Published in final edited form as:

J Med Chem. 2011 April 28; 54(8): 2646–2657. doi:10.1021/jm101334y.

Exploring the Size Limit of Templates for Inhibitors of the M2 Ion Channel of Influenza A Virus

María D. Duque[‡], Chunlong Ma^{#,§}, Eva Torres[‡], Jun Wang[†], Lieve Naesens[⊥], Jordi Juárez-Jiménez^Δ, Pelayo Camps[‡], F. Javier Luque^Δ, William F. DeGrado[†], Robert A. Lamb^{§,‡}, Lawrence H. Pinto[#], and Santiago Vázquez^{*,‡}

[‡] Laboratori de Química Farmacèutica (Unitat Associada al CSIC), Facultat de Farmàcia, and Institute of Biomedicine (IBUB), Universitat de Barcelona, Av. Diagonal 643, Barcelona E-08028, Spain

[#] Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208-3500

[†] Department of Chemistry and Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059

[⊥] Rega Institute for Medical Research, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

^Δ Departament de Fisicoquímica, Facultat de Farmàcia, and Institut de Biomedicina (IBUB), Universitat de Barcelona, Av. Diagonal 643, E-08028, Barcelona, Spain

[§] Department of Molecular Biosciences, Northwestern University, Evanston, Illinois 60208-3500

[‡] Howard Hughes Medical Institute, Northwestern University, Evanston, Illinois 60208-3500

Abstract

Amantadine inhibits the M2 proton channel of influenza A virus, yet its clinical use has been limited by the rapid emergence of amantadine-resistant virus strains. We have synthesized and characterized a series of polycyclic compounds designed as ring-contracted or ring-expanded analogs of amantadine. Inhibition of the wild-type (wt) M2 channel and the A/M2-S31N and A/M2-V27A mutant ion channels were measured in *Xenopus* oocytes using two-electrode voltage clamp (TEV) assays. Several bisnoradamantane and noradamantane derivatives inhibited the wt ion channel. The compounds bind to a primary site delineated by Val27, Ala30 and Ser31, though ring-expansion restricts the positioning in the binding site. Only the smallest analog **8** was found to inhibit the S31N mutant ion channel. The structure-activity relationship obtained by TEV assay was confirmed by plaque reduction assays with A/H3N2 influenza virus carrying wt M2 protein.

Keywords

Amantadine; cage compounds; influenza A virus; M2 proton channel; drug design

*Address correspondence to this author. Tel: +34 934024533.; Fax: +34 934035941. svazquez@ub.edu.

Supporting Information Available. Figures S1-S5 (modeling studies) and S6 (CD titration assay). B3LYP/6-31G(d) optimized geometries of Amt and **24**, and their RESP partial charges. Table S1: Solvent Interaction Energies (SIE). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

The M2 protein of the influenza A virus forms a pH-gated proton channel that is essential for virus replication.^{1–2} The influenza virus enters its target cells by receptor-mediated endocytosis, which is followed by acid-induced fusion of the viral and endosomal membranes. This fusion event is mediated by a conformational change of the influenza hemagglutinin proteins, triggered by the low pH in the endosome lumen.³ The endosomal pH also activates the membrane-spanning A/M2 protein, which acts as a proton channel to conduct protons into the virion interior. As a result of decreased intravirion pH, the viral ribonucleoprotein is uncoated and transferred, after viral fusion with the endosome, to the cytoplasm.⁴ In addition, it has been shown that, for some influenza strains, the A/M2 proton channel function is required for preventing a premature hemagglutinin conformation transition when newly synthesized viral proteins are trafficked through the *trans*-Golgi network.⁵

The highly conserved H₃₇xxxW₄₁ motif in the transmembrane (TM) domain of the A/M2 protein is responsible for its channel activity and proton selectivity.^{6–7} The channel function of wt A/M2 is efficiently inhibited by two FDA approved drugs, amantadine (Amt) and rimantadine, as well as by a few new compounds such as **1** (BL-1743) and its derivatives **2** and **3** (Chart 1).^{8–10} Naturally arising single amino acid substitutions associated with Amt resistance, such as L26F, V27A, A30T, S31N and G34E, are all located outside of the H₃₇xxxW₄₁ motif.^{11–14} Extensive studies suggest that these residues contribute to delineate the binding pocket for the inhibitors,^{15–21} although Schnell and Chou showed that rimantadine can also bind to Asp44 of M2 TM in detergent solutions; this residue is found at the external lipid-facing helix-interface, and this finding has been taken to imply an allosteric mechanism.²² Even though the precise nature of the binding site of the inhibitors has been subject of an intense debate, a series of functional evidences supports the binding to the interior of the channel. (1) A chimeric channel comprised of A/M2 residues 24–36 embedded in an Amt-insensitive channel shows 85% inhibition by Amt.²³ (2) Amt and the known pore-residing inhibitor **1** compete for inhibition of the channel.²⁴ (3) Mutation of Asp44 to each of seven amino acids does not reduce Amt inhibition.^{18,25} Recently, a solid state (ss) NMR study and,¹⁵ independently, a series of surface plasmon resonance experiments,²⁶ showed that the external lipid-facing Asp44 site has much lower affinity than the luminal site. These results suggest that drug binding within the pore is pharmacologically relevant.

Amt-resistant mutants are frequently detected among the two currently circulating subtypes of influenza A virus, A/H3N2 and A/H1N1, with the S31N mutation being observed in more than 90% of influenza A isolates in certain years.^{27–28} More recently, the pandemic H1N1 swine flu virus, which is Amt resistant, was the cause of global and significant morbidity and mortality.^{29–30} Thus, there is an urgent need for the development of novel anti-influenza drugs that are effective against the most common Amt-resistant mutants.^{27–28}

This study reports novel scaffolds designed to bind to the high affinity site of the A/M2 channel. We have found that the wt channel can be inhibited by several polycyclic compounds covering an *a priori* unexpectedly large diversity in size. We have identified a promising compound, **8**, that is capable of inhibiting the M2-S31N mutant ion channel.

Chemistry

Although Amt and rimantadine are well known antiviral drugs and their structure-activity relationships have been previously examined,^{8,31–50} quite surprisingly few ring-contracted or ring-expanded analogs have been synthesized.³² Moreover, little effort has been paid to investigate the influence of polycyclic scaffolds on the inhibitory activity,^{33,51–56} the

guanidine **1** and related spiro-piperidine compounds being a notable exception (Chart 1).^{9,10,24} Recently, we have described the synthesis and characterization of ring-contracted analogs of Amt and rimantadine, such as noramantadine derivatives **4-7**, and bisnoradamantane derivatives **8** and **9**.⁵⁷ We have also synthesized 2-oxa-analogs of Amt, such as **10** and **11** (Chart 2).⁵⁸ We tested these compounds against the influenza A/H1N1 and A/H3N2 subtypes in Madin-Darby Canine Kidney (MDCK) cells and found that primary amine **4** showed reasonable antiviral activity.

To further explore the impact of the polycyclic ring size on the inhibition of the A/M2 channel, we have now synthesized larger analogs of Amt using pentacyclic and hexacyclic scaffolds. The pentacyclo[6.4.0.0.2¹⁰.0^{3,7}.0^{4,9}]dodecane scaffold has been used in the synthesis of polycyclic compounds of theoretical interest, such as dodecahedrane.⁵⁹ To the best of our knowledge, nevertheless, it has never been applied to the synthesis of compounds with potential biological interest. Starting from the diacid **12**,⁶⁰⁻⁶¹ we synthesized monoacid **13**, using a high-yielding synthetic sequence previously described by our group.⁶² Schmidt reaction of acid **13** led to pentacyclic amine **14** in 82% yield. On the other hand, reaction of acid **13** with SOCl₂ followed by reaction of the acyl chloride with NH₄OH led to amide **17** that was reduced with LiAlH₄ to primary amine **18** (Scheme 1).

In order to explore the effect of the alkylation on the biological activity, **14** was treated with acetaldehyde and NaCNBH₃ to obtain tertiary amine **15** in 78% yield, while treatment of **18** with ethyl chloroformate followed by reduction with LiAlH₄, led to secondary amine **19** in 41% overall yield. In order to study the effect of the basicity of the nitrogen atom on the inhibitory activity, we synthesized acetamidine **16** by reaction of amine **14** with methyl acetimidate. Unfortunately, several attempts to synthesize the corresponding guanidine were unsuccessful (Scheme 1).

The synthesis of the conformationally more rigid pyrrolidine-based derivatives **21**, **22** and **24** started from the reaction of diacid **12** with urea at 180 °C for 30 minutes to yield imide **20**, which was subsequently reduced with LiAlH₄ to give secondary amine **21** in 36% overall yield. Catalytic hydrogenation of **21** quantitatively furnished amine **22**. Besides, cyclopropanation of the two C=C bonds of **20** led to imide **23** that was reduced with LiAlH₄ to give amine **24** in 61% overall yield (Scheme 2).

All the new amines **14**, **15**, **18**, **19**, **21**, **22** and **24** and acetamidine **16** were fully characterized as hydrochlorides through their spectroscopic data and elemental analyses.

Pharmacological activity and structure-activity relationships

Inhibition of wt and amantadine-insensitive A/M2 ion channels

The inhibitory activity of the compounds was tested on A/M2 channels expressed in *Xenopus* oocytes using the TEV technique. All inhibitors were initially tested at 100 μM; those that inhibited the wt A/M2 channel activity by more than 80% were chosen for measurement of their IC₅₀. The results are given in Table 1 and Figure 1.

Amt inhibited wt A/M2 channel with an IC₅₀ of 16.0 μM in an isochronic (2 min) inhibition assay. Replacement of a methylene unit by an oxygen atom at position 2 of the adamantane ring (compounds **10** and **11**) led to a reduction in the inhibitory activity (IC₅₀ = 29.2 and 22.9 μM, respectively). Most strikingly, ring-contraction of the Amt ring to noradamantane derivatives led to more active compounds. Thus, primary amine **4** showed an IC₅₀ of 7.1 μM, while guanidine **6**, with an IC₅₀ of 2.4 μM, was almost an order of magnitude more potent than Amt. The racemic rimantadine analog **7** showed an IC₅₀ of 12.7 μM. Kolocouris et al. have previously found that in several adamantane derivatives, alkylation of the amine

group caused a reduction of anti-influenza A virus activity.^{40,43–47} This trend was also observed in going from compound **4** to its monomethylated derivative, **5** (IC₅₀ of 17.1 μM). For this reason, no additional alkylated compounds were considered.

A second ring-contraction led to bisnoradamantane derivatives, such as **8** and **9**. These smaller compounds behaved in a similar way as the bigger analogs. Thus, **8** showed an IC₅₀ of 17.0 μM, similar to that of Amt, while **9** showed an IC₅₀ of 7.2 μM, which compares with the inhibitory activity of **4**. Taken together, these results suggest that the A/M2 channel can be inhibited by compounds smaller than Amt. This observation is important, as most of the structure-activity relationships previously carried out on Amt analogs have concentrated on adamantane-substituted compounds with overall larger structures than Amt. Amongst all the new compounds tested, **8** was the only one able to inhibit A/M2 S31N channel with an IC₅₀ of 252 μM, slightly higher than that of Amt (IC₅₀ = 200 μM). Also, at 100 μM, **8** inhibited the A/M2 V27A channel activity by 10.6%, quite similar to Amt (10.8%).

Taking into account that the bisnoradamantane derivative **8** showed an interesting activity against wt and S31N A/M2 channels, compounds **14**, **15**, **16**, **18** and **19** were synthesized. While being derivatives of the pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodecane, a larger polycyclic scaffold, their structures also contain a bisnoradamantane subunit. Unfortunately, they performed poorly in the M2 inhibition experiments, with the diethylated derivative **15** being, not surprisingly, the least active compound in this series.

It has been suggested that in designing Amt analogs, pyrrolidine or piperidine derivatives may result in a more favorable orientation inside the M2 channel pore as compared to freely rotating alkylamine chains.⁴⁸ Thus, we tested conformationally more rigid amines **21**, **22** and **24**. In sharp contrast with the pentacyclic derivatives from Scheme 1, diene **21** was shown to be able to inhibit wt A/M2 channel by more than 80%, showing an IC₅₀ of 33.5 μM. While hydrogenation of the two C=C double bonds led to a much less potent compound, bis-cyclopropanation slightly increased the potency, derivative **24** showing an IC₅₀ of 24.0 μM. Interestingly, **24** (at a concentration of 100 μM) inhibited the A/M2 V27A channel activity by 17.7%, which is superior to Amt (10.8%), although much less than **3** (53.4%).²⁴

Molecular modeling

To examine the effect of ring-expansion on the binding to the A/M2 ion channel we performed computational studies with **24**. Prior to studying the binding of **24**, however, we performed combined docking calculations and restrained molecular dynamics (MD) using the experimental data available for Amt. Previous X-ray²⁰ and ssNMR¹⁵ studies have suggested that Amt mainly binds to the region of the channel comprising residues Val27 and Ser31, and that the amino group is oriented towards the C-terminal end of the pore. Our modeling studies, undertaken with an initial orientation of Amt in which the amino group was directed toward the C-terminus, agreed with this binding mode, as seen in snapshots sampled in a 40 ns restrained MD simulation (Figure 2, top left). The axis of Amt deviated ~7° from the pore axis, and the center of the cage deviated ~0.3 Å from the plane defined by the four Ser31 C_α atoms (see Figure S1 in Supporting Information). These findings agree with the structural features derived from ssNMR data (tilt angle of ~13°, and height from the Ser31 C_α plane close to 0 Å; 15), and with the binding mode of Amt found in MD simulations of the channel at low pH conditions.⁶³ Remarkably, three water molecules were on average hydrogen-bonded to the amino group along the trajectory, thus enabling the formation of contacts with the dense network of water molecules surrounding the His37 box.⁶⁴

Docking computations led to an alternative binding mode for Amt in which the amino group was hydrogen-bonded to both the hydroxyl group of Ser31 and the carbonyl group of Val27 of the same TM chain. Using this alternative docked pose as a starting structure, a slight readjustment of Amt was observed at the beginning of the MD trajectory, leading to the loss of the hydrogen bond with Val27, even though the amino group retained its hydrogen bonding with the hydroxyl group of Ser31 (Figure 2, top right; see also Figure S2 in Supporting Information). Moreover, the amino group became hydrogen-bonded with up to two water molecules during the trajectory. Interestingly, the axis of Amt deviated around 52° from the pore axis, in agreement with the minor fraction of M2-bound Amt observed from ssNMR data, found to have a tilt angle of 54.7°. ¹⁵

We also performed an MD simulation starting with Amt oriented along the axis but with its amino group pointing toward the N-terminus. In this case, both the tilt angle of Amt (~37°) and the deviation from the Ser31 C_α plane (1.2 Å) clearly differed from those of the ssNMR data. Solvent Interaction Energy (SIE) calculations were performed to check the interaction free energy of the three binding modes. The results (see Supporting Information) revealed that the interaction free energies differ by 0.9 kcal/mol, which is less than the error estimated for those calculations (around 1.3 kcal/mol). Under these circumstances, the use of the structural parameters such as the tilt angle and the separation from the plane defined by Ser31 C_α atoms, which are directly derived from experimental NMR data, seems to be better suited to discriminate between the different binding modes of Amt. On the basis of these results, the two binding modes shown in Figure 2 are suggested to be representative of the major and minor fractions of the Amt-bound A/M2 ion channel.

The chemical nature of the skeleton of the ring-contracted compounds not only retain the amphiphilic nature of Amt, but also have a moderate impact on the overall shape of the compounds. Thus, the van der Waals surface and volume of those compounds vary from 205 Å² and 147 Å³ for **4** to 231 Å² and 162 Å³ for **9**, which compare with values of 202 Å² and 146 Å³, respectively, for Amt. Accordingly, such a chemical resemblance should enable the ring-contracted compounds to mimic the binding mode of Amt in the M2 channel, thus leading to small variations in the IC₅₀ values (from 7.1 to 17.1 μM, which compare with the IC₅₀ of 16 μM determined for Amt). At this point, it is worth noting that the replacement of one of the carbon atoms in β position in Amt by an oxygen atom has a larger effect on the inhibitory activity, as noted in the IC₅₀ value of 29.2 μM determined for the oxa-analog **10**. This effect can likely be attributed to two factors: i) the lack of a suitable partner in the transmembrane helix that could interact with the lone pairs of the oxygen atom (see Figure S3 in Supporting Information), and ii) a slightly worse fit of the region filled by Amt, as suggested by the smaller surface (189 Å²) and volume (138 Å³) of **10** compared to Amt.

In light of the preceding discussion, the similar inhibitory activity determined for Amt and the ring-expanded compound **24** (IC₅₀ = 24.0 μM) is totally unexpected, as the chemical structure of this latter compound leads to the largest increase in the molecular size (by around 36%) relative to Amt (van der Waals surface and volume of 261 Å² and 198 Å³ for **24**). It is worth noting that previous studies have already reported larger compounds with inhibitory activity at the A/M2 channel, as illustrated by compounds **1-3** in Chart 1. However, the expansion of the chemical skeleton took place along the molecular axis, which should increase the volume filled by the compound in the interior of the channel. In contrast, the ring-expansion strategy presented here occurs along the normal to the main axis, and it can be accomplished without a significant reduction in inhibitory potency even though such a chemical modification would prevent the ring-expanded compound to adopt a binding mode that mimics the arrangement of Amt in the channel. Thus, attempts to accommodate compound **24** in the same region occupied by Amt with the amino group toward the C-

terminus failed, due to severe steric clashes with the residues in the TM helices that surrounded the compound.

Docking calculations were then used in conjunction with MD simulations in order to explore the binding mode of compound **24**. Inspection of the docked poses indicated that **24** was able to bind the primary site through hydrogen bonds with Ser31 and Val27. Subsequent refinement by restrained MD simulations showed a slight readjustment of the binding mode, as **24** was found to retain the direct hydrogen bond to Ser31, but a water molecule was bridging the amino group and the carbonyl unit of Val27 in a neighboring TM chain (Figure 3, left). In addition, the large size of the cage imposed a distinct positioning in the binding site to alleviate steric clashes in the interior of the channel, and the main axis of the fused ring deviated around 40° from the pore axis. In turn, this binding mode facilitated the formation of van der Waals contacts with the side chain of Ala30. To further check the binding mode proposed for **24**, two additional MD simulations were run by placing this compound in distinct starting orientations (see Molecular Modeling in Experimental Section, and Figure S4 in Supporting Information). In both cases the arrangement of **24** at the end of the simulations mimicked the binding mode shown in Figure 2, as noted in the close similarity of the compound found in the three independent trajectories (see Figure S5 in Supporting Information), thus giving support to the tentative binding mode of the ring-expanded derivative.

Overall, even though molecular modeling results are consistent with the region delineated by residues Val27, Ala30 and Ser31 being the major binding site of **24**, its binding is more constrained than that of Amt, leading to different arrangements of these compounds in the pore (Figure 3, right). Accordingly, we expect that the structure-activity relationships for ring-expanded analogs will differ considerably from those for Amt. In particular, it is worth noting the high asymmetry in the environment around the two cyclopropane rings of **24**. Thus, in one case the cyclopropane unit is oriented toward the hydrophobic ring formed by the four Val27. However, the other unit is embedded in the region filled by the hydrating water molecules placed around the protonated amine of Amt. Thus, even though a weak C–H···O hydrogen bond could be formed between the cyclopropane unit and the carbonyl group of Ala30, the disruption of the network of water interactions above the His ring can be expected to have a net destabilizing effect for the binding of **24**. Accordingly, it can be envisaged that breaking of the internal symmetry of **24** through the incorporation of polar groups in one of the cyclopropane units should facilitate the formation of favorable interactions with water molecules, and therefore lead to an enhancement in the inhibitory activity.

Activity in plaque reduction assays

The most potent compounds were tested for inhibition of influenza virus containing the wt A/M2 ion channel by plaque reduction assays (Figure 4). Plaque formation of wt influenza virus (strain A/Udorn/72) was strongly inhibited by Amt, **4-9** and **24** at 5 µM compound concentrations, in agreement with electrophysiological recordings.

Binding of selected amines

We also assessed the binding of selected drugs to the TM domain of wt A/M2 protein (M2TM, residues 22-46), using a spectroscopic assay that relies upon changes in the CD spectrum of M2 induced by binding of drugs (Table 1 and Figure S6 in the Supporting Information). All drugs bound with a stoichiometry of approximately one drug per tetramer. At pH 7.4, bisnoradamantane amine **8** was found to bind to the tetrameric form of M2TM with low micromolar binding constant ($K_d = 4.7 \mu\text{M}$), being approximately equipotent with Amt ($K_d = 3.9 \mu\text{M}$).

Cytotoxicity evaluation

All the synthesized amines were evaluated for cytotoxicity in MDCK cells, using a formazan-based cell viability assay. The concentration producing 50% cytotoxicity (CC_{50}) was higher than 500 μ M (the highest concentration tested) for Amt, **8**, **10**, **11**, **14**, **16** and **21**. Low cytotoxicity was also observed for compounds **4**, **7**, **15** and **19** (CC_{50} values: 357; 256; 119 and 224 μ M, respectively). Compounds **9** and **24** had intermediate toxicity, their CC_{50} values being 79 and 61, respectively. Finally, compounds **5**, **6** and **18** displayed relatively high cytotoxicity, since their CC_{50} values were <19.4, 10.5 and 12.0 μ M, respectively.

Conclusions

The present work demonstrates that novel scaffolds exist for compounds that are capable of effective inhibition of the M2 ion channel of influenza A virus, and can in fact inhibit the channel more effectively than amantadine. These scaffolds contain ring-expanded and ring-diminished variants of adamantane and are not inherently cytotoxic. The work confirms previous studies that found the binding site for amantadine to lie within the channel pore with the amino group directed toward the C-terminal of the helical bundle and suggests that ring-expanded compounds do not bind in a similar manner.

Experimental Section

Plasmid, mRNA synthesis, and microinjection of oocytes

The cDNA encoding the influenza A/Udorn/72 (A/M2) was inserted into pGEM3 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.⁶⁵

Two-electrode voltage clamp analysis

Macroscopic membrane current was recorded 48-72 hrs after injection as described previously.²⁴ The tested compounds were applied at pH 5.5 at various concentrations 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. Membrane currents were analyzed with pCLAMP 10.0 software package (Axon Instruments, Sunnyvale, CA).

Plaque reduction assay

Confluent monolayers of MDCK cells were incubated with serially diluted virus samples (A/Udorn/72) in DMEM with 1% bovine serum albumin (BSA) for 1 h at 37 °C. The inocula 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 *N*-acetyltrypsin (NAT, 1.0 μ g/ml). To examine the effect of the compounds on plaque formation, MDCK cell monolayers were preincubated with DMEM supplemented with the compound (5 μ M) at 37 °C for 30 min, and virus samples were preincubated with DMEM plus 1% BSA with the compound (5 μ M) at 4 °C for 30 min before infection. At 2 days after infection, the monolayers were fixed and stained with naphthalene black dye solution (0.1% naphthalene black, 6% glacial acetic acid, 1.36% anhydrous sodium acetate).

Cytotoxicity assays

The compound cytotoxicity was assessed as described.⁶⁶ Briefly, exponentially growing MDCK cells were grown in 96-well plates and test compounds were added in serial

dilutions. 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 compounds concentrations causing 50% cytotoxicity (CC₅₀).

CD titrations

CD titrations were conducted using materials and procedures described in a previous study.²⁴ Synthetic M2TM 22-46 (62.5 μM) was dissolved in DPC micelles (detergent concentration, 2.5 mM) potassium phosphate buffer (10mM), pH 7.4. For titrations, the drug was added from concentrated solutions in the same buffer. Spectra were recorded in a 1 mm path length cuvette using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD) at 25°C with 1 nm step scans (4 sec acquisitions; 3 accumulations). The ellipticity at 222±1 nm was averaged to improve signal-to-noise and corrected for dilution factor.

Chemical Synthesis. General Methods

Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp or a Büchi B-540 melting point apparatus. 300 MHz ¹H/75.4 MHz ¹³C NMR spectra, 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 or Thermo Nicolet Nexus spectrophotometers. Absorption values are expressed as wave-numbers (cm⁻¹); only significant absorption bands are given. 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 a purity ≥95% as evidenced by their elemental analyses.

(Pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodec-8-yl)amine Hydrochloride (14·HCl)

To a solution of known acid **13** (2.50 g, 12.2 mmol) in CH₂Cl₂ (25 mL), conc H₂SO₄ (4 mL) was added and the mixture was heated to 50 °C. Then, sodium azide (1.69 g, 26.0 mmol) was added portionwise and the heating was continued for 1.5 h. The mixture was cooled to 0 °C (ice-water bath), ice (25 g) was added and 2 N NaOH was added until basic pH. The two layers were separated and the aqueous layer was extracted with EtOAc (4×20 mL). The combined organic extracts were dried with anhyd. Na₂SO₄, filtered, treated with excess of HCl in Et₂O, and concentrated in vacuo to give the hydrochloride of **14** (2.25 g, 82% yield). An analytical sample of **14·HCl** was obtained as a white solid by crystallization from methanol/Et₂O, mp >242 °C (dec.); IR (KBr) ν 3200-2500 (max at 2948, 2926, 2882 and 2786, N-H, ⁺N-H and C-H st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.49 (s, 1 H, 9-H), 1.60–1.70 [complex signal, 6 H, 5(11)-H_α, 5(11)-H_β and 6(12)-H_α], 1.71–1.79 [complex signal, 2 H, 6(12)-H_β], 2.32 [s, 2 H, 4(10)-H], 2.40 [s, 4 H, 1(7)-H and 2(3)-H]; ¹³C NMR (100.6 MHz, CD₃OD) δ 21.9 [CH₂, C6(12)], 24.6 [CH₂, C5(11)], 48.6 [CH, C2(3)], 52.1 (CH, C9), 54.7 [CH, C4(10)], 56.3 [CH, C1(7)], 64.5 (C, C8). MS (EI), m/e: 175 (M⁺, 17), 147 (100), 146 (19), 132 (16), 119 (32). Anal. Calcd. for C₁₂H₁₇N·HCl·0.1H₂O (213.54): C 67.49, H 8.59, N 6.56. Found C 67.49, H 8.68, N 6.56.

***N,N*-Diethyl(pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodec-8-yl)amine Hydrochloride (15·HCl)**

To a solution of **14·HCl** (275 mg, 1.3 mmol) in MeOH (10 mL), NaBH₃CN (95%, 172 mg, 2.6 mmol), acetic acid (0.4 mL) and acetaldehyde (0.22 mL, 3.9 mmol) were added and the mixture was stirred for 4 h at room temperature. A second portion of NaBH₃CN (95%, 86 mg, 1.3 mmol) and acetaldehyde (0.11 mL, 1.95 mmol) were added and the mixture was stirred for an additional 18 h at room temperature. The mixture was concentrated in vacuo, water (15 mL) was added, the suspension was made basic with 2 N NaOH and extracted with EtOAc (4×10 mL). The combined organic extracts were washed with brine (2×25 mL), dried with anhyd. Na₂SO₄, filtered, treated with excess of HCl in Et₂O, and concentrated in vacuo to give the hydrochloride of **15** (269 mg, 78% yield). An analytical sample of **15·HCl** was obtained as a white solid by crystallization from MeOH/Et₂O, mp 206–207 °C; IR (KBr) ν 3100–2800 (max at 2956 and 2864, C–H st), 2700–2400 (max at 2624, 2549 and 2470, ⁺N–H st) cm^{–1}; ¹H NMR (500 MHz, CD₃OD) δ 1.45 [t, *J* = 7.5 Hz, 6 H, N(CH₂CH₃)₂], 1.66–1.76 [complex signal, 6 H, 5(11)–H _{β} , 6(12)–H _{α} and 6(12)–H _{β}], 1.91–1.96 [complex signal, 3 H, 5(11)–H _{α} and 9–H], 2.39 [complex signal, 4 H, 2(3)–H and 4(10)–H], 2.72 [s, 2 H, 1(7)–H], 3.41–3.50 [complex signal, 4 H, N(CH₂CH₃)₂], 4.85 (s, NH); ¹³C NMR (100.6 MHz, CD₃OD) δ 11.5 [CH₃, N(CH₂CH₃)₂], 23.8 [CH₃, C6(12)], 23.9 [CH₂, C5(11)], 48.2 [CH, C2(3)], 48.7 [CH₂, N(CH₂CH₃)₂], 51.5 (CH, C9), 54.3 [CH, 4(10)], 56.3 [CH, 1(7)], 79.4 (C, C8). MS (EI), *m/e* (%): 231 (M⁺, 48), 216 (23), 203 (100), 202 (32), 188 (33), 175 (30), 136 (30). Anal. Calcd. for C₁₆H₂₅N·HCl·0.15H₂O (270.54): C 71.03, H 9.80, N 5.18, Cl 13.10. Found C 70.87, H 9.73, N 5.30, Cl 13.31.

***N*-(Pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodec-8-yl)acetamidine Hydrochloride (16·HCl)**

A suspension of **14·HCl** (250 mg, 1.18 mmol), Et₃N (0.5 mL, 3.59 mmol) and methyl acetimidate hydrochloride (259 mg, 2.37 mmol) in THF (8 mL) was stirred at room temperature for 24 h. The precipitate was filtered and washed with THF and Et₂O. The filtrate was taken in H₂O (5 mL), 5 N NaOH was added til basic pH and the suspension was stirred at room temperature for 1 h. The solid was filtered, washed with H₂O (3×5 mL), taken in EtOAc (10 mL), dried with anhyd. Na₂SO₄, filtered, treated with excess of HCl in Et₂O, and concentrated in vacuo to give the hydrochloride of **16** (124 mg, 42% yield) as a white solid. An analytical sample of **16·HCl** was obtained as a white solid by crystallization from EtOAc/Et₂O, mp >273 °C (dec.); IR (KBr) ν 3500–2800 (max at 3368, 3135, 2940 and 2866, N–H, ⁺N–H and C–H st), 1670 and 1636 (C=N st) cm^{–1}; ¹H-NMR (500 MHz, CD₃OD) δ : 1.45–1.60 [complex signal, 4 H, 6(12)–H _{α} and 6(12)–H _{β}], 1.60–1.66 [complex signal, 5 H, 5(11)–H _{α} , 5(11)–H _{β} and 9–H], 2.27 [s, 2 H, 4(10)–H], 2.32 (s, 3 H, CH₃), 2.38 [broad s, 2 H, 2(3)–H], 2.87 [t, *J* = 3.5 Hz, 2 H, 1(7)–H], 7.62 (s, 1 H, NH), 9.16 (s, 1 H) and 9.34 (s, 1 H) (=NH₂⁺); ¹³C-NMR (100.6 MHz, CD₃OD) δ : 19.4 (CH₃, CH₃), 22.1 [CH₂, C6(12)], 25.1 [CH₂, C5(11)], 48.6 [CH, C2(3)], 52.8 [CH, C1(7)], 53.7 [CH, 4(10)], 55.4 (CH) and 55.5 (CH) (C9), 68.5 (C) y 68.6 (C) (C8), 166.0 [C, CH₃(C=NH)NH]. MS (EI), *m/e* (%): 217 (23), 216 (M⁺, 100), 201 (70), 199 (28), 198 (31), 188 (31), 160 (46), 159 (53), 147 (100), 146 (44), 137 (37), 132 (28), 130 (27), 129 (27), 119 (43), 109 (34), 91 (31), 80 (33), 77 (26), 59 (32). Anal. Calcd. for C₁₄H₂₀N₂·HCl·1.1H₂O (272.60): C 61.68, H 8.58, N 10.28, Cl 13.01. Found C 61.52, H 8.76, N 10.22, Cl 13.44.

Pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodecane-8-carboxamide (17)

A solution of acid **13** (1.00 g, 4.90 mmol) in SOCl₂ (20 mL, 0.27 mol) was heated under reflux for 2 h. The solution was concentrated in vacuo, taken in toluene (5 mL) and concentrated in vacuo (twice). A yellow oil, corresponding to the acyl chloride, was obtained. A solution of this oil in CH₂Cl₂ (5 mL) was cooled to 0 °C, NH₄OH (25% aq. solution, 45 mL) was added dropwise, and the mixture was stirred at room temperature overnight. The mixture was extracted with CH₂Cl₂ (4×30 mL). The aqueous layer was acidified with concd. HCl, extracted with ethyl acetate (3×30 mL), dried with anhydrous

Na₂SO₄, filtered, and concentrated in vacuo to give starting acid **13** (40 mg). On the other hand, the organic phase of CH₂Cl₂ was washed with brine (2x30 mL), dried with anhydrous Na₂SO₄, filtered and concentrated in vacuo to give the amide **17** (894 mg, 94% yield based in recovered starting material) as a white solid. An analytical sample of **17** was obtained by crystallization from CH₂Cl₂/hexane, mp 173-174 °C; IR (KBr) ν 3500-3150 (max at 3481, 3391, 3344, 3206 and 3187, N-H st), 300-2750 (max at 2973, 2943, 2908 and 2868 C-H st), 1651 (C=O st), 1607 (N-C=O st) cm⁻¹; ¹H-NMR (500 MHz, CDCl₃) δ 1.45-1.67 [complex signal, 8 H, 5(11)-H _{α} , 5(11)-H _{β} , 6(12)-H _{α} and 6(12)-H _{β}], 1.84 [t, J = 2.5 Hz, 1 H, 9-H], 2.23 [broad s, 2 H, 4(10)-H], 2.30 [m, 2 H, 2(3)-H], 2.39 [broad s, 2 H, 1(7)-H], 5.43 (broad signal, 1 H) and 5.96 (broad signal, 1 H) (CONH₂); ¹³C-NMR (100.6 MHz, CDCl₃) δ : 23.0 [CH₂, C6(12)], 24.1 [CH₂, C5(11)], 49.0 [CH, C2(3)], 50.0 (CH, C9), 53.5 [CH, C4(10)], 58.1 [CH, C1(7)], 59.4 (C, C8), 176.5 (C, CO). MS (EI), m/e (%): 204 (15), 203 (M⁺, 100), 159 (57), 117 (28), 115 (21), 91 (34), 79 (21), 77 (24). Anal. Calcd. for C₁₃H₁₇NO (203.28): C 76.81, H 8.43, N 6.89. Found C 76.76, H 8.52, N 6.85.

[(Pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodec-8-yl)methyl]amine Hydrochloride (18·HCl)

To a stirred solution of amide **17** (598 mg, 2.94 mmol) in anhyd THF (40 mL) at 0 °C, LiAlH₄ (375 mg, 95% purity, 9.40 mmol) was carefully added. When the addition was finished, the suspension was heated under reflux for 18 h. The mixture was cooled to 0 °C (ice-water bath), treated with 10 N NaOH (5 mL) and stirred at room temperature for 1 h. The obtained solid was filtered through Celite in vacuo and washed with CH₂Cl₂ (3x25 mL). The filtrate was dried with anhyd Na₂SO₄, filtered, treated with excess of HCl in Et₂O and concentrated in vacuo to give the hydrochloride of **18**. Crystallization from MeOH/Et₂O gave pure **18·HCl** as a white solid (396 mg, 60% yield), mp >255 °C (dec.); IR (KBr) ν 3200-2700 (max at 2980, 2940 and 2861, N-H, ⁺N-H and C-H st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.14 [t, J = 2.5 Hz, 1 H, 9-H], 1.47-1.66 [complex signal, 8 H, 5(11)-H _{α} , 5(11)-H _{β} , 6(12)-H _{α} and 6(12)-H _{β}], 2.20 [broad s, 4 H, 1(7)-H and 4(10)-H], 2.33 [m, 2 H, 2(3)-H], 3.11 (s, 2 H, CH₂NH₂), 4.86 (broad signal, 3 H, NH⁺₃); ¹³C-NMR (100.6 MHz, CD₃OD) δ 23.1 [CH₂, C6(12)], 25.0 [CH₂, C5(11)], 38.5 (CH₂, CH₂NH₂), 49.2 [CH, C2(3)], 51.0 (C, C8), 53.0 (CH, C9), 53.7 [CH, C4(10)], 54.4 [CH, C1(7)]. MS (EI), m/e: 190 (16), 189 (M⁺, 17), 173 (30), 172 (100), 157 (35), 144 (52), 143 (37), 131 (38), 130 (41), 129 (68), 128 (32), 117 (45), 115 (41), 109 (34), 108 (30), 107 (30), 106 (35), 105 (33), 94 (54), 92 (36), 91 (71), 80 (47), 79 (46), 77 (41). Anal. Calcd. for C₁₃H₁₉N·HCl·0.33H₂O (231.77): C 67.37, H 8.99, N 6.04, Cl 15.30. Found C 67.51, H 9.22, N 6.00, Cl 15.44.

N-Methyl[(pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodec-8-yl)methyl]amine Hydrochloride (19·HCl)

To a stirred solution of **18·HCl** (225.8 mg, 1.0 mmol), Et₃N (0.55 mL, 3.76 mmol) in anhyd Et₂O (10 mL) at 0 °C, methyl chloroformate (0.18 mL, 1.94 mmol) was added dropwise. When the addition was finished, the solution was stirred at room temperature for 18 h. Water (15 mL) was added and the two layers were separated. The aqueous layer was extracted with EtOAc (3x10 mL). The combined organic extracts were washed with H₂O (1x10 mL), with 2 N HCl (2x10 mL), dried with anhyd Na₂SO₄, filtered and concentrated in vacuo to give the expected carbamate. This residue dissolved in anhyd THF (2 mL) was added dropwise to a suspension of LiAlH₄ (265 mg, 7.0 mmol) in anhyd THF (7 mL) at 0 °C. When the addition was finished, the suspension was heated under reflux for 20 h. The mixture was cooled to 0 °C (ice-water bath), treated with 5 N NaOH (3 mL) and stirred at room temperature for 1 h. The obtained solid was filtered through Celite in vacuo and washed with CH₂Cl₂ (3x10 mL). The filtrate was dried with anhyd Na₂SO₄, filtered, treated with excess of HCl in Et₂O and concentrated in vacuo to give the hydrochloride of **19**. Crystallization from MeOH/Et₂O gave pure **19·HCl** as a white solid (98.3 mg, 41% yield), mp >280 °C (dec.); IR (KBr) ν 3000-2750 (max at 2938, 2873, 2824 and 2754, N-H, ⁺N-H and C-H st) cm⁻¹; ¹H-NMR (500 MHz, CD₃OD) δ 1.15 [broad s, 1 H, 9-H], 1.48x1.67

[complex signal, 8 H, 5(11)-H α , 5(11)-H β , 6(12)-H α and 6(12)-H β], 2.18 [broad s, 2 H, 4(10)-H], 2.21 [broad s, 2 H, 1(7)-H], 2.34 [d, $J = 1.5$ Hz, 2 H, 2(3)-H], 2.76 (s, 3 H, CH₃), 3.21 (s, 2 H, CH₂N), 4.85 (s, NH₂⁺); ¹³C-NMR (100.6 MHz, CD₃OD) δ 23.1 [CH₂, C6(12)], 25.0 [CH₂, C5(11)], 34.9 (CH₃, CH₃NH), 48.9 (CH₂, CH₂N), 49.3 [CH, C2(3)], 50.8 (C, C8), 53.3 (CH, C9), 54.0 [CH, C4(10)], 54.3 [CH, C1(7)]. MS (EI), m/e (%): 203 (M⁺, 15), 172 (100), 157 (44), 144 (52), 143 (38), 131 (26), 130 (30), 129 (49), 117 (25), 106 (31), 94 (59), 91 (48), 80 (69), 79 (33), 44 (81). Anal. Calcd. for C₁₄H₂₁N·HCl (239.78): C 70.13, H 9.25, N 5.84, Cl 14.79. Found C 70.37, H 9.30, N 5.81, Cl 14.91.

3-Azahexacyclo[7.6.0.0^{1,5}.0^{5,12}.0^{6,10}.0^{11,15}]pentadeca-7,13-diene-1,2-dione (**20**)

A mixture of diacid **12** (2.44 g, 10.0 mmol) and urea (3.00 g, 97% purity, 50.0 mmol) was heated slowly to 135 °C. When the mixture melted it was heated to 180 °C for 30 min and cooled. Water (50 mL) was added and the suspension was extracted with CH₂Cl₂ (6×30 mL). The combined organic extracts were washed with brine (1×45 mL), dried with anhyd Na₂SO₄, filtered and concentrated in vacuo to dryness to give imide **20** as a white solid (952 mg, 42% yield). An analytical sample of **20** was obtained by crystallization from CH₂Cl₂, mp >245 °C (dec.); IR (KBr) ν 3187 (N-H), 1751 and 1718 (C=O st) cm⁻¹; ¹H NMR (500 MHz, CDCl₃) δ 2.87 [m, 2 H, 10(11)-H], 3.46 [m, 4 H, 6(9,12,15)-H], 6.07 [t, $J = 2.0$ Hz, 4 H, 7(8,13,14)-H], 7.96 (broad s, 1 H, NH); ¹³C NMR (100.6 MHz, CDCl₃) δ 62.4 [CH, C6(9,12,15)], 64.4 [CH, C10(11)], 68.3 [C, C1(5)], 132.2 [CH, C7(8,13,14)], 175.5 (C, CO). MS (EI), m/e (%): 225 (M⁺, 4), 160 (100), 153 (31), 152 (18). Anal. Calcd. for C₁₄H₁₄NO₂ (225.24): C 74.65, H 4.92, N 6.22. Found C 74.29, H 4.97, N 6.31.

3-Azahexacyclo[7.6.0.0^{1,5}.0^{5,12}.0^{6,10}.0^{11,15}]pentadeca-7,13-diene Hydrochloride (**21·HCl**)

To a stirred solution of imide **20** (1.45 g, 6.42 mmol) in anhyd THF (50 mL) at 0 °C, LiAlH₄ (2.44 g, 64.2 mmol) was carefully added. When the addition was finished, the suspension was heated under reflux for 72 h. The mixture was cooled to 0 °C (ice-water bath), treated with 10 N NaOH til basic pH and stirred at room temperature for 1 h. The obtained solid was filtered in vacuo through Celite and washed with CH₂Cl₂ (3×25 mL). The filtrate was dried with anhyd Na₂SO₄, filtered and evaporated in vacuo. The obtained solid residue was taken in Et₂O (20 mL) and treated with excess of HCl in Et₂O to give the hydrochloride of **21**. An analytical sample of **21·HCl** was obtained as a white solid by crystallization from MeOH/Et₂O, mp >240 °C (dec.); IR (KBr) ν 3160, 3060, 2974, 2949, 2883, 2836 and 2736 (NH₂⁺ st) cm⁻¹; ¹H NMR (500 MHz, DMSO-d₆) δ 2.77 (m, 2 H, 10(11)-H), 2.97 [s, 4 H, 2(4)-H₂], 3.11 [pseudo q, $J = 2.5$ Hz, 4 H, 6(9,12,15)-H], 4.86 (s, NH₂), 6.19 [t, $J = 2.0$ Hz, 4 H, 7(8,13,14)-H]; ¹³C NMR (100.6 MHz, DMSO-d₆) δ 36.3 [CH₂, C2(4)], 53.3 [CH, C6(9,12,15)], 55.3 [CH, C10(11)], 63.3 [C, C1(5)], 125.4 [CH, C7(8,13,14)]. MS (EI), m/e (%): 197 (M⁺, 55), 196 (35), 168 (24), 167 (26), 165 (27), 156 (30), 153 (25), 152 (30), 132 (64), 131 (91), 130 (100), 118 (48), 117 (38), 115 (51). Anal. Calcd. for C₁₄H₁₅N·HCl (233.74): C 71.94, H 6.90, N 5.99, Cl 15.17. Found C 71.80, H 7.08, N 5.91, Cl 15.47.

3-Azahexacyclo[7.6.0.0^{1,5}.0^{5,12}.0^{6,10}.0^{11,15}]pentadecane Hydrochloride (**22·HCl**)

A suspension of **21·HCl** (150 mg, 0.64 mmol) and 5% Pd/C (50% in water, 10 mg) in absolute EtOH (20 mL) was hydrogenated at 1 atm for 48 h. The suspension was filtered, the residue was washed with EtOH and the filtrate was concentrated in vacuo to give **22·HCl** as a white solid (150 mg, 99% yield). An analytical sample of **22·HCl** was obtained by crystallization from MeOH/Et₂O, mp >240 °C (dec.); IR (KBr) ν 2938, 2871, 2793 and 2765 (NH₂⁺ st) cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ 1.62–1.68 [complex signal, 8 H, 7(8,13,14)-H α and 7(8,13,14)-H β], 2.24 [m, 4 H, 6(9,12,15)-H], 2.55 (broad s, 2 H, 10(11)-H), 3.11 [s, 4 H, 2(4)-H₂], 4.86 (s, NH₂); ¹³C NMR (100.6 MHz, CD₃OD) δ 22.3 [CH₂, C7(8,13,14)], 41.5 [CH₂, C2(4)], 51.7 [CH, C10(11)], 56.1 [CH, C6(9,12,15)], 62.2 [C, C1(5)]. MS (EI), m/e (%): 201 (M⁺, 100), 186 (23), 184 (36), 169 (50), 156 (27), 155 (27),

143 (32), 129 (45), 106 (70), 91 (50). Anal. Calcd. for $C_{14}H_{19}N \cdot HCl \cdot 0.4H_2O$ (244.98): C 68.64, H 8.56, N 5.72, Cl 14.47. Found C 68.59, H 8.56, N 5.56, Cl 14.41.

3-Azaoctacyclo[8.7.0.0^{1,5}.0^{5,13}.0^{6,11}.0^{7,9}.0^{12,17}.0^{14,16}]heptadecane-2,4-dione (**23**)

To a suspension of imide **20** (2.50 g, 11.1 mmol) and $Pd(OAc)_2$ (62.8 mg, 0.28 mmol) in CH_2Cl_2 (5 mL) an ethereal solution of diazomethane (freshly prepared from *N*-methyl-*N*-nitrosourea, 10 g, 9.7 mmol) was added and the mixture was stirred at room temperature. Two further additions of ethereal solution of diazomethane were performed to achieve total conversion (1H NMR control). The suspension was filtered, $NaHCO_3$ (saturated aq. solution, 100 mL) was added and the mixture was stirred at room temperature overnight. The organic layer was separated, dried with anhyd Na_2SO_4 , filtered and evaporated in vacuo to give the imide **23** as a pale yellow solid (2.66 g, 95% yield). An analytical sample of **23** was obtained by crystallization from CH_2Cl_2 /pentane, mp 277–278 °C (dec.); IR (KBr) ν 3225 (NH st), 1751, 1721 and 1698 (C=O st) cm^{-1} . 1H NMR (500 MHz, $CDCl_3$) δ 0.13 [dt, 2 H, $J = 5.5$ Hz, $J' = 7.5$ Hz, 8(15)- H_{endo}], 0.26 [dt, 2 H, $J = 5.5$ Hz, $J' = 3.3$ Hz, 8(15)- H_{exo}], 1.11 [dd, 4 H, $J = 7.5$ Hz, $J' = 3.3$ Hz, 7(9,14,16)-H], 1.93 [m, 2 H, 11(12)-H], 2.81 [dd, 4 H, $J = 2.0$ Hz, $J' = 0.5$ Hz, 6(10,13,17)-H], 8.28 (s, 1 H, NH); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 2.1 [CH_2 , C8(15)], 9.6 [CH, C7(9,14,16)], 39.1 [CH, C11(12)], 55.2 [CH, C6(10,13,17)], 66.3 [C, C1(5)], 176.6 [C, C2(4)]. MS (EI), m/e (%): 253 (M^+ , 21), 187 (24), 182 (20), 175 (31), 174 (26), 167 (32), 166 (23), 165 (48), 152 (27), 148 (30), 128 (28), 115 (38), 104 (25), 91 (38), 79 (100), 77 (61). Anal. Calcd. For $C_{16}H_{15}NO_2$ (253.30): C 75.87, H 5.97, N 5.53. Found C 75.64, H 5.77, N 5.49.

3-Azaoctacyclo[8.7.0.0^{1,5}.0^{5,13}.0^{6,11}.0^{7,9}.0^{12,17}.0^{14,16}]heptadecane Hydrochloride (**24**·HCl)

To a stirred solution of imide **23** (763 mg, 3.01 mmol) in anhyd THF (28 mL) at 0 °C, $LiAlH_4$ (1.14 g, 30.1 mmol) was carefully added. When the addition was finished, the suspension was heated under reflux for 72 h. The mixture was cooled to 0 °C (ice-water bath), treated with 10 N NaOH til basic pH and stirred at room temperature for 1 h. The obtained solid was filtered in vacuo through Celite and was washed with CH_2Cl_2 (3×25 mL). The filtrate was dried with anhyd Na_2SO_4 , filtered and evaporated in vacuo. The obtained solid residue was taken in EtOAc/Et₂O and treated with excess of HCl in Et₂O to give the hydrochloride of **24** as a solid (506 mg, 64% yield). An analytical sample of **24**·HCl was obtained by crystallization from CH_2Cl_2 /pentane, mp 242–243 °C (dec.); IR (KBr) ν 3446 (NH st) cm^{-1} . 1H NMR (500 MHz, CD_3OD) δ 0.19 [dt, 2 H, $J = 5.0$ Hz, $J' = 7.2$ Hz, 8(15)- H_{endo}], 0.43 [dt, 2 H, $J = 5.0$ Hz, $J' = 3.0$ Hz, 8(15)- H_{exo}], 1.13 [dd, 4 H, $J = 7.2$ Hz, $J' = 3.1$ Hz, 7(9,14,16)-H], 1.89 [m, 2 H, 11(12)-H], 2.37 [broad s, 4 H, 6(10,13,17)-H], 3.29 [s, 4 H, 2(4)- H_2]; ^{13}C NMR (100.6 MHz, CD_3OD) δ 3.2 [CH_2 , C8(15)], 10.3 [CH, C7(9,14,16)], 39.7 [CH, C11(12)], 41.9 [CH_2 , C2(4)], 54.8 [CH, C6(10,13,17)], 66.5 [C, C1(5)]. MS (EI), m/e (%): 226 (19), 225 (M^+ , 100), 224 (74), 170 (20), 165 (21), 146 (27), 145 (31), 144 (70), 132 (22), 130 (30), 129 (25), 128 (26), 118 (38), 117 (45), 115 (41), 91 (38), 80 (41), 79 (44), 77 (39). Anal. Calcd. For $C_{16}H_{19}N \cdot HCl \cdot 1.25H_2O$ (284.31): C 67.59, H 7.98, N 4.93, Cl 12.47. Found C 67.42, H 7.75, N 4.87, Cl 12.80.

Molecular modeling

Docking studies and restrained molecular dynamics (MD) simulations were combined to explore the structural features of the binding of Amt and the ring-expanded compound **24** to the A/M2 ion channel. The PDB file 2kqt was used as template of the channel, as it provides the ssNMR structure of the M2TM region in DMPC bilayers bound to deuterated Amt.¹⁵ Thus, it accounts for conformational changes in the transmembrane helices induced upon ligand binding. The structure of the channel was refined by energy minimization using AMBER⁶⁷ and the parmff99SB⁶⁸ force field by imposing suitable positional restraints to the backbone atoms (see below). The whole channel lumen was considered as the binding site in

docking computations. The ligands were built up with MOE (Chemical Computing Group, Montreal). According to the expected basicity for primary and secondary amines at physiological pH, both Amt and **24** were modeled in the protonated form. The geometry was refined by energy minimization with the MMFF94 force field.⁶⁹ Docking simulations were carried out using rDock.^{70–71} The best 50 poses for each ligand were considered to examine the binding of the compounds in the luminal part of the channel.

Restrained molecular dynamics (MD) simulations were performed for three different models of Amt, which were built up by placing this compound with the amino group oriented towards the i) C- and ii) N-terminus of the pore, and iii) starting from the docked pose. Likewise, three distinct starting orientations were also considered for compound **24**: i) the docking pose, ii) by placing the main axis of the molecule aligned with the axis of the pore in the Amt binding site, and iii) by placing **24** below the plane defined by Ala30, with the main axis normal to the pore axis and the amino group pointing towards the C-terminus (see Figure S4 in Supporting Information). Capping (acetyl, *N*-methyl) groups were added to the four helices in the ssNMR structure. TIP3P water molecules⁷² were added to fill the luminal region paying particular attention to the positioning of the hydrating water molecules around the His37 box, as they have been recently identified in a X-ray structure of the channel (PDB entry 3LBW).⁶⁴ Correspondingly, only two His37 were protonated.⁶⁴ The geometry of Amt and **24**, both in their protonated state, was optimized at the B3LYP/6-31G(d) level and partial charges were derived using the RESP procedure.⁷³ Standard atom types defined in the AMBER force field were assigned to the ligand.

The geometry of the system was minimized in four steps. First, the position of hydrogen atoms was optimized using 3000 steps of steepest descent algorithm. Then, water molecules were refined through 2000 steps of steepest descent followed by 3000 steps of conjugate gradient. Next, the position of the ligand and water molecules were optimized with 2000 steps of steepest descent and 4000 steps of conjugate gradient. Finally, the whole system was optimized with 3000 steps of steepest descent and 7000 steps of conjugate gradient. Thermalization of the system was performed in five steps of 100 ps, increasing the temperature from 50 to 298 K. A restrain of 25 kcal mol⁻¹ Å⁻² was applied through all the stages to backbone atoms of the helices, and to the side chains of His37 and Trp41. Then, a 250 ps MD simulation was run at 298 K reducing progressively the restraint of the backbone atoms to 10 kcal mol⁻¹ Å⁻², and to 5 kcal mol⁻¹ Å⁻² for the heavy atoms in the side chain of Trp41. The addition of the restraints is necessary to preserve the global conformational state of the channel in the presence of bound ligand. Then, a series of restrained trajectories were run using a time step of 1 fs, SHAKE for those bonds containing hydrogen atoms, and a cutoff of 11 Å for nonbonded interactions. In particular, three 40 ns MD simulations were run for Amt, each starting from one of the three different binding modes. For **24**, two trajectories were run for 40 ns (binding modes i and ii; see above), and in a third case (binding mode iii) the trajectory was continued up to 58 ns, when convergence in the binding mode of the ligand relative to the orientation adopted in the other two MD simulations was found (see Figure S5 in Supporting Information).

Finally, the Solvent Interaction Energies (SIE) technique developed by Purisima and co-workers was used to estimate the interaction free energies for the different binding modes of Amt (see Table S1 in Supporting Information).^{74–75}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

MDD, ET, SV, JJ-J and FJL thank the Spanish Ministerio de Ciencia e Innovación (FPU fellowship to MDD; grants CTQ2008-03768 and SAF2008-0559) and the Generalitat de Catalunya (FI fellowship to ET; grant SCG-2009-294) for financial support. LN acknowledges financial support from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO No. 9.0188.07) and the Geconcerteerde Onderzoeksacties (GOA/10/014), and the technical assistance from L. Persoons and W. van Dam. The research was supported by NIH Research Grants R01 AI-57363 (LHP) and U01 AI-1074571 (LHP, WFD and RAL).

a Abbreviations

Amt	amantadine
BSA	bovine serum albumin
CD	circular dichroism
DMEM	Dulbecco's modified eagle medium
DMPC	dimyristoylphosphatidylcholine
DPC	dodecylphosphocholine
MDCK	Madin-Darby Canine Kidney
MD	molecular dynamics
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 <i>H</i> -tetrazolium
PBS	phosphate buffered saline
PDB	Protein Data Bank
ssNMR	solid state Nuclear Magnetic Resonance
TEV	two-electrode voltage clamps
TM	transmembrane
wt	wild-type

References

1. Lamb, RA.; Holsinger, LJ.; Pinto, LH. The influenza A virus M₂ ion channel protein and its role in the influenza virus life cycle.. In: Wimmer, E., editor. Receptor-Mediated Virus Entry into Cells. Cold Spring Harbor Press; Cold Spring Harbor, New York: 1994. p. 303-321.
2. Lamb RA, Zebedee SL, Richardson CD. Influenza virus M₂ protein is an integral membrane protein expressed on the infected-cell surface. *Cell*. 1985; 40:627–633. [PubMed: 3882238]
3. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Ann. Rev. Biochem.* 2000; 69:531–569. [PubMed: 10966468]
4. Zhimov OP. Solubilization of matrix protein M1/M from virions occurs at different pH for orthomyxo- and paramyxoviruses. *Virology*. 1990; 176:274–279. [PubMed: 2158693]
5. Grambas S, Hay AJ. Maturation of influenza A virus hemagglutinin--estimates of the pH encountered during transport and its regulation by the M2 protein. *Virology*. 1992; 190:11–18. [PubMed: 1529523]
6. Hu J, Fu R, Nishimura K, Zhang L, Zhou H-X, Busath DD, Vijayvergiya V, Cross TA. Histidines, heart of the hydrogen ion channel from influenza A virus: Toward an understanding of conductance and proton selectivity. *Proc. Natl. Acad. Sci. U S A*. 2006; 103:6865–6870. [PubMed: 16632600]
7. Pinto LH. The M2 proton channels of influenza A and B viruses. *J. Biol. Chem.* 2006; 281:8997–9000. [PubMed: 16407184]

8. Lagoja IM, De Clercq E. Anti-influenza virus agents: synthesis and mode of action. *Med. Res. Rev.* 2008; 28:1–38. [PubMed: 17160999]
9. Tu Q, Pinto LH, Luo G, Shaughnessy MA, Mullaney D, Kurtz S, Krystal M, Lamb RA. Characterization of inhibition of M₂ ion channel activity by BL-1743, an inhibitor of influenza A virus. *J. Virol.* 1996; 70:4246–4252. [PubMed: 8676445]
10. Wang J, Cady SD, Balannik V, Pinto LH, DeGrado WF, Hong M. Discovery of spiro-piperidine inhibitors and their modulation of the dynamics of the M2 proton channel from influenza A virus. *J. Am. Chem. Soc.* 2009; 131:8066–8076. [PubMed: 19469531]
11. Bright RA, Shay DK, Shu B, Cox NJ, Klimov AI. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. *Jama.* 2006; 295:891–894. [PubMed: 16456087]
12. Cady SD, Mishanina TV, Hong M. Structure of amantadine-bound M2 transmembrane peptide of influenza A in lipid bilayers from magic-angle-spinning solid-state NMR: the role of Ser31 in amantadine binding. *J. Mol. Biol.* 2009; 385:1127–1141. [PubMed: 19061899]
13. Cady SD, Luo W, Hu F, Hong M. Structure and function of the influenza A M2 proton channel. *Biochemistry.* 2009; 48:7356–7364. [PubMed: 19601584]
14. Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, Shu Y, Gubareva LV, Cox NJ, Klimov AI. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J. Infect. Dis.* 2007; 196:249–257. [PubMed: 17570112]
15. Cady SD, Schmidt-Rohr K, Wang J, Soto CS, DeGrado WF, Hong M. Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. *Nature.* 2010; 463:689–692. [PubMed: 20130653]
16. Chen H, Wu Y, Voth GA. Proton transport behavior through the influenza A M2 channel: insights from molecular simulation. *Biophys. J.* 2007; 93:3470–3479. [PubMed: 17693473]
17. Duff KC, Gilchrist PJ, Saxena AM, Bradshaw JP. Neutron diffraction reveals the site of amantadine blockade in the influenza A M2 ion channel. *Virology.* 1994; 202:287–293. [PubMed: 7516598]
18. Jing X, Ma C, Ohigashi Y, Oliveira FA, Jardetzky TS, Pinto LH, Lamb RA. Functional studies indicate amantadine binds to the pore of the influenza A virus M2 proton-selective ion channel. *Proc. Natl. Acad. Sci. U S A.* 2008; 105:10967–10972. [PubMed: 18669647]
19. Khurana E, Dal Peraro M, DeVane R, Vemparala S, DeGrado WF, Klein ML. Molecular dynamics calculations suggest a conduction mechanism for the M2 proton channel from influenza A virus. *Proc. Natl. Acad. Sci. U S A.* 2009; 106:1069–1074. [PubMed: 19144924]
20. Stouffer AL, Acharya R, Salom D, Levine AS, Di Costanzo L, Soto CS, Tereshko V, Nanda V, Stayrook S, DeGrado WF. Structural basis for the function and inhibition of an influenza virus proton channel. *Nature.* 2008; 451:596–599. [PubMed: 18235504]
21. Yi M, Cross TA, Zhou HX. A secondary gate as a mechanism for inhibition of the M2 proton channel by amantadine. *J. Phys. Chem.* 2008; 112:7977–7979.
22. Schnell JR, Chou JJ. Structure and mechanism of the M2 proton channel of influenza A virus. *Nature.* 2008; 451:591–595. [PubMed: 18235503]
23. Ohigashi Y, Ma C, Jing X, Balannick V, Pinto LH, Lamb RA. An amantadine-sensitive chimeric BM2 ion channel of influenza B virus has implications for the mechanism of drug inhibition. *Proc. Natl. Acad. Sci. U S A.* 2009; 106:18775–18779. [PubMed: 19841275]
24. Balannik V, Wang J, Ohigashi Y, Jing X, Magavern E, Lamb RA, DeGrado WF, Pinto LH. Design and pharmacological characterization of inhibitors of amantadine-resistant mutants of the M2 ion channel of influenza A virus. *Biochemistry.* 2009; 48:11872–11882. [PubMed: 19905033]
25. Balannik V, Carnevale V, Fiorin G, Levine BG, Lamb RA, Klein ML, DeGrado WF, Pinto LH. Functional studies and modeling of pore-lining residue mutants of the influenza a virus M2 ion channel. *Biochemistry.* 2010; 49:696–708. [PubMed: 20028125]
26. Rosenberg MR, Casarotto MG. Coexistence of two adamantane binding sites in the influenza A M2 ion channel. *Proc. Natl. Acad. Sci. U S A.* 2010; 107:13866–13871. [PubMed: 20643947]
27. Bright RA, Medina MJ, Xu X, Pérez-Oronoz G, Wallis TR, Davis XM, Povinelli L, Cox NJ, Klimov AI. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated

- worldwide from 1994 to 2005: a cause for concern. *Lancet*. 2005; 366:1175–1181. [PubMed: 16198766]
28. Saito R, Sakai T, Sato I, Sano Y, Oshitani H, Sato M, Suzuki H. Frequency of amantadine-resistant influenza A viruses during two seasons featuring cocirculation of H1N1 and H3N2. *J. Clin. Microbiol.* 2003; 41:2164–2165. [PubMed: 12734269]
 29. Hien TT, Boni MF, Bryant JE, Ngan TT, Wolbers M, Nguyen TD, Truong NT, Dung NT, Ha do Q, Hien VM, Thanh TT, Nhu le NT, Uyen le TT, Nhien PT, Chinh NT, Chau NV, Farrar J, van Doorn HR. Early pandemic influenza (2009 H1N1) in Ho Chi Minh City, Vietnam: a clinical virological and epidemiological analysis. *PLoS Med.* 2010; 7:e1000277. [PubMed: 20502525]
 30. Pebody R, McLean E, Zhao H, Cleary P, Bracebridge S, Foster K, Charlett A, Hardeid P, Waight P, Ellis J, Birmingham A, Zambon M, Evans B, Salmon R, McMenamin J, Smyth B, Catchpole M, Watson J. Pandemic Influenza A (H1N1) 2009 and mortality in the United Kingdom: risk factors for death, April 2009 to March 2010. *Euro Surveill* 15. 2010
 31. Geluk HW, Schut J, Schlattmann JLMA. Synthesis and antiviral properties of 1-adamantylguanidine. A modified method for preparing *t*-alkylguanidines. *J. Med. Chem.* 1969; 12:712–715. [PubMed: 5793173]
 32. Aldrich PE, Hermann EC, Meier WE, Paulshock M, Prichard WW, Snyder JA, Watts JC. Antiviral agents. 2. Structure-activity relationships of compounds related to 1-adamantanamine. *J. Med. Chem.* 1971; 14:535–543. [PubMed: 5091970]
 33. Lundahl K, Schut J, Schlattmann JLMA, Paerels GB, Peters A. Synthesis and antiviral activities of adamantane spiro compounds. 1. Adamantane and analogous spiro-3'-pyrrolidines. *J. Med. Chem.* 1972; 15:129–132. [PubMed: 4332673]
 34. Van Hes R, Smit A, Kralt T, Peters A. Synthesis and antiviral activities of adamantane spiro compounds. 2. *J. Med. Chem.* 1972; 15:132–136. [PubMed: 5008237]
 35. Mathur A, Beare AS, Reed SE. In vitro antiviral activity and preliminary clinical trials of a new adamantane compound. *Antimicrob. Agents Chemother.* 1973; 4:421–426. [PubMed: 4364762]
 36. Aigami K, Inamoto Y, Takaishi N, Hattori K, Takatsuki A, Tamura G. Biologically active polycycloalkanes. 1. Antiviral adamantane derivatives. *J. Med. Chem.* 1975; 18:713–721. [PubMed: 1171241]
 37. Kolocouris N, Foscolos GB, Kolocouris A, Marakos P, Pouli N, Fytas G, Ikeda S, De Clercq E. Synthesis and antiviral activity of some aminoadamantane derivatives. *J. Med. Chem.* 1994; 37:2896–2902. [PubMed: 8071937]
 38. Kolocouris N, Kolocouris A, Foscolos GB, Fytas G, Neyts J, Padalko E, Balzarini J, Snoeck R, Andrei G, De Clercq E. Synthesis and antiviral activity of some new aminoadamantane derivatives. 2. *J. Med. Chem.* 1994; 37:2896–2902. [PubMed: 8071937]
 39. Scholtissek C, Quack G, Klenk HD, Webster RG. How to overcome resistance of influenza A viruses against adamantane derivatives. *Antiviral Res.* 1998; 37:83–95. [PubMed: 9588841]
 40. Stamatiou G, Foscolos GB, Futas G, Kolocouris A, Kolocouris N, Pannecouque C, Witvrouw M, Padalko E, Neyts J, De Clercq E. Heterocyclic rimantadine analogues with antiviral activity. *Bioorg. Med. Chem.* 2003; 11:5485–5492. [PubMed: 14642592]
 41. Stylianakais I, Kolocouris A, Kolocouris N, Fytas G, Foscolos GB, Padalko E, Neyts J, De Clercq E. Spiro[pyrrolidine-2,2'-adamantanes]: synthesis, anti-influenza virus activity and conformational properties. *Bioorg. Med. Chem. Lett.* 2003; 13:1699–1703. [PubMed: 12729645]
 42. Zoidis G, Kolocouris N, Foscolos GB, Kolocouris A, Fytas G, Karayannis P, Padalko E, Neyts J, De Clercq E. Are the 2-isomers of the drug rimantadine active anti-influenza A agents?. *Antiviral Chem. Chemother.* 2003; 14:153–164.
 43. Setaki D, Tataridis D, Stamatiou G, Kolocouris A, Foscolos GB, Fytas G, Kolocouris N, Padalko E, Neyts J, De Clercq E. Synthesis, conformational characteristics and anti-influenza virus A activity of some 2-adamantylsubstituted azacycles. *Bioorg. Chem.* 2006; 34:248–273. [PubMed: 16879857]
 44. Zoidis G, Fytas C, Papanastasiou I, Foscolos GB, Fytas G, Padalko E, De Clercq E, Naesens L, Neyts J, Kolocouris N. Heterocyclic rimantadine analogues with antiviral activity. *Bioorg. Med. Chem.* 2006; 14:3341–3348. [PubMed: 16439137]

45. Tataridis D, Fytas G, Kolocouris A, Fytas C, Kolocouris N, Foscolos GB, Padalko E, Neyts J, De Clercq E. Influence of an additional 2-amino substituent of the 1-aminoethyl pharmacophore group on the potency of rimantadine against influenza virus A. *Bioorg. Med. Chem. Lett.* 2007; 17:692–696. [PubMed: 17113287]
46. Kolocouris N, Zoidis G, Foscolos GB, Fytas G, Prathalingham SR, Kelly JM, De Clercq E. Design and synthesis of bioactive adamantane spiro heterocycles. *Bioorg. Med. Chem. Lett.* 2007; 17:4358–4362. [PubMed: 17588747]
47. Zoidis G, Tsotinis A, Kolocouris N, Kelly JM, Prathalingham SR, Naesens L, De Clercq E. Design and synthesis of bioactive 1,2-annulated adamantane derivatives. *Org. Biomol. Chem.* 2008; 6:3177–3185. [PubMed: 18698478]
48. Kolocouris A, Spearpoint P, Martin SR, Hay AJ, López-Querol M, Sureda FX, Padalko E, Neyts J, De Clercq E. Comparisons of the influenza virus A M2 channel binding affinities, anti-influenza virus potencies and NMDA antagonistic activities of 2-alkyl-2-aminoadamantanes and analogues. *Bioorg. Med. Chem. Lett.* 2008; 18:6156–6160. [PubMed: 18947998]
49. Zoidis G, Kolocouris N, Naesens L, De Clercq E. Design and synthesis of 1,2-annulated adamantane piperidines with anti-influenza virus activity. *Bioorg. Med. Chem.* 2009; 17:1534–1541. [PubMed: 19195900]
50. Zarubae VV, Golod EL, Anfimov PM, Shtro AA, Saraev VV, Gavrilov AS, Logvinov AV, Kiselev OI. Synthesis and anti-viral activity of azolo-adamantanes against influenza A virus. *Bioorg. Med. Chem.* 2010; 18:839–848. [PubMed: 20006516]
51. Whitney JG, Gregory WA, Kauer JC, Roland JR, Snyder JA, Benson RE, Hermann EC. Antiviral agents. I. Bicyclo[2.2.2]octan- and -oct-2-enamines. *J. Med. Chem.* 1970; 13:254–260. [PubMed: 5418498]
52. Aigami K, Inamoto Y, Takaishi N, Hattori K, Takatsuki A, Tamura G. Biologically active polycycloalkanes. 1. Antiviral adamantane derivatives. *J. Med. Chem.* 1975; 18:713–721. [PubMed: 1171241]
53. Inamoto Y, Aigami K, Takaishi N, Fujikura Y, Ohsugi M, Ikeda H, Tsuchihashi K. Biologically active polycycloalkanes. 6. Antiviral 1-tricyclo[4.3.1.1^{2,5}]undecyl derivatives. *J. Med. Chem.* 1979; 22:1206–1214. [PubMed: 92566]
54. Al-Nakib W, Higgins PG, Willman J, Tyrrell DAJ, Swallow DL, Hurst BC, Rushton A. Prevention and treatment of experimental influenza A virus infection in volunteers with a new antiviral ICI 130685. *J. Antimicrob. Chemother.* 1986; 18:119–129. [PubMed: 3531141]
55. García A, Teso E, García A, de la Moya S, Rodríguez ME, Martínez P, Subramanian LR, García A. Synthesis of substituted 1-norbornylamines with antiviral activity. *J. Med. Chem.* 1995; 38:4474–4477. [PubMed: 7473574]
56. Hu W, Zeng S, Li C, Jie Y, Li Z, Chen L. Identification of hits as matrix-2 protein inhibitors through the focused screening of a small primary amine library. *J. Med. Chem.* 2010; 53:3831–3834. [PubMed: 20394375]
57. Camps P, Duque MD, Vázquez S, Naesens L, De Clercq E, Sureda FS, López-Querol M, Camins A, Pallàs M, Prathalingam SR, Kelly JM, Romero V, Ivorra D, Cortés D. Synthesis and pharmacological evaluation of several ring-contracted amantadine analogs. *Bioorg. Med. Chem.* 2008; 16:9925–9936. [PubMed: 18954995]
58. Duque MD, Camps P, Profire L, Montaner S, Vázquez S, Sureda FS, Mallol J, López-Querol M, Naesens L, De Clercq E, Prathalingam SR, Kelly JM. Synthesis and pharmacological evaluation of (2-oxaadamant-1-yl)amines. *Bioorg. Med. Chem.* 2009; 17:3198–3206. [PubMed: 19251424]
59. Paquette LA. Dodecahedranes and allied spherical molecules. *Chem. Rev.* 1989; 89:1051–1065.
60. McNeil D, Vogt BR, Sudol JJ, Theodoropoulos S, Hedaya E. Multiple cycloaddition reaction of 9,10-dihydrofulvalene. New approach to 3,4,7-methenocyclopenta[a]pentalene derivatives. *J. Am. Chem. Soc.* 1974; 96:4673–4674.
61. Taylor RT, Pelter MW, Paquette LA. Domino Diels-Alder reaction: 3,3a,3b,4,6a,7a-hexahydro-3,4,7-metheno-7H-cyclopenta[a]pentalene-7,8-dicarboxylic acid. *Org. Synth.* 1990; 68:198–205.

62. Camps P, Lukach AE, Rossi RA. Synthesis of Several Halobisnoradamantane Derivatives and Their Reactivity through the $S_{RN}1$ Mechanism. *J. Org. Chem.* 2001; 66:5366–5373. [PubMed: 11485457]
63. Khurana E, DeVane RH, Dal Peraro M, Klein ML. Computational study of drug binding to the membrane-bound tetrameric M2 peptide bundle from influenza A virus. *Biochim. Biophys. Acta.* 2011; 1808:530–537. [PubMed: 20385097]
64. Acharya R, Carnevale V, Fiorin G, Levine BG, Polischuk AL, Balannik V, Samish I, Lamb RA, Pinto LH, DeGrado WF, Klein ML. Structure and mechanism of proton transport through the transmembrane tetrameric M2 protein bundle of the influenza A virus. *Proc. Natl. Acad. Sci. USA.* 2010; 107:15075–15080. [PubMed: 20689043]
65. Ma C, Soto CS, Ohigashi Y, Taylor A, Bournas V, Glawe B, Udo MK, DeGrado WF, Lamb RA, Pinto LH. Identification of the pore-lining residues of the BM2 ion channel protein of influenza B virus. *J. Biol. Chem.* 2008; 283:15921–15931. [PubMed: 18408016]
66. Vanderlinden E, Göktas F, Cesur Z, Froeyen M, Reed ML, Russell CJ, Cesur N, Naesens L. Novel inhibitors of influenza virus fusion: structure-activity relationship and interaction with the viral hemagglutinin. *J. Virol.* 2010; 84:4277–4288. [PubMed: 20181685]
67. Case, DA.; Darden, TA.; Cheatham, TE., III; Simmerling, CL.; Wang, J.; Duke, RE.; Luo, R.; Merz, KM.; Pearlman, DA.; Crowley, M.; Walker, RC.; Zhang, W.; Wang, B.; Hayik, S.; Roitberg, A.; Seabra, G.; Wong, KF.; Paesani, F.; Wu, X.; Brozell, S.; Tsui, V.; Gohlke, H.; Yang, L.; Tan, C.; Mongan, J.; Hornak, V.; Cui, G.; Beroza, P.; Mathews, DH.; Schafmeister, C.; Ross, WS.; Kollman, PA. AMBER, version 9. University of California; San Francisco: 2006.
68. Hornak V, Abel R, Okur A, Strockbine B, Roitberg A, Simmerling C. Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins: Struct., Funct., Bioinf.* 2006; 65:712–725.
69. Halgren TA. MMFF VI. MMFF94s option for energy minimization studies. *J. Comput. Chem.* 1999; 20:720–729.
70. Morley SD, Afshar M. Validation of an empirical RNA-ligand scoring function for fast flexible docking using RiboDock. *J. Comput.-Aided Mol. Des.* 2004; 18:189–208. [PubMed: 15368919]
71. Barril X, Hubbard RE, Morley SD. Virtual screening in structure-based drug discovery. *Mini-Rev. Med. Chem.* 2004; 4:779–791. [PubMed: 15379645]
72. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 1983; 79:926–935.
73. Bayly CI, Cieplak P, Cornell W, Kollman PA. A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* 1993; 97:10269–10280.
74. Naïm M, Bhat S, Rankin KN, Dennis S, Chowdhury SF, Siddiqi I, Drabik P, Sulea T, Bayly CI, Jakalian A, Purisima EO. Solvated interaction energy (SIE) for scoring protein-ligand binding affinities. 1. Exploring the parameter space. *J. Chem. Inf. Model.* 2007; 47:122–133. [PubMed: 17238257]
75. Cui Q, Sulea T, Schrag JD, Munger C, Hung M-N, Naïm M, Cygler M, Purisima EO. Molecular Dynamics-solvated interaction energy studies of protein-protein interactions: the MP1-p14 scaffolding complex. *J. Mol. Biol.* 2008; 379:787–802. [PubMed: 18479705]

Chart 1.
Structures of amantadine, rimantadine, and **1** (BL-1743), **2** (3-azaspiro[5,5]undecane) and **3** (spiro[5.5]undecan-3-amine).

Chart 2.
Structures of ring-contracted and oxa-analogs of amantadine.

Scheme 1.

Synthesis of new amantadine analogs with the pentacyclo[6.4.0.0^{2,10}.0^{3,7}.0^{4,9}]dodecane scaffold.

Scheme 2.
Synthesis of polycyclic pyrrolidine derivatives.

Figure 1.

Dose-response curves on A/M2 *wt* and A/M2 S31N proton channels. Isochronic inhibition curves were generated for selected compounds which caused, in initial inhibition experiments with one compound concentration of 100 μ M, more than 80% inhibition of A/M2 *wt* channel activity and more than 20% of S31N mutant proton channel activity. The inhibition efficacy of the compounds were assessed by comparing the residual membrane current (*I*) after 2 min application of the compound to the maximum membrane current (*I*₀) before the application of the compound at pH 5.5. For each compound, 7 to 9 concentrations were used, and 3 oocytes were used at each concentration. The dose-response relationship was applied to the results by Origin 6 software, and best fit IC₅₀ values are listed in Table 1.

Figure 2. Structural models of putative binding modes of amantadine (orange) in the A/M2 ion channel suggested to account for the major (left) and minor (right) fractions identified by NMR data for amantadine bound to the channel.¹⁵ Residues Val27, Ser31, His37 and Trp41 are shown in stick. For the sake of clarity, one transmembrane helix is not shown.

Figure 3.

(*Left*) Structural model of the putative binding mode of compound **24** (blue) in the A/M2 ion channel (Residues Val27, Ser31, His37 and Trp41 are shown in stick). (*Right*) Superposition of the binding modes of Amt and **24** (residues Val27, Ala30, Ser31 and His37 are shown in stick; hydrating waters of the protonated amine of Amt are shown as red spheres). For the sake of clarity, one transmembrane helix is not shown.

Udorn A/M2 wt

Figure 4.

Plaque reduction assay with A/M2 wt influenza virus. The inhibitory effect of the compounds on influenza A virus replication was evaluated by plaque formation in MDCK cells in the presence or absence of the compounds (5 μ M) as described in the experimental section. Plaque counts (number of plaques per well): no drug, 78; amantadine, 10; **4**, 8; **5**, 14; **6**, 20; **7**, 6; **8**, 12; **9**, 3; and **24**, 32.

Table 1

Inhibitory effect of the synthesized compounds on A/M2 wt, S31N and V27A proton channels functions.^{a,b}

Compound	A/M2 wt (mean ± SE)		A/M2 S31N (mean ± SE)		A/M2 V27A (mean ± SE)	
	Inhibition by 100μM for 2 min (%)	IC ₅₀ (μM)	Inhibition by 100μM for 2 min (%)	IC ₅₀ (μM)	Inhibition by 100μM for 2 min (%)	IC ₅₀ (μM)
Amantadine	91.0 ± 2.1	16.0 ± 1.2	35.6 ± 1.5	199.9 ± 13.5	10.8 ± 2.0	ND
4	97.1 ± 0.1	7.1 ± 0.4	0	ND	0	ND
5	92.2 ± 1.2	17.1 ± 1.3	0	ND	0	ND
6	97.2 ± 0.7	2.4 ± 0.1	0	ND	0	ND
7	93.2 ± 1.0	12.7 ± 0.9	0	ND	0	ND
8	93.6 ± 0.9	17.0 ± 1.0	22.1 ± 0.2	252.2 ± 13.2	10.6 ± 0.7	ND
9	97.3 ± 0.4	7.2 ± 0.3	0	ND	17.6 ± 1.8	ND
10	88.2 ± 0.3	29.2 ± 1.2	0	ND	0	ND
11	91.7 ± 0.7	22.9 ± 1.4	0	ND	0	ND
14	4.8 ± 2.8	ND	0	ND	0	ND
15	0	ND	0	ND	0	ND
16	9.3 ± 2.4	ND	0	ND	2.2 ± 1.3	ND
18	12.8 ± 1.0	ND	0	ND	0	ND
19	14.7 ± 1.8	ND	0	ND	5.0 ± 2.2	ND
21	82.5 ± 0.8	33.5 ± 1.6	0	ND	9.2 ± 1.0	ND
22	39.7 ± 1.0	ND	0	ND	14.8 ± 0.5	ND
24	86.5 ± 0.8	24.0 ± 2.1	0	ND	17.7 ± 0.5	ND

ND, Not Determined.

^a Isochronic (2 min) values for IC₅₀ are given.

^b K_D was obtained for selected compounds by global fitting of circular dichroism (CD) data of ligand titration to wt A/M2TM (22-46) using Igor Pro (wavemetrics). See text and supporting information for details. K_D values: amantadine = 3.9 μM; **8** = 4.7 μM; **10** = 91.6 μM; **21** = 74.9 μM; **24** = 231.5 μM.