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2. Inhibitors of the M2 channel of influenza A virus

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Abstract. Influenza is a highly contagious, major respiratory tract disease affecting millions of people each year. At present, two classes of antivirals are available: the neuraminidase inhibitors and the M2 proton channel blockers amantadine and rimantadine. However, rapid emergence of M2 blockers resistance makes imperative the development of new anti-influenza drugs. In the last few years several groups have synthesized and evaluated several analogs of amantadine. While several of them are active against wild-type M2 channel only a few are able to inhibit the mutant ion channels that lead to amantadine-resistance.

Introduction

Influenza is a worldwide epidemic that causes substantial morbidity and mortality. Of the three types of influenza viruses, A, B and C, influenza A and B

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cause seasonal epidemics. Moreover, influenza A viruses are responsible for sporadic pandemics that usually cause higher mortality rates than seasonal influenza epidemics. The most severe pandemic, the “Spanish flu”, occurred in 1918, is thought to have killed more individuals than any disease outbreak in history, resulting in approximately 40 million deaths worldwide [1]. More recent pandemics in 1957 (“Asian flu”, H2N2 strain) and 1968 (“Hong Kong flu”, H3N2 strain) were not as deadly, yet influenza remains a grave health hazard [2]. For example, in the United States, according to the Center for Disease Control and Prevention (CDC), influenza and its complications are currently the leading cause of death due to any infectious disease. In fact, in 2009, a new influenza virus (“swine flu”, H1N1 strain) originated a new pandemic that caused much concern, although, thankfully, was not as deadly as initially thought [3]. In addition, H5N1 viruses (“bird flu”), which are also currently worldwide circulating, are extremely virulent in humans but have not acquired the ability for efficient human-to-human transmission yet [4].

Influenza A viruses infect a wide range of avian and mammalian hosts, unlike influenza B viruses, which infect only humans. Influenza A and B viruses are enveloped negative-strand segmented RNA viruses. The envelope of influenza A viruses contains two different surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [5]. Influenza A viruses are categorized into antigenic HA and NA subtypes: 16 HA (H1–H16) and 9 NA (N1–N9) antigenic subtypes have been identified so far. For example, the 2009 “swine flu” is an H1N1 virus because it contains a H1 subtype HA and a N1 subtype NA.

The major influenza A subtypes that have infected humans during seasonal epidemics are H1N1, H2N2 and H3N2. Within a subtype, different strains arise as a result of point mutations in a process known as ‘genetic drift’. These new strains cause seasonal epidemics. A new pandemic can emergence by two different mechanisms: by direct transmission from animals, usually birds, to humans, as happened in 1918 with the “Spanish flu”; or through reassortment of an avian influenza virus with a human influenza virus, as occurred in 1957 with the “Asian flu” (H2N2) and, again, in 1968 with the “Hong Kong flu” (H3N2) [6]. The H1N1 virus of the 2009 “swine flu” is an apparent reassortment of four endemic strains of influenza: one from humans, one from birds, and two from pigs, a fact that further exemplifies the versatility of the influenza A virus [3]. When a new pandemic starts, the HA of the new strain differs substantially from recent HAs of seasonal influenza A viruses and, consequently, most of the human population lacks immunological protection against this virus, resulting in a pandemic.

There are two different strategies for combating influenza: vaccination and chemotherapy. The primary defense against influenza A has been

vaccination with inactivated or live-attenuated virus. However, vaccination effectiveness is limited due to the antigenic drifts and shifts that the influenza virus undergoes from year to year, and new influenza vaccines must be designed every year by predicting the genetic drift of seasonal influenza A. Antivirals have also been used for both prophylactic and therapeutic treatments during seasonal epidemics [5b]. Additionally, antivirals are particularly important at the beginning of a fast-spreading pandemic because the timely production of sufficient amounts of an effective vaccine is difficult. Current antivirals are directed against the M2 ion-channel protein of the influenza A virus (amantadine and rimantadine, Fig. 1) and the NA of the influenza A and B virus (zanamivir and oseltamivir) [8]. However, many influenza virus strains have developed resistance to adamantanes and/or oseltamivir (the only orally bioavailable NA inhibitor), highlighting a major health risk [9]. For example, after four decades of effective use of amantadine, resistance by influenza viruses of the A/H3N2 subtype currently exceeds 90% in the United States, and virus mutants are as fit as the wild-type (wt) virus. The situation is even worst with the new 2009 pandemic H1N1 influenza. In both strains, the basis for resistance is a single Ser to Asn amino acid replacement (S31N) in the matrix M2 ion channel, which interferes with the drug's ability to block M2 ion channel activity and viral replication [10].

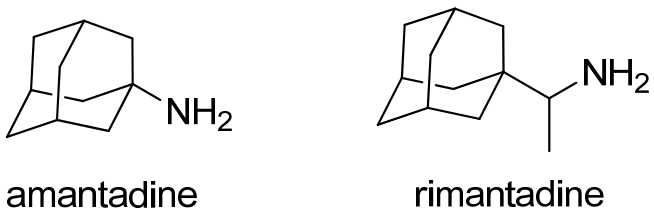


Figure 1. The structures of the anti-influenza A drugs amantadine and rimantadine.

Another important problem encountered in the administration of amantadine and rimantadine is related to the central nervous system side effects of both drugs. In fact, amantadine has been used in the treatment of Parkinson’s disease, although its antiparkinsonian effect is poorly understood [11]. The side effects of rimantadine are analogous, but somewhat less pronounced than those of amantadine [12].

The appearance of pandemic H1N1 and highly pathogenic avian influenza viruses of the H5N1 subtype being able to infect humans, the emergence of resistances, and the side effects of amantadine and rimantadine reveal the urgent need for the development of new antiviral drugs [13].

In this review, we will focus on the design of new amantadine analogs targeting the matrix M2 ion channel of influenza A virus.

1. The M2 protein

The influenza A virus M2 protein is a homotetrameric protein containing four parts: a short unstructured N-terminal extracellular domain, important for incorporation into the virion; a transmembrane helix that is necessary for tetramerization, proton conductance and drug-binding; a cytoplasmic amphiphilic helix, involved in cholesterol-binding, membrane localization, budding and scission; and a disordered tail that interacts with the matrix protein M1 [14].

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 HA proteins, triggered by the low pH in the endosome lumen [15]. The transmembrane region of the M2 protein forms a pH-activated channel that selectively conducts protons along a chain of water molecules and ionizable sidechains, including His37, playing an essential role for viral replication equilibrating the pH of the virus interior with that of the acidic endosome. When the endosome is acidified, His37 residues in the transmembrane region of M2 become protonated, leading to the opening of the M2 channel and to a proton influx from the endosome into the virus interior [16]. The acidification of the virus interior enables the release of the viral RNA into the host's cytoplasm after membrane fusion has taken place. In addition, it has been shown that, for some strains of influenza A virus, the M2 proton channel function is required for preventing a premature HA conformation transition when newly synthesized viral proteins are trafficked through the trans-Golgi network [17].

The replication of the influenza A virus can be stopped by inhibiting the activity of the M2 channel, using amantadine and rimantadine.

Although the role of the M2 protein as the target for amantadine and rimantadine has been known for more than twenty years [18], only very recent functional, structural [19] and computational [20] studies have revealed that the drugs inhibit proton conduction by binding to an aqueous cavity adjacent to M2's proton-selective filter, thereby blocking access of proton to the filter [14]. These recent works enable novel insights into the adamantanes-resistance and provide a solid basis for structure-based drug design.

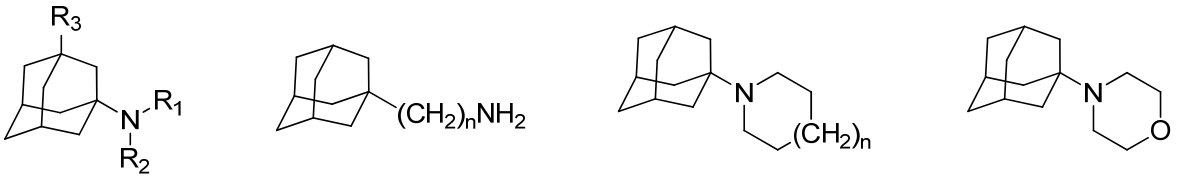
The number of drug-resistant variants of influenza A M2 channel is limited by the very conserved nature of the binding site within the channel. Thus, only a few amantadine-resistant mutations, namely V27A, L26F and

S31N, have been widely observed in transmissible strains of the virus in the past eight decades for which a genetic record is available [21], although other mutations can easily be observed *in vitro* [22]. The mutations that cause the greatest decrease in inhibition, S31N and V27A, increase the polarity of pore-lining residues.

2. 1-Substituted-adamantanes

Amantadine and rimantadine exhibit their inhibitory activity at micromolar concentrations. Rimantadine has a superior intrinsic antiviral activity compared to amantadine, but peak plasma levels of rimantadine are 2-3 fold lower than those achieved with amantadine when given at the same dose [23]. Amantadine was initially licensed in USA in 1966. Interestingly, for many years, amantadine was mainly used in western countries, while rimantadine was used in the former USSR and eastern European countries [24].

Both drugs are rather old, therefore it is not a great surprise that hundreds of derivatives have been synthesized and pharmacologically tested. In fact, soon after the publication of the antiviral activivity of amantadine by du Pont de Nemours' researchers [25], several amantadine derivatives were synthesized and evaluated as anti-influenza agents [26]. Most of these analogs were alkylaminoalkyl derivatives of adamantane (Fig. 2), although some derivatives featuring aditional polar groups, such as alcohols, amines, ethers, or derivatives lacking an amino group were also synthesized and tested (Fig. 3).



R_1, R_2 = H, methyl, ethyl, allyl, propargyl, propyl, butyl, etc.; R_3 = H, methyl, phenyl, etc.; n = 0, 1, 2

Figure 2. Alkylamantadines and related compounds.

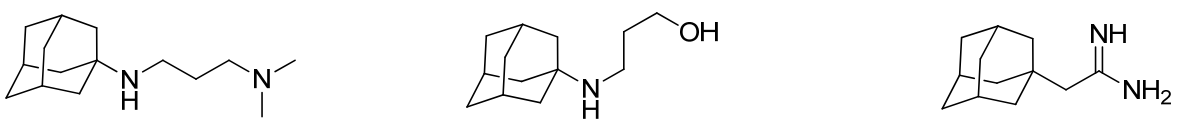


Figure 3. Amantadine analogs featuring polar groups.

Although several of these compounds displayed anti-viral activities similar to that of amantadine, they showed cross-resistance with amantadine and rimantadine, so their therapeutical interest is rather low.

Worthy of note, while amantadine is, for the most part, excreted without metabolism [27], rimantadine is extensively metabolized by hydroxylation before excretion in the urine [23c,28]. Manchand and coworkers reported the synthesis of three hydroxylated metabolites of rimantadine and showed that 2-hydroxyrimantadine was as active against several influenza A virus as amantadine, while the 3- and the 4-hydroxy derivatives showed only very modest inhibitory activity (Fig. 4). Unfortunately, rimantadine-resistant strains exhibited cross-resistance to the 2-hydroxyamantadine [29].

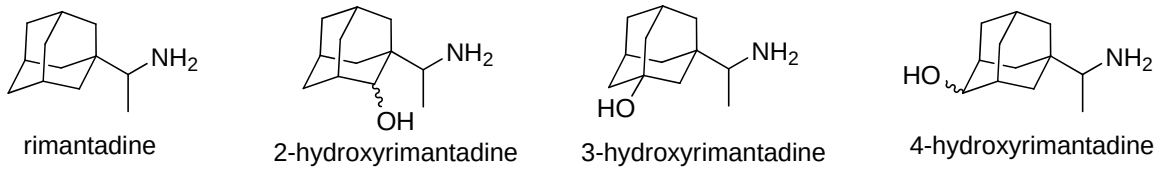


Figure 4. Rimantadine and its hydroxylated metabolites.

The antiviral activity of 2-hydroxyrimantadine was the first example of a trend that has also been observed in much more recent work using amantadine and other polycyclic systems, that is, the introduction of polar groups in the polycyclic scaffold is tolerated, but does not enhance the potency of amantadine and related aminopolycyclic derivatives. For example, in 2011, Wang *et al*. described that the aminoalcohol **3** showed an IC_{50} = 16 μM against the wt M2 channel from influenza A virus, exactly the same value than that reported for amantadine [30]. Surprisingly, they found that 1-adamantanol, **1**, and 2-methyl-2-adamantanol, **2**, showed IC_{50} values very close to that of amantadine, while the 3-amino-1-adamantanol, **4**, showed to be inactive (Fig. 5).

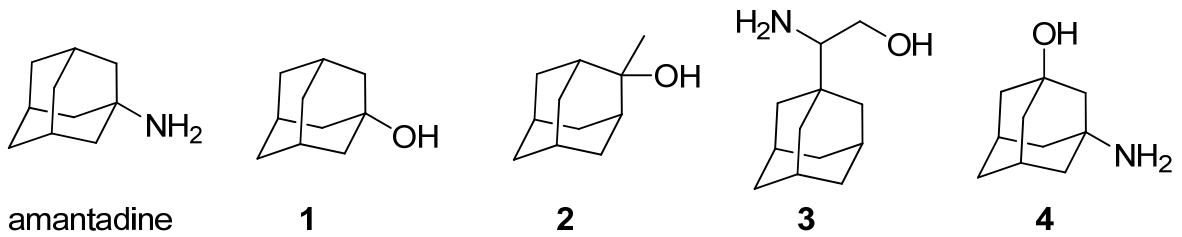


Figure 5. Several hydroxylated analogs of amantadine. IC_{50} values (against wt M2 channel): amantadine (16 μM), **1** (20 μM), **2** (14 μM), **3** (16 μM), **4** (not active).

Kolocouris' group has reported the synthesis and anti-influenza activity of a series of heterocyclic rimantadine analogs (Fig. 6) [31]. The aziridine and the azepine derivatives were much less active than amantadine, while azetidine **6** (R_1=H), pirrolidines **7a** (R_1=R_2=H) and **7b** (R_1=H, R_2=CH_3), and piperidine **8** (R_1=H) showed to be more potent than amantadine and rimantadine against the influenza A_2/Japan/305/57 (H2N2) strain. Compounds **6** and **7b** also showed good inhibitory activity against the influenza A/Hong Kong/68 (H3N2) strain. While amantadine displayed IC_{50} of 42 and 6 μM against A_2/Japan/305/57 and A/Hong Kong/68, respectively, the most potent compound within this series, **7b**, showed IC_{50} of 1.6 and 1.8 μM against A_2/Japan/305/57 and A/Hong Kong/68, respectively. The introduction of an additional alkyl group in the nitrogen atom caused a dramatic reduction in anti-influenza activity.

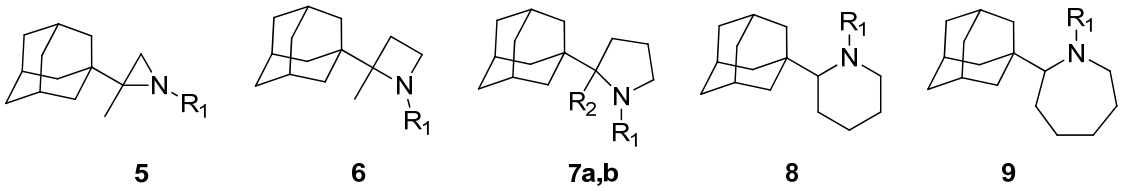


Figure 6. Heterocyclic rimantadine analogs.

Later on, the same group reported analogs of rimantadine featuring a second nitrogen atom, the aim of this modification being the incorporation of additional hydrogen bonding interactions with the M2 protein [32]. Although the presence of this second amino group was compatible with anti-influenza activity, the new analogs were not more potent than rimantadine. Thus, compounds **10** and **11** (Fig. 7) displayed EC_{50} of 18.3 and 24.1 μM, respectively, against A/Hong Kong/68 (H3N2) strain, very similar values to that of rimantadine (EC_{50} = 19.1 μM).

Figure 7. Rimantadine analogs featuring a second nitrogen atom.

As previously stated, amantadine interferes with the ion channel function of the M2 protein of influenza A virus at low micromolar concentrations. Interestingly, a second mechanism of action of amantadine, at least in some influenza A strains, is on the hemagglutinin, at concentrations around 100 times

higher. Theoretically, an amantadine derivative able to simultaneously interact with both targets at the same concentrations should have a reduced probability to develop resistance. In this case, two mutations, one in each target protein would be necessary at once. With this aim, Scholtissek and coworkers reported the synthesis and evaluation of forty adamantane derivatives and tested them against the influenza A/Singapore/1/57 (H2N2) strain [33]. They found several analogs active against this strain and also against A/Swine/1976/31 and A/Udorn/307/72, although all the products were inactive against A/WSN/33, which is amantadine-resistant. Most of the active compounds at low micromolar concentrations (e. g., **12-14**) interacted with the M2 protein; the corresponding escape mutants produced with them had amino acid replacements at positions 27, 30 or 31 of the M2 protein. Interestingly, they found two compounds, **15** and **16** (Fig. 8), able to interact with both the ion channel and the hemagglutinin at about the same concentration. It was expected that in order to become resistant the virus should mutate both proteins. However, the resistant mutants to these compounds showed mutations only in the HA protein [33].

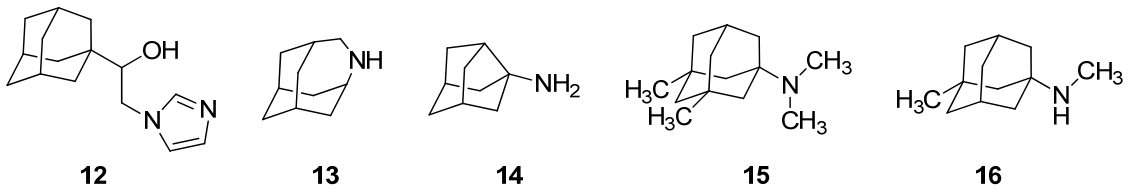


Figure 8. Some of the amantadine analogs reported by Scholtissek and coworkers.

Very recently, Zarubaev and coworkers have reported the synthesis and the anti-influenza activity of a series of di-, tri- and tetrazole derivatives of amantadine. Interestingly, several compounds were active against the amantadine-resistant influenza A/Puerto Rico/8/34 strain, which bears the S31N mutation in its M2 channel. Tetrazoles such as **17**, **18** and **19** (Fig. 9), showed micromolar values of EC_{50} and higher selectivity index (SI) than rimantadine [34]. It remains to be clarified if the target of these adamantane derivatives is the M2 channel of the influenza virus.

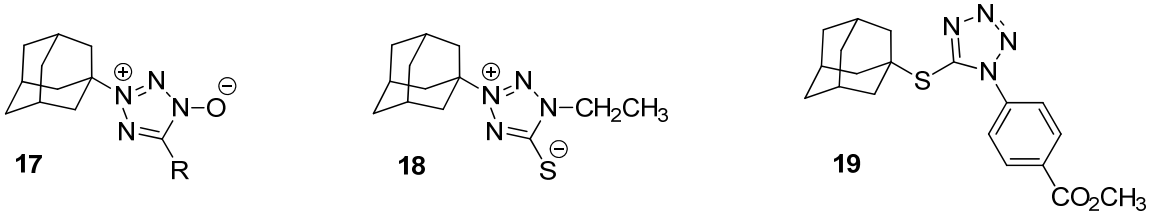
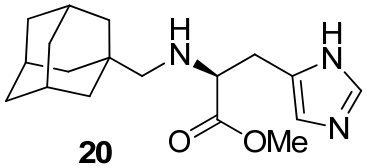


Figure 9. Tetrazolo-adamantanes with anti-influenza A virus activity.

Finally, Zhang *et al.*, have reported that an *L*-histidine derivative of adamantane, **20** (Fig. 10), was able to inhibit the wt, the S31N, and the double mutant S31N/L26I M2 channels of avian H5N1 influenza expressed in cell lines of transformed HEK 293. The IC_{50} of **20** against the wt, the S31N mutant and the double mutant S31N/L26I channels were 5.84, 10.96 and 9.77 μM, respectively [35]. However, these data were not confirmed with viral inhibition assays.

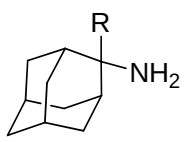


20

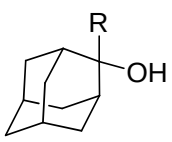
Figure 10. Structure of histidine derivative **20**.

3. 2-Substituted-adamantanes

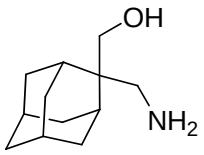
2-Amantadine is only moderately active against influenza virus. The anti-viral activity improved by the incorporation of a 2-ethyl or 2-*n*-propyl group, although the introduction of a methyl group in C-2 diminished the activity. Interestingly, 2-methyl-2-adamantanol, **2a**, showed an EC_{50} of 3 μM against influenza A/Japan/305/57 (H2N2) strain, very similar to the EC_{50} of amantadine against this strain (1.1 μM) [36]. As previously stated, **2a** inhibits the wt M2 channel of influenza A with an IC_{50} of 14 μM [30]. In 2010, Kolocouris' group reported that several adamantanaminoalcohols such as **23** and **24** (Fig. 11) had potent anti-influenza activity. For example, aminoalcohol **23**, displayed submicromolar activity against the influenza A /Hong Kong/7/87 (H3N2) strain [37].



21a-c



2a-c



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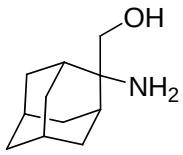

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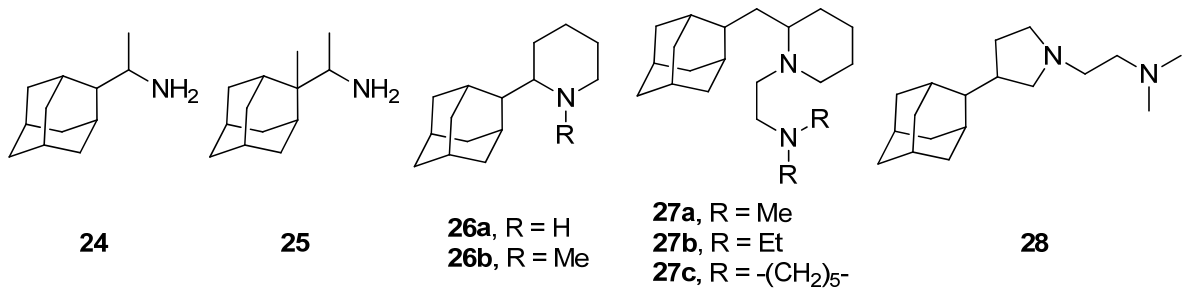
Figure 11. 2,2-Disubstituted adamantanes. a, R = methyl; b, R = ethyl; c, R = *n*-propyl.

Although 2-amantadine is only moderately active against influenza virus, the 2-isomer of rimantadine, **24** (Fig. 12), was found to be 4 times more potent than rimantadine against the influenza A_2/Japan/305/57 (H2N2) strain. This

finding led to Kolocouris' and De Clercq's groups to investigate the antiviral activity of several 2-alkyl and 2-cycloalkyl analogs of rimantadine [38]. They found that alkylation of the nitrogen atom reduced the anti-viral activity as did the introduction of a methyl group in the C-2 of the adamantane, as in **25**. Unfortunately, **24** was much less potent against X-31, a reassortant influenza A H3N2 strain (A/Hong Kong/1/68 with A/Puerto Rico/8/34) carrying the S31N mutation.

They also investigated the activity of 2-(2-adamantyl)piperidines, 2-(2-adamantylmethyl)piperidines and 3-(2-adamantyl)pyrrolidines. In these series they found that while the alkylation of the nitrogen atom reduced the activity, as in going from **26a** to **26b**, the introduction of a further nitrogen atom two carbon away from the heterocyclic ring, as in **27a-c** or **28** led to high anti-viral potency. For example, compounds **27a-c** showed EC_{50} between 3 and 7 μM, against the X-31 strain, much lower than amantadine (EC_{50} = 49 μM) or rimantadine (EC_{50} = 19 μM). Taking into account the size of the diamines, it seems like the M2 receptor site can accommodate cages much larger than the adamantane. Unfortunately, the selectivity index (SI) of these compounds was much lower than that of amantadine or rimantadine [39].



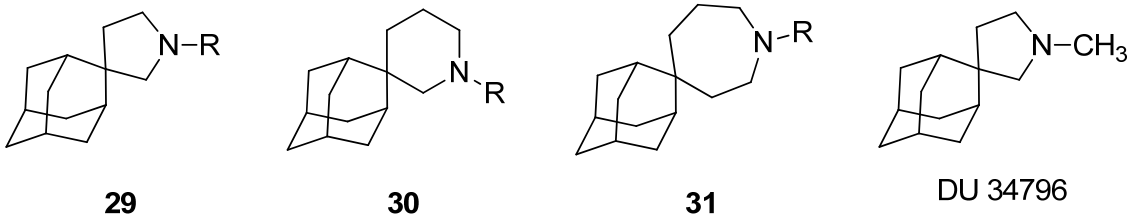
24 **25** **26a**, R = H; **26b**, R = Me **27a**, R = Me; **27b**, R = Et; **27c**, R = -(CH2)5- **28**

Figure 12. 2-Substituted analogs of rimantadine.

4. Azaspiroadamantanes

A unique kind of 2-substituted adamantanes is the group of the azaspiroadamantanes, because several of these derivatives have very potent anti-influenza activity.

Forty years ago, researchers at N. V. Philips-Duphar synthesized a series of azaspiroadamantanes (Fig. 13) [40]. Several of these amantadine analogs showed anti-influenza activity and, in fact, one of them, DU 34796, that had an antiviral spectrum *in vitro* wider than that of amantadine and was more potent than amantadine against mouse influenza, entered clinical trials, although finally the drug was not further developed [41]. The main problem of these compounds was, once again, the cross-resistance with amantadine and rimantadine.

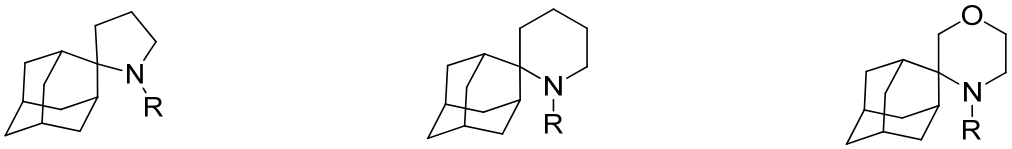


29 **30** **31** DU 34796

Figure 13. Spiro adamantane derivatives synthesized by N. V. Philips-Duphar. R is a lower alkyl group.

As 2-adamantanamine is only moderately active against influenza virus, the antiviral activity of the aforementioned derivatives points out that a carbon substituent in the vicinity of the 2-adamantyl carbon leads to a remarkable increase in antiviral activity. We will see further examples of this behaviour in the following paragraphs and also in different analogs that will be shown in the next sections.

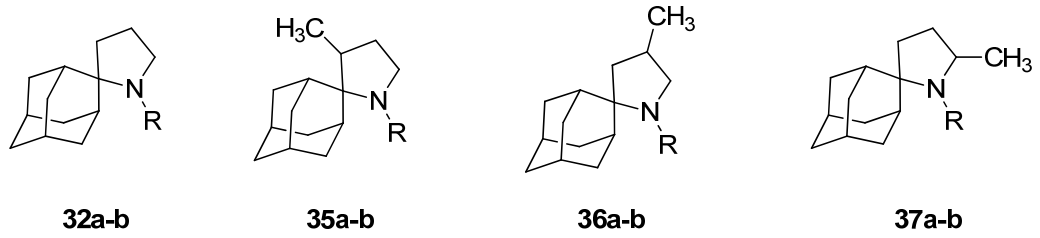
In the nineties, Kolocouris' group, successfully revisited the topic, synthesizing several azaspiro- and oxazaspiro-adamantanes, such as those shown in Fig. 14. The compounds were examined against several influenza A strains (H1N1, H2N2 and H3N2) by De Clercq's group. Interestingly, against a H2N2 strain, the compound **32b** was found to be up to 230 times more active than amantadine. Although **32b** showed a SI of 714 *in vitro*, unfortunately, it proved rather toxic *in vivo*. Worthy of note, the change of a methylene unit by an oxygen atom was compatible with anti-influenza activity, although these oxa-analogs were less active than amantadine [42].



32a-d **33a-c** **34a-c**

Figure 14. Azaspiro- and oxazaspiro-adamantane derivatives. a, R = H; b, R = methyl; c, R = ethyl; d, R = cyclopropylmethyl.

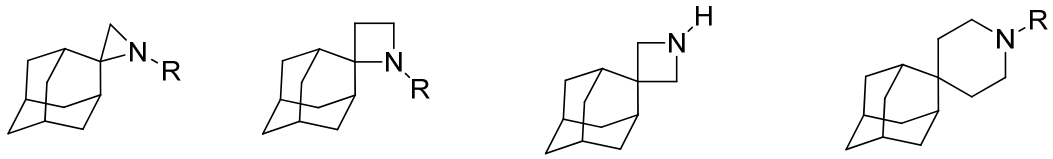
Later on, with the aim of improving the antiviral activity, Kolocouris's group explored the introduction of a methyl group in the pyrrolidine ring of **32a** and **32b** (Fig. 15). While the introduction of a methyl in either C-3 or C-4 of the pyrrolidine ring of **32a** and **32b** led to slightly less active compounds, introduction of a methyl in C-5 of the pyrrolidine was optimal for biological activity against H2N2 strain. Unfortunately, all pyrrolidines had lower SI than amantadine [43].



32a-b **35a-b** **36a-b** **37a-b**

Figure 15. *C*-methyl derivatives of azaspiroadamantanes **32a-b**. a, R = H; b, R = methyl.

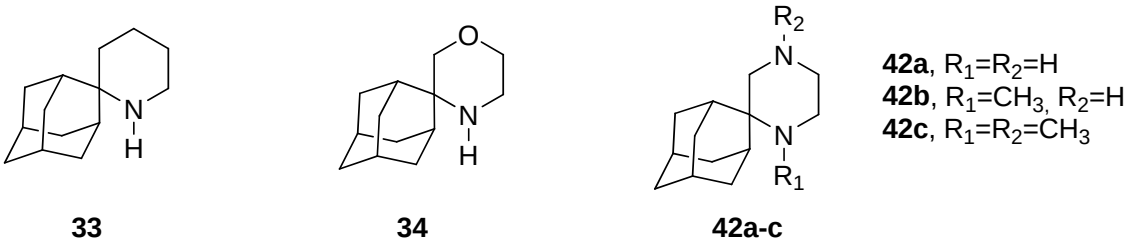
More recently, Kolocouris et al. have completed this series with the synthesis of ring-contracted and ring-expanded analogs of **32a** (Fig. 16) [44]. Azaspiro derivatives **38-41** were synthesized and tested against an H3N2 strain of influenza A. Whereas aziridine derivatives **38a,b** were less potent than amantadine, azetidines **39a,b** and **40**, and the piperidine derivatives **41a,b** were more potent than amantadine. Piperidine **41a**, the most potent of them, showed significant anti-influenza A virus activity, being 12-fold more active than amantadine and about 2-fold more active than rimantadine. Azetidine **36a**, while being slightly less potent than **41a** showed a better SI (694 vs 106). Methyl substitution at the nitrogen atom of all heterocycles caused reduction in anti-influenza virus A potency.



38a-b **39a-b** **40** **41a-b**

Figure 16. Ring-contracted and ring-expanded analogs of **32**. a, R = H; b, R = methyl.

Very recently, Kolocouris' group has reported the synthesis of several spiropiperazines of general structure **42** (Figure 17) [45]. These compounds can be regarded as analogs of **33** and **34** featuring an additional nitrogen atom. The main aim of this approach was to introduce a further group able to establish additional hydrogen bonds within the channel. However, piperazine derivative **42a** was three times less active than spiropiperidine **33** or amantadine. Moreover, *N*-methylation of **42a** to **42b** and **42c** further reduced the activity, probably by hampering the hydrogen bonding ability of the ligand, **42c** being inactive against influenza A/HongKong/68 (H3N2). No significant antiviral effect was observed against the amantadine resistant influenza A/WSN/33 (H1N1) strain. Notwithstanding the introduction of a second nitrogen atom was negative in this spiroadamantanes, other series of adamantane derivatives increased their potency with the introduction of a second amino group, as we have already seen in section 2.



33 **34** **42a-c**

Figure 17. Spiropiperidine 33 and analogs featuring an additional heteroatom.

Finally, it should be noted that, recently, Kolocouris and coworkers have reported the binding constants of some spiroadamantanes against the M2 channel of the influenza A/chicken/Germany/27 (H7N7, Weybridge strain), expressed in *E. coli* [36]. The binding affinity of spiropiperidine **33** was in the submicrolar range (K_d = 0.39 μM), very similar to that of amantadine (K_d = 0.32 μM), although much higher than that of rimantadine (K_d = 0.016 μM). Spiropirrolidines **32a**, **32b**, and **37b** displayed binding affinities in the micromolar range (1.16, 2.93, and 1.5 μM, respectively).

Unfortunately, sometimes it is difficult to compare the anti-viral activity of the different adamantane derivatives. This is, at least partly, a reflection of the time-span lasting more than four decades in which these compounds were synthesized and tested. For example, while spiropiperidines **30**, published in 1972 [40d], and **33a-c**, published in 1996 [42b], were tested against the influenza A_2/Japan, an H2N2 strain, **41a-b**, described in 2007 [44], were tested against the influenza A/HongKong/7/87, an H3N2 strain. As the activity of amantadine against these strains is different, it is difficult a quantitative comparison between the activity of all these compounds. Moreover, sometimes the description of the antiviral potency is not very accurate. For example, van Hes and coworkers, in describing the antiviral activity of **30**, only reported "*activity comparable to that of amantadine or better*" without stating a value for the IC_{50} [40d].

In order to investigate the SAR for their compounds, Kolocouris and coworkers have reported a conformational analysis study by a combination of NMR spectroscopy and theoretical calculations. They found that, in general, for the most active compounds the amine nitrogen atom lies in a distance of 1.5 to 2.5 Å away from the 2-adamantyl carbon [47].

5. Aminospiroadamantanes

As part of its monumental work in adamantane chemistry, Kolocouris' group has also reported the synthesis of several aminospiroadamantanes such

as those shown in Fig. 18. These compounds retain the pharmacophore group of rimantadine in the C-2 position of the adamantane ring. Compounds **43b** and **44a** showed to be more than 100 times more active than amantadine when tested against the influenza A_2/Japan/305/57 (H2N2) strain [42a] with SI of 83 and 24, respectively.

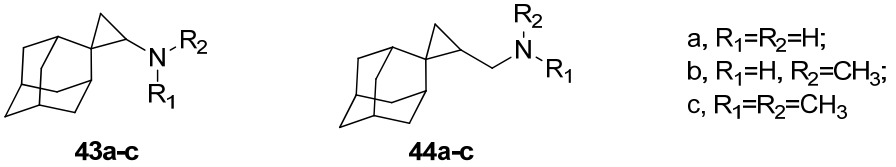


Figure 18. Aminospiroadamantanes **43** and **44**.

Analogs of **43** featuring a cyclobutyl or cyclopentyl ring have also been synthesized and tested against A_2/Japan/305/57 (H2N2) and X-31 (H3N2, with S31N in the M2 protein) strains [38]. Cyclobutyl derivatives **45** had similar potency against the H3N2 strain than rimantadine. Ring enlargement resulted in spirocyclopentane analogs **46** which were less potent than their cyclobutane analogs. When tested against the X-31 strain, all the new compounds showed to be less potent than rimantadine (Fig. 19).

Overall, in going from cyclopropyl analogs **43** to cyclopentyl derivatives **46**, it appears as if increasing the carbon crowding around the spiro carbon leads to compounds with reduced antiviral potency. However, it must be taken into account that Philips-Duphar researchers reported, in the 1970s, that the cyclopentyl derivative **47** had, against the A_2/Japan/305/57 (H2N2) strain, an antiviral activity of the same order of DU 34796, that, as we have already stated, entered clinical trials [40d]. Moreover, very recently, DeGrado's group disclosed in a patent the structure of the cyclohexyl derivative **48**, somehow related to spiropiperidine **41a**. Compound **48**, when tested against the wt M2 channel of influenza A virus expressed in oocytes of *Xenopus laevis*, showed an IC_{50} of 18.7 μM, very similar to that of amantadine (IC_{50} = 16 μM) and was slightly less active than rimantadine (IC_{50} = 10.8 μM). As rimantadine, **48** was inactive against the mutant S31N. However, compound **48** revealed to be a submicromolar inhibitor of the clinically important mutant V27A (IC_{50} = 0.31 μM) and also showed to be active against the mutant L26F (IC_{50} = 5.6 μM). To the best of our knowledge, **48** is the most potent compound ever reported against the mutant V27A [48]. Unfortunately, there is no data regarding the activity of compounds **43-47** against the V27A mutant M2 channel. Worthy of note, analog **49** has not been synthesized yet.

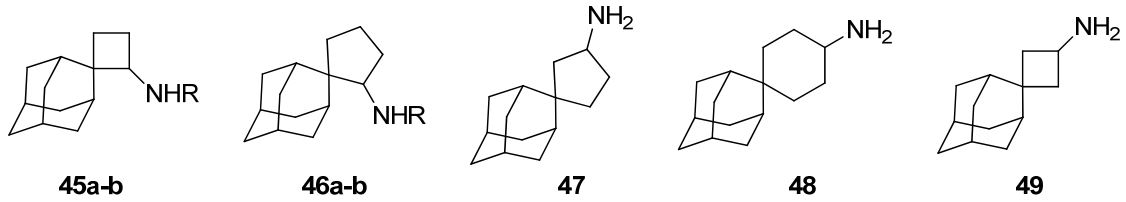


Figure 19. Aminospiroadamantanes **45**-**48** and unkown compound **49**. a, R = H; b, R = methyl.

Taken together the antiviral acitivity of compounds **43** to **48**, it seems that the distance and orientation between the nitrogen atom and the adamantyl cage is more important than the steric hindrance around the spiro carbon atom.

6. 1,2-Annulated adamantane derivatives

In the earlier 1970s, several patents by Squibb claimed anti-influenza activity for a series of 1,2-annulated adamantanopyrrolidines of general structure **50** (Fig. 20), although no much details regarding biological activity were given [49]. Nearly forty years later, Kolocouris's group synthesized several adamantanopyrrolidines **51**-**52**, the related compound **53** and 1,2-annulated adamantanopiperidines of general structures **54**-**56** and tested them against influenza A/Hong Kong/7/87 (H3N2) strain [50].

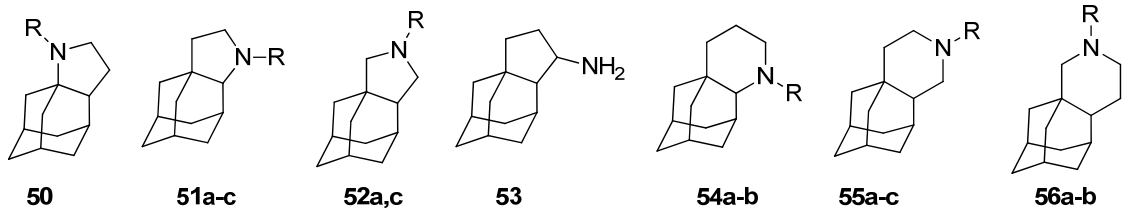


Figure 20. 1,2-Annulated adamantane derivatives **50**-**56**. a, R = H; b, R = methyl; c, R = ethyl.

The compounds **52a** and **56a** elicited submicromolar activities (IC_{50} of 0.5 and 0.6 μM, respectively) and a SI of 732 and 200, respectively, being equipotent to rimantadine (IC_{50} = 0.36 μM). Compounds **51** and **54**, with the nitrogen atom attached directly to the C-2 position of the adamantane ring, showed low micromolar activities (between 2 and 8 μM), similar to that of amantadine (2.0 μM).

As previously seen in other series, these results showed that a large lipophilic moiety in the vicinity of adamantane skeleton is compatible with good anti-viral activity, that moving the amine nitrogen atom away from the

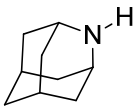
2-adamantyl carbon atom enhaces activity (compare **52a**, IC_{50} = 0.6 μM, with **51a**, IC_{50} = 2.2 μM), and that *N*-alkylation reduced the potency (compare **52a**, IC_{50} = 0.5 μM, with **52c**, IC_{50} = 2.4 μM, or **56a**, IC_{50} = 0.6 μM, with **56b**, IC_{50} > 500 μM).

In closing sections 2 to 5, it should be mentioned that in 2009, K.-C. Chou published a fragment-based quantitative structure-activity relationship (FB-QSAR) study with 34 substituted adamantanes. His main conclusion was that position 2 of the adamantane was more sensitive to substitution than position 1 [51].

7. 2-Azaadamantanes and (2-oxaadamant-1-yl)amines

Geigy has claimed that 2-azaadamantane, **57** (Fig. 21), first described in 1964 by Stetter et al. [52], displayed antiviral activity against three different influenza A H2N2 strains: A/Bethesda/10/63, A/Taiwan/1/62 and A/Singapore/1/57, but not further progress was published later [53].

More recently, we have found that replacement of the methylene unit of C-2 in amantadine by an oxygen atom to obtain (2-oxaadamant-1-yl)amine, **58**, reduced the antiviral activity [54].



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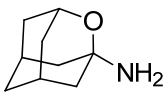


58

Figure 21. 2-azaadamantane and (2-oxaadamant-1-yl)amines.

8. BL-1743 and related compounds

In 1995, Bristol-Myers Squibb's researchers carried out a high-throughput screen based on the ability of inhibitors to reverse the toxicity associated with M2 channels expressed in the yeast *Saccharomyces cerevisiae* membranes. They found an azaspiro[5.5]undecane derivative, BL-1743, able to efficiently inhibit the activity of wt influenza A M2 channels (Fig. 22) [55]. The mechanism of action of BL-1743 was further characterized by electrophysiological methods. BL-1743 was also able to inhibit the AM2 channel expressed in *Xenopus* oocytes, as determined using the two-electrode voltage clamp (TEV) technique. It was found that the majority of M2 sequences isolated from influenza viruses resistant to amantadine were also resistant to BL-1743, which suggests that BL-1743 binds competitively with

amantadine. Interestingly, the kinetics of channel inhibition by BL-1743 were more rapid, showing a fast onset of inhibition as well as a reasonably rapid reversal of inhibition following removal of the compound [56]. This behavior contrasts with that of amantadine, whose second-order rate constant for the onset of inhibition is much slower than the diffusion-controlled rate, and whose off-rate is essentially irreversible on the minute to hour time scale of the experiment. The Hill coefficient for inhibition was 1.0, which is consistent with the binding ratio of one BL-1743 per AM2 tetramer [56].

It should be noted that twenty years before the discovery of BL-1743, A. H. Robins Company Inc., in a US patent [57], claimed anti-influenza activity for a series of aminospiranes that were already known from older literature [58]. Compounds **59-61** (Fig. 22) protected chicken embryos against influenza A/Taiwan/1/64 (H2N2) strain better or similarly than amantadine. No information about the activity of these compounds against amantadine-resistant strains was disclosed.

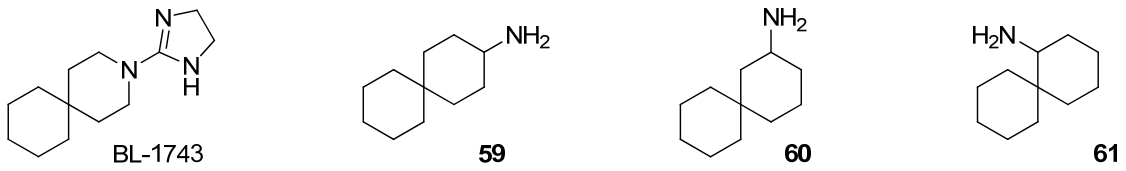


Figure 22. BL-1743 and related aminospiro[5.5]undecanes.

Taking into account the recent determination of the 3-D structure of the M2 ion channel of influenza A virus [19], and the structural difference between BL-1743 and the amantadine class of compounds, in 2008 Pinto's and DeGrado's groups started a SAR study of this scaffold with the aim of discovering new inhibitors of amantadine-resistant mutants [59].

Interestingly, spiropiperidine **62a** (Fig. 23), an analog of BL-1743 lacking the imidazoline group, had an IC_{50} of 0.9 μM against the influenza A wt M2 channel expressed in the *Xenopus* oocytes membrane, which is more than one order of magnitude more potent than amantadine (IC_{50} = 16 μM) and represents a more than 45-fold increase in potency relative to BL-1743 (IC_{50} = 45.3 μM). Alkylation of **62a** with a methyl group to **62b** reduced the potency (IC_{50} = 20.6 μM), and alkylation with larger groups as in **62c** led to inactive compounds. Several *N*-heteroarylmethyl derivatives of **62** were also inactive. Worthy of note, solid-state NMR data indicated that **62a** interacts with influenza A M2 channel differently from amantadine, affecting a longer stretch of the transmembrane helix and immobilizing the G34-I35 region. Ring-contracted analogs **63** and **64** were also active (IC_{50} = 8.1 and 12.0 μM, respectively)

although were less potent than **62a**. Dithiene **65** was moderately active (IC_{50} = 37.6 μM), while ketal **66** was inactive [59a]. Finally, it should be noted that **62a** can be seen as a simplified analog of Kolocouris' spiroadamantane **41a** (Figure 16), a compound that, when tested against influenza A/Hong Kong/7/87 (H3N2) strain, was found to be 12-fold more active than amantadine [44].

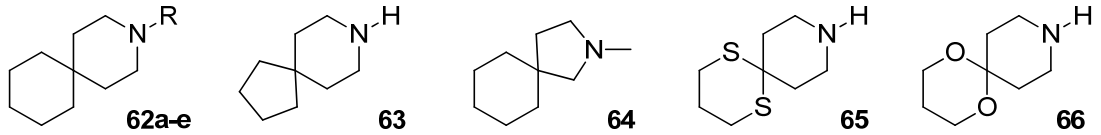


Figure 23. Spiropiperidine **62** and related compounds. a, R = H; b, R = methyl; c, R = methylcyclopropyl; d, R = methyl-2-pyridyl; e, R = methyl-2-imidazolyl.

Moving the nitrogen atom out of the spiro-ring led to the aforementioned amine **59**. DeGrado's group found that **59** had an IC_{50} of 12.6 μM, very similar to amantadine. Analogs **67** and **68** were also active (IC_{50} = 15.7 and 14.6 μM, respectively), while more complex derivatives, such as **69** were inactive (Fig. 24) [59b]. Interestingly, while **59** was less potent than **62a** against the wt channel of influenza A, **59** was active against the amantadine-resistant L26F and V27A mutants (IC_{50} = 30.6 and 84.9 μM, respectively) and also inhibited replication of recombinant mutant viruses bearing these mutations in plaque reduction assays. However, **59** was inactive against the S31N mutant. It is interesting to compare the structure and the activity of **59** with the spiroadamantane **48** (Fig. 19). While **59** and **48** displayed very similar activities against the wt channel (IC_{50} = 12.6 and 18.7 μM, respectively), **48** is much more potent against the amantadine-resistant mutants (V27A, IC_{50} = 0.3 μM; L26F, IC_{50} = 5.6 μM) [48].

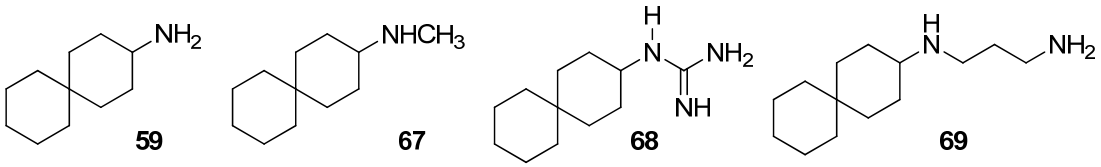


Figure 24. Spiro[5.5]undecan-3-amine **59** and related compounds.

9. Ring-contracted adamantane analogs

For the wt M2 protein, the diameter of the hole made from four Ser31 of separate trans-membrane chains is about 8 Å. However, after the mutation of residue 31 from Ser to Asn, the diameter of this hole was reduced to 6.32 Å [20i]. As the X-ray structure of the M2-amantadine complex shows that

amantadine is located in the hole between Ser31 and Ala34, the mutation of Ser to Asn leaves less space for amantadine entering or being stabilized [19d].

Taking into account this reduction in the space available for binding, we synthesized a series of ring-contracted amantadine and rimantadine analogs, featuring noradamantane and bisnoradamantane scaffolds (Fig. 25). Several derivatives showed low micromolar inhibitory activities of the wt M2 channel ranging from IC_{50} = 2.4 μM for guanidine **72** to IC_{50} = 17 μM for **71** and **75**. The activity was confirmed by plaque reduction assays with influenza A/Udorn/72 (H3N2) strain, carrying wt M2 protein and, for **70** it was also confirmed in an assay of inhibitory effect on virus replication using influenza A/Hong Kong/7/87 (H3N2) strain [60]. However, only bisnoradamantane derivative **74** showed to be moderately active against the S31N channel (IC_{50} = 252 μM), being less potent than amantadine (IC_{50} = 200 μM) [60b]. Several bisnoradamantanes carrying additional rings were also studied and some of them showed to be slightly less potent than amantadine. For example, pyrrolidine derivative **76**, had IC_{50} = 24 μM against the wt channel of influenza A.

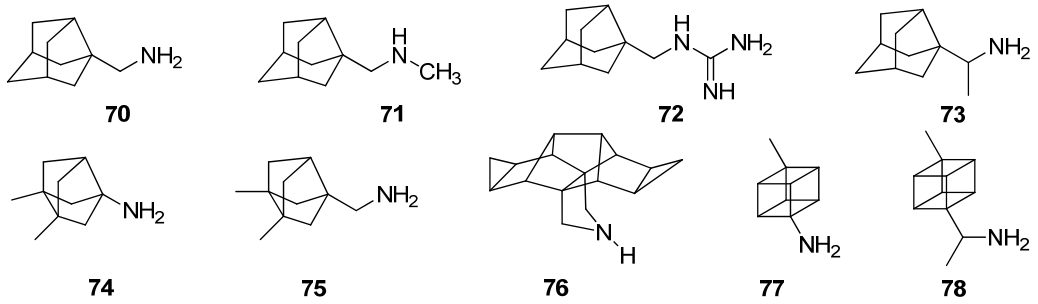


Figure 25. Ring-contracted analogs of amantadine and rimantadine.

Cubylamines also can be regarded as ring-contracted analogs of amantadine and rimantadine. In 1971, Du Pont de Nemours, claimed in a patent anti-influenza activity for several cubane derivatives, such as 4-methylcubane-1-amine, **77**, and α,4-dimethylcubane-1-methylamine, **78** (Fig. 25). When mice infected with the influenza A/Ann Arbor/2/60 (H2N2) strain were treated with the rimantadine analog **78**, there was a 70% survival rate as compared with 20% survivors in the infected, non-treated control animals [61].

10. Aminobicyclo[2.2.1]heptanes, aminobicyclo[2.2.2]octanes and related compounds

As early as in 1969, Smith Kline & French disclosed that bicyclo[2.2.1]heptanes **79-81** (Fig. 26) had anti-influenza activity. They

reported that compound **80**, at oral and subcutaneous doses of 25-100 mg/kg, caused a 35-80% and a 30-75% increase in survival of mice infected with the influenza A/Ann Arbor/2/60 (H2N2) strain, and with a swine strain of influenza A, respectively [62]. Although no further details have been published in the western literature related to the antiviral activity of **79** or **80**, later, Russian researchers found that an isomeric mixture of **81** and **82** effectively inhibited replication of influenza viruses and this mixture, as its hydrochloride, known as deitiforin, has been in used as antiviral in the former USSR for several years [63]. As an anti-influenza drug, deitiforin is equal to rimantadine from the standpoint of the protective effect in the treatment of influenza infection, and it can not only efficiently supress virus-specific growth, but can also selectively act on virus-infected cells. It has been found that influenza A/Victoria/35/72 (H3N2) strain resistant to deitiforin mutated the M2 protein in 3 amino acids: Met14Leu, Ala30Val and Met59Leu [64]. Interestingly, compound ICI 130685, which can be regarded as a derivative of **81** with further rings, advanced into clinical trials, but was not approved for clinical use [65].

García Martínez and coworkers have reported that several 1-norbornylamines were also endowed with potent anti-influenza activity. Secondary amines **83** and **84** were more potent than amantadine and showed very high SI [66].

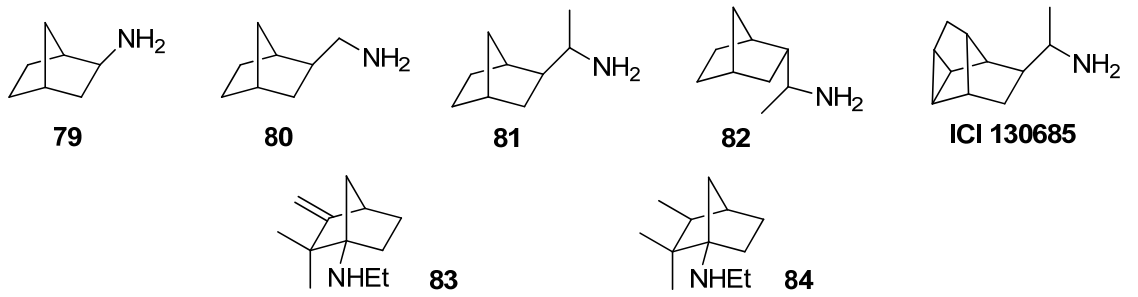
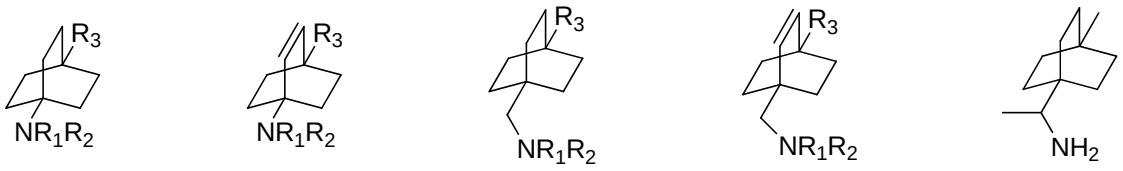


Figure 26. Bicyclo[2.2.1]heptanes with anti-influenza activity.

In 1969, DuPont de Nemours & Co, also claimed anti-influenza activity for a series of bicyclo[2.2.2]octan-1-amines, **85**, bicyclo[2.2.2]oct-2-en-1-amines, **86**, bicyclo[2.2.2]octane-1-methylamines, **87**, and bicyclo[2.2.2]oct-2-ene-1-methylamines, **88** [67]. They tested the compounds in mice using the influenza A/swine/S-15 strain and found that the unsaturated cage amines were similar in antiviral activity to their saturated counterparts. As seen in other families of polycyclic amines, substitution on the amino group with alkyl groups decreased the anti-viral activity. The addition of a methyl group in C-4 of the bicyclo[2.2.2]octane was optimal but inclusion of a larger group

reduced the activity. Finally, the presence of α-alkyl groups in the bicyclo[2.2.2]octane-1-methylamine series enhances antiviral activity. Overall, rimantadine analog **89** was the most active compound.



85 **86** **87** **88** **89**

Figure 27. Bicyclo[2.2.2]octan-1-amine, **85**, and related compounds.

Interestingly, Inamoto and co-workers reported the synthesis of several tricyclo[5.3.1.$0^{3,8}$]undecane (4-homoisotwistane) derivatives, such as amines **90** and **91**. 4-Homoisotwistanes can be seen as bicyclo[2.2.2]octane derivatives carrying an additional ring. Amines **90** and **91** were quite active against the Newcastle disease virus, which is sensitive to amantadine and is the causal agent of a bird disease that, when infecting humans, causes influenza-like symptoms. However, they did not test these compounds against influenza virus [68].

90 **91**

Figure 28. Tricyclo[5.3.1.$0^{3,8}$]undecane derivatives.

11. Other polycycloalkanes with anti-influenza activity

Finally, in this section we will discuss several unrelated polycyclic structures that have shown anti-influenza activity. For example, there are several amines derived from the pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane that have been biologically tested. In the 1970s, Smith, Kline & French, reported that amine **92** (Fig. 29) showed marginal activity against influenza A/Ann Arbor, while its isomer **93** was inactive [69]. Very recently, DeGrado and coworkers have found that amine **94** inhibited the activity of the wt M2 channel of influenza A expressed in oocytes of *Xenopus laevis*, with an IC_{50} = 8 μM, lower than that of amantadine (IC_{50} = 16 μM) and rimantadine (IC_{50} = 10.8 μM). As seen in other polycyclic derivatives, the addition of a

hydroxyl group, as in **95**, was compatible with inhibitory activity (IC_{50} = 24 μM), but not increased the potency [30].

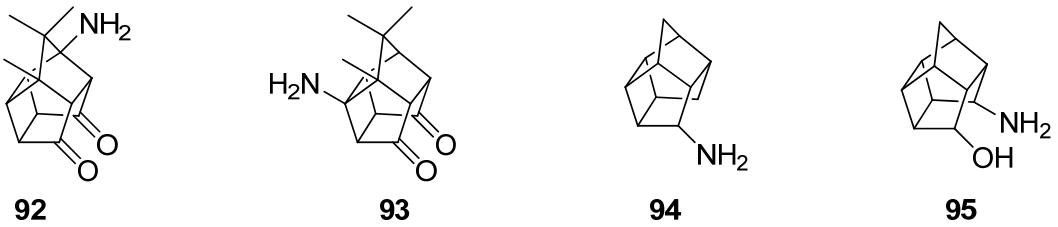


Figure 29. Derivatives of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane.

In 2010, Hu et al. carried out the screening of a small primary amine library as M2 protein inhibitors. They reported that linear alkyl amines, aromatic amines and unsubstituted monocyclic amines were inactive. However, they found five compounds, **14**, previously studied by Scholtissek [33], and **96-99** (Fig. 30), with similar activities to that of amantadine. Isopinocamphenylamine **99**, the most potent inhibitor, was three times more active than amantadine (IC_{50} = 1.4 μM vs IC_{50} = 6.0 μM) for viral inhibition of the influenza A/Hong Kong/8/68 (H3N2) strain [70]. Encouraged by these results, the same group has very recently published a small library of derivatives of **99** obtained by keeping the scaffold constant and modifying the amino functionality. The compounds were evaluated for viral inhibiton against influenza A/WS/33 (H1N1), amantadine resistant, and influenza A/Hong Kong/8/68 (H3N2), amantadine sensitive. Although there was no inhibition of the amantadine resistant strain, most of the compounds exhibited antiviral inhibition as good as amantadine against the amantadine sensitive strain. Compound **100** (IC_{50} = 0.09 μM) was nearly 240-fold more potent than amantadine against wt influenza A virus [71].

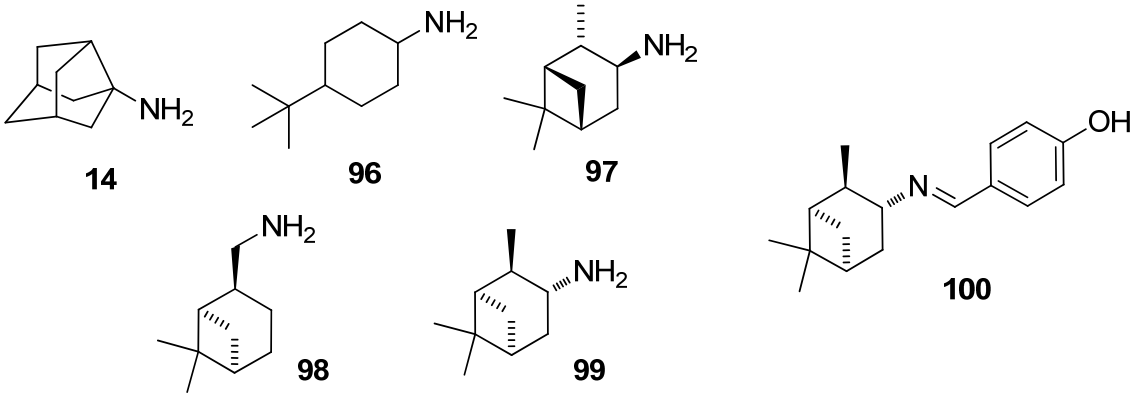


Figure 30. Primary amine inhibitors of M2 channel and derivative **100**.

Finally, DeGrado and coworkers tested the inhibitory activity against wt M2 channel of a series of branched and polycyclic amines (Fig. 21) [30]. Surprisingly, branched alkyl amine **101** was nearly as active as amantadine (IC_{50} = 21 μM vs IC_{50} = 16 μM), tricyclic amine **102**, showed higher activity than amantadine (IC_{50} = 9 μM) and four homoadamantane derivatives, **103**-**105** and **13** showed similar activity as amantadine, suggesting that the M2 channel can accommodate a wide range of structural diversity and that is insensitive to minor scaffold modifications, so long as the shape of the molecule conforms to the M2 cavity. All these compounds were found to be less potent or inactive against V27A and/or S31N mutant channels, probably as a consequence of the higher polarity of the mutant channels [30].

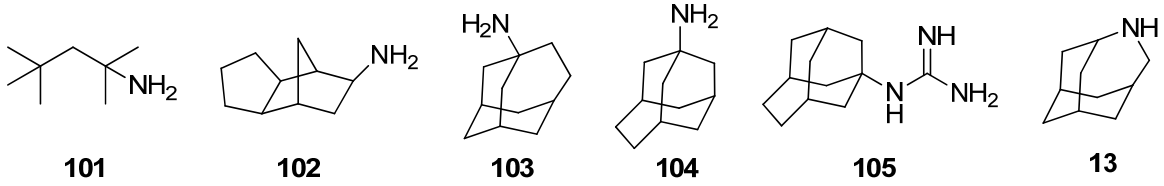


Figure 31. Several inhibitors of M2 channel.

12. Conclusion

Although amantadine and rimantadine have been in clinical use for many years and hundreds of analogs have been tested as anti-influenza agents, the results obtained so far are a bit disappointing. While several active compounds have been found, occasionally having more potency than amantadine and rimantadine, cross-resistance with both drugs is still an unresolved issue. The above notwithstanding, the recent structural, functional, and computational studies carried out with M2 protein have opened the door to the rational design of new inhibitors [72], and, very recently, some derivatives have shown promising activity against the V27A amantadine-resistant mutant [30,48,59]. The S31N mutant is still even a major challenge.

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