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Design and synthesis of pinanamine derivatives as anti-influenza A M2 ion channel inhibitors

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ABSTRACT

The adamantanes are a class of anti-influenza drugs that inhibit the M2 ion channel of the influenza A virus. However recently, the clinical effectiveness of these drugs has been called into question due to the emergence of adamantane-insensitive A/M2 mutants. Although we previously reported (1R,2R,3R,5S)-3-pinanamine **3** as a novel inhibitor of the wild type influenza A virus M2 protein (WT A/M2), limited inhibition was found for adamantane-resistant M2 mutants. In this study, we explored whether newly synthesized pinanamine derivatives were capable of inhibiting WT A/M2 and selected adamantane-resistant M2 mutants. Several imidazole and guanazole derivatives of pinanamine were found to inhibit WT A/M2 to a comparable degree as amantadine and one of these compounds **12** exhibits weak inhibition of A/M2-S31N mutant and it is marginally more effective in inhibiting S31N M2 than amantadine. This study provides a new insight into the structural nature of drugs required to inhibit WT A/M2 and its mutants.

1. Introduction

Over the past century influenza epidemics and pandemics have profoundly impacted on human morbidity, mortality, and the economy. In 1918–1919, the outbreak of "Spanish flu" (H1N1) resulted in the death of at least 20 million people (Kilbourne, 2006). Today, influenza still remains one of the major virulent infectious diseases known to mankind. The impact of influenza has not just been confined to humans with outbreaks of highly pathogenic avian influenza (HPAI) of various subtypes occurring frequently in poultry over the past decade (Munster et al., 2005). Since 2003, the HPAI subtype A (H5N1) has had a devastating impact on domestic or wild birds in many parts of South East Asia, Europe, the Middle East and parts of Africa. Alarmingly, this strain of virus is also transmissible to humans with more than 230 people so far infected, of whom over 50% have died (WHO, 2006). More recently, the H1N1 influenza (swine flu) outbreak swept Mexico and other parts of the world prompting the WHO to issue a pandemic warning (Yeh et al., 2010). Thus, there is a clear need for novel and efficient anti-influenza therapeutics. Although vaccination is the ideal way to prevent influenza virus infection, the preparation of a new vaccine requires more than 6 months (Couzin-Frankel, 2009), and is subject to seasonal flu strains. Clinically approved antiviral drugs on the other hand are not typically dependent on these factors. Currently, the only known anti-influenza A drugs are neuraminidase inhibitors (zanamivir and oseltamivir) and M2 ion channel blockers (amantadine and rimantadine, Fig. 1, De Clercq, 2006). A problem with both classes of drug is the development of drug-resistant strains to influenza A. This is particularly relevant for amantadine and rimantadine and reports of drug resistance to the neuraminidase inhibitor, oseltamivir (Tamiflu) also surfaced in 2009–2010 flu season (Baz et al., 2009). Therefore there is an urgent need to develop alternative anti-influenza agents.

The influenza A virus M2 protein (A/M2) plays an essential role in viral replication. The A/M2 ion channel is 97 amino acids in size but it is the membrane-spanning portion (residues 22–46) that is required for its proton transport activity (Pinto et al., 1997; Sakaguchi et al., 1997). Two high-resolution structures containing the transmembrane portion of the A/M2 protein showed that the A/M2 protein forms a homotetrameric proton selective channel (Stouffer et al., 2008; Schnell and Chou, 2008). The virus is known to enter the host cell by receptor-mediated endocytosis and the low pH of the endosome activates the A/M2 channel to allow an influx of protons. Viral RNA and other viral genetic material are

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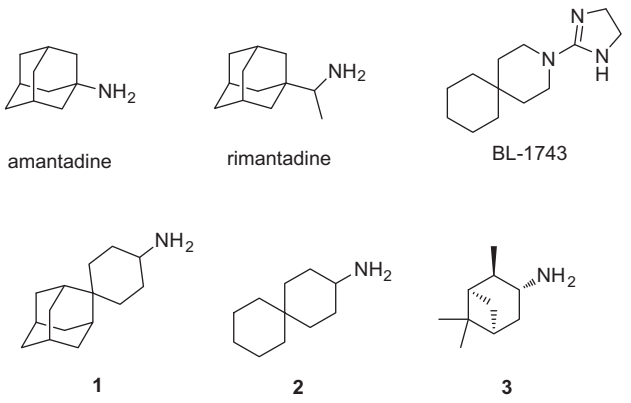


Fig. 1. Reported A/M2 channel inhibitors.

then released to the cytoplasm to allow for replication (Zhirnov, 1990; Martin and Heleniust, 1991). A/M2 also equilibrates the pH between the Golgi lumen and the cytoplasm and prevents a premature hemagglutinin conformation transition (Ciampor et al., 1992; Grambas and Hay, 1992). A variety of mutations of A/M2 can lead to amantadine insensitive in vitro, and S31N, V27A, and L26F are common phenotypes (Abed et al., 2005; Brown et al., 2010; Li et al., 2009; Bright et al., 2005; Saito et al., 2003). Although inhibitors targeting wild type (WT) and A/M2 mutants are well reported, amantadine and its derivatives can only block WT A/M2 (Davies et al., 1964; Aldrich et al., 1971; Kolocouris et al., 1994, 1996, 1999; Zoidis et al., 2006, 2009). Some nonadamantane-based compounds, spiroadamantane **1**, BL-1743 analogs such as **2** (Fig. 1), and **4** (Fig. 2) were recently reported to inhibit the A/M2-WT and both L26F and V27A mutants (Wang et al., 2009; Balannik et al., 2009; Wang et al., 2011a,b), however inhibitors of the most common mutant S31N are less prevalent (Duque et al., 2011). Therefore, the design of inhibitors that can block the S31N mutant of A/M2 is a priority.

It was previously noted by our laboratory that (1R,2R,3R,5S)-3-pinanamine (pinanamine **3**, Fig. 1) was more potent than amantadine in inhibiting WT A/M2 (Hu et al., 2010a,b) and by maintaining this scaffold and modifying the amino functionality, several additional compounds were found to be highly potent inhibitors (Zhao et al., 2011). However, none of these compounds could inhibit adamantane-insensitive A/M2 mutants. In this study, surface plasmon resonance (SPR) experiments (Rosenberg and Casrotto, 2010) were conducted to measure the binding affinity of **3** to the WT A/M2 ion channel and a series of novel pinanamine derivatives involving linking the alkali group to this scaffold has been designed and synthesized, and their capacity to inhibit A/M2 assessed using a number of physiological and biophysical assays. We have found that the WT A/M2 can be blocked by several compounds comprising imidazole or guanazole groups and among them, compound **12** can also inhibit the A/M2-S31N.

2. Materials and methods

2.1. Experimental chemistry

All commercially available compounds and solvents were reagent grade and were used without further treatment unless otherwise noted. Reactions were monitored by TLC using Qing Dao Hai Yang GF_{254} silica gel plates (5 × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) by either spraying with an ethanol solution of Ninhydrin or by treatment with iodine gas. Compounds were purified silica gel column chromatography which performed on silica gel (200–300 mesh) from Qing Dao Hai Yang and characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS, NMR spectra were recorded on a Bruker NMR AVANCE 400 (400 MHz) or a Bruker NMR AVANCE 500 (500 MHz), and the NMR reagents $CDCl_3$ and DMSO-d_6 were used as internal standards. Chemical shifts (δ) were recorded in part per million and coupling constants (J) in hertz (Hz). MS data were measured on an Agilent MSD-1200 ESI-MS system.

2.1.1. Inhibitor synthesis

Detailed inhibitor synthesis and characterization can be found in the Supplementary data.

2.2. Biological experiments

2.2.1. Patch clamp assay

The inhibitors were tested via patch clamp assay using A/M2 expressed 293Trex cells and membrane currents were recorded as in a previous report (Hu et al., 2010a,b).

2.2.2. Plaque reduction assay

A monolayer of MDCK cells were infected with 0.01 MOI influenza A viruses for 1 h at 37 °C. The inoculums were then removed, and the cells were washed twice with phosphate-buffered saline

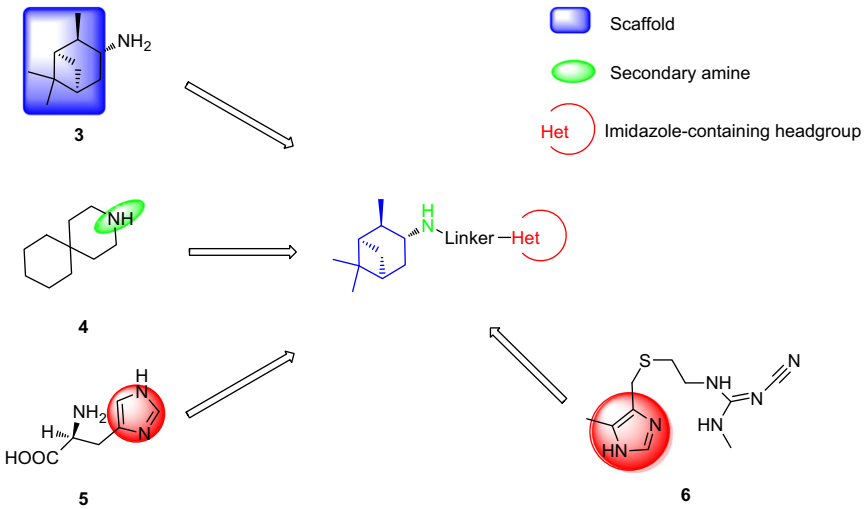


Fig. 2. Design strategy for imidazole-containing compounds.

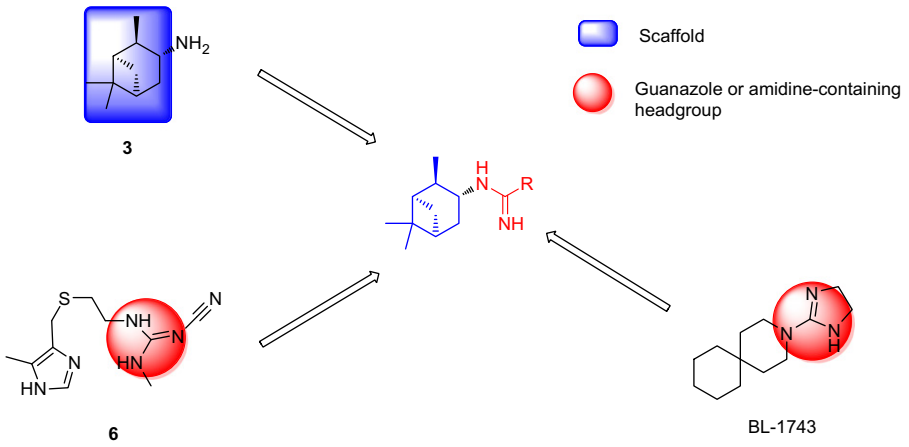


Fig. 3. Design strategy for guanidine or amidine compounds.

(PBS). The cells were then overlaid with 1% agar DMEM-containing amantadine or one of the synthesized compounds in the presence of 2 μg/mL trypsin and 0.3% BSA. Two to 3 days after infection, the monolayers were fixed and stained with 0.1% crystal violet solution.

2.2.3. Viral inhibition assay

MDCK cells were grown to confluence in 96-well microtiter plates, the medium was removed, and the cells were covered with 50 μL of medium containing various amounts of amantadine or one of the synthesized compounds in the presence of 1 mg/mL TPCK and 0.3% BSA. The plates were then incubated at 37 °C for 30 min. Fifty microliters, equal to approximately 0.01 MOI of influenza A viruses were then added to the plates. After incubation in 5% CO_2 at 37 °C for 72 h, 10 μL of CCK-8 reagent were added to each well, and the mixture was incubated for 3 h. The A450 was then measured using an UVstar-Microplates Synergy HT. Data were analyzed using GraphPad Prism 5.

2.2.4. Cytotoxicity assays

MDCK cells were grown as monolayers in 96-well plates after seeding for 18–24 h. The medium was removed and rinsed twice with Hanks' solution and the compounds were serially diluted in 100 μL medium containing 0.3% BSA. After incubating for 72 h at 37 °C in a humidified 5% CO_2 incubator, 5 μL CCK-8 reagent in 50 μL medium was added to each well and the absorbance was measured at 450 nm using a UVstar-Microplates Synergy HT plate reader. The CC_{50} values were calculated by nonlinear regression using GraphPad Prism 5.

2.2.5. M2 peptide synthesis and purification

The wild-type M2 peptide (A/Udorn/72 strain) was synthesized using solid phase chemistry on a Symphony/Multiplex multiple peptide synthesizer using FMOC protection. The sequence is SSDPLVVAASIIGILHLILWILDRL.

Peptides were produced by Kerry McAndrew (Bio-Molecular Resource Facility) at the Australian National University and received as a freeze-dried powder. The purification of the M2 peptide was performed on a C4 reverse phase HPLC column and purified using a Waters HPLC system (Empower-2 software) as previously described (Rosenberg and Casrotto, 2010).

2.2.6. Production of liposomes and SPR experiments

Liposomes were produced by organic solvent extraction. DMPC was dissolved in chloroform at a concentration of 10 mg/ml and was removed using a Buch™ rotary evaporator at room temperature. The peptide was dissolved in TFA added to the lipid, which was again removed using the rotary evaporator. A lipid to peptide ratio of 50:1 was used in experimental samples. PBS was added to the film, and incubated at 37 °C for 30 min and extruded using a Lipex Biomembranes nitrogen powered extruder. Liposomes were produced at a concentration of 0.1 μM by extrusion through a Nucleopore membrane. Assessment of liposome size and quality was performed using dynamic light scattering measurements carried out on a Malvern Instruments Zetasizer Nano ZS. Electron microscopy images were also taken to confirm the formation of liposomes. SPR experiments and analysis were conducted as previously described (Rosenberg and Casrotto, 2010).

3. Results and discussion

3.1. Theoretical basis and chemical design

The His-x-x-x-Trp motif is essential for formation of the pH-sensitive proton channel in the transmembrane (TM) domain of the A/M2 protein (Hu et al., 2006). It has been shown that the acidification of the His_{37} side chains plays a crucial role in the activation of the A/M2, and additional studies have suggested evidence of a cation–π interaction between the protonated imidazole ring

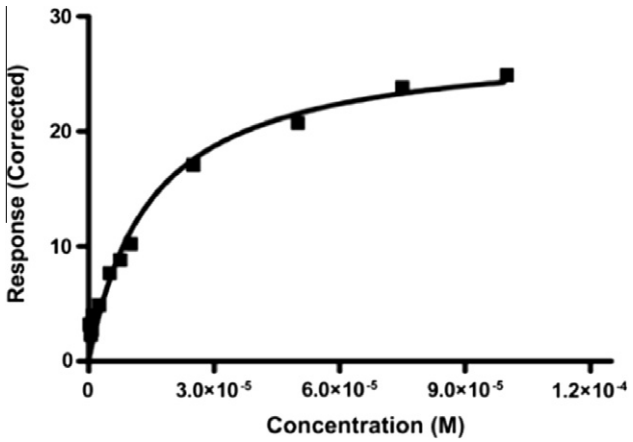
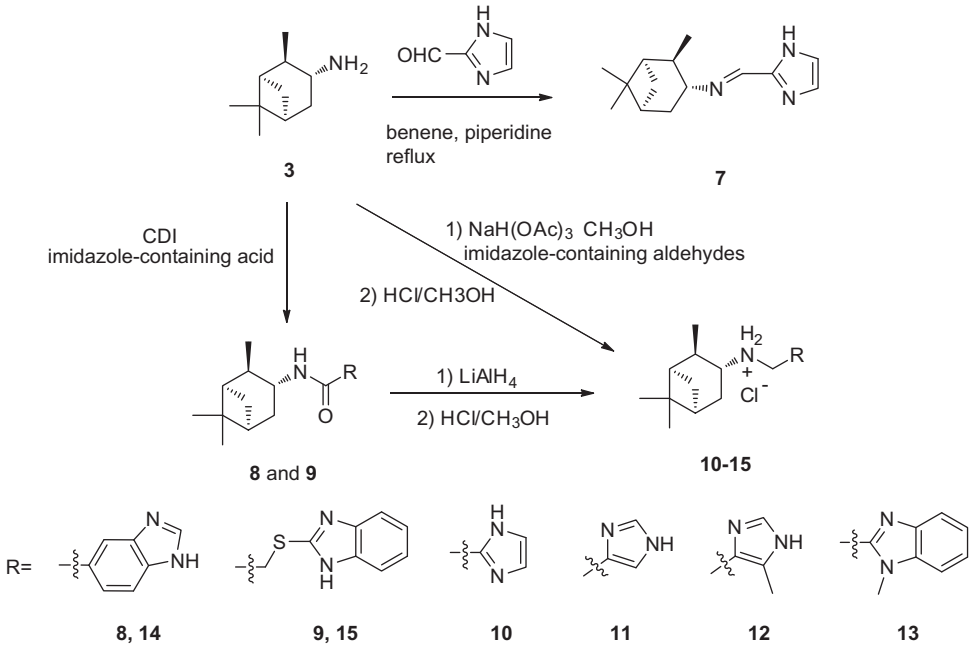


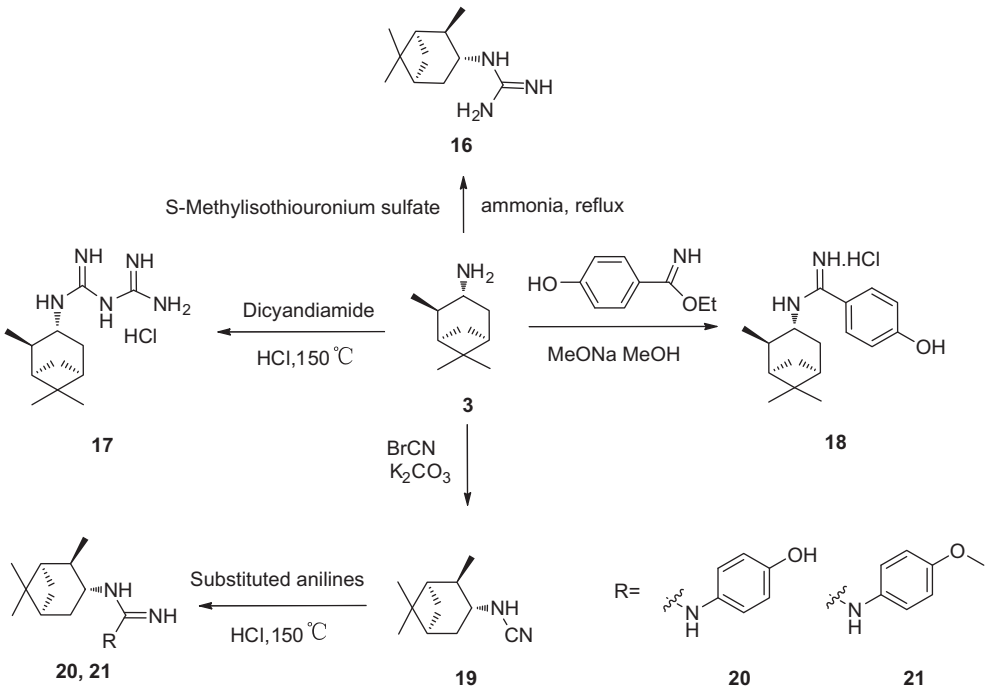
Fig. 4. Representative SPR dose–response curve for compound **3** at concentrations 0.1–100 μM. Curves represent the equilibrium-binding data fitted to a previously described equation[35]. The mean equilibrium-binding affinity for compound 3 is 14. 7 μM (Std. Dev. 2.5 μM, $n = 4$).

of His_{37} and the indole group of Trp_{41} (Wang et al., 1995; Okada et al., 2001; Witter et al., 2008; Takeuchi et al., 2003; Hu et al., 2010a,b). Thus the role of the His_{37} imidazole ring in H^+ ion channel transport provides an important clue for inhibiting H^+ transport and based on the pore-binding model, we predict that compounds containing an alkali group such as an imidazole ring or a guanazole could potentially decrease the protonation of His_{37}. To test the hypothesis, two known compounds containing L-histidine (**5**, Fig. 2) and cimetidine (**6**, Fig. 2) were selected for A/M2 inhibition testing by using patch clamp assay on 293Trex. Interestingly, both of them were found to inhibit WT A/M2 at a concentration of 100 μM (Table S1).

A common feature of all known M2 inhibitors is the presence of a primary amine headgroup connected to a hydrophobic scaffold (Wang et al., 2011a,b; Zhao et al., 2011). In this study, we have maintained the pinanamine scaffold and linked a secondary amine and an imidazole or guanazole as a headgroup. The design strategies are shown in Figs. 2 and 3.



Scheme 1. Synthetic scheme for the preparation of imidazole-containing compounds.



Scheme 2. Synthetic scheme for preparation guanidine or amidine derivatives of pinanamine.

3.2. Measurement of the WT M2 and pinanamine binding affinity by SPR

The inhibition of M2 peptide ion channels with compound **3** has been previously examined using a variety of techniques including patch clamp and viral inhibition. Given the diverse nature of these assays, another affinity binding measurement was performed using steady-state surface plasmon resonance (SPR). A binding constant of ~14 μM was observed for compound **3** with A/M2 (Fig. 4), a value which is consistent with results obtained from patch clamp and viral inhibition (Hu et al., 2010a,b). This binding affinity suggests that like amantadine, the most likely binding site location for the pinanamine scaffold is the pore of the M2 channel (Rosenberg and Casrotto, 2010).

Table 1
Inhibitory activity of compounds on M2 ion channel conductance detected by patch clamp.

Compound	A/M2 wt Inhibition by 100 μM (%)	A/M2 S31N Inhibition by 100 μM (%)
7	92.4	0
10	34.2	0
11	83.3	0
12	95.8	26.7
13	23.0	0
14	88.2	0
15	15.7	0
16	81.3	0
17	94.5	0
18	91.9	0
20	62.3	10.4
21	34.8	0
Amantadine	94.0	<10[a]

[a] <10% Indicates that when 10% channel activity was inhibited, more than 1 mM compounds were needed.

Table 2
Inhibition efficiency of selected compounds on influenza A virus.

Compound	A/M2 WT[a] IC_{50} (μM)	A/M2 S31N[b] IC_{50} (μM)
7	0.227 ± 0.051[c]	ND[d]
11	6.656 ± 0.320	ND
12	1.862 ± 0.930	80 ± 6.5
14	2.680 ± 1.840	ND
16	2.121 ± 1.288	ND
17	0.230 ± 0.050	ND
18	2.005 ± 0.728	ND
Amantadine	0.528 ± 0.150	102 ± 11.0

[a] Using influenza A/M2-WT virus (A/Hong Kong/68 (H3N2) strain).
[b] Using influenza A/M2-S31N mutant virus (A/WSN/33 (H1N1) strain).
[c] IC_{50} = mean ± SD.
[d] ND, not determined.

3.3. Chemistry

In order to further explore the impact of the imidazole group on the inhibition of the A/M2 channel, we embarked upon the synthesis and characterization of imine-based compounds. The imidazole-containing imine **7** (Zhao et al., 2011) was the first set of compounds synthesized. Subsequently, a reductive amination of **3** with different imidazole-containing aldehydes using $NaBH(OAc)_3$ in methanol (Li et al., 2007), followed by treatment with HCl/CH_3OH provided the salts of **10–13** with yields of 60–70%. Another two compounds, **14** and **15**, were obtained via the reduction of corresponding amides **8** and **9**. First, the acid was activated with *N,N'*-carbonyldiimidazole, and then treated with **3** to afford the amide (Vaidyanathan et al., 2004) **8** and **9**, which were subsequently reduced with $LiAlH_4$ to give the secondary amine. In the final step, the products were treated with HCl/CH_3OH gave rise to **14** and **15** (Stead et al., 2008) in 41% and 50% overall yields, respectively (Scheme 1).

To explore the effect of the guanazole on the biological activity, a series of amidine and guanidine compounds were prepared (Scheme 2). Nucleophilic substitution of *S*-methylisothiouronium with **3** generated compound **16**. Synthesis of **17** was achieved by the acid-catalyzed addition of **3** to dicyandiamide (Wilson et al., 1991). The same process was used afforded to **20** and **21** via the addition substituted anilines to **19**. Commercially available ethyl 4-hydroxybenzimidate hydrochloride was reacted with **3** in the presence of sodium methoxide afford **18** (Arstad et al., 2006) with a yield of 64.9%.

3.4. Pharmacological activity

The inhibitory properties of the compounds were measured using the patch clamp technique with WT and mutant A/M2 expressed in 293Trex cells. All inhibitors were initially tested at 100 μM. For those compounds that inhibited the WT A/M2 channel activity by more than 80%, IC_{50} values were then obtained using the viral inhibition assays. The results are given in Tables 1 and 2 and Fig. 5.

It had been previously found that an unsubstituted guanidine compound of spiro-piperidine could inhibit the A/M2-WT channel activity by 87% at 100 μM (Wang et al., 2009). This level of inhibition was also observed for compound **16** when a guanadino group was incorporated into the pinanamine scaffold. Compound **16** inhibited the A/M2-WT channel activity by 81.3% and exhibited an IC_{50} value of 2.1 μM as measured by the viral inhibition assay. Replacement of the primary amido group of **16** by a 4-hydroxyphenyl which according to our previous study (Zhao et al., 2011), could act as a more favorable group, gives rise to the amidine compound **18** with an inhibition constant (IC_{50}) of 2.1 μM. The corresponding substituted guanidine **20**, however, shows slightly

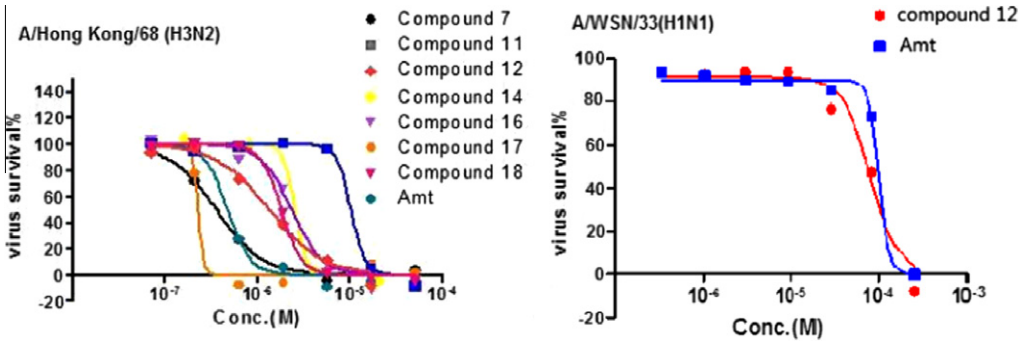


Fig. 5. Dose–response curves for selected compounds on influenza A virus strains harboring A/M2-WT and A/M2-S31N, respectively.

weaker A/M2-WT inhibition (62.3%), but interestingly, was able to inhibit A/M2-S31N channel activity by 10.4% at 100 μM, which is slightly higher than that of Amt (<10%). The replacement of a hydroxyl with a methoxy group (compound **21**) led to a marked loss of potency at 100 μM (34.8%), and a complete loss of A/M2-S31N channel inhibition. This may indicate that the hydrophilicity

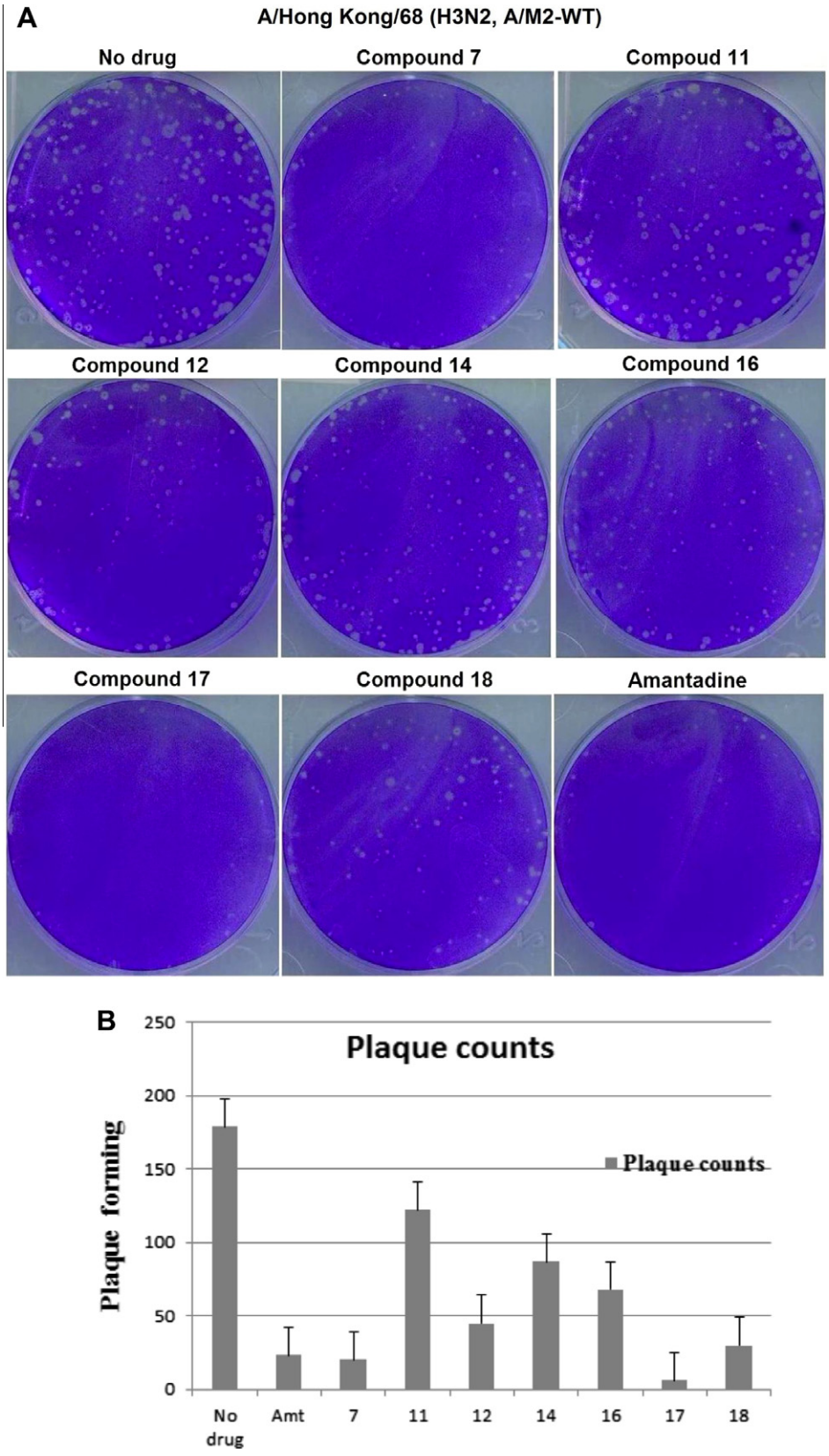


Fig. 6. Reduction of plaque formation by A/Hong Kong//68 (H3N2, A/M2-WT) in MDCK cells upon treatment with selected inhibitors. (A) Viral plaque formation in MDCK cells in the presence of each compound (2 μM). (B) Number of plaques per well: no drug, 179; amantadine, 23; **7**, 20; **11**, 122; **12**, 45; **14**, 87; **16**, 68; **17**, 6 and **18**, 30.

of the polar headgroup is an important determinant in A/M2-S31N channel inhibition.

The bi-guanidine based compound, **17** inhibited A/M2-WT by 94.5% at 100 μM and was 10-fold more potent than **16** (IC_{50} = 0.23 μM vs 2.12 μM). Indeed, compound **17** was the most potent inhibitor in this series due possibly to its moderate hydrophobicity and polarity compared with **16** and **18**. For this reason, no additional substitute guanidine compounds were considered.

The viral inhibition assays demonstrate that the imine compounds are excellent inhibitors (Zhao et al., 2011). Not surprisingly, compound **7**, made up of a pinanamine scaffold linked to a 2- imidazole group with a C=N double bond, retained its inhibitory properties. In contrast, compound **10** was a poor inhibitor of M2 (34.2%) which is consistent with reported data featuring similar compounds (Wang et al., 2009). Disappointingly, linking the secondary amine to an *N*-substitute 2-benzimidazole group or adding a sulfur atom in the linker (**13** and **15)** lead to much less potent compounds that inhibited the A/M2-WT channel activity by 23.0% and 15.7%, respectively.

Taking the above result into consideration, an alternative design strategy was adopted by incorporation of a 5-benzimidazole headgroup (compound **14**). The potency for this compound was significantly increased to apptoximately 88% inhibition of the A/M2-WT channel (IC_{50} = 2.680 μM). Encouraged by this result, compound **11** was synthesized. This compound is distinct from **10** in that it included a 4-imidazole headgroup derivative. It also proved much more potent with an IC_{50} of 6.65 μM although it was less than **14** which may be explained by the decreased basicity of the headgroup. Adding an methyl to the imidazole group of **11**, gave rise to a more basic compound **12**, which was shown to be able to inhibit WT A/M2 by more than 95% and possessed an IC_{50} of 1.86 μM. It is feasible to speculate that the activity of compound **12** was greater than **11** and **14**, because of its intermediate hydrophobic and basic property. Surprisingly, 26.7% of the A/M2-S31N channel activity was inhibited by **12** which is an improvement to amantadine. A similar result was shown in viral inhibition of corresponding influenza A virus (IC_{50} = 80 vs 102 μM).

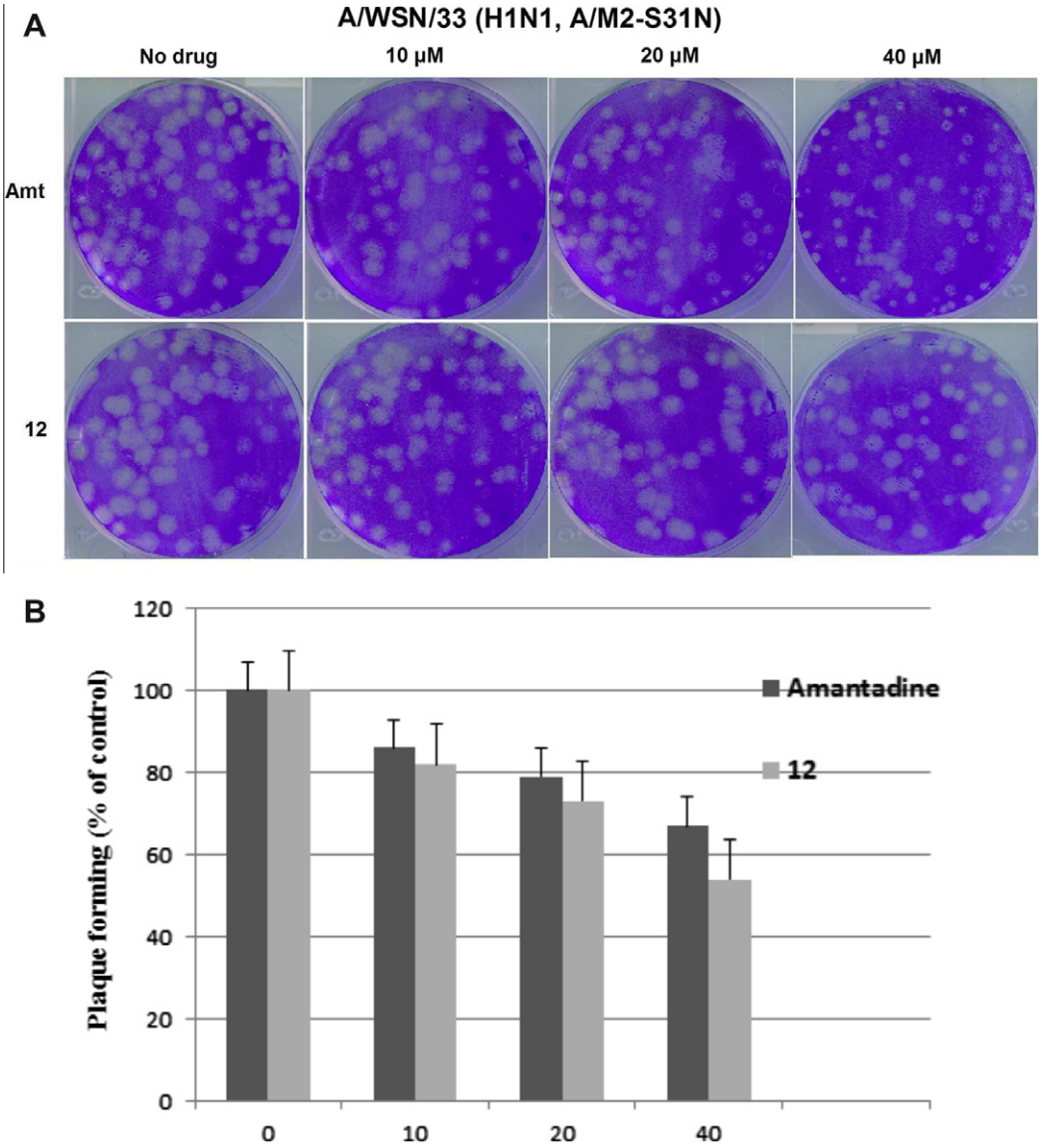


Fig. 7. Effects of selected compounds on A/WSN/33 virus were evaluated by plaque formation on MDCK cells in the presence or absence of these compounds. MDCK cells were infected with virus at approximate 70 plaque forming units/well for amantadine and 61 for **12**. (A) Both the size and the number of plaque formation of S31N influenza virus were reduced by compound **12** at the concentration of 20 and 40 μM. (B) Quantification of viral plaque formation following treatment with serial dilutions of the compound.

3.5. Viral inhibition assay

Selected compounds were chosen for measurement of their IC_{50} using a viral inhibition assay. The inhibitory action was assessed by measuring the survival of cells infected with the influenza A virus. Therefore the addition of a potent M2 inhibitor should in principle, rescue the cell by inhibiting the replication of the virus. The inhibitory property of the compounds were assessed using a A/Hong Kong/68 (H3N2, WT) and A/WS/33 virus, which is a natural S31N variant (Judd et al., 1997; Sidwell and Smee, 2000; Abed et al., 2005).

3.6. Activity in plaque reduction assays

The effectiveness of the inhibitor compounds against the influenza A virus was confirmed by plaque reduction assays. Both the size and the number of plaque formation of the WT influenza virus (A/Hong Kong/68) were significantly reduced by Amt, **7**, **12**, **14**, **16**, **17**, **18**, at 2 μM concentration (Fig. 6). The plaque formation of A/M2-S31N influenza virus (A/WS/33) was inhibited by Amt and **12** at concentrations ranging from 10 to 40 μM which is consistent with viral inhibition assay shown in Fig. 7.

3.7. Cytotoxicity evaluation

All of the synthesized chemicals were tested for cytotoxicity in MDCK cells. Low cytotoxicity was observed for compounds **13** and **14** (CC_{50} of 215 and 230 μM, respectively), and **15** and **21** had intermediate toxicity with CC_{50} values of 24 and 30 μM, respectively. The CC_{50} values of other compounds were higher than 250 μM (the highest concentration tested).

4. Conclusion

We confirmed that the compounds containing a pinanamine scaffold bind with micromolar affinity to the A/M2-WT ion channel suggesting that like amantadine, this class of compound binds to the pore region of the channel. This study indicated that linking a secondary amine to an imidazole or guanazole may further increase the inhibition of A/M2 channel activity. Compound **12** was identified to be a novel inhibitor against the A/M2-WT and A/M2-S31N mutant channels and in the case of the S31N mutant displays improved inhibition compared to amantadine. Compound **12** therefore represents a step forward in developing a drug that is sensitive to A/M2-WT and its associated mutants and signifies a starting point for future optimization of novel influenza A inhibitors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.09.001.

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