** or the 2-methyl-2-adamantylamine derivative **9** which were conjugated with 2,4-dihydroxylphenyl group (compound **8**) or 2-hydroxy-4-methoxyphenyl group (compounds **7**, **9**) are micromolar (compound **7**) or low micromolar (compounds **8, 9**) inhibitors of M2 WT virus, respectively, and had no activity against M2 mutant viruses.

(b) In compounds **12–19**, the 2-propyl-2-adamantylamine was conjugated with 2-, 3-, 4-hydroxylphenyl group (compounds **12–14**) or 2,4-dihydroxylphenyl group (compound **16**) or 2-hydroxy-4-methoxyphenyl group (compound **15**) or 5-methyloxazol-3-yl group (compound **17**) or 5-phenyloxazol-3-yl group (compound **18**). Between **12**–**14**, only the 2-propyl-2-adamantylamine – 4-hydroxylphenyl conjugate **14**, in which the hydroxyl substituent of the hydroxyphenyl group is in p-position, was low micromolar inhibitor against M2 WT and L27F viruses compared to the totally inactive compounds **12**, **13**, having the hydroxyl group in o- or m-position. Compounds **17** or **18** which are conjugates of the 2-propyl-2-adamantylamine with 5-methyloxazol-3-yl or 5-phenyloxazol-3-yl group, respectively, were inactive compounds, while **15** or **16** which are conjugates of the 2-propyl-2-adamantylamine with 4-di-hydroxylphenyl or 2-hydroxy-4-methoxyphenyl group, respectively, were low micromolar inibitors of M2 WT virus and micromolar of M2 L26F virus. It is worth to note that compound **15** inhibited with a micromolar concentration also the M2 G34E virus.

(c) The imine precursors **10**, **11** of amines **15**, **16**, respectively, were low micromolar inhibitors of the M2 WT virus and had 10-fold lower (compound **10**) or 4-fold lower activity (compound **11**) against M2 L26F virus and no activity against other M2 mutant viruses. Compound **19** with an *N*-(2-aminoacetyl) group connected to 2-propyl-2-adamantylamine was a low micromolar inhibitor only of the M2 WT virus.

(d) In compounds **30**, **31**, a 3-substituted amantadine was conjugated with 2-hydroxy-4-methoxyphenyl group. The 3-iPr-amantadine – 2-hydroxy-4-methoxyphenyl conjugate **30** was a micromolar inhibitor of M2 WT, A30T and G34E viruses while the 3-F-amantadine – 2-hydroxy-4-methoxyphenyl conjugate **31** was inactive.

(e) In compounds **20**, **24**, **25** or **21–23**, **26–29**, rimantadine or a rimantadine variant (with a CR_2 bridge between adamantyl and amino group and R = Me, Et, Pr), respectively, was conjugated with the 2-hydroxy-4-methoxyphenyl group. The rimantadine variant – aryl conjugates **21–23**, **28, 29** had low micromolar or sub-micromolar potency against both M2 WT and L26F viruses, while **20**, **24**, **25**, and **27** were also very potent against the M2 WT virus and only **20**, **27** had potency against the M2 L26F virus.

(f) In compounds **32**, **33**, **34**, diamantylamine, triamantylamine or (1-adamantyl)benzenamine, respectively, were conjugated with 2-hydroxy-4-methoxyphenyl as aryl group. Compounds **32**, **33** were submicromolar inhibitors of M2 WT and micromolar inhibitors of the M2 S31N and M2 L26F viruses; compound **32** inhibited also the M2 A30T virus, while compound **34** was a low micromolar inhibitor of only M2 WT.

(g) In compounds **35**–**37** or **38**, **39**, a primary *tert*-alkylamine or a polycyclic cage amine, respectively, was conjugated with 2-hydroxy-4-methoxyphenyl group. The primary *tert*-alkylamine – aryl conjugates **35–37** and the polycyclic cage amine – aryl conjugates **38** and **39** were inactive against all viruses.

(h) The structure of the aryl group in the studied conjugate compounds affected the inhibitory potency. For example, the conjugation of the 2-propyl-adamantylamine with 5-phenyloxazol-3-yl in compound **18** did not afford a potent compound against all viruses, while the conjugation with 2-hydroxy-4-methoxyphenyl provided the potent inhibitor **16** endowed with submicromolar potency against M2 WT virus and low micromolar activity against M2 L26F virus. Importantly, the amantadine – 5-phenyloxazol-3-yl conjugate **5** (M2WJ352 in Ref. [12]) was inactive against the M2 WT but was a low micromolar inhibitor against the M2 S31N virus while the amantadine – 2-hydroxy-4-methoxyphenyl conjugate **2** was a low micromolar inhibitor against both the M2 WT and M2 S31N viruses. Compounds **2** or **5** were inactive against all other mutant M2 viruses. While the amantadine – 2-bromothiophenyl conjugate **6** [14] (Fig. 1) was potent against M2 WT and M2 S31N viruses [14], when this aryl group was conjugated with a CR_2-rimantadine variant (R = methyl) in **29**, the potency against M2 S31N virus was lost but **29** showed submicromolar inhibitory potency against M2 WT virus and low micromolar against M2 L27F virus (Table 1).

From the *in vitro* antiviral assays, the rimantadine variant – 2-hydroxy-4-methoxyphenyl conjugate **21**, diamantylamine – 2-hydroxy-4-methoxyphenyl conjugate **32** and triamantylamine – 2-hydroxy-4-methoxyphenyl conjugate **33** stand out as the best anti-influenza agents tested here, with EC_{50} ranging from submicromolar to low micromolar against the M2 WT virus while being not toxic *in vitro* (Table 1) compared to previously developed compounds **2**, **5**, having CC_{50} values ~ 50 μM and 68 μM *in vitro*. As regards viruses carrying mutant M2 channels, compound **21** inhibited M2 L26F, S31N, G34E viruses, compound **33** inhibited M2 L26F, S31N viruses and compound **32** inhibited also M2 L26F, SN31, A30T viruses at micromolar concentrations, with an $EC_{50} < 20$ μM. Of particular relevance, these three selected compounds, **21**, **32** and **33**, were more potent against the highly prevalent M2 S31N virus than the previously described amantadine – 2-hydroxy-4-methoxyphenyl conjugate **2**.

To further characterize the antiviral profile, we selected 7 compounds for testing them against the two influenza A virus isolates of pandemic 2009H1N1, Jena/8178/09 (Jena/8178) and A/California/07/09 (Calif/07), both carrying the mutant M2 S31N channel and being currently epidemic strains (Table 2). WSN/33 (M2 S31N) virus is resistant to amantadine, amantadine variants and those amantadine – aryl conjugates that can not block M2 S31N-mediated proton current. However, there are M2 S31N influenza viruses, e.g. Calif/07 that can be inhibited by amantadine variants [24–26] or amantadine – aryl conjugates [19] with a mechanism other than M2 channel pore blockage as we previously reported [19,24–26]. To examine this case, we tested representatively the amantadine variant – conjugates **20**, **21**, **24**, **27**, **32** and **33** having *in vitro* low micromolar to submicromolar activities against the M2 WT virus, while **21**, **32** and **33** were potent also against the M2 S31N virus (Table 1). We also tested the 4-(1-adamantyl)benzenamine (**62**) (shown in Scheme S1) as an amantadine variant. As it was expected **62** did not inhibit *in vitro* the M2 S31N virus [27]. The antiviral testing results in Table 2 showed that compounds **21**, **32** and **33** were also active *in vitro*

Table 2
In vitro potency (EC_{50}, μM) of seven selected amantadine variant – conjugates and the amantadine variant **62** against influenza A(H1N1) Calif/07 and Jena/8178/09 viruses.[a]

Compound	EC_{50} (μM)	
	Jena/8178/09	**Calif/07**
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) benzenamine (62)	37.82 ± 1.54	**19.88 ± 7.35**

[a] Measured in triplicate.
[b] n.a. = non active.

against Calif/07 and Jena/8178/09 viruses with similar potencies compared to the M2 S31N (WSN/33) virus shown in Table 1. Additionally, we observed that compounds **20** and **24**, which did not inhibit the M2 S31N (WSN/33) virus, inhibited Calif/07 and Jena/8178/09 viruses, while the amantadine variant **62** exhibited activity against Calif/07 in agreement with previous observations. [19,24–26], All compounds were much less potent than oseltamivir, which was a submicromolar virus inhibitor against all strains in Table 1 or Table 2.

2.2.2. Electrophysiology

We explored using EP if the M2 channel-mediated proton current can be blocked by the amantadine variant – aryl conjugates **21**, **32** and **33** with the promising multiple strain-antiviral potency according to the *in vitro* testing. We also performed experiments for the amantadine – aryl conjugate **2** which inhibited both M2 WT and M2 S31N viruses *in vitro* [13]. The blocking effect against full length-M2 protein was determined with a two-electrode voltage clamp (TEVC) assay at 2 min [12] (Table 3).

As is shown in Table 3, compound **2** blocked efficiently both M2 WT (64%) and M2 S31N (59%) channels [12,19]. Compound **21** was an efficient blocker of both M2 WT (80.0%) and M2 L26F (89.9%) channels and moderately blocked the M2 V27A channel (20.5%). Compound **32** only blocked efficiently the M2 WT (81%) channel and moderately the M2 L26F (24%) and V27A (21%) channels. Compound **33** was a triple M2 channel blocker; it blocked the M2 WT (81%), M2 L26F (37%) and M2 V27A (48.8%) channels. With TEVC assay we validated that M2 in the corresponding M2 mutant strains is the drug target of *in vitro* antiviral activity of compounds **21**, **32** and **33**. Compared to our previous paper [19], the TEVC results for **2**, **21**, **32**, **33** against M2 WT are in good agreement while the values against M2 S31N were improved. The results for 2 were also similar with values in ref. [12]. In ref. [19] we also showed how **21**, **32** and **33** bind the M2 WT and M2 S31N pore using MD simulations and binding free energy calculations (in Figs. S1 and S2 the binding profile of these compounds inside M2 WT or M2 S31N channel pore is described; see also in the Supporting Information the paragraph entitled as *Mechanism of mutant M2 channel blockage*) [19]. For compound **5** the binding profile has been studied using solution NMR in micelles and MD simulations [11] and solid state NMR [20] and for compound **6** the binding profile has been studied with solution NMR in micelles and MD simulations [14].

Interestingly, it has been previously observed that few compounds blocked M2 channel at 2 min time point in EP without showing *in vitro* anti-influenza A activity [18,28] while some compounds that were unable to block the M2 channel in EP experiments showed *in vitro* anti-influenza A activity [15,16,25,26]. Here, we provide further examples of both behaviours, as following:

(1) A striking observation was that compound **33** blocked in EP the M2 V27A channel but showed a weak inhibition *in vitro* of the M2 V27A virus. It has been previously shown that the percentage of channel blockage at 2 min time point can be an accurate predictor for the antiviral activity only for fast k_{on} and slow k_{off} compounds [18,28]. Thus, compound **33**, being non-potent *in vitro* can be a slow k_{on}, slow k_{off} against the M2 V27A channel, since the antiviral potency depends on the K_d (=k_{off}/kon) value rather that on the percentage of inhibition at the 2 min time point.

(2) According to the EP results, amantadine variant – aryl conjugates **21**, **32** and **33** blocked the M2 WT and M2 L26F channels but did not block the M2 S31N or M2 A30T channels. However, compounds **21** and **33** inhibited *in vitro* the three M2 WT, S31N, L26F viruses, while **32** inhibited *in vitro* the four M2 WT, S31N, L26F, A30T viruses. The observation that amantadine – or amantadine variant – or pinanamine – aryl conjugates inhibited the influenza A virus replication in cell culture, without blocking the M2 channel in EP has also previously observed by us [18,25,25] and other groups [15], underlying an additional mechanism of antiviral activity [24,26]

Table 3
%-Block of full-length Udorn after 2 min wash-in M2 current by amantadine – aryl conjugate **2** and amantadine variant – aryl conjugates **21**, **32** and **33**.[a,b]

Compound	WT	L26F	V27A	A30T	S31 N	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 AM2 current at listed concentrations (+/-s.e.m.).
[b] Three replicates were used for measurements at 100 μM.

Drug metabolism and pharmacokinetics (DMPK) assays. Taking into account the good anti-influenza A *in vitro* profile of the amantadine variant – aryl conjugates **21**, **32** and **33**, we perfomed some preliminary drug metabolism and pharmacokinetics. Gratifyingly, the three compounds did not significantly inhibit the hERG channel. The microsomal stability of **21**, **32** and **33** in rat and mouse microsomes was very low (see Supporting information), likely because of hydroxylation of bridgehead positions of the polycyclic core. Indeed, it is known that adamantane-based drugs are hydroxylated *in vitro* and in recent studies of amantadine-aryl conjugates, the 3-hydroxy-amantadine was used to improve the metabolic stability instead of amantadine [17]. Regarding permeability in Caco-2 cells, the assay revealed that **21** and **33** had good permeability but low recovery (likely due to metabolism) while no recovery was observed for **32**. Finally, the three compounds inhibited CYP2C19 and CYP2D6 in the low micromolar range (while they did not inhibit other cytochromes, e.g. CYP1A2, CYP2C9, CYP3A4). See the Supporting Information for further details and the experimental procedures for the DMPK assays.

3. Conclusion

The TEVC results showed that compound **21** was an efficient blocker of the M2 WT and M2 L26F channels, compound **32** blocked efficiently the M2 WT channel and compound **33** blocked the M2 WT, L26F and V27A channels. We observed that these compounds can inhibit *in vitro* viruses without blocking the M2 channel by EP. This has also previously observed [15,18,26,28], underlying an additional mechanism of antiviral activity [24,26]. DMPK studies showed that the properties of these compounds need further optimization.Thus, these results clearly indicate that although a good *in vitro* anti-influenza A activity profile has been found for **21**, **32** and **33** further medicinal chemistry work is compulsory in order to advance this family of compounds to *in vivo* assays. Nevertheless, this work adds to the anti-influenza A SARs of the second generation adamantane-based drugs.

4. Experimental part

The biological assays are described in the Supporting Information. Representative synthetic procedures for few compounds in Scheme 1 are described below:

Compound 7 (Procedure A). Reaction of 2-aminoadamantane **40** (88 mg, 0.583 mmol) and 2-hydroxy-4-methoxybenzaldehyde (74 mg, 0.486 mmol) 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 $COCH_3$); HRMS (m/z): [M + H^+] calcd for $C_{18}H_{25}NO_2$ 287.1885, experimental 287.1890.

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, 2xCH_3), 1.34–1.56 (m, 8H, 2x$CH_2CH_2CH_3$), 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, CH_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$CH_2CH_2CH_3$), 29.3 (3,5,7-adamantyl C), 35.9 (2x$CH_2CH_2CH_3$), 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.

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AK designed this research project and with MS supervised the research. CT did most of the ligands synthesis for her PhD thesis; ALT in SV lab and GL, CL in AK lab prepared some additional compounds. AH and PS generated the mutant viruses with reverse genetics and did the antiviral testing in the MS lab. CM performed the EP measurements in JW lab. JB carried out DMPK studies. AK wrote the manuscript, ALT, SV, MS revised it and AK thoroughly revised it.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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