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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

Marta Barniol-Xicota[‡], Sabrina Gazzarrini[#], Eva Torres[‡], Yanmei Hu^{⊥,†}, Jun Wang^{⊥,†}, Lieve Naesens[§], Anna Moroni[#], and Santiago Vázquez^{‡,*}

[‡]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 Biosciences and National Research Council (CNR) Biophysics Institute (IBF), University of Milan, Via Celoria 26, 20133 Milan, Italy

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

[†]BI05 Institute, The University of Arizona, Tucson, Arizona 85721, United States

[§]Rega Institute for Medical Research, KU Leuven, B-3000 Leuven, Belgium

Abstract

New insights on the amantadine resistance mechanism of the V27A mutant were obtained through the study of novel, easily accessible 4-(1- and 2-adamanty) piperidines, identified as dual binders of the wild-type and V27A mutant M2 channels of influenza A virus. Their antiviral activity and channel blocking ability were determined using cell-based assays and two-electrode voltage clamp (TEVC) technique on M2 channels, respectively. In addition, electrophysiology experiments revealed two interesting findings: i) these inhibitors display a different behaviour against the wild-type *versus* V27A mutant A/M2 channels and, ii) the compounds display antiviral activity when have k_d equal or smaller than 10^{-6} while do not exhibit antiviral activity when k_d is 10^{-5} or higher although may show blocking activity in the TEV assay. Thus caution must be taken when predicting antiviral activity based on percent channel blockage in electrophysiological assays. These findings provide experimental evidence of the resistance mechanism of the V27A mutation to wild-type inhibitors, previously predicted *in silico*, offer an explanation for the lack of antiviral activity of compounds active in the TEV assay, and may help design new and more effective drugs.

*Corresponding Author: Phone: +34 934024533. svazquez@ub.edu.

ASSOCIATED CONTENT

Supporting Information.

The supporting information is available free of charge on the ACS Publications website at DOI:

Additional electrophysiological experiments. Plaque reduction assay with A/WSN/33 N31S/V27A (H1N1) virus. Elemental analysis data of the new compounds. (PDF)

Molecular formula string and some data (CSV)

Graphical Abstract

Keywords

Amantadine; drug design; influenza A virus; M2 proton channel; electrophysiology

Introduction

The target of amantadine (Amt) and rimantadine, two well-known clinically approved anti-influenza drugs,¹ is the M2 protein of influenza A virus (A/M2), a 97-residue integral membrane protein that forms an homotetrameric proton selective transmembrane channel with a critical role in the virus replication cycle.^{2–11} Unfortunately, most of the circulating strains are Amt-resistant,¹² which has compelled the Centers for Disease Control and Prevention to deter its use.¹³ Amt-resistant influenza strains possess one or more mutations in the M2 channel protein.^{14,15} Although several mutations appear to be viable *in vitro*,¹⁶ a recent analysis of 31,251 M2 protein sequences revealed that the most frequent Amt-resistant mutations in the circulating strains occur at position 31 (S31N) (~ 95%), followed by position 27 (V27A), while other mutations (L26F, A30T, G34E and L38F) were rare.¹² Although in this analysis the second most prevalent mutant, V27A, was only observed in about 1% of the sequences, other reports have pointed out that the V27A mutant occurred in 10–77% of influenza virus isolates, depending on the viral strain and season.^{17,18} Several findings add functional relevance to the V27A mutation: (i) among the most prevalent mutations, V27A is the only one proven to originate from drug selection pressure;¹⁹ (ii) while the S31N and L26F mutants are sensitive, to some extent, to Amt, this drug is completely ineffective against the V27A mutant M2 channel;¹⁶ (iii) recent studies have noticed an increased frequency of the Amt-resistant V27A/S31N double mutant;²⁰ and, (iv) finally, a virus harboring this V27A/S31N double mutant form of M2 displayed significantly higher mortality compared to wild-type (wt) virus in a mouse model.²¹

The design of new M2 inhibitors that address the problem of drug resistance is currently of utmost importance. Accordingly, efforts have been recently made to design small molecule inhibitors that target the drug-resistant forms of the influenza A/M2 proton channel.^{22,23} While numerous compounds targeting the wt channel have been synthesized,^{24–30} few dual inhibitors of the wt and either the V27A,^{31–36} or the S31N mutants,^{37–44} were only recently identified.

In 2011, Wang *et al.* used a computationally-driven design for the synthesis and pharmacological evaluation of the spiroadamante **1**, a triple inhibitor of the wt, V27A and

L26F mutant channels, that features a primary amine.^{32,36} Taking into account that the synthesis of **1** involves six steps from 2-adamantanone and that potent inhibition of the wt and V27A mutant channels was also achieved with secondary amines,^{33–35} we wondered whether the synthetically more accessible analogs of **1**, such as the piperidine derivatives **2** and **3** described herein, would have similar channel blockage and antiviral activity as compound **1**. Of note, it is known that Amt (1-aminoadamantane) is, in some influenza A strains, more potent than its 2-isomer (2-aminoadamantane).^{45–46} Specifically, the comparison of **2** and **3** would shed light on which position of the adamantane scaffold is more suitable for designing potent V27A inhibitors (Chart 1).

Results and discussion

Herein we describe the synthesis and pharmacological evaluation of the aforementioned piperidine derivatives **2** and **3** and some related amines and guanidines. The activity of the compounds has been tested against the wt M2 channel as well as the V27A and S31N mutant forms in the two-electrode voltage clamp (TEVC) assay. Several novel compounds were low micromolar blockers of the wt A/M2 channel and/or the V27A A/M2 variant. Surprisingly, while their blocking abilities in the wt M2 channel nicely correlated with their antiviral activity in a wt-containing influenza A virus, there was a lack of correlation between their V27A channel blockage and antiviral activity of a V27A-containing influenza A virus. Only the 1-adamantyl substituted derivative **2** showed minimal antiviral activity against a virus carrying the V27A A/M2 mutant channel, despite its potent channel blockage. Interestingly, electrophysiology experiments revealed that our dual inhibitors display distinct channel affinities, which translate in two opposite binding patterns,⁴⁷ explaining the puzzling antiviral activities observed. Overall, these findings add valuable insights into the drug-resistance mechanism postulated for the V27A mutation.^{48–49}

Chemistry

First, we undertook the synthesis of the 4-(1-adamantyl)piperidine, **2**, and its related compounds. Togo and coworkers had previously reported the synthesis of a mixture of the (1-adamantyl)pyridines **5** and **6** by radical decarboxylation of 1-adamantanecarboxylic acid, **4**, in the presence of pyridine.⁵⁰ In our hands, compounds **5** and **6** were obtained in a ratio 3 : 1 in 36% overall yield. Both isomers were easily separated by column chromatography. Catalytic hydrogenation of **5** and **6** gave the corresponding piperidines **2** and **8** in 97 and 99% yield, respectively. Kolocouris and coworkers previously reported the synthesis of **8** through a different route, and reported its activity against an H2N2 strain with a wt A/M2 channel.⁵¹ For this reason, we also evaluated compound **8** for M2 channel inhibition (see below), although its basic nitrogen atom must adopt a different orientation in the channel compared to known inhibitors.⁵²

While the reaction of amine **2** with 1*H*-pyrazole-1-carboxamide furnished guanidine **7** in 76% yield, several attempts directed towards the synthesis of the corresponding guanidine derived from **8** were unsuccessful, probably reflecting the greater steric congestion around this nitrogen atom (Scheme 1).⁵³

In order to synthesize piperidines **3** and **12**, we followed the pioneering work of Kolocouris *et al.* for the synthesis of the related 2-(2-adamantyl)piperidine,⁵⁴ but starting from 4-pyridyl lithium instead of 2-pyridyl lithium. The addition of 4-pyridyl lithium –synthesized *in situ* from air and light sensitive 4-bromopyridine–, to 2-adamantone, **9**, furnished the expected tertiary alcohol **10** in 70% yield. Catalytic hydrogenation of **10** or its hydrochloride under several conditions did not give the pure alcohol **11**, but a complex mixture of **3**, **11** and **12** along with other minor impurities, presumably arising from isomerization or rearrangement.⁵⁴ By crystallization from CH₂Cl₂, we were able to isolate the pure alcohol **11** from **3** and **12**. Several unsuccessful attempts were made to elicit **3** and **12** from the mother liquors (containing **3**, **12** and remaining **11**) by crystallization or column chromatography, but without success. Therefore, we decided to proceed directly to piperidine **3** by treating the mixture with a dehydrating agent, securing the conversion of **11** to **12**, followed by catalytic hydrogenation to solely yield the compound **3**. Of note, after several fruitless dehydration trials with the acknowledged Burgess' reagent or anhydrous oxalic acid, which was successfully applied to a very related alcohol,⁵⁵ we finally were able to dehydrate the tertiary alcohol in very high yields using an extremely inexpensive, facile and fast procedure reported by Álvarez-Manzaneda *et al.*,⁵⁶ which involves the use of a mixture of SOCl₂ and pyridine in CH₂Cl₂. The catalytic hydrogenation of the remaining mixture of **3** and **12**, furnished the piperidine **3** in nearly quantitative yield (Scheme 2).

Since water molecules in the M2 channel are believed to play an important role in assisting the binding of the amino group of the putative inhibitor,^{9,15,57} we decided to check the effect of introducing a tertiary amine in compound **3**. This was accomplished through the reductive alkylation of **3**, the assumption being that the ethyl derivative **13** should be less potent as M2 channel inhibitor than the secondary amine **3**. The reductive alkylation of **3** with acetaldehyde and NaBH₃CN in methanol in the presence of acetic acid led to **13**.

On the other hand, since the lumen of the V27A M2 protein is wider than that of the wt channel,^{58–59} size-expanded Amt derivatives may lead to better inhibition of the V27A mutant channel.^{31–36} Taking into account the poor inhibitory activity displayed by the first series (see discussion of inhibitory activity of **3** and **13** below), a second group of piperidine derivatives was envisaged from 2-adamantanone, **9**. The second series featured an increased length thanks to the introduction of an additional carbon atom between the piperidine and the adamantyl group. The addition of the organolithium derivative of 4-picoline to 2-adamantanone furnished alcohol **14** in 90% yield. The subsequent catalytic hydrogenation to the piperidine hydrochloride **15** elapsed in excellent yields. The dehydration of **15** using Álvarez-Manzaneda's procedure⁵⁶ led to alkene **16** in quantitative yield. Upon catalytic hydrogenation, **16** smoothly furnished **17** in 79% yield. Finally, guanidines **19** and **20** were synthesized from **16** and **17**, respectively, by reaction with 1*H*-pyrazole-carboxamide hydrochloride. The tertiary amine **18** was prepared through a reductive alkylation of **17** with formaldehyde in 84% yield (Scheme 2).

Inhibition of the wt A/M2 ion channel and amantadine-resistant mutant forms

The inhibitory activity of the compounds was tested on A/M2 channels expressed in *Xenopus laevis* oocytes, using the TEVC technique. All inhibitors were initially tested at

100 μ M. In the next step, the IC₅₀ values for the compounds that inhibited the V27A channel by more than 60% were obtained using an isochronic (2 min) inhibition assay. The results are given in Table 1. As reference, Amt inhibited the wt A/M2 channel with an IC₅₀ of 16.0 μ M, while displaying much lower activity against the S31N mutant channel (IC₅₀ of 200 μ M),⁶⁰ and being totally inactive against the V27A mutant.³³

For the wt M2 channel and any given scaffold, the guanidine derivative was more potent than the corresponding amine for the pairs **19** versus **16**, and **20** versus **17**, although such a difference was less apparent for the pair of compounds **7** versus **2**. Similar trends were also observed from the inhibitory data determined against the V27A mutant. Thus, the presence of the guanidinium fragment presumably offers a better anchoring to the inhibitors in the interior of the wt M2 channel, though this effect is more relevant for compounds with a non-linear structural scaffold, as found in **16** and **17**. These results are in line with those previously reported for other sets of molecules.^{33–34}

Moving the adamantane ring from position 2, as found in **8** and in the family of compounds reported by Kolocouris *et al.*,^{51,54} to position 4 of the piperidine, as in the rest of our new inhibitors, had a clear incremental effect on the inhibitory activity for both wt and V27A channels. Indeed, the inhibitory effect increases from 48.1 (**8**) to 92% (**2**) in the wt M2 channel, and from 10.1 (**8**) to 85.7% (**2**) in the V27A mutant M2 channel. On the other hand, the insertion of an extra carbon atom between the adamantane and piperidine rings (**3** versus **17**) seemed to have little influence on the inhibitory potency. A larger effect, however, was achieved in both channels when moving the anchoring point of the piperidine ring from the C-2 position of adamantane, as in **3**, to the C-1 position, as in **2** and **7**. This change triggered a substantial increase of the inhibitory activity against both wt and V27A channels, as noted in the 11- and 17-fold reduction of the IC₅₀ observed between compounds **3** and **2**. Unluckily, none of the tested molecules had activity against the S31N channel (<10%) (data not shown).

The electrophysiological inhibition assay for wt and mutant channels routinely uses a fixed time of 2 min to calculate the isochronal inhibitory constant (IC₅₀). As the amino alcohols **11** and **15** showed to be very poor inhibitors under these conditions, together with the fact that the bindings were far from saturated at 2 min, additional experiments were performed at longer times, taking **11** as a representative example. Even after 10 min, **11** inhibited only 30% of the wt channel current (data not shown). This observation suggests that the introduction of a hydroxyl group in the middle part of the molecule is deleterious for activity (**11** versus **3** or **15** versus **17**). Nevertheless, after incubation for 6 min compound **8** led to more than 90% inhibition of the wt channel (Figure 2D). This potent inhibition by compound **8** agrees with its activity in the cell-based antiviral assay (see below), in which the compound is applied for longer incubation times to achieve equilibrium. In contrast, the inhibition of the V27A M2 mutant channel with **8** was already at steady-state after just 1 min (Figure 2H).

Antiviral activity and cytotoxicity in cell culture

The anti-influenza virus activity of the compounds was determined in MDCK cells using two different assays, i.e. a 24-h virus yield assay (based on RT-qPCR quantification of virus in the supernatant), and a 72-h virus plaque reduction assay (PRA), both with the A/HK/7/87 virus (H3N2 subtype), which carries a wt M2 protein (Table 2). Guanidine derivatives **7**, **19** and **20** did not display antiviral activity (data not shown). In the PRA (Figure 1), amines **2** and **8** and amino alcohol **15** were found to be active, with **2** being equally potent as Amt ($EC_{50} = 0.14 \mu\text{M}$ for the two compounds in the PRA assay). Considering that compound **11**, an aminoalcohol like **15**, did not block the wt M2 channel in the TEVC assay even at long times, the antiviral activity of **15** in cell culture suggests the involvement of another mechanism of action, an observation that we previously made for other adamantane derivatives.^{46,61–62} In the case of compound **8**, its greater potency in the PRA compared to the electrophysiological method relates to its slow A/M2 binding kinetics (see Figure 2 and Table 3). Of note, the antiviral activity determined for **8** ($EC_{50} = 1.0 \mu\text{M}$) is very similar to the EC_{50} value of $3.3 \mu\text{M}$ that was reported for this compound by Kolocouris et al. in an assay with an H2N2 virus strain.⁵¹ In addition, the antiviral activity of **2** and **8** (but not compound **15**) was confirmed in our virus yield assay (Table 2). This antiviral effect was seen at concentrations well below the cytotoxic concentrations, since compound **2** had a selectivity index (ratio of CC_{50} to EC_{50} based on 72-h compound exposure time) of 128, and compound **8** was devoid of cytotoxicity at $100 \mu\text{M}$, the highest concentration tested.

The anti-influenza virus activity of compounds **2**, **3**, **7** and **19**, that inhibited the V27A channel (Table 1), was also determined in MDCK cells using a 46-h virus PRA with the A/WSN/33 virus (H1N1 subtype), which carries a N31S/V27A M2 protein. Compound **8**, that did not inhibit the V27A channel (Table 1 and Figure 2H) but showed antiviral activity against the A/HK/7/87, was also assayed. Surprisingly, in addition to **8**, compounds **3**, **7** and **19** were inactive and only a weak activity was found for compound **2** that clearly did not match its high potency in the TEVC assay (Table 1 and Figure S1).

Inhibition kinetics of the wt A/M2 ion channel and amantadine-resistant mutant forms

To further characterize the inhibitory effects on wt and V27A channels and rationalize the antiviral results, the current kinetics of the most potent channel blockers **2**, **7**, **8**, **19** and **20** were determined and compared with the profile obtained for Amt (Figure 2 and Table 3; see also results for **19** and **20** in SI). Analysis of the curves for the wt channel (left panels in Figure 2) showed that the forward rate constant (k_{on}) for association and inhibition by compound **7** ($654 \pm 51 \text{ M}^{-1} \text{ s}^{-1}$) was around 2.3-fold faster than the values obtained for compound **2** ($259 \pm 29 \text{ M}^{-1} \text{ s}^{-1}$) and Amt ($293 \pm 37 \text{ M}^{-1} \text{ s}^{-1}$). At saturating compound concentrations ($100 \mu\text{M}$), the slow k_{off} -rate found for compounds **2** and **8** identified them as strong binders, since that resembles the behavior of Amt, which has a k_{off} -rate of $3 \times 10^{-4} \text{ s}^{-1}$ (Figure 2A–B).⁴⁷ Assuming that the inhibition by compounds **2** and **8** might be partially reversible,⁴⁷ we performed several experiments at low inhibitor concentrations for obtaining the K_{off} -rate, but we found that, *within the time frame of the experiments* (ca. 50–60 min), the inhibition by both compounds was essentially irreversible (see Figure S4).³⁶ Contrarily, compound **7** dissociated from the channel after 5 min, an observation that may explain the

failure to display antiviral activity in the PRA despite being able to block the channel (Figure 2C). This result is consistent with the 33-fold slower k_{off} found for Amt ($3 \times 10^{-4} \text{ s}^{-1}$)⁴⁷ relative to compound **7** (10^{-2} s^{-1}) (Table 3). Compounds **19** and **20**, with rapid k_{on} and k_{off} values, displayed the same weak binding affinity as **7** (see Figures S2 and S3).

In the case of the V27A mutant channel (right panels in Figure 2), compounds **2** and **7** had higher affinity (IC_{50} of 3.6 ± 0.6 and $16.2 \pm 1.7 \text{ }\mu\text{M}$, respectively) compared with Amt ($IC_{50} > 500 \text{ }\mu\text{M}$)³³ and compound **8** ($IC_{50} > 500 \text{ }\mu\text{M}$) (Table 1). Despite **2** and **7** showed greater k_{on} rate constants compared to Amt and **8**, which allowed them to display channel blockade at the 2 min assay (unlike Amt or **8**), all four compounds have similar k_{off} . Hence, although compounds **2** and **7** can bind the V27A channel (Table 1), i. e., they show a fast association that allows to follow the inhibition of the proton current, the weak binding affinity facilitates dissociation during washing with drug-free pH 8.5 buffer, rendering the channel functional again (Figure 2E–H and Table 3).^{36,47} This behavior agrees with the resistance mechanism proposed for Amt in the V27A mutant.⁴⁹ Interestingly, the case of compound **2** clearly exemplifies the different outcomes in the wt and V27A channels: although this compound has similar IC_{50} values (4.1 and $3.6 \text{ }\mu\text{M}$, respectively, Table 1), the faster k_{off} in the mutated channel, probably reflecting a worse fitting to the wider pore,^{58–59} allows the compound to be washed away, revoking the channel blockade. This fact correlates with the antiviral activity assays (see above), justifying that some compounds with promising V27A mutant M2 channel blockade activities fail to behave as good antivirals likely due to fast dissociation (fast k_{off} rate).

Conclusions

Several adamantane derivatives have been synthesized and tested as potential inhibitors of the influenza A virus. The novel 4-(1-adamantyl)piperidine **2** and its guanidine derivative **7**, were low micromolar blockers of the wt and V27A mutant M2 channels. The following SAR trends were observed: (i) alkylation of a secondary amine to a tertiary amine reduced the activity (**3** vs **13** and **17** vs **18**); (ii) switching the adamantane ring from position 2 to position 4 of the piperidine, triggered faster association rate to the channel (**8** vs **2**); (iii) moving the piperidine ring from the C-2 position of the adamantane, as in **3**, to the C-1 position, as in **2**, led to an increase in the inhibitory activity on both channels. The inhibitory activity of **2** was confirmed in cell-based antiviral (virus plaque reduction and virus yield) assays. Of note, the most potent compound in the antiviral assays, amine **2**, is synthetically accessible in just two steps from commercially available precursors and has a selectivity index of 128 (Table 2).

Remarkably our electrophysiological experiments have revealed some unexpected differences among structurally very similar compounds. Namely, amine **2** is a strong binder of the wt channel ($IC_{50} = 3.6 \text{ }\mu\text{M}$, Table 1, see also Figure 2B), while its binding being fast and weak in the V27A channel (Figure 2F). Its guanidine derivative, **7**, behaves in both channels as a fast and weak binder (Figures 2C and 2G), meanwhile the compound **8**, its 2-piperidine isomer, shows a very slow but strong binding with the wt M2 channel (Figure 2D) and no remarkable binding at all with the V27A mutant channel (Figure 2H).

Building up from these findings we can conclude that: (1) M2 channel blockers with potent influenza A antiviral activity usually show steady binding to the channel ($k_{on} \gg k_{off}$),⁴⁷ (2) caution has to be taken when defining M2 inhibitors solely by isochronic inhibition assays at 2 min, M2 channel blockers are very slow binders, so the IC₅₀ depends on the amount of time the drug is exposed to the target. In our case a good correlation between the TEVC and the antiviral activity for the amine **2** (IC₅₀ = 4.1 μM and EC₅₀ = 0.14 μM, respectively) was observed in inhibiting wt M2 (because in this case $k_{on} \gg k_{off}$). Nevertheless, while all the guanidine derivatives were pointed out to be potent dual inhibitors of the M2 and V27A channels by TEVC assays, their inactivity in the plaque reduction assays proved the TEVC predictions to be completely wrong. On top of that, the TEVC assay failed, under the standard 2 min conditions, to identify **8**, shown to be one of the most promising antivirals synthesized in this work (see Tables 1 and 2). (3) The weak affinity observed in our compounds for the V27A A/M2 mutant channel, goes in line with the proposed drug resistance mechanism by in silico^{11,59,64–65} and structural⁵⁸ approaches. This is the first time the drug resistance mechanism of the V27A A/M2 mutant channel is experimentally evidenced. Hence, our findings not only reinforce the previous in silico hypothesis but also lead to a more profound understanding of the influenza A virus resistance mechanism and to potential avenues for M2 inhibitor design.

Experimental section

Chemical Synthesis

General Methods—Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp. 400 MHz ¹H/100.6 MHz ¹³C NMR spectra, and 500 MHz ¹H NMR spectra were recorded on Varian Gemini 300, Varian Mercury 400, and Varian Inova 500 spectrometers, respectively. The chemical shifts are reported in ppm (δ scale) relative to internal tetramethylsilane, and coupling constants are reported in Hertz (Hz). Assignments given for the NMR spectra of the new compounds have been carried out on the basis of DEPT, COSY ¹H/¹H (standard procedures), and COSY ¹H/¹³C (gHSQC and gHMBC sequences) experiments. IR spectra were run on Perkin-Elmer Spectrum RX I spectrophotometer. Absorption values are expressed as wave-numbers (cm⁻¹); only significant absorption bands are given. The GC/MS analysis was carried out in an inert Agilent Technologies 5975 gas chromatograph equipped with an Agilent 122–5532 DB-5MS 1b (30 m × 0.25 mm) capillary column with a stationary phase of phenylmethylsilicon (5% diphenyl – 95% dimethylpolysiloxane), using the following conditions: initial temperature of 50 °C (1 min), with a gradient of 10 °C / min up to 300 °C, and a temperature in the source of 250 °C. *Solvent Delay* (SD) of 4 minutes and a pressure of 7,35 psi. Column chromatography was performed on silica gel 60 AC.C (35–70 mesh, SDS, ref 2000027). Thin-layer chromatography was performed with aluminum-backed sheets with silica gel 60 F₂₅₄ (Merck, ref 1.05554), and spots were visualized with UV light and 1% aqueous solution of KMnO₄. The analytical samples of all of the new compounds which were subjected to pharmacological evaluation possessed purity 95% as evidenced by their elemental analyses.⁶⁶

Synthesis of 4-(1-adamantyl)piperidine hydrochloride, 2·HCl—A solution of compound **6** (830 mg, 3.9 mmol) and PtO₂ at 10% (83 mg) in methanol (100 mL) containing few drops of HCl was hydrogenated at room temperature, at a pressure of 30 atm until no more hydrogen absorption was observed. The resulting suspension was filtered and the residue washed with methanol. The solvent was removed under vacuo to obtain amine **2·HCl** (970 mg, 97% yield) as a white solid. The analytical sample was obtained by crystallization from 2-propanol, mp 272–273 °C (dec). IR (KBr) 3674, 2900, 2846, 2728, 2671, 2503, 1597, 1446, 1396, 1344, 1075, 1023, 960 cm⁻¹. ¹H-NMR (500 MHz, CD₃OD) δ 1.20 (tt, *J* = 12.3 Hz, *J'* = 3.2 Hz, 1 H, 4-H), 1.49 [qd, *J* = 14.0 Hz, *J'* = 4.0 Hz, 2 H, 3(5)-H_{ax}], 1.57 [d, *J* = 2.6 Hz, 6 H, 2' (8', 9')], 1.68 (d, *J* = 11.3 Hz, 3 H) and 1.77 (d, *J* = 12.1 Hz, 3 H) [4' (6', 10')-H_{exo} and H_{endo}], 1.94 [bs, 2 H, 3(5)-H_{eq}], 1.99 [bs, 3 H, 3' (5', 7')-H], 2.91 [dt, *J* = 13.1 Hz, *J'* = 2.9 Hz, 2 H, 2(6)-H_{ax}], 3.42 [dt, *J* = 12.4 Hz, *J'* = 2.2 Hz, 2 H, 2(6)-H_{eq}]. ¹³C-NMR (125.7 MHz, CD₃OD) δ 23.6 [CH₂, C3(5)], 30.1 [CH, C3' (5', 7')], 35.0 (C, 1'), 38.3 [CH₂, C4' (6', 10')], 40.3 [CH₂, C2' (8', 9')], 45.8 (CH, C4), 46.0 [CH₂, C2(6)]. MS (EI, m/e (%); main ions: 219 (M⁺, 100), 218 (21), 205 (60), 163 (15), 135 (C₁₀H₁₅⁺, 83), 93 (27), 91 (19), 85 (83), 84 (50), 83 (21), 79 (33), 77 (16), 58 (18), 57 (48), 56 (25), 55 (21).

4-(Adamantan-2-yl)piperidine hydrochloride, 3·HCl—SOCl₂ (0.42 mL, 5.8 mmol) was slowly added to a mixture of **3**, **11** and **12** (850 mg; ca 3.9 mmol) and pyridine (0.84 mL) in anhydrous CH₂Cl₂ (8 mL) at -60 °C. The reaction mixture was stirred at this temperature under argon atmosphere for 30 min and quenched with sat. aq. Solution of NaHCO₃ (1 mL) and the cooling bath was removed. The mixture was poured into diethyl ether-water (90 mL : 20 mL) and the phases were shaken and separated. The organic phases were reunited, washed with brine, dried over Na₂SO₄ and concentrated to dryness under reduced pressure. The yellow solid residue (a mixture of **3** and **12**, GC/MS) was solved in methanol containing few drops of conc. HCl and hydrogenated with Pd/C (460 mg) at room temperature and 1 atm pressure of H₂, for 2 hours. After filtration of the catalyst, the solvent was concentrated under reduced pressure and the white solid obtained was washed with pentane, giving 183 mg (21% yield) of the piperidine **3·HCl**. An analytical sample was obtained by crystallization from dichloromethane, mp 252–254 °C. IR (ATR) 3311, 2931, 2911, 2850, 2820, 2785, 2724, 2501, 1598, 1451, 1423, 1302, 1279, 1041, 983 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 1.21–2.02 (complex signal, 20 H, adamantyl-H, 4-H and 3(5)-H₂), 2.91 [td, *J* = 12.4 Hz, *J'* = 3.2 Hz, 2 H, 2(6)-H_{ax}], 3.34 [broad d, 2 H, *J* = 12.4 Hz, 2(6)-H_{eq}]. ¹³C NMR (100.6 MHz, CDCl₃) δ 28.6 [CH₂, C3(5)], 29.1 (CH) and 29.5 (CH) (C5' and C7'), 29.7 [CH, C1' (3')], 32.7 [CH₂, C4' (9') or C8' (10')], 34.6 (CH, C4), 39.3 (CH₂, C6'), 40.2 [CH₂, C8' (10') or C4' (9')], 45.8 [CH₂, C2(6)], 50.4 (CH, C2'). GC/MS (EI, m/e (%); main ions: 219 (M⁺, 17), 204 (100), 174 (20), 162 (93), 84 (32), 82 (18), 79 (20), 57 (22), 56 (27). HRMS-ESI+ m/z [M+H]⁺ calcd for [C₁₅H₂₅N+H⁺]: 220.2060, found: 220.2059.

4-(1-adamantyl)pyridine, 5 and 2-(1-adamantyl)pyridine, 6—A solution of 1-adamantanecarboxylic acid, **4**, (11.35 g, 63.0 mmol), pyridine (5.1 mL, 63.0 mmol), bis[(trifluoroacetoxy)iodo]benzene (9.34 g, 21.8 mmol) in anhydrous benzene (100 mL) was heated under reflux overnight. The reaction was cooled down and the organic phase was

washed with a 10 N aqueous solution of NaOH (2×100 mL) and the aqueous layer was washed with AcOEt (2×100 mL). The combined organic extracts were washed with a 1 N solution of HCl (3×100 mL) and the aqueous was neutralized to pH = 14 with a 10 N solution of NaOH. Then, the aqueous layer was extracted with AcOEt (3×100 mL) and the organic layer was dried over anhydrous Na_2SO_4 , filtered and concentrated under vacuo to obtain a mixture of pyridines **5** and **6**. This mixture was purified by column chromatography (SiO_2). Compound **5** was obtained (CH_2Cl_2 :MeOH 98.5:1.5, 830 mg, 9.3% yield) as a beige solid. Compound **6** was obtained (CH_2Cl_2 :MeOH 99.5:0.5, 830 mg, 27.4% yield) as a white solid. The spectroscopical data of both compounds matched the previously reported.⁵⁰

Synthesis of *N*-amidyl-4-(1-adamantyl)piperidine hydrochloride, 7·HCl—In a 10 mL round-bottomed flask equipped with a condenser and a magnetic stirrer, a suspension of amine **2·HCl** (484 mg, 1.9 mmol), 1*H*-pyrazole-1-carboxamide hydrochloride (333 mg, 2.3 mmol), anhydrous triethylamine (0.47 mL) in acetonitrile (5 mL) was prepared. The reaction was heated at 70 °C for 6 hours. The reaction was cooled down and stored at 4 °C overnight. The suspension was filtered out and the filtrate washed with cold acetonitrile obtaining the guanidine **7·HCl** as a beige solid (428 mg, 76% yield). The analytical sample was obtained by crystallization from 2-propanol, mp > 300 (dec.). IR (KBr) 3112, 2898, 2845, 1639, 1597, 1526, 1446, 1345, 1195, 1160, 967, 743 cm^{-1} . ^1H -NMR (400 MHz, CD_3OD) δ 1.18 (tt, $J = 12.4$ Hz, $J' = 3.2$ Hz, 1 H, 4-H), 1.31 [qd, $J = 13.2$ Hz, $J' = 3.6$ Hz, 2 H, 3(5)- H_{ax}], 1.57 [d, $J = 2.4$ Hz, 6 H, 2' (8', 9')], 1.68 (d, $J = 12.0$ Hz, 3 H) and 1.76 (d, $J = 12.0$ Hz, 3 H) [4' (6', 10')- H_{exo} and H_{endo}], 1.83 [dd, $J = 13.2$ Hz, $J' = 1.6$ Hz, 2 H, 3(5)- H_{eq}], 1.98 [bs, 3 H, 3' (5', 7')-H], 2.98 [td, $J = 13.2$ Hz, $J' = 2.0$ Hz, 2 H, 2(6)- H_{ax}], 3.93 [dt, $J = 13.2$ Hz, $J' = 2.4$ Hz, 2 H, 2(6)- H_{eq}]. ^{13}C -NMR (100.6 MHz, CD_3OD) δ 26.1 [CH_2 , C3(5)], 30.1 [CH , C3' (5', 7')], 35.0 (C, 1'), 38.3 [CH_2 , C4' (6', 10')], 40.5 [CH_2 , C2' (8', 9')], 47.5 (CH, C4), 47.6 [CH_2 , C2(6)], 157.4 (C, C=NH). MS (EI), m/e (%); main ions: 261 (M^+ , 2), 219 (13), 135 ($\text{C}_{10}\text{H}_{15}^+$, 30), 129 (96), 128 (100), 127 (38), 101 (11), 93 (17), 91 (16), 88 (16), 87 (17), 86 (15), 85 (15), 79 (23), 77 (12), 75 (13), 74 (14), 67 (11), 57 (24), 56 (18), 55 (16).

Synthesis of 2-(1-adamantyl)piperidine hydrochloride, 8·HCl—From a solution of compound **6** (2.25 g, 10.5 mol) and PtO_2 at 10% (225 mg) in MeOH (100 mL) containing few drops of HCl and following the same procedure than the previously mentioned for the synthesis of **2·HCl**, amine **8·HCl** (2.67 g, 99% yield) was obtained as a white solid, 262–265 °C (dec). IR (ATR) 3392, 2911, 2855, 2754, 2698, 2481, 1585, 1431, 1413, 1375, 1342, 1304, 1276, 1054, 1016, 971, 814 cm^{-1} . ^1H -NMR (400 MHz, CD_3OD) δ 1.40–2.04 [complex signal, 18 H, 3- H_2 , 4- H_2 , 5- H_2 , 2' (8', 9')- H_2 , 4' (6', 10')- H_2], 2.06 [broad s, 3 H, 3' (5', 7')-H], 2.71 (dd, $J = 11.6$, $J' = 2.4$ Hz, 1 H, 2-H), 3.00 (dt, $J = 13.0$ Hz, $J' = 3.2$ Hz, 1 H, 6- H_{ax}), 3.41 (dm, $J = 13.0$ Hz, 1 H, 6- H_{eq}). ^{13}C -NMR (100.6 MHz, CD_3OD) δ 23.6 (CH_2), 23.8 (CH_2) and 23.9 (CH_2) (C3, C4 and C5), 29.6 [CH , C3' (5', 7')], 36.0 (C, 1'), 37.6 [CH_2 , C4' (6', 10')], 39.1 [CH_2 , C2' (8', 9')], 47.6 (CH_2 , C6), 46.0 (CH, C2). HRMS-ESI⁺ m/z [$\text{M}+\text{H}$]⁺ calcd for [$\text{C}_{15}\text{H}_{25}\text{N}+\text{H}$]⁺: 220.2060, found: 220.2061.

2-(4-Pyridyl)adamantan-2-ol, 10—To a 2.5 M solution of *n*-butyllithium in hexane (11.7 mL, 29.25 mmol) was added dropwise with stirring at –75 °C, a solution of the released base,

4-bromopyridine (5.048 g, 31.95 mmol) in anhydrous ether (80 mL) under argon atmosphere, giving a fuchsia light solution that was further stirred at $-65\text{ }^{\circ}\text{C}$ for 20 min. Afterwards a solution of 2-adamantanone (4 g, 26.6 mmol) in anhydrous ether (60 mL) and THF (2 mL) was added dropwise. The stirring was continued for 3 hours, after which the pink mixture was then allowed to gradually warm to ambient temperature and left stirring overnight. The reaction mixture was quenched with water (40 mL) and poured into a 5 N HCl solution (40 mL) under ice-cooling. The solution was stirred for 20 minutes and the acidic aqueous phase were separated ($2 \times 40\text{ mL}$) and basified with NaOH 5N (100 mL) over an ice bath. The resultant solid was filtered while cold under vacuum and washed with cold pentane and dried to give **10** was obtained (4.3 g, 70% yield) as a white solid, $184\text{--}186\text{ }^{\circ}\text{C}$. IR (ATR) 2906, 2860, 1686, 1600, 1539, 1451, 1410, 1345, 1276, 1241, 983, 842 cm^{-1} . ^1H NMR (400 MHz, CD_3OD) δ 1.56 (broad d, $J = 12.8\text{ Hz}$, 2 H, 6- H_2), 1.74–1.93 [complex signal, 8 H, 4(9)- H_2 and 8(10)- H_2], 2.48 [d, $J = 12.4\text{ Hz}$, 2 H, 5(7)-H], 2.61 [broad s, 2 H, 1(3)-H], 8.20 [d, $J = 6.6\text{ Hz}$, 2 H, 3' (5')-H], 8.82 [d, $J = 6.6\text{ Hz}$, 2' (6')-H]. ^{13}C NMR (100.6 MHz, CD_3OD) δ 28.1 (CH) and 28.6 (CH) (C5 and C7), 33.5 (CH_2) and 35.5 (CH_2) [C4(9) and C8(10)], 35.9 [CH, C1(3)], 38.3 (CH_2 , C6), 75.8 (C, C2), 125.9 [CH, C3' (5')], 143.1 [CH, C2' (6')], 168.3 (C, C4'). MS (EI), m/e (%); main ions: 229 (M^+ , 29), 212 (14), 211 [$(\text{M}-\text{H}_2\text{O})^+$, 72], 151 (100), 109 (20), 106 (32), 93 (20), 91 (16), 81 (30), 80 (50), 79 (93), 78 (23), 77 (16), 67 (17).

2-(4-Piperidiny)adamantan-2-ol, 11—A solution of the aminoalcohol **10** (980 mg, 4.27 mmol) in absolute ethanol, was hydrogenated over PtO_2 (100 mg) at room temperature and 1 atm pressure for 24 hours. After filtration in order to remove the catalyst, it was concentrated under reduced pressure and the white solid obtained, washed with pentane, giving 930 mg (approx. 93%) of a mixture of **11**, **3** and **12**. An analytical sample of **11** was obtained by crystallization from dichloromethane, $\text{mp} > 231\text{ }^{\circ}\text{C}$ (dec.); IR (ATR) 3427, 2906, 2850, 2794, 2729, 2663, 2501, 1694, 1590, 1451, 1433, 1410, 1357, 1327, 1287, 1137, 1054, 1034, 991, 950, 928, 756 cm^{-1} . ^1H NMR (400 MHz, CDCl_3) δ 1.44–1.94 (complex signal, 16 H, adamantyl-H and 3' (5')- H_2), 2.08 (tt, $J = 11.6\text{ Hz}$, $J' = 3.6\text{ Hz}$, 1 H, 4'-H), 2.13 [d, $J = 12.4\text{ Hz}$, 2 H, adamantyl-H], 2.4–2.7 (very broad signal, 2 H, NH and OH), 2.67 [td, 2 H, $J = 12.4\text{ Hz}$, $J' = 2.8\text{ Hz}$, 2' (6')- H_{ax}], 3.37 [broad d, 2 H, $J = 12.4\text{ Hz}$, 2' (6')- H_{eq}]. ^{13}C NMR (100.6 MHz, CDCl_3) δ 24.2 [CH_2 , C3' (5')], 26.9 (CH) and 27.2 (CH) (C5 and C7), 33.0 [CH_2 , C4(9) or C8(10)], 33.6 [CH, C1(3)], 33.7 [CH_2 , C8(10) or C4(9)], 38.1 (CH, C4'), 38.2 (CH_2 , C6), 46.4 [CH_2 , C2' (6')], 75.0 (C, C2). GC/MS (EI), m/e (%); main ions: 235 (M^+ , 9), 234 (42), 219 (18), 218 (100), 216 (28), 151 (24). HRMS-ESI $^+$ m/z [$\text{M}+\text{H}$] $^+$ calcd for $[\text{C}_{15}\text{H}_{25}\text{NO}+\text{H}^+]$: 236.2009, found: 236.2011.

Synthesis of 4-(adamantan-2-yl)-1-ethylpiperidine hydrochloride, 13-HCl—To a solution of **3-HCl** (100 mg, 0.39 mmol) in water (5 mL) was added a 10 N aqueous solution of NaOH. It was then extracted with EtOAc ($3 \times 5\text{ mL}$) and the organic phase was dried over Na_2SO_4 , filtered and concentrated under vacuo (yellowish oil, 85 mg, quantitative yield). This oil was dissolved in MeOH (5 mL) and sodium cyanoborohydride (73.5 mg, 1.17 mmol), acetaldehyde (21 mg, 0.48 mmol) and glacial acetic acid (0.1 mL) were added to the solution. The solution was stirred at room temperature for 8 hours and a second portion of sodium cyanoborohydride (73.5 mg, 1.17 mmol) and acetaldehyde (21 mg, 0.48 mmol) were

added. The yellow solution was further stirred at room temperature overnight. It was concentrated under vacuo and the yellow oil was solved in water (5 mL) and extracted with EtOAc (3 × 5 mL). The organic phase was dried over Na₂SO₄, filtered and concentrated under vacuo. Column chromatography of the solid (silica gel, hexane/EtOAc mixtures) gave the pure amine as an off-white solid (84 mg, 76%). An analytical sample of the hydrochloride was obtained by adding an excess of Et₂O·HCl to a solution of **13** in EtOAc followed by filtration of the obtained solid, mp > 300 °C (dec.); IR (ATR) 3336, 2911, 2850, 2643, 2531, 1648, 1453, 1433, 1393, 1244, 1107, 976 cm⁻¹. ¹H NMR (400 MHz, CD₃OD) δ 1.35 (t, *J* = 7.4 Hz, 3 H, NCH₂CH₃), 1.58 [d, *J* = 12.4 Hz, 2 H, 4' (9')-H_a or 8' (10')-H_a], 1.74 [d, *J* = 10.4 Hz, 2 H, 8' (10')-H_a or 4' (9')-H_a], 1.76–1.95 [complex signal, 13 H, 3(5)-H₂ and 9-adamantyl-H], 2.09 (m, 1 H) and 2.12 (m, 1 H) (1'-H and 3'-H), 2.93 [td, *J* = 12.6 Hz, *J'* = 2.4 Hz, 2 H, 2(6)-H_{ax}], 3.15 (q, *J* = 7.4 Hz, 2 H, NCH₂CH₃), 3.58 [dm, *J* = 12.6 Hz, 2 H, 2(6)-H_{eq}]. ¹³C NMR (100.6 MHz, CD₃OD) δ 9.6 (CH₃, NCH₂CH₃), 28.9 [(CH₂, C3(5))], 29.1 (CH) and 29.4 (CH) (C5' and C7'), 29.9 [CH, C1' (3')], 32.7 [CH₂, C4' (9') or C8' (10')], 34.2 (CH, C4), 39.2 (CH₂, C6'), 40.1 [CH₂, C8' (10') or C4' (9')], 50.2 (CH, C2'), 53.3 (CH₂, NCH₂CH₃), 53.7 [CH₂, C2(6)]. GC/MS (EI), *m/e* (%); main ions: 247 (M⁺, 10), 233 (18), 232 (100), 58 (23). HRMS-ESI⁺ *m/z* [M+H]⁺ calcd for [C₁₇H₂₉N+H⁺]: 248.2373, found: 248.2374.

Synthesis of 2-(pyridin-4-ylmethyl)adamantan-2-ol, 14—To a solution of 4-picoline (3.1 g, 33.3 mmol) in anhydrous THF (25 mL) at -20 °C, *n*-butyllithium (13.4 mL, 2.5 N in hexanes, 33.5 mmol) was added dropwise. When the addition was over, the mixture was allowed to reach room temperature and a solution of 2-adamantanone (5.0 g, 33.3 mmol) in anhydrous THF (18 mL) was added dropwise during 15 minutes and left stirring for 2 hours. After quenching with water (5 mL), the THF was evaporated. Diethyl ether was added, layers were separated and the aqueous one was washed with water (1 × 15 mL) and extracted with HCl 5N (3 × 15 mL). The aqueous extract was basified with solid Na₂CO₃. A white solid precipitated that was filtered, washed with water and dried to give pure **14** (7.3 g, 90% yield), mp 202–203 °C (dec.). IR (ATR) 3721, 3215, 2906, 1691, 1603, 1423, 1345, 1236, 1170, 1001, 958, 829, 753 cm⁻¹. ¹H NMR (400 MHz, CD₃OD) δ 1.53 [d, *J* = 12.6 Hz, 2 H, 4(9)-H_a or 8(10)-H_a], 1.62 [broad s, 2 H, 1(3)-H], 1.74–1.85 (complex signal, 5 H, 6-H₂, 8(10)-H_a or 4(9)-H_a and 5-H or 7-H], 1.91 (m, 1 H, 7-H or 5-H), 2.15 [broad d, *J* = 10.8 Hz, 2 H, 8(10)-H_b or 4(9)-H_b], 2.24 (broad d, *J* = 12.6 Hz, 2 H, 4(9)-H_b or 8(10)-H_b), 3.04 (s, 2 H, 2C-CH₂), 7.34 [m, 2 H, 3' (5')-H], 8.39 [m, 2 H, 2' (6')-H]. ¹³C NMR (100.6 MHz, CD₃OD) δ 28.8 (CH) and 28.9 (CH) (C5 and C7), 33.9 (CH₂) and 35.5 (CH₂) [C4(9) and C8(10)], 38.0 [CH, C1(3)], 39.6 (CH₂, C6), 44.6 (CH₂, 2C-CH₂), 76.1 (C, 2C), 128.0 [CH, C3' (5')], 149.1 [CH, C2' (6')], 150.4 (C, C4'). HRMS-ESI⁺ *m/z* [M+H]⁺ calcd for [C₁₆H₂₁NO+H⁺]: 244.1696, found: 244.1693.

Synthesis of 2-(piperidin-4-ylmethyl)adamantan-2-ol hydrochloride, 15·HCl—A solution of **14** (3.0 g, 12.3 mmol) and conc HCl (6 mL, 72 mmol) in methanol (50 mL), was hydrogenated over PtO₂ (50 mg) at room temperature and 1 atm for 5 days in which H₂ was recharged. After filtration in order to remove the catalyst, it was concentrated under reduced pressure, obtaining the desired product **15·HCl** as a white solid in quantitative yield. mp > 300 °C (dec.) IR (ATR) 3407, 2901, 2845, 2789, 2729, 2516, 2156, 1595, 1448, 1350, 1282,

1254, 1127, 1044, 1019, 1006, 958, 930 cm^{-1} . ^1H NMR (400 MHz, CDCl_3) δ 1.47 [m, 2 H, 3' (5')- H_{ax}], 1.54 [m, 2 H, 4(9)- H_a or 8(10)- H_a], 1.68 (d, $J = 5.6$ Hz, 2 H, 2C- CH_2), 1.70–1.94 (complex signal, 11 H, 5-H, 7-H, 4'-H, 1(3)-H, 6- H_2 , 4(9)- H_b or 8(10)- H_b and 8(10)- H_a or 4(9)- H_a], 2.02 [dm, $J = 13.2$ Hz, 2 H, 3' (5')- H_{eq}], 2.29 [dm, $J = 12.0$ Hz, 2 H, 8(10)- H_b or 4(9)- H_b], 2.98 [td, $J = 12.4$ Hz, $J' = 3.2$ Hz, 2 H, 2' (6')- H_{ax}], 3.34 [broad d, 2 H, $J = 12.4$ Hz, 2' (6')- H_{eq}]. ^{13}C NMR (100.6 MHz, CDCl_3) δ 28.9 (CH) and 29.9 (CH) (C5 and C7), 30.1 (CH, C4'), 32.0 [CH_2 , C3' (5')], 33.8 [CH_2 , C4(9) or C8(10)], 35.6 [CH_2 , C8(10) or C4(9)], 38.4 [CH, C1(3)], 39.5 (CH_2 , C6), 45.1 (CH_2 , C2- CH_2), 45.4 [CH_2 , C2' (6')], 76.4 (CH, C2). HRMS-ESI⁺ m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{16}\text{H}_{27}\text{NO}+\text{H}^+]$: 250.2165, found: 250.2170.

Synthesis of 4-[(adamantan-2-ylidene)methyl]piperidine hydrochloride, 16-HCl

— SOCl_2 (0.42 mL, 5.78 mmol) was slowly added to a solution of **15**·HCl (1.0 g, 3.50 mmol) and pyridine (0.84 mL) in dry CH_2Cl_2 (8 mL) at -60°C . The reaction mixture was stirred at this temperature under argon atmosphere for 30 min and quenched with saturated aqueous solution of NaHCO_3 (1 mL) and the cooling bath was removed. The mixture was poured into diethyl ether-water (90 mL : 20 mL) and the phases were shaken and separated. The aqueous layer was further extracted with diethyl ether twice. The organic phases were joined, washed with brine, dried over Na_2SO_4 and evaporated to give **16** as a white solid in quantitative yield. An analytical sample of the hydrochloride was obtained by adding an excess of $\text{Et}_2\text{O}\cdot\text{HCl}$ to a solution of **16** in diethyl ether, mp $> 300^\circ\text{C}$ (dec.). IR (ATR) 3397, 2921, 2845, 2718, 2658, 2541, 2506, 1663, 1625, 1595, 1527, 1469, 1443, 1421, 1241, 1100, 1077, 976, 930, 849, 806 cm^{-1} . ^1H -NMR (400 MHz, CD_3OD) δ : 1.44 [dq, 2 H, $J = 4.0$ Hz, $J' = 12.6$ Hz, 3(5)- H_{ax}], 1.68–1.82 (complex signal, 6 H, 3(5)- H_{eq} , 4' (10')- H_a and 8' (9')- H_a], 1.87 [broad s, 2 H, 5' (7')-H], 1.88–1.96 (complex signal, 6 H, 6'- H_2 , 4' (10')- H_b and 8' (9')- H_b], 2.30 (broad s, 1 H, 1'-H), 2.51 (m, 1 H, 4-H), 2.85 (broad s, 1 H, 3'-H), 2.88 [dt, 3 H, $J = 12.8$ Hz, $J = 2.8$ Hz, 2(6)- H_{ax}], 3.26 [dm, 2 H, $J = 12.8$ Hz, 2(6)- H_{eq}], 4.90 (d, 1 H, $J = 8.8$ Hz, C2'=CH). ^{13}C -NMR (100.6 MHz, CD_3OD) δ : 29.9 [CH, C5' (7')], 31.8 [CH_2 , C3(5)], 33.2 (CH, C4), 34.1 (CH, C3'), 38.2 (CH_2 , C6'), 40.2 [CH_2 , C4' (10') or C8' (9')], 40.9 [CH_2 , C8' (9') or C4' (10')], 41.9 (CH, C1'), 45.4 [CH_2 , C2(6)], 121.1 (CH, C2'=CH), 149.8 (C, C2'). GC/MS (EI), m/e (%); main ions: 231 (M^+ , 5), 83 (10), 82 (100), 57 (12). HRMS-ESI⁺ m/z $[\text{M}+\text{H}]^+$ calcd for $[\text{C}_{16}\text{H}_{25}\text{N}+\text{H}^+]$: 232.2060, found: 232.2062.

Synthesis of 4-[(adamantan-2-yl)methyl]piperidine hydrochloride, 17-HCl—A

solution of **16** (230 mg; 1 mmol) in methanol containing few drops of conc HCl, was hydrogenated over Pd/C (5% Pd, 23 mg) at room temperature and 1 atm pressure, for 2 hours. After filtration in order to remove the catalyst, it was concentrated under reduced pressure and the white solid obtained, washed with pentane, giving **17**·HCl (183 mg, 68% yield), mp $> 278^\circ\text{C}$ (dec.). IR (ATR) 3397, 2906, 2845, 2718, 2496, 1595, 1448, 1385, 1271, 1100, 1072, 973, 953 cm^{-1} . ^1H -NMR (400 MHz, CD_3OD) δ : 1.35 [m, 2 H, 3(5)- H_{ax}], 1.45 (t, 2 H, $J = 7.6$ Hz, Ad- CH_2 -piperidine), 1.56 [d, 2 H, $J = 12.8$ Hz, 4' (9')- H_a or 8' (10')- H_a], 1.64 (m, 1 H, 4-H), 1.67 [s, 2 H, 1' (3')-H], 1.75–1.98 (complex signal, 14 H, 2'-H, 5'-H, 7'-H, 3(5)- H_{eq} , 8' (10')- H_b or 4' (9')- H_b , 6'- H_2 , 8' (10')- H_a or 4' (9')- H_a], 2.97 [tm, 2 H, $J = 12.8$ Hz, 2(6)- H_{ax}], 3.37 [dm, 2 H, $J = 12.8$ Hz, 2(6)- H_{eq}]. ^{13}C -NMR (100.6

MHz, CD₃OD) δ : 29.5 (CH) and 29.7 (CH) (C5' and C7'), 30.4 [CH₂, C3(5)], 32.4 (CH, C4), 32.6 [CH₂, C4' (9) or C8' (10')], 33.4 [CH, C1' (3')], 39.4 (CH₂, C6'), 40.2 (CH₂, Ad-CH₂-piperidine), 40.3 [CH₂, C8' (10') or C4' (9')], 41.9 (CH, C2'), 45.4 [CH₂, C2(6)]. GC/MS (EI), m/e (%); tr 20.5 min: 233 (M⁺, 11), 219 (16), 218 (100), 98 (31), 85 (95), 84 (77), 82 (22), 79 (20), 67 (18), 57 (39), 56 (30), 55 (18). HRMS-ESI⁺ m/z [M+H]⁺ calcd for [C₁₆H₂₇N+H⁺]: 234.2216, found: 234.2215.

4-[(adamantan-2-yl)methyl]-N-methylpiperidine, 18·HCl—In a 250 mL round bottom flask was added **17·HCl** (123 mg, 0.5 mmol), NaBH₃CN (94.2 mg, 1.5 mmol), formaldehyde (37% aqueous solution, 0.13 mL, 1.5 mmol) and glacial acetic acid (0.1 mL) and left stirring, covered with a CaCl₂ tube at 30 °C overnight. The mixture was concentrated in vacuo, the residue was suspended in water (10 mL) and the solution was made basic with aqueous solution of 2 N NaOH and extracted with EtOAc (1×50 mL) and dichloromethane (2 × 50 mL). The combined organic extracts were dried, filtered and concentrated under vacuum. The corresponding hydrochloride, **18·HCl**, was made by adding an ethereal solution of HCl to a solution of the residue in EtOAc and purified by crystallization from diethyleter (104 mg, 73% yield), mp 265–267 °C. IR (ATR) 3417, 3362, 2906, 2850, 2663, 2546, 1615, 1464, 1451, 1256, 1067, 955 cm⁻¹. ¹H-NMR (400 MHz, CD₃OD) δ : 1.44 (t, 2 H, *J* = 6.8 Hz, Ad-CH₂-piperidine), 1.45 [m, 2 H, 3(5)-H_{ax}], 1.55 [broad d, 2 H, *J* = 12.8 Hz, 4' (9')-H_a or 8' (10')-H_a], 1.59 (m, 1 H, 4-H), 1.66 [s, 2 H, 1' (3')-H], 1.74–1.82 (complex signal, 6 H, 2'-H, 5'-H or 7'-H, 8(10)-H_a or 4(9')-H_a, 6'-H₂), 1.84–2.00 (complex signal, 7 H, 8(10)-H₂ or C4(9)-H₂), 7'-H or 5'-H, 3(5)-H_{eq}], 2.83 (s, 3 H, NCH₃), 2.70 [broad t, 2 H, *J* = 12.8 Hz, 2(6)-H_{ax}], 3.47 [broad d, 2 H, *J* = 12.8 Hz, 2(6)-H_{eq}]. ¹³C-NMR (100.6 MHz, CD₃OD) δ : 29.5 (CH) and 29.6 (CH) (C5' and C7'), 31.3 [CH₂, C3(5)], 31.8 (CH, C4), 32.5 [CH₂, C4' (9) or C8' (10')], 33.3 [CH, C1' (3')], 39.4 (CH₂, C6'), 39.9 (CH₂, Ad-CH₂-piperidine), 40.3 [CH₂, C8' (10') or C4' (9')], 42.0 (CH, C2'), 44.1 (CH₃, N-CH₃), 56.0 [CH₂, C2(6)]. HRMS-ESI⁺ m/z [M+H]⁺ calcd for [C₁₇H₂₉N+H⁺]: 248.2373, found: 248.2374.

Synthesis of 4-[(adamantan-2-ylidene)methyl]piperidine-1-carboximidamide hydrochloride, 19·HCl—In a 10 mL round-bottomed flask equipped with a condenser and a magnetic stirrer, a suspension of amine **16** (131 mg, 0.56 mmol), 1*H*-pyrazole-1-carboximidine hydrochloride (86.3 mg, 0.59 mmol), anhydrous triethylamine (0.12 mL, 0.86 mmol) in acetonitrile (3 mL) was prepared. The reaction was heated at 70 °C for 6 hours. The reaction was cooled down and stored at 4 °C overnight. The suspension was filtered out and the filtrate washed with cold pentane obtaining the guanidine **19·HCl** as a white solid (131 mg, 88% yield). mp 266–272 °C (dec.). IR (ATR) 3245, 3139, 2901, 2850, 1648, 1614, 1512, 1441, 1342, 1168, 1122, 960 cm⁻¹. ¹H-NMR (400 MHz, CD₃OD) δ : 1.35 [m, 2 H, 3(5)-H_{ax}], 1.65–1.79 (complex signal, 6 H, 3(5)-H_{eq}, 4' (10')-H_a and 8' (9')-H_a], 1.88 [broad s, 2 H, 5' (7')-H], 1.88–1.96 (complex signal, 6 H, 6'-H₂, 4' (10')-H_b and 8' (9')-H_b), 2.29 (broad s, 1 H, 1'-H), 2.58 (m, 1 H, 4-H), 2.87 (broad s, 1 H, 3'-H), 3.12 [dt, 3 H, *J* = 13.6 Hz, *J* = 2.8 Hz, 2(6)-H_{ax}], 3.85 [dm, 2 H, *J* = 13.6 Hz, 2(6)-H_{eq}], 4.90 (d, 1 H, *J* = 8.8 Hz, C2'=CH). ¹³C-NMR (100.6 MHz, CD₃OD) δ : 30.0 [CH, C5' (7')], 33.4 [CH₂, C3(5)], 34.1 (CH, C3'), 34.2 (CH, C4), 38.2 (CH₂, C6'), 40.2 [CH₂, C4' (10') or C8' (9')], 40.9 [CH₂, C8' (9) ' or C4' (10')], 41.9 (CH, C1'), 46.9 [CH₂, C2(6)], 121.0 (CH,

C2'=CH), 149.7 (C, C2'), 157.5 (C, C=NH). HRMS-ESI⁺ m/z [*M*+H]⁺ calcd for [C₁₇H₂₇N₃+H⁺]: 274.2278, found: 274.2275.

4-[(adamantan-2-yl)methyl]piperidine-1-carboximidamide hydrochloride, 20·HCl

—In a 10 mL round-bottomed flask equipped with a condenser and a magnetic stirrer, a suspension of amine **17·HCl** (83 mg, 0.3 mmol), 1*H*-pyrazole-1-carboximidamide hydrochloride (54.2 mg, 0.37 mmol), anhydrous triethylamine (0.1 mL) in acetonitrile (3 mL) was prepared. The reaction was heated at 70 °C for 6 hours. The reaction was cooled down and stored at 4 °C overnight. The suspension was filtered out and the filtrate washed with cold acetonitrile obtaining the guanidine **20·HCl** as a white solid (60 mg, 64% yield). mp 268–270 °C (dec.). IR (ATR) 3306, 3220, 3103, 2906, 2845, 1633, 1453, 1347, 1160, 1092, 963, 821, 723 cm⁻¹. ¹H-NMR (400 MHz, CD₃OD) δ: 1.19 [dq, 2 H, *J* = 13.6 Hz, *J'* = 4.0 Hz, 3(5)-H_{ax}], 1.32 (t, 2 H, *J* = 7.6 Hz, Ad-CH₂-piperidine), 1.55 [broad d, 2 H, *J* = 12.4 Hz, 4' (9')-H_a or 8' (10')-H_a], 1.64 (m, 1 H, 4-H), 1.67 [broad s, 2 H, 1' (3')-H], 1.75–1.98 [complex signal, 13 H, 3(5)-H_{eq}, 2'-H, 5'-H, 6'-H₂, 7'-H, 4' (9')-H_b or 8' (10')-H_b, 4' (9')-H₂ or 8' (10')-H₂], 3.06 [td, 2 H, *J* = 13.6 Hz, *J'* = 2.8 Hz, 2(6)-H_{ax}], 3.87 [dm, 2 H, *J* = 13.6 Hz, 2(6)-H_{eq}]. ¹³C-NMR (100.6 MHz, CD₃OD) δ: 29.5 (CH) and 29.7 (CH) (C5' and C7'), 32.6 [CH₂, C4' (9) or C8' (10')], 33.1 [CH₂, C3(5)], 33.4 [CH, C1' (3')], 33.9 (CH, C4), 39.4 (CH₂, C6'), 40.3 (CH₂, Ad-CH₂-piperidine), 40.4 [CH₂, C8' (10') or C4' (9')], 42.0 (CH, C2'), 47.2 [CH₂, C2(6)], 157.5 (C, C=N). HRMS-ESI⁺ m/z [*M*+H]⁺ calcd for [C₁₇H₂₉N₃+H⁺]: 276.2434, found: 276.2437.

Plasmid, mRNA synthesis, and microinjection of oocytes

The cDNA encoding the influenza A/Udorn/72 (A/M2) was inserted into pSUPER vector for the expression on oocyte plasma membrane. A/M2 S31N and A/M2 V27A mutants were generated by QuikChange site-directed mutagenesis kit (Agilent Technologies). The synthesis of mRNA and microinjection of oocytes have been described previously.⁶⁷

Two-electrode voltage clamp analysis

Macroscopic membrane current was recorded 24–72 hours after injection as described previously.³¹ Oocytes were perfused at room temperature in Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.3 NaNO₃, 0.71 CaCl₂, 0.82 MgCl₂, and 15 HEPES for pH 8.5 or 15 MES for pH 5.5 at a rate of 2 mL/min. The tested compounds were dissolved in DMSO and applied (100 μM) at pH 5.5 when the inward current reaches maximum. The compounds were applied for 2 min, and residual membrane current was compared with the membrane current before the application of compounds. The compounds were typically applied for 2 min, and residual membrane current was compared with the membrane current before the application of compounds. Membrane currents were recorded at -20 mV and analyzed with pCLAMP 10.0 software package (Axon Instruments, Sunnyvale, CA).

Antiviral assays

The anti-influenza virus activity in Madin-Darby canine kidney (MDCK) cells was determined by plaque reduction (PRA) and virus yield assays. For the PRA, influenza virus A/HK/7/87 (A/H3N2) was incubated (1 h at 4°C) with different concentrations of the

compounds, and then added to confluent MDCK cells in 12-well plates. After 1 h incubation at 35 °C, excess virus was removed and replaced by fresh medium containing the compounds and 0.8% agarose. After 72 h incubation, plaques were visualized by cell fixation with 3.7% formaldehyde and staining with 0.1% crystal violet. For the virus yield assay, MDCK cells grown in 96-well plates were infected with influenza virus A/HK/7/87, and at the same time the test compounds were added in serial dilutions. After 24 h incubation at 35 °C, supernatants were harvested to determine the virus yield by RT-qPCR.^{63,68} In parallel, mock-infected cell cultures were used to determine the compounds' cytotoxicity. After 72 h incubation at 35 °C, cell viability was measured by the formazan-based MTS assay and the OD values were used to calculate the compound concentrations causing 50% cytotoxicity (CC₅₀).⁶⁸

The PRA with A/WSN/33 N31S/V27A (H1N1) virus was carried out similarly as previously described.³² Briefly, MDCK cells monolayers in 6-well plates were infected with virus (~100 PFU/well). After adding the inoculums, cells were kept in 4 °C for 1 h to synchronize the infection, then cells were moved to 37 °C incubator for 1 h. The inoculums were removed, and the cells were washed with phosphate buffered saline (PBS). The cells were then overlaid with DMEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) and NAT (2.0 µg/ml). To examine the effect of the drugs on plaque formation, at 46 h after infection, the monolayers were fixed and stained with 0.2% crystal violet in 20% methanol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Amt	amantadine
ATR	Attenuated Total Reflectance
MDCK	Madin-Darby Canine Kidney
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
RT-qPCR	quantitative real-time reverse transcription polymerase chain reaction
TEVC	two-electrode voltage clamp
wt	wild-type

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Figure 1.

Activity of the compounds in the influenza virus plaque reduction assay. MDCK cells were infected with influenza virus (strain A/HK/7/87; 30 PFU per well) in the presence of the test compounds. After 72 h incubation, plaques were visualized by crystal violet staining. VC: mock-treated virus control; CC: uninfected cell control.

Figure 2.

Inhibition of the A/M2 wt channel (derived from the A/Udorn/307/72 H3N2 virus strain) and V27A mutant form, using the TEVC technique in *Xenopus* oocytes. Oocytes were bathed in Barth solution at pH = 8.5 and pH = 5.5. Current was clamped at -20 mV and the indicated compounds were applied at 100 μ M in pH 5.5 solution once the inward current reached maximum amplitude in A/M2 wt channel (A-D) and V27A mutant channel (E-H).

Scheme 1.

Synthesis of (1-adamantyl)piperidines **2** and **8** and guanidine **7**.^a

^aReagents and conditions: a: pyridine, [bis(trifluoroacetoxy)iodo]benzene, anh. benzene, reflux, overnight, **5**, 9%; **6**, 27%; b: H₂, PtO₂, MeOH, 30 atm, 97% yield for **2**; 99% yield for **8**; c: 1H-pyrazole-1-carboxamide hydrochloride, anh. Et₃N, acetonitrile, reflux, 6 h, 76% yield.

Scheme 2.

Synthesis of 4-(2-adamantyl)piperidines and related compounds from 2-adamantanone, **9**.^a

^aReagents and conditions: **a**: 4-pyridyl lithium; Et₂O/THF, -65 °C to rt, 70% yield; **b**: 1 atm H₂, PtO₂, ethanol, rt, 24 h, 93% yield of a mixture of **3**, **11** and **12**; **c**: 1) SOCl₂, pyridine, anh. CH₂Cl₂, -60 °C, 30 min, 2) 1 atm H₂, Pd/C, methanol, HCl, rt, 2 h, 63% overall yield; **d**: acetaldehyde, NaCNBH₃, AcOH, methanol, rt, 24 h, 76% yield; **e**: 1) 4-picoline, anh THF, *n*-BuLi; 2) **9**, 2 h, rt, 90% yield; **f**: 1 atm H₂, PtO₂, HCl, methanol, 5 days, > 99% yield; **g**: SOCl₂, pyridine, anh. CH₂Cl₂, -60 °C, 30 min, > 99% yield; **h**: 1*H*-pyrazole-1-carboxamide hydrochloride, anh Et₃N, acetonitrile, 70 °C, 6 h, 88% yield for **19**, 64% yield for **20**; **i**: 1 atm H₂, Pd/C, methanol, HCl, rt, 2 h, 68% yield; **j**: formaldehyde (37% aqueous solution), NaCNBH₃, AcOH, rt, 18 h, 73% yield.

Chart 1.

Structures of Amt, rimantadine, spiroadamantane **1** and novel analogs **2–3**.

Table 1Inhibitory effect of the synthesized compounds on proton channel function of wt or V27A mutant A/M2.^{a,b}

Compound	A/M2 wt (mean ± SE)		A/M2 V27A (mean ± SE)	
	Inhibition by 100 µM for 2 min (%)	IC ₅₀ (µM)	Inhibition by 100 µM for 2 min (%)	IC ₅₀ (µM)
Amantadine	91.0 ± 2.1	16.0 ± 1.2	8.9 ± 1.0	>500 ³²
2	92.0 ± 1.4	4.1 ± 0.5	85.7 ± 1.5	3.6 ± 0.6
3	70.7 ± 2.4	45.3 ± 2.3	61.9 ± 2.3	60.6 ± 5.1
7	91.8 ± 1.0	1.9 ± 0.2	79.3 ± 2.8	16.2 ± 1.7
8	48.1 ± 3.4 ^c	ND	10.1 ± 0.6	>500
11	10.9 ± 0.8 ^d	ND	13.1 ± 0.6	ND
13	43.9 ± 1.0	ND	23.5 ± 2.1	ND
15	18.4 ± 2.8	ND	14.6 ± 1.9	ND
16	64.0 ± 4.3	ND	44.2 ± 1.9	ND
17	76.7 ± 4.0	ND	47.3 ± 3.5	ND
18	48.9 ± 10.7	ND	29.2 ± 0.9	ND
19	89.9 ± 0.9	6.8 ± 1.4	63.0 ± 0.9	46.0 ± 1.1
20	94.7 ± 1.2	4.2 ± 0.1	77.8 ± 1.5	21.7 ± 2.6

^aThe activity of the inhibitors was measured using the TEVC technique on A/M2 channels expressed in *Xenopus* oocytes; percentage of inhibition was the mean of at least three experiments. For IC₅₀ experiments, 7–9 concentrations were measured, and, at each concentration, experiments were run at least three times.

^bIsochronic (2 min) values for IC₅₀ are given.

^cThe inhibition for 4 min was ca 75% and > 90% after 6 min of treatment.

^dThe inhibition for 7 min was ca 25% and ca 30% after 10 min. See text and supporting information for details. ND, Not determined.

Table 2Antiviral activity in influenza virus-infected MDCK cells.^a

Compound	Antiviral activity (μM) against A/HK/7/87 (wt M2)		Cytotoxicity (μM)
	EC ₅₀ (PRA) ^b	EC ₉₉ (Virus yield) ^c	CC ₅₀ ^d
2	0.14	1.7	18
3	ND	50	>100
7	>50	>2	10
8	1.0	3.5	>100
11	>100	76	>100
13	>10	>10	25
15	10	>50	>50
16	>5	>10	25
17	>10	>2	15
18	>10	>2	6.2
19	>5	>2	19
20	>10	>2	3.8
Amantadine	0.14	0.51	>500
Rimantadine	0.016	1.1	>500

^aMDCK: Madin-Darby canine kidney cells; virus strain: A/HK/7/87 (A/H3N2 carrying wt M2). None of the compounds displayed activity against the A/PR/8/34 strain bearing the S31N mutant M2 channel (data not shown).

^bEC₅₀ in the virus plaque reduction assay (PRA): concentration at which the plaque number is reduced by 50% compared to untreated virus control.

^cEC₉₉: compound concentration giving 2-log₁₀ reduction in virus yield, as determined by quantifying the virus in the supernatant at 24 h post infection, using an RT-qPCR based method.⁶³

^dCC₅₀: 50% cytotoxic concentration, as determined by the MTS cell viability test in uninfected cells exposed to the compounds during 72 h. Values shown are the mean of 2–3 determinations. ND, not determined.

Table 3

Rate constants for association (k_{on}) and dissociation (k_{off}) of the compounds to/from the A/M2 wt or V27A mutant channels.^a

Compound	A/M2 wt (mean $\pm$ SE)		V27A (mean $\pm$ SE)	
	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	k_{off} (s^{-1})	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	k_{off} (s^{-1})
Amantadine	293 $\pm$ 37 (4)	3 $\times$ 10 ⁻⁴ (ref 44)	360 $\pm$ 18 (4)	16 $\times$ 10 ⁻² (1)
2	259 $\pm$ 29 (4)	ND	1369 $\pm$ 149 (9)	6 $\times$ 10 ⁻² $\pm$ 0.006 (5)
7	654 $\pm$ 51 (5)	10 ⁻² $\pm$ 0.001 (3)	1028 $\pm$ 167 (9)	14 $\times$ 10 ⁻² $\pm$ 0.041 (3)
8	119 $\pm$ 11 (3)	ND	595 $\pm$ 48 (4)	4 $\times$ 10 ⁻² (1)

^a Currents from Figure 2 were fitted to a single exponential function ($y = e^{-t/\tau} + \text{constant}$ with $r > 0.94$) and the time constant τ was used to calculate the forward rate constant after addition of compound ($k_{\text{on}} = 1/[\text{compound}] \tau$) and the reverse rate constant during the washout at pH 5.5 ($k_{\text{off}} = 1/\tau$).⁴⁷ Number of tested oocytes in brackets. ND, not determined.