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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

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ABSTRACT

The new 2-alkyl-2-aminoadamantanes and analogues **4–10** were designed and synthesized by simplification of the structure of the potent anti-influenza virus A spiranic aminoadamantane heterocycles **2** and **3**. The aim of the present work was to examine the effects of bulky and extended lipophilic moieties attached to amantadine **1** on binding to the M2 channel and the resulting antiviral potency. The binding affinities of the compounds to the M2 protein of influenza virus A/chicken/Germany/27 (Weybridge strain; H7N7) were measured for the first time using an assay based on quenching of Trp-41 fluorescence by His-37 protonation, and their antiviral potencies were evaluated against the replication of influenza virus A H2N2 and H3N2 subtypes and influenza virus B in MDCK cells. Of the various 2-alkyl-2-aminoadamantanes, and analogues, spiro[piperidine-2,2′-adamantane] **3** had the strongest M2 binding and antiviral potency, which were similar those of amantadine **1**. The relative binding affinities suggested that the rigid carbon framework provided by the pyrrolidine or piperidine rings results in a more favorable orientation inside the M2 channel pore as compared to large, freely rotating alkyl groups. The aminoadamantane derivatives exhibited similar NMDA antagonistic activity to amantadine **1**. A striking finding was the antiviral activity of the adamantanols **4**, and **6**, which lack any NMDA antagonist activity.

Influenza presents a severe threat to public health. More casualties were inflicted in Europe in the twentieth century by influenza than any other infectious disease.[1] Furthermore, although the cumulative impact of recurrent annual epidemics is greater than that of the infrequent pandemics, the specterre of another influenza pandemic caused by H5N1 'Bird Flu' or other subtype highlights the importance of anti-influenza drugs as a first line of defense against such a threat. The H5N1 avian influenza virus that emerged in 1996 in southern China, and caused the deaths of six of the 18 infected individuals in Hong Kong during 1997, are now endemic in domestic poultry in many countries and have spread across three continents, Asia, Africa and Europe.[2] From late 2003 to date, the virus has infected 385 humans with 243 (63%) fatalities.[2c] Infections by other influenza virus A subtypes also pose a threat. Another highly pathogenic avian influenza virus subtype H7N7 which caused some 89 mild infections in the Netherlands in 2003 also caused the death of a veterinarian, while in the same year H9N2 viruses were isolated from individuals with mild influenza.[2] The M2 protein of influenza virus A is the target of one of two classes of anti-influenza drugs that could help to control influenza infections.[3]

Amantadine **1** (Scheme 1) was the first anti-influenza drug to be developed. It inhibits the function of the M2 proton channel of influenza virus A involved in virus entry and uncoating at micromolar concentrations.[4,5] Acidification of the virion, mediated by the M2 H^+ channel during endocytosis, causes the dissociation of viral ribonucleoprotein from the matrix protein, a critical early step in initiating virus replication. In addition, during infection by some highly pathogenic influenza viruses, including H5 and H7 subtypes, it regulates the pH of the *trans* Golgi to assist maturation of the haemagglutinin (HA) and the release of progeny virus from the cell.[11,4b] During the past fourteen years we have synthesized many potent aminoadamantane derivatives.[6,7] The proton-

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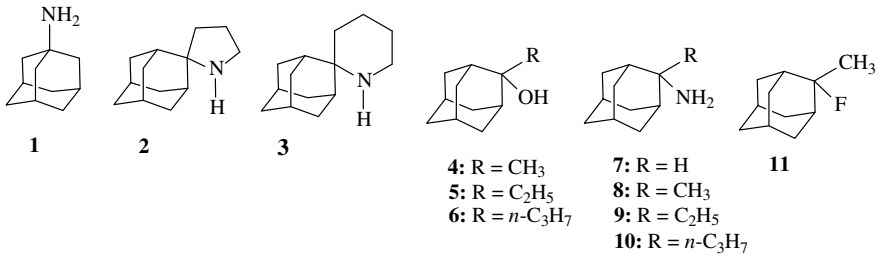


Scheme 1.

ated form of these compounds are considered to block the tetrameric M2 ion channel pore,[8] formed by its transmembrane domain M2TM,[9] and hence, its proton transport function.[10]

The desired property of new synthetic aminoadamantane derivatives is of course the effective inhibition of virus replication. However, in order to design effective inhibitors and those active against amantadine-resistant mutants, the mode of interaction of the class of compounds with their receptor should be systematically examined at the molecular level. Although we have tested the in vitro potencies of several aminoadamantane derivatives in cell culture[7] our group has never applied procedures that measure directly changes in physicochemical properties of the target protein which result from drug–receptor interaction. Only recently we examined how these molecules interact with the transmembrane channel of the M2 protein using suitable probe molecules and NMR spectroscopy.[12]

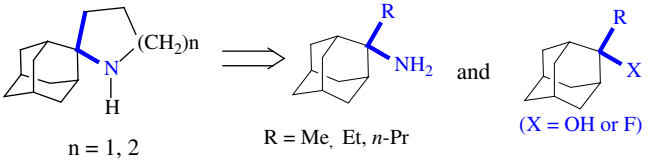
A first approach to assessing the stereoelectronic characteristics of the ligand and to map the complementary M2 receptor binding site is to measure the binding constants of different derivatives. In this paper we report the binding affinities of a series of aminoadamantane compounds to the M2 protein of influenza virus A/chicken/Germany/27 (H7N7 'Weybridge' strain), using an assay based on the quenching of tryptophan-41 fluorescence by histidine-37 protonation in the M2 channel pore (Table 1).[13]

Design pathway. The spiropyrrolidine **2**[6,7b] and the spiropiperidine **3**[6,7c] represent two of the most active anti-influenza virus A M2 agents ever synthesized; the selectivity index of the spiro[piperidine-2,2′-adamantane] **3** (SI > 1034) is the highest among aminoadamantanes or other cage structure amines tested till now. In a general sense, compounds **2** or **3** are 2-adamantanamines (2-amantadines) substituted at the 2-position with a carbon chain. Simplification of this structure leads to the design of simple 2-alkyl-2-amantadines and analogues not previously synthesized or tested (Scheme 2).

Table 1
Binding constants[a] of some amino adamantane derivatives[b] and analogues to influenza virus A M2 protein[c]

Compound	K_d (μM)[c]
2-Me-2-AdOH, **4**	No effect
2-*n*-Pr-2-AdOH, **6**	No effect
2-Me-2-AdF, **11**	>26
2-$AdNH_2$, **7**	2.36
2-Me-2-$AdNH_2$, **8**	3.60
2-Et-2-$AdNH_2$, **9**	6.70
2-*n*-Pr-2-$AdNH_2$, **10**	8.71
Spiro[pyrrolidine-2,2′-adamantane], **2**	1.16
Spiro[piperidine-2,2′-adamantane], **3**	0.39
Amantadine, **1**	0.32

[a] Dissociation constants were obtained from the kinetics of inhibition, under equilibrium conditions, of quenching of Trp-41 fluorescence of purified M2 by His-37 protonation at pH 5.[13]
[b] Compounds **1–3**, **7**, **8–10** were tested as hydrochloride salts.
[c] Purified M2 protein of influenza virus A/chicken/Germany/27 (H7N7; 'Weybridge' strain), expressed in *E. coli*.

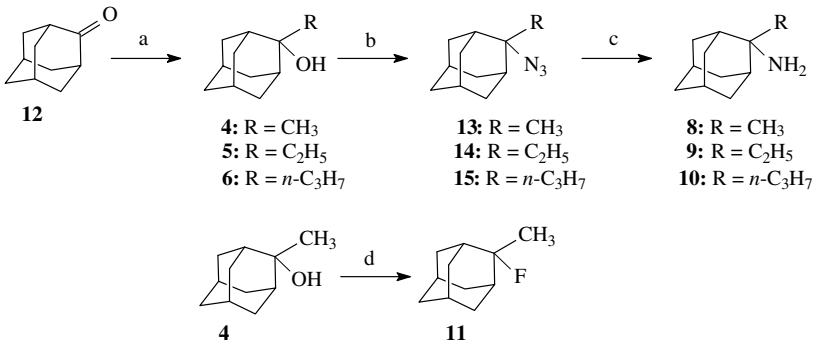


Scheme 2. Design pathway of the new compounds **4–11** based on simplification of the lead structures **2** (*n* = 1) or **3** (*n* = 2).

The amantadine **1**—receptor complex is probably stabilized through formation of hydrogen bonding between the drug's ammonium group and residues, probably His-37 or Ser-31, within the M2 acceptor site.[8,10] We examined the effect on M2 binding exerted by replacing the amino group with the hydroxyl group (compounds **4–6**) or a fluorine group (compound **11**); the hydroxyl group could possibly participate as donor or acceptor and fluorine as acceptor in hydrogen bonding interaction with the receptor. The binding constants of the compounds depicted in Scheme 1 were determined (Table 1).

Synthesis. The tertiary alcohol **4** was obtained after treating adamantanone **12** with CH_3MgI. This is not the preferred method for the preparation of alcohols **5** and **6** due to the bulky adamantanone **12** and to the soft carbanion character of the Grignard reagent making the hydride addition a competitive reaction to the alkyl addition; thus 2-adamantanol is also formed along with the tertiary alcohols **5** or **6**.[14] The preparation was effected by the reaction of the relevant organolithium reagent with adamantanone **12**.[14] The azides **13–15** were obtained by reaction of the alcohols **4–6** with $NaN_3/H_2SO_4/CHCl_3$/rt using a concentration of 78% w/w rather than the 57% w/w reported in the literature, which failed to yield **13**[15] quantitatively. The amines **8–10** were prepared by means of $LiAlH_4$/ether/rt reduction of the azides **13–15**. The fluoride **11** was prepared after treating the tertiary alcohol **4** with DAST[16] in CH_2Cl_2 at −78 °C (Scheme 3).[17]

Binding affinity to influenza virus A M2 protein. The binding affinities[13] of compounds **4–11** to purified M2 protein of the influenza virus Weybridge strain are depicted in Table 1. 2-Amantadine **7** (K_d = 2.36 μM) has a 7.4-fold smaller binding constant compared to that of amantadine drug **1** (K_d = 0.32 μM). Spiropiperidine **3** with a K_d of 0.39 μM exhibited a binding affinity similar as to that of amantadine **1**. The reduction in ring size by one methylene results in spiropyrrolidine **2** with a 3-fold lower binding affinity (K_d = 1.16 μM), but 2-fold higher when compared to the binding affinity of 2-amantadine **7**. Thus, spiropyrrolidine **2** and spiropiperidine **3**, which can be considered to be 2-alkyl-2-aminoadamantanes (Scheme 2), exhibited a 3-fold and 6-fold stronger binding, respectively, with respect to 2-amantadine **7**. The effect of attaching simple alkyl groups at the 2-position of 2-amantadine **7** was striking, since it did not result in a more stable complex, as was expected from the higher affinities of spiropyrrolidine **2** and spiropiperidine **3** with respect to 2-amantadine **7**; the methyl, ethyl and



Scheme 3. Reagents and conditions: (a) RMgI, ether, THF, rt, 2 h then NH_4Cl/H_2O for **4,** or RLi, Ar, THF, 0 °C, 2 h then NH_4Cl/H_2O for **5**, **6** (quant.); (b) NaN_3, H_2SO_4 78% w/w, $CHCl_3$, 0 °C then rt (66–77%); (c) $LiAlH_4$, ether, rt, 24 h (74–83%); (d) DAST, CH_2Cl_2, Ar, −78 °C (69%).

n-propyl substitutions (compounds **8**, **9** and **10** respectively) reduced the affinity by 1.5-, 2.8- and 3.7-fold, respectively, with respect to 2-amantadine **7**. These findings suggest that the rigid carbon framework in the pyrrolidine and piperidine rings of **2** and **3** fit better, than a free rotating group, into a lipophilic pocket in the M2 receptor. The van der Waals interactions together with appropriate hydrogen bonding appear to result in an overall more favorable orientation of the molecule inside the M2 proton channel. Future structural studies and docking simulations should help to understand the molecular interactions through which these aminoadamantane derivatives (**2**, **3**, **7–10**) bind to M2 channel.

The adamantanols **4** and **6** or the fluoride **17**, having a hydroxyl or a fluorine group, respectively, did not inhibit the low pH-dependent quenching of Trp-41. It therefore seems that the presence of a protonated amino group is crucial for favorable interaction with the receptor using this assay.

NMDA blocking activity. One of the most serious side-effects of amantadine **1** is its CNS-activity, reflected in its anti-Parkinsonism activity, partly due to action on dopamine reuptake and partly through blockade of the NMDA receptor, which influences dopamine release.[18] Also, memantine, which is the most important NMDA antagonist in the aminoadamantane series, is thought to act by blockade of the NMDA channel in exerting its neuroprotective action.[19] Thus, although in developing successful anti-influenza therapeutic agents this activity should be minimized, the ability to antagonize NMDA receptors could result in new leads for further development as neuroprotective agents. For these reasons, the NMDA receptor blocking activity of some representative compounds was evaluated by measuring their ability to inhibit the intracellular calcium increase, induced either by NMDA or by the non-selective agonist glutamate, in in vitro cultures of neurons.[20]

There was a fairly good correlation between results using glutamate or NMDA to stimulate the intracellular calcium increase in the neurons. The potency of all the compounds was increased using the more specific agonist NMDA. The results of Table 2 showed that all compounds were less active than memantine. The next most actives agents were compounds **3** and **10** being approximately 4.5-fold less active than memantine using glutamate as the agonist. These results showed that the attachment of the amino group to a tertiary carbon of the adamantane ring (1-position) is more favorable for the antagonistic activity than the insertion of the amino group at a secondary adamantane carbon position (2-position). However, the insertion of a carbon chain at the 2-position (compounds **2**, **3**, **8**, **10**) boosts the activity; in these compounds the amino group is therefore connected with a tertiary carbon of the adamantane system. Compared to 2-adamantanamine **7**, the 2-methyl substitution (compound **8**, IC_{50} = 64.4 μM using NMDA) resulted in a 3.4-fold enhancement in activity while the 2-propyl substitution (compound **10**) increased only slightly antagonistic activity. The similarities in the activities of the 2-propyl derivative **10** and spiropyrrolidine **2** suggest that there is not a clear advantage of a freely rotating 2-carbon substituent over a rigid structure. The similarities in the antagonistic activities of the aminoadamantane derivatives **2**, **3** ,**8**, **10** against NMDA receptors to that of amantadine, may suggest that they will also cause similar CNS side-effects derived from the interaction with these receptors.

Table 2
Inhibitory activity of glutamate- or NMDA-induced intracellular calcium increase (both 100 μM, in the presence of glycine 10 μM) in cultures of rat cerebellar granule neurons

Compound[a]	Glutamate (100 μM)	NMDA (100 μM)
Alcohols: **4**, **6**	>350	>350
2-$AdNH_2$, **7**	>350	219.4
2-Me-2-$AdNH_2$, **8**	305.8	64.4
2-*n*-Pr-2-$AdNH_2$, **10**	257.8	61.3
Spiropyrrolidine, **2**	314.0	50.6
Spiropiperidine, **3**	239.3	19.0
Amantadine, **1**	358.4	75.0
Memantine	55.4	1.5

[a] Compounds **1–3**, **7**, **8**, **10** were tested as hydrochloride salts.

Table 3
Anti-influenza virus A activity and cytotoxicity of aminoadamantane derivatives[a] and analogues in influenza A and B infected MDCK cells[b]

Compound	EC_{50} (μM)[c] Influenza			MCC_{50} (μM)[d]
	A/H_2N_2[b]	A/H_3N_2[b]	B[b]	
2-Amantadine, **7**	2.8	—	Inactive	>1333
2-Me-2-AdOH, **4**	3.0	10.8	Inactive	>1506
2-*n*-Pr-2-AdOH, **6**	<2.1	—	Inactive	>1289
2-Me-2-$AdNH_2$, **8**	3.5	<2.0	Inactive	>1240
2-Et-2-$AdNH_2$, **9**	<1.9	<1.9	Inactive	>1159
2-*n*Pr-2-$AdNH_2$, **10**	<1.7	<1.7	Inactive	>1088
Amantadine, **1**	1.1	<0.9	Inactive	>1333
Spiro[pyrrolidine-2,2′-adamantane], **2**	1.8	1.8	Inactive	>1099
Spiro[piperidine-2,2′-adamantane], **3**	1.0	—	241	>1034

[a] The aminoadamantanes **1–3**, **7–10** were tested as hydrochlorides.
[b] Abbreviations and strains used: MDCK: Madin–Darby canine kidney cells; influenza virus A H2N2: A/Japan/305/57; influenza virus A H3N2: X31(A/Hong Kong/1/68 x A/Puerto Rico/8/34 reassortant); influenza B: B/Hong Kong/5/72.
[c] Effective concentration, or concentration required to reduce virus-induced cytopathogenicity by 50%.
[d] Minimum cytotoxic concentration, or concentration required to cause a microscopically detectable alteration of normal cell morphology. All the data represent mean values for at least two separate experiments.

However, the replacement of the amino group with a hydroxyl group (compounds **4**, **6**) abolished activity.

Anti-influenza virusal potency. The potencies of the aminoadamantane derivatives **1–3**, **7**, **8–10** and alcohols **4**, **6** in inhibitoring in vitro replication of influenza virus A H_2N_2 and H_3N_2 subtypes and influenza B viruses, was determined using previously reported methods (Table 3).[21] All compounds were shown to be active against the A/H_2N_2 and H_3N_2 strains, with corresponding low toxicity, and the amines **2**, **3**, **9**, **10** were almost equipotent to amantadine **1**. All compounds tested were inactive at low concentrations against influenza virus B in accordance with their putative mode action, i.e., their interaction with influenza virus A M2 protein, but inactivity against the influenza B BM2 protein.[22] Compound **3** reduced influenza virus B cytopathogenicity but only at high concentrations. The latter effect is not specific since high concentrations of amines generally inhibit the low pH-induced, HA-mediated membrane fusion involved in uncoating of influenza virus during endocytosis.[23]

Unexpectedly in view of their apparent lack of M2 binding in our in vitro assay, the alcohols **4** and **6** exhibited marked inhibitory activities against influenza virus A (but not B), which for A/Japan/305/57(H2N2) were equivalent to those for the corresponding amines, though lower for X-31(H3N2). These characteristics are consistent with a similar specific mode of action of the alcohols and amines against influenza virus A. It is therefore unlikely that the alcohols **4** and **6** exert their inhibitory activity through a mechanism which is different from inhibition of the M2 channel, and it is likely that the apparent inconsistencies in the results from the two types of experiment are due to differences in the experimental conditions. The fluorescence M2 binding assay was conducted at pH 5 in the presence of low concentrations of detergent (0.1% LDAO),[13] which may result in lower concentrations of the water insoluble alcohols being available for interaction with the M2 protein. In this respect, recent preliminary results from electrophysiological measurements have shown that the alcohol **4** does inhibit proton currents in M2-expressing mouse erythroleukaemia (MEL) cells,[24] although the M2 specificity of the effect has yet to be determined.

The antiviral activities (Table 3) indicate that the 2-ethyl, 2-propyl, spiropyrrolidine and spiropiperidine derivatives **9**, **10**, **2**, **3** exhibited a small increase in potency with respect to the 2-methyl derivative **8**; it appears that enlargement of the alkyl group increases anti-influenza virus A potency. However, the results in Table 1 suggest that increase in size of the freely rotating alkyl group reduces affinity of binding to M2. Thus, as for the alcohols, the relative binding affinities are not directly comparable to the relative antiviral potencies in cell culture; while spiropyrrolidine **2** had a 3.6-fold lower binding constant than amantadine **1** their in vitro potencies were equivalent. In mechanistic terms, in order to inhibit virus replication amantadine **1** must first be solvated in the lipid bilayer[25] prior to the blockade of the M2 proton channel inside the acidic endosome or *trans* Golgi in which, like other acidotropic amines, amantadine **1** concentrates. Thus, the observed anti-influenza virus A potencies of the aminoadamantane derivatives can be considered to result from a combination of their ability to reach the site of interaction with the receptor, via membrane penetration, and binding affinity.

The major conclusions from this study can be summarized as follows: (a) Transposition of the amino group from adamantane C-1 in amantadine **1** to C-2 in 2-amantadine **7** resulted in a lower binding affinity to the M2 channel. For the active 2-alkyl-2-aminoadamantane analogues **8–10**, which have an extended lipophilic substituent, the binding affinity was reduced by increasing the size of the rotatable alkyl group. In contrast, on going from 2-methyl-2-adamantanamine **8** to the pyrrolidine **2** or the piperidine **3** derivatives, the additional lipophilic moiety boosted the binding affinity; it therefore appears that compounds **2** and **3** bind to M2 protein in a more favorable orientation. Of the 2-alkyl-2-aminoadamantane analogues, spiro[piperidine-2,2′-adamantane] **3** had the stronger binding and antiviral potency, which were similar to those of amantadine **1**. The present SAR study indicates that large and extended lipophilic moieties in the vicinity of adamantane carbon C-2 are compatible with biological activity and suggests that there is a complementary acceptor group/site within the lumen of the M2 channel pore. Thus, the *potency of the new compounds can be rationalized in terms of a larger lipophilic cavity inside the lumen of the M2 channel that can accommodate larger entities than the adamantyl group*. (b) It is apparent that for a series of aminoadamantane compounds, the relative binding affinities to M2 protein are not directly comparable to the relative antiviral potencies in cell culture. (c) The aminoadamantane derivatives tested did not differ from amantadine **1** in their NMDA antagonistic activity. (d) Adamantanols **4**, **6** were found to be active anti-influenza virus A agents without having antagonistic activity against the NMDA receptor in the cell-based assay. The simultaneous presence of anti-influenza virus A activity with potentially no (or fewer) CNS side effects are of particular interest and merit further investigation.

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