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CONCISE ARTICLE

# Identification of Camphor Derivatives as Novel M2 Ion Channel Inhibitors of Influenza A Virus

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**Amantadine derivatives have been the only drugs marketed as M2 inhibitors of influenza A for decades. The identification of pinanamine as a novel M2 inhibitor suggests that M2 ion channels can accommodate more types of hydrophobic scaffolds. Herein, we further investigated M2 ion channels and identified camphor derivatives as new types of M2 inhibitors. Compound 18 was found to be more potent than amantadine against wild-type influenza virus. The molecular docking revealed that compound 18 occupies more space in the M2 ion channel than amantadine and thus exhibited enhanced activity.**

Influenza A viruses (IAVs) are easily transmitted and adversely affect the respiratory system, therefore they are a severe threat to human health. Every year, influenza epidemics result in the deaths of individuals and economic losses. There have been three influenza pandemics over the last century with high mortality rates for humans.[1] In recent years, new strains of IAV have emerged, such as the highly pathogenic avian influenza (HPAI) H5N1 strain (avian flu) and H1N1 influenza (swine flu).[2] In 2013, there was an outbreak of a new H7N9 avian influenza virus in China.[3] These sudden and unpredictable outbreaks pose a severe threat to the health of animals and humans. New and efficient therapeutics to combat IAV infections are urgently needed. Currently, there are two classes of antiviral drugs for treating human IAV infections: M2 ion channel inhibitors (amantadine and rimantadine, Fig. 1), and neuraminidase inhibitors (oseltamivir and zanimivir).[4] However, some strains of IAV have become resistant to existing drugs, in particular the M2 ion channel inhibitors, which have been limited to clinical use by the FDA.[5] There are also some issues with neuraminidase inhibitors: zanimivir needs to be administered by inhalation, while there have been an increasing number of reports regarding resistance to orally bioavailable oseltamivir.[6,7] Alternative antivirals should be developed to deal with this situation.

Amantadine has been successfully used for over three decades to block the M2 protein of IAV. M2 is a proton-selective ion channel protein in the viral envelope of IAV comprising 97 amino acids, and plays an essential role in viral replication.[8-10]. The ion channel is a homotetramer consisting of four M2 units; mutations in the M2 channel lead to amantadine resistance.[11-15] Side effects on the central nervous system (CNS) are another drawback of M2 ion channel inhibitors, thereby limiting their use.[16-18] All known M2 ion channel inhibitors contain a hydrophobic scaffold linked to a polar head group. Inhibitors containing the adamantine-based scaffold, such as compounds **1**–**3** (Fig. 1), target M2 proteins containing wild-type (WT), L26F, V27A or S31N mutations.[19-22] Some compounds carrying large cage-based scaffolds inhibit WT M2 and/or V27A mutants;[23-26] however, A/M2-V27A inhibitor **4** (Fig. 1) exhibits some cytotoxicity.[25] Investigating novel non-adamantine-based M2 inhibitors is of interest in the treatment of IAV infections.

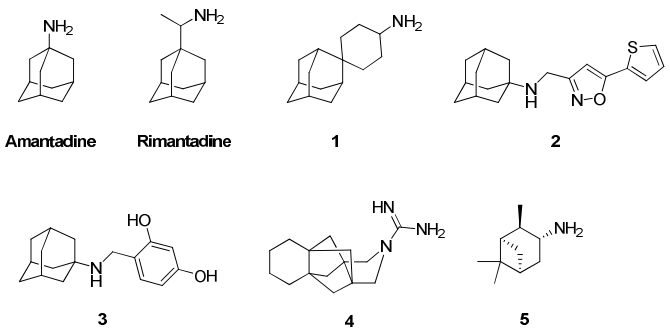



**Fig. 1** Structures of amantadine, rimantadine and other compounds that target mutated M2 ion channel proteins.

Compound **5** (Fig. 1), known as (1*R*,2*R*,3*R*,5*S*)-3-Pinanamine (Pinanamine), was found to be more active than amantadine with respect to WT A/M2 inhibition.[27] Linking a secondary amine to a methylimidazole group on the pinanamine scaffold (Compound **5**, Fig. 2) could further increase the inhibition of A/M2 channel activity. For the S31N mutant, this resulted in improved inhibition compared with that by amantadine.[28] Encouraged by this promising result, we considered using strategies of classical pharmacophore modification (green shade, Fig. 2) and scaffold extending and transition approaches for optimization of novel M2 inhibitors (blue shade, Fig. 2). On one hand, by pharmacophore modification, several more potent compounds inhibiting mutant amantadine-resistance strains have been discovered, these results led us to consider to explore the druggability of these small-molecule towards discovering new candidates for anti-influenza A therapy. On the other hand, as an initial step of this research pipeline, it is important to explore the hydrophobic scaffold of pinanamine and investigate their activity targeting WT-M2 to identify dominant scaffold.

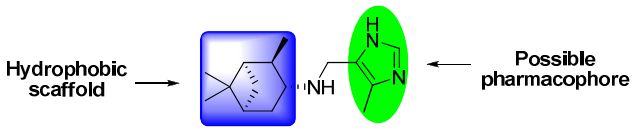
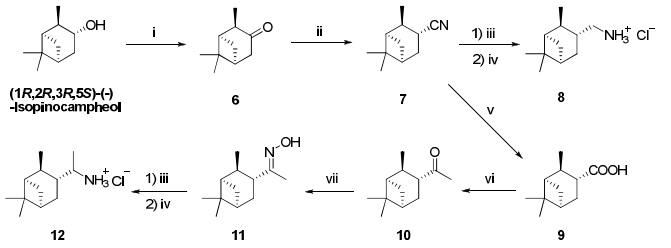


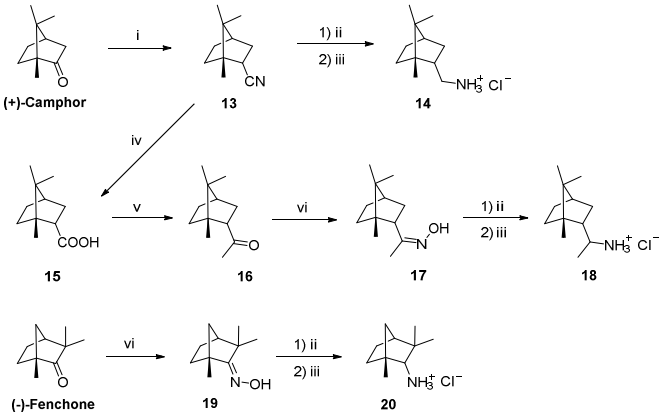

**Fig. 2** Design strategies for optimization.

Bicyclo [2.2.1] heptanes (compound **21**)[29] and its analogues[30], borneol amines, and its derivatives based on the camphor scaffold were reported had anti-influenza activity.[31-33] In this study, we extended the hydrophobic scaffold of pinanamine and borneol amines with camphor scaffold and designed some novel scaffold amino compounds which inhibit the WT M2 ion channel of influenza A virus. Among them, the pinanamine analogue **18**, methyl-substitued camphor ethylamine, exhibited potent activity against WT influenza A virus. The docking computations demonstrate that **18** can bind within the channel pore and its amino group directed toward the C-terminal. And **18** was found to be able to occupy larger space in M2 channel than amantadine, this may enhance its activity.

**Design and Synthesis of New M2 Inhibitors.** To explore the impact of pinanamine analogs, we extended its hydrophobic framework, along with the similar scaffolds camphor and fenchone. Synthetic routes are shown in Schemes 1 and 2. Commercially available (1*R*,2*R*,3*R*,5*S*)-(-)-isopinocampheol was oxidized by pyridinium dichromate (PDC) in $CH_2Cl_2$ to give compound **6**.[34] Reductive cyanation of **6** with tosylmethylisocyanide (TosMIC) gave nitrile **7** with high stereoselectivity,[35,36] which was followed by reduction with $LiAlH_4$ to obtain methylene-inserted pinanamine. This was treated with $HCl/CH_3OH$ to give hydrochloride **8** with a 44 % yield over three steps. Compound **7** could be hydrolyzed to acid **9**,[37] which was treated with $CH_3Li$ to produce methyl ketone **10**.[20] Oxime **11** was prepared by reacting **10** with hydroxylamine; this was followed by $LiAlH_4$ reduction and salification, and **11** was converted to mixture of two isomers[38] of methyl-substituted ethyl amine **12** with a 24.3 % overall yield. To extend the camphor and fenchone scaffold, the same reactions were used and gave the mixture of two isomers of compounds **18** and **20** respectively, except for the cyanation of (+)-camphor, where we acquired a higher yield by heating to 45°C.




**Scheme 1** Synthesis of frame-extending pinanamine analogs. Reagents and conditions: (i) PDC, $CH_2Cl_2$; (ii) TosMIC, tBuOK, DMSO, room temperature; (iii) $LiAlH_4$, THF, room temperature; (iv) $HCl/CH_3OH$; (v) $H_2SO_4$, $CH_3COOH$; (vi) $CH_3Li$, $Et_2O$, 0°C; (vii) Pyridine, $NH_3OH \cdot HCl$.




**Scheme 2** Synthesis of pinanamine scaffold analogs. Reagents and conditions: (i) TosMIC, tBuOK, DMSO, 45°C; (ii) $LiAlH_4$, THF, room temperature; (iii) $HCl/CH_3OH$; (iv) $H_2SO_4$, $CH_3COOH$; (v) $CH_3Li$, $Et_2O$, 0°C ; (vi) Pyridine, $NH_3OH \cdot HCl$.

**Pharmacological Activity.** We obtained 50% inhibitory concentration ($IC_{50}$) values using viral inhibition assays (Table 1). Pinanamine (**5**) is a potent inhibitor of the WT M2 ion channel. We extended the length of **5** by inserting a methylene; corresponding compound **8** led to a marked loss of potency ($IC_{50}$ = 2.47 *vs*. 0.12 $\mu$M). The methyl-substituted analog of **8**,

compound **12**, had a similar structure compared with rimantadine but little increase in activity. Rimantadine showed an obvious improvement in antiviral activity compared with amantadine. Based on our findings, we believe it would be disadvantageous to extend the length of the amino and pinana-framework linker.

Examination of 2-aminonorbornane hydrochloride (**21**) and its *exo* isomer (**22**) showed a scaffold with a bridge-ring similar to **5**, but different from **5** as it had four- and six-member rings with two five-member rings available (Fig. 3).[39] The potencies of **21** and **22** were significantly decreased, but allowed us to conclude that this kind of borneol scaffold should be used. Adding three methyl groups on bridge carbon atoms yielded a much more potent compound, bornylamine (**23**), with an $IC_{50}$ of 0.49 μM. Bornylamine inhibited AIV virus to a comparable degree as amantadine; moving the two methyl groups of the bridge to the β carbon atom (compound **20)** led to a marked loss of activity. This indicates that the two methyl groups of the bridge are necessary. Encouraged by potential compound **23**, we extended the linker of the scaffold and amino group. Compound **14** was a little more potent with an $IC_{50}$ of 0.29 μM. Its potency was further enhanced by adding a methyl group on the linker, producing the methyl-substituted camphor ethylamine **18** with an $IC_{50}$ of 0.10 μM. Compound **18** was five-fold more active than amantadine and as potent as pinanamine.

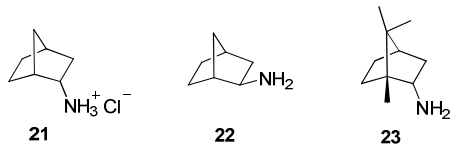
All compounds were also tested for their inhibition of amantadine-resistant strain (A/WSN/33, M2-S31N), but unfortunately, no effects were shown.

**Table 1** Inhibition efficiency of compounds on IAV

| Compound | A/M2 WT[a] $IC_{50}$ (μM)[b] | Compound | A/M2 WT[a] $IC_{50}$ (μM)[b] |
|---|---|---|---|
| **8** | 2.47 ± 0.54 | **21** | 17.40 ± 4.36 |
| **12** | 1.93 ± 0.62 | **22** | 12.40 ± 2.01 |
| **14** | 0.29 ± 0.10 | **23** | 0.49 ± 0.16 |
| **18** | 0.10 ± 0.05 | **5** | 0.12 ± 0.02 |
| **20** | 15.70 ± 0.30 | **amantadine** | 0.50 ± 0.16 |

[a] A/M2-WT virus (A/Hong Kong/68, strain H3N2)

[b] $IC_{50}$ is presented as mean ± SD.




21 22 23

**Fig. 3** Structures of some borneol amines.

**Patch clamp assays.** To test the inhibitory properties of compounds targeting the M2 ion channel, patch clamp assays were applied to 293Trex cells with WT A/M2. All inhibitors were tested at 100 μM and the inhibitory activity (%) of each compound as found (Fig. 4). These results were consistent with those from the viral inhibition assays.

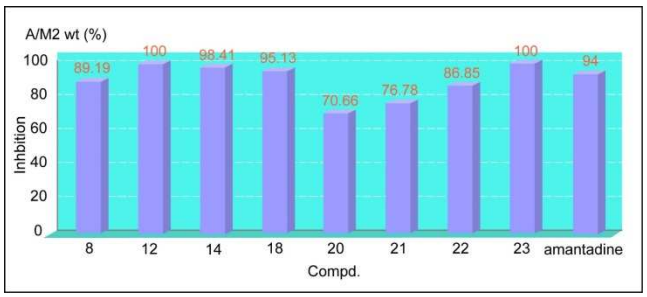



**Fig. 4** Inhibition of M2 WT ion channel conductivity by various compounds as determined by patch clamp assays.

**Plaque Reduction Assays.** The effectiveness of compound **18** against IAV was confirmed by plaque reduction assays. The size and number of plaques for the WT influenza virus (A/Hong Kong/68) were significantly reduced by **18** at 1 and 0.2 μM (Fig. 5).

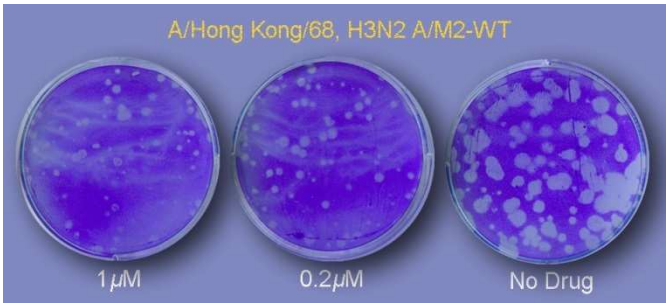



**Fig. 5** Plaque reduction assays for compound **18** on WT influenza virus (A/Hong Kong/68).

**Molecular Docking.** We conducted molecular docking studies to investigate the binding of **18** to the A/M2 WT ion channel. SSNMR and X-ray studies of amantadine binding to the M2 ion channel showed that amantadine binds to residues 27–31 of the transmembrane (TM) domain, and its amino group was oriented towards the C-terminus.[40, 41] A molecular docking simulation showed that the tilt angle of amantadine and the deviation from the Ser31 Cα plane was clearly different from those determined by SSNMR.[23] Using docking computations, we found that the docking conformation of amantadine agreed with the molecular docking simulation performed by Duque[23] (Fig. S1A), with a similar conformation of compound **18** identified (Fig. S1B and C). A stick model showed that a C=O…H hydrogen bond might form between the protonated amino and carbonyl group of Ala30 (Fig. 6A), and **18** had a closer distance (2.5 Å) to Ala30 of the M2 channel than that of amantadine (3.4 Å). This might enhance the strength of the hydrogen bond from 18 (Fig. 6B). A space-filling model indicated that 18 could occupy more room in the M2 channel than amantadine, which might enhance its activity (Fig. 7).

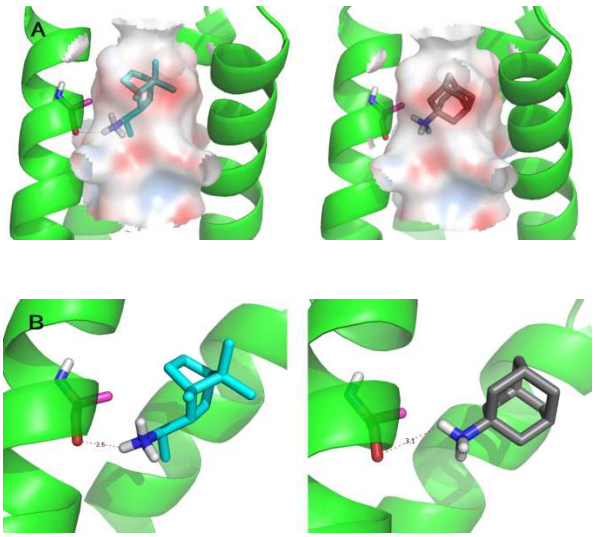



**Fig. 6** Stick model of the molecular docking study. A: Docking conformation of **18** (left) and amantadine (right). B: Distance between the protonated amino and carbonyl group of Ala30 for **18** (left) and amantadine (right). Residue Ala30 is shown as a stick. For clarity, one of the transmembrane helices is not shown.

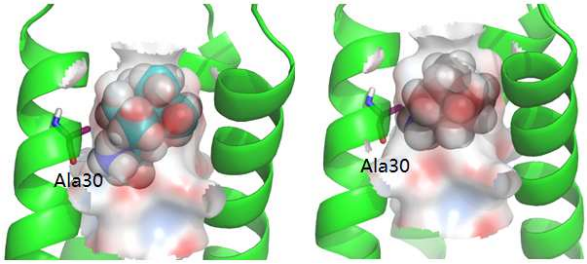



**Fig. 7** Space-filling models of **18** (left) and amantadine (right). For clarity, one of the transmembrane helices is not shown.

**Evaluation of cytotoxicity.** All compounds were tested for cytotoxicity in MDCK cells. The minimum cytotoxic concentration (MCC) of these compounds was higher than 300 μM.

## Conclusions

In order to generate new type of M2 inhibitors other than amantadine derivatives, more hydrophobic scaffolds have been investigated in this study. Camphor derivatives were identified to be a new class of M2 inhibitors with moderate activity. Among of them, compound **18** was found to be 5 times more active than Amantadine. The molecular docking revealed that compound **18** occupies more space in the M2 channel than amantadine, and thus exhibits enhanced activity. We conclude that **18** represents a new series of anti-influenza virus drugs and could be developed as backup scaffolds for a new generation of M2 inhibitors.

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## Notes and references

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† Electronic Supplementary Information (ESI) available: Biological experiments, general method of chemistry, synthesis of target compounds, intermediate products, and their characterization. Docking conformation of amantadine and compound **18**. See DOI: 10.1039/c000000x/

‡ These authors contribute equally

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