European Journal of Medicinal Chemistry 46 (2011) 52–57

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Discovery of highly potent agents against influenza A virus

Xin Zhao [a], Chufang Li [a], Shaogao Zeng [a], Wenhui Hu [a,b,*]

[a] Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Avenue, Guangzhou Science Park, Guangzhou 510530, People's Republic of China
[b] State Key Laboratory of Respiratory Disease, Guangzhou, Guangdong 510120, People's Republic of China

ARTICLE INFO

Article history:
Received 10 August 2010
Received in revised form
8 October 2010
Accepted 9 October 2010
Available online 16 October 2010

Keywords:
Hit-to-lead optimization
Influenza A virus
SAR study

ABSTRACT

We previously reported several new M2 inhibitors as active as amantadine against influenza A virus and validated by three types of *in vitro* assays. Herein, we further modified one of the most potent hits in a viral inhibition assay and conducted structure–activity relationship studies on this scaffold. As a result, compound **8e** was identified to be the most potent inhibitor against wild-type influenza A virus, being nearly 240-fold more active than amantadine.

1. Introduction

Influenza is always a major worldwide health threat to humankind. In the face of the recent outbreak of highly pathogenic avian influenza (H5N1) in Southeast Asia and H1N1 influenza (swine flu) around the world [1,2], there is a great need for anti-influenza therapeutics. However, very few effective drugs are available to combat the influenza virus. Amantadine (**1**) and rimantadine (**2**) (Fig. 1), which target the influenza A virus M2 (matrix-2 protein) ion channel, have long been available for both the prophylaxis and therapy of influenza A viral infections, but their use has been limited because of the rapid emergence of drug resistance and, particularly for amantadine, the occurrence of central nervous system (CNS) side effects [3]. Over the past decade, nearly all reported M2 inhibitors have been amantadine derivatives, such as compound **3** (Fig. 1) [4–9]. Compound **4** is one of the very few examples of nonadamantane-based M2 inhibitors that have been reported [10–12]. Therefore, there is an increasingly urgent need to discover new types of M2 inhibitors for the development of new anti-influenza drugs.

Recently, we reported the identification of several new hits as M2 inhibitors through the focused screening of a small primary amine library [13]. The hits were as active as amantadine against wild-type influenza A virus as determined by three kinds of assays, including cell-based, viral inhibition and patch clamp assays. Among them, compound **5** was the most potent inhibitor and was three times more active than amantadine for viral inhibition ($IC_{50} = 1.363$ μM vs. 5.960 μM).

Encouraged by these results, we decided to modify the hit to further increase its potency. By keeping the scaffold constant and modifying the amino functionality, 14 analogs were made and evaluated for viral inhibition, as assessed by A/WS/33 (H1N1, amantadine resistant) and A/Hong Kong/8/68 (H3N2, amantadine sensitive) viruses [14,15]. Most of the compounds in this study exhibited antiviral inhibition as good as amantadine, and compound **8e** was identified to be the most potent; it was nearly 240-fold more potent than amantadine.

2. Chemistry

The synthesis of (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine amido derivatives **7a**–**7g** is shown in Scheme 1. Initially, commercially available compound **5** was *N*-acylated with methyl chloroformate or acetyl chloride to give **6a** and **6b**, which were then reduced with $LiAlH_4$. Following reduction, salification with HCl/CH_3OH gave **7a** and **7b** [16]. On the other hand, compounds **7c**–**g** were obtained through a one-pot synthesis in which compound **5** was first coupled with different aldehydes or ketones. This synthesis was then followed by hydrogenation with $NaBH(OAc)_3$ in methanol [17] and finally treated with HCl/CH_3OH to provide salts **7c**–**g**.

Abbreviations: M2, matrix-2 protein; SAR, structure–activity relationship.

* Corresponding author. Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Avenue, Guangzhou Science Park, Guangzhou 510530, People's Republic of China. Tel.: +86 20 32015211; fax: +86 20 32015299.
E-mail address: hu_wenhui@gibh.ac.cn (W. Hu).

0223-5234/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2010.10.010

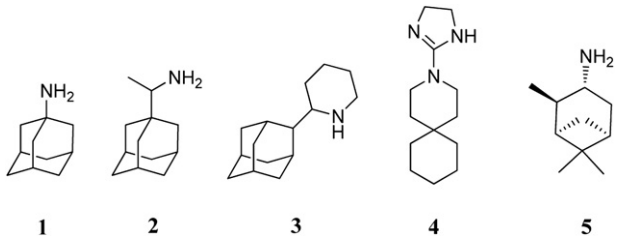


Fig. 1. Reported M2 inhibitors.

The preparation of imines **8a**–**g** is shown in Scheme 2. The starting material **5** was condensed with various aromatic aldehydes [18] to easily provide compounds **8a**–**g** with a yield of 64–90%.

3. Results and discussion

3.1. Viral inhibition assay

All the synthesized compounds were evaluated for their inhibition of influenza A virus. The inhibitory activity was measured by the survival of cells infected with the influenza A virus because a potent M2 inhibitor can rescue the cell by inhibiting the replication of the virus. The results showed that the inhibition ability of the compounds in Table 1 were decreased or even lost completely. While all the compounds except for **8d** in Table 2 were more active than amantadine, **8e** was found to be the most potent one at an inhibition level 240-fold higher than amantadine ($IC_{50} = 0.088$ μM) (for inhibition data, see Fig. 2).

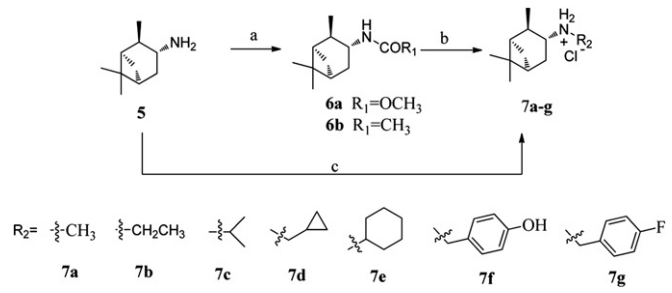
3.2. 3.2. Structure–activity relationship (SAR) studies

The mechanism of M2 inhibitors is to block proton flow in the M2 ion channel and thus inhibit viral replication [19,20]. In our initial investigations, the amine functionality was recognized as the pharmacophore and the alkyl ring was the scaffold. Therefore, we decided to modify the primary amine of compound **5** while keeping the bicyclic ring constant. In the beginning, the primary amine was changed to a secondary amine (Table 1) by the addition of a diverse set of alkyl groups, such as methyl (**7a**), ethyl (**7b**), isopropyl (**7c**), cyclopropylmethyl (**7d**) and cyclohexylmethyl (**7e**). All of them displayed activity lower than adamantanamine. The attachment of aromatic rings, such as 4-hydroxylbenzyl and 4-fluorobenzyl (**7f** and **7g**), made this trend worse, as the compounds completely lost their viral inhibition.

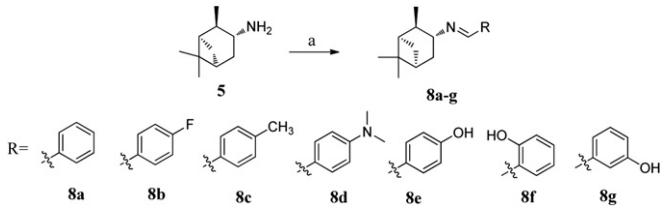
The previous structure–activity relationships of adamantanamine [4] showed that there was no significant difference between the *N*-mono and *N*-dialkyl derivatives. This reminded us that we might need a strategy other than the classic optimization process to improve the activity. Because the Schiff base, like compound **8a**, was so easy to make from the condensation of compound **5** with aldehydes, a series of compounds were rapidly synthesized and screened (Table 2). Compound **8a** shows a significantly improved activity compared with the compounds in Table 1; it was found to be three times more potent than amantadine.

We further optimized this series by placing diverse substituents on the phenyl ring: 4-fluorophenyl substituted compound **8b** was the exact analog of **7g**, but the data (16.3 μM vs. >200 μM) clearly demonstrated that the C=N bond was much more favorable for inhibition than a C–H bond. In addition, neither 4-methylphenyl (**8c**) nor 4-*N*,*N*-dimethylphenyl (**8d**) increased the activity. As we expected, 4-hydroxyphenyl exhibited highly potent inhibition (0.088 μM), which was more than 200 times that of adamantanamine. The switch of hydroxyl group on the phenyl ring, such as 2-hydroxyphenyl (**8f**) and 3-hydroxyphenyl (**8g**), did not retain the previously observed excellent inhibition, but these compounds were still more active than amantadine.

All the secondary amines prepared here exerted decreased activity, especially the aromatic analogs, which completely lost their inhibition ability. However, all the imine compounds listed here, except **8d**, were more active than amantadine.

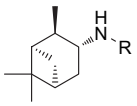


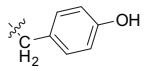
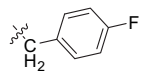
Scheme 1. Synthesis of compounds **7a**–**g**. (a) Acyl chloride, Et_3N, rt, yield 90%; (b) (i) $LiAlH_4$, THF, rt, yield 60–66%; (ii) HCl, CH_3OH, rt, yield 100%; (c) (i) aldehyde or ketone, $NaBH(OAc)_3$, CH_3OH, rt, yield 30–78%; (ii) HCl, CH_3OH, rt, yield 100%.



Scheme 2. Synthesis of compounds **8a**–**g**. (a) Substituted arylaldehyde, benzene, piperidine, reflux, yield 64–90%.

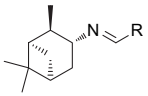
Table 1
In vitro[a] anti-influenza A virus activity of compounds **7a**–**e**



Compound	R	IC_{50} (μM)
7a	$-CH_3$	23.54
7b	$-CH_2CH_3$	>200
7c	$-CH(CH_3)_2$	>200
7d		28.48
7e		43.68
7f		>200
7g		>200
1		21.11

[a] Using influenza A/Hong Kong/8/68 (H3N2) strain.

Table 2
In vitro[a] anti-influenza A virus activity of compounds **8a**–**g**



Compound	R	IC_{50} (μM)
8a	Ph	7.78
8b	Ph–4-F	16.30
8c	Ph–4-CH_3	13.91
8d	Ph–4-$N(CH_3)_2$	38.36
8e	Ph–4-OH	0.088
8f	Ph–2-OH	20.28
8g	Ph–3-OH	5.99
1		21.11

[a] Using influenza A/Hong Kong/8/68 (H3N2) strain.

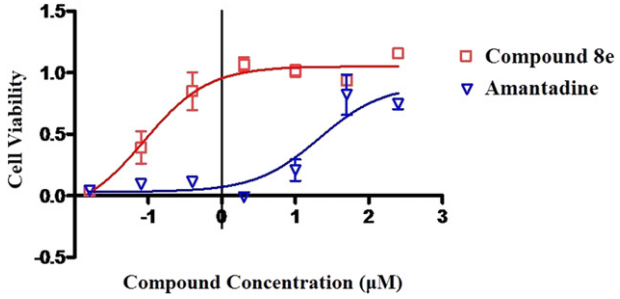


Fig. 2. Inhibition efficiency of amantadine and **8e** on A/Hong Kong/8/68.

3.3. Plaque reduction assay

The inhibitory effect of compound **8e** was further assessed in a plaque reduction assay using influenza A viruses. The photos in Fig. 3A show that plaque formation by wild-type influenza virus (A/Hong Kong/8/68) was inhibited by amantadine **1** and compound **8e** at concentrations ranging from 0.01 to 10 μM. From the results shown in Fig. 3B, compound **8e** was an obviously potent inhibitor.

4. Conclusion

We have identified a highly potent anti-influenza A agent through a general modification of hit **5** followed by a viral inhibition assay. From the study, compound **8e** was identified as the most active inhibitor against wild-type influenza A virus. The IC_{50} value for this compound was found to be 0.088 μM, which was nearly 240-fold more potent than amantadine in the same assay. Although there was no inhibition of mutant virus observed by any of the compounds listed here, compound **8e** might be a novel chemical probe for mechanism studies and further investigation of the M2 ion channel.

5. Experimental procedure

5.1. 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

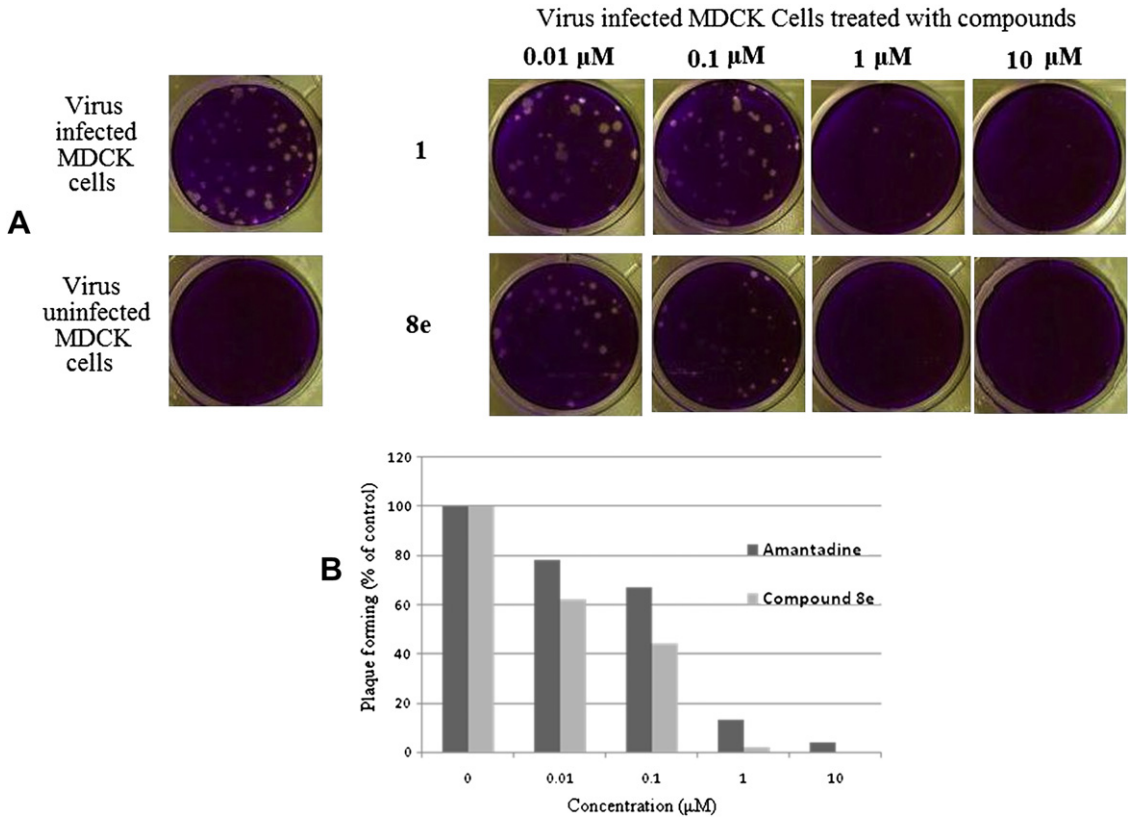


Fig. 3. Reduction of plaque formation by A/Hong Kong/8/68 (H3N2, amantadine sensitive) in MDCK cells upon treatment with **1** and **8e**. MDCK cells were infected with virus at approximate 45 plaque forming units/well. (A) The effect of **1** and **8e** on viral plaque formation. (B) Quantification of viral plaque formation following treatment with serial dilutions of the compound.

Hai Yang GF_{254} silica gel plates (5 × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) and by spraying with an ethanol solution of 2,4-DNP or Ninhydrin or by being fumed with iodine steam. Silica gel column chromatography was performed on silica gel (200–300 mesh) from Qing Dao Hai Yang. NMR spectra were recorded on a Bruker NMR AVANCE 400 (400 MHz) or a Bruker NMR AVANCE 500 (500 MHz), and nuclear magnetism reagents were used as internal standards. Chemical shifts (δ) were recorded in parts per million and coupling constants (J) in hertz (Hz). MS data were measured on an Agilent MSD-1200 ESI-MS system.

5.1.1. General synthetic procedure for amines (method A; Scheme 1)

To a solution of (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine (1 g, 6.5 mmol) in CH_3OH (20 mL), aldehyde or ketone (1.5 equiv, 9.8 mmol) was added. The reaction mixture was stirred at rt for 1 h, followed by addition of sodium triacetoxyborohydride (5.5 g, 26 mmol). The reaction mixture was then stirred for 10 h and quenched by the addition of H_2O and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed two times with brine, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by silica gel column chromatography and treated with saturated HCl/CH_3OH (20 mL), then evaporated to dryness. The insoluble material was washed with diethyl ether (3 × 20 mL) to afford the secondary amines as the corresponding hydrochloride salts.

5.1.2. General synthetic procedure for Schiff bases (method B; Scheme 2)

To a solution of (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine (1 g, 6.5 mmol) in benzene (20 mL), the aromatic aldehydes (4.35 mmol) and one drop of piperidine were added. The solution was heated at reflux with a Dean–Stark trap condenser until no further water appeared. Then, the reaction solution was concentrated and purified by flash silica gel column chromatography to obtain the desired Schiff bases.

5.1.3. Ethyl ((1R,2R,3R,5S)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-yl) carbamate (**6a**)

A solution of methyl chloroformate (0.92 g, 9.8 mmol) in CH_2Cl_2 was added dropwise to a solution of (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine (1 g, 6.5 mmol) and Et_3N (1.03 mL) in CH_2Cl_2 (20 mL) in an ice bath. The reaction mixture was stirred for 1 h, quenched by the addition of H_2O and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed two times with brine, dried (Na_2SO_4) and evaporated to dryness. The residue was purified by silica gel column chromatography. The product was a white powder and was directly used in the next step. Yield: 90%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 0.82 (d, 1H, J = 10.0 Hz), 1.02 (s, 3H), 1.12 (d, 3H, J = 6.8 Hz), 1.21 (s, 3H), 1.52–1.57 (m, 1H), 1.73–1.81 (m, 3H), 1.92–1.94 (m, 1H), 2.36–2.41 (m, 1H), 2.58 (m, 1H), 3.66 (s, 3H), 3.97 (br s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 20.63, 23.28, 27.96, 35.22, 37.45, 38.33, 41.57, 46.36, 47.72, 49.78, 51.86, 156.82; ESI-MS: calculated for $C_{12}H_{21}NO_2$ ($M + H^+$): 212.30, found: 212.2.

5.1.4. N-((1R,2R,3R,5S)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl) acetamide (**6b**)

Synthesis of **6b** was accomplished using the same procedure described for **6a**. Compound **6b** was obtained as a white powder. Yield: 90%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 0.81 (d, 1H, J = 10.0 Hz), 1.04 (s, 3H), 1.10 (d, 3H, J = 7.2 Hz), 1.21 (s, 3H), 1.47–1.52 (m, 1H), 1.65 (br s, 1H), 1.73 (t, 1H, J = 4.0 Hz), 1.82 (t, 1H, J = 8.0 Hz), 1.94–1.95 (m, 1H), 1.98 (s, 3H), 2.39–2.42 (m, 1H), 2.60 (m, 1H), 4.25 (m, 1H), 5.37 (br s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 20.68, 23.32, 23.61, 27.97, 35.30, 37.37, 38.37, 41.59, 46.49, 47.78, 47.84, 169.27; ESI-MS: calculated for $C_{12}H_{21}NO$ ($M + H^+$): 196.30, found: 196.1.

5.1.5. (1R,2R,3R,5S)-N,2,6,6-Tetramethylbicyclo[3.1.1]heptan-3-amine hydrochloride (**7a**)

A solution of **6a** (1 g, 4.73 mmol) in anhydrous THF (5 mL) was added dropwise to a stirred suspension of $LiAlH_4$ (0.54 g, 14.2 mmol) in anhydrous THF (20 mL) at 0 °C. The resulting solution was stirred for 10 h at reflux. The solution was then cooled to 0 °C and filtered following the portionwise addition of $Na_2SO_4 \cdot 10H_2O$ (5.0 g). The collected solids were washed with CH_2Cl_2 (2 × 20 mL). The combined organics were evaporated to dryness and treated with saturated HCl/CH_3OH (20 mL) then evaporated to dryness, and the insoluble material was washed with diethyl ether (3 × 20 mL) to afford the corresponding hydrochloride as a white powder. Yield: 60%; ^{1}HNMR (400 MHz, D_2O) δ ppm: 0.82 (d, 1H, J = 10.4 Hz), 0.87 (s, 3H), 1.10 (d, 3H, J = 7.2 Hz), 1.15 (s, 3H), 1.68–1.73 (m, 1H), 1.81–1.84 (m, 1H), 1.95–2.03 (m, 2H), 2.35–2.46 (m, 2H), 2.64 (s, 3H), 3.38–3.40 (m, 1H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 20.09, 22.62, 26.66, 30.77, 30.99, 32.77, 37.91, 40.37, 40.72, 46.93, 57.84; ESI-MS: calculated for $C_{11}H_{21}N$ ($M + H^+$): 168.29, found: 168.2.

5.1.6. (1R,2R,3R,5S)-N-Ethyl-2,6,6-trimethylbicyclo[3.1.1]heptan-3-amine hydrochloride (**7b**)

Synthesis of **7b** was accomplished using the same procedure described for **7a**. Compound **7b** was obtained as a white powder. Yield: 66%; ^{1}HNMR (400 MHz, DMSO-d_6) δ ppm: 0.92 (s, 3H), 1.14 (d, 3H, J = 7.2 Hz), 1.20 (s, 3H), 1.25 (t, 3H, J = 6.8 Hz), 1.31 (d, 1H, J = 10.0 Hz), 1.77–1.85 (m, 2H), 1.94 (br s, 1H), 2.08 (t, 1H, J = 6.0 Hz), 2.25–2.34 (m, 2H), 2.97 (br s, 2H), 3.35 (br s, 1H), 8.68 (br s, 1H), 8.96 (br s, 1H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 10.66, 20.10, 22.61, 26.62, 31.40, 32.65, 37.91, 40.37, 40.72, 40.96, 46.96, 56.32; ESI-MS: calculated for $C_{12}H_{23}N$ ($M + H^+$): 182.32, found: 182.2.

5.1.7. (1R,2R,3R,5S)-N-Isopropyl-2,6,6-trimethylbicyclo[3.1.1] heptan-3-amine (**7c**)

Method A; yellow powder; yield: 71%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 0.95 (s, 3H), 1.23 (s, 3H), 1.24 (d, 3H, J = 7.2 Hz), 1.27 (s, 1H), 1.44 (d, 3H, J = 6.4 Hz), 1.58 (d, 3H, J = 6.4 Hz), 1.69 (d, 1H, J = 10.4 Hz), 1.81 (t, 1H, J = 5.6 Hz), 2.00 (t, 1H, J = 2.8 Hz), 2.16–2.22 (m, 1H), 2.29–2.34 (m, 2H), 2.42–2.45 (m, 1H), 3.36–3.41 (m, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 18.51, 19.91, 21.16, 23.71, 27.48, 31.52, 32.52, 38.99, 40.51, 41.00, 47.67, 48.23, 54.10; ESI-MS: calculated for $C_{12}H_{25}N$ ($M + H^+$): 196.34, found: 196.1.

5.1.8. (1R,2R,3R,5S)-N-(Cyclopropylmethyl)-2,6,6-trimethylbicyclo [3.1.1]heptan-3-amine hydrochloride (**7d**)

Method A; white powder; yield: 73%; ^{1}HNMR (400 MHz, D_2O) δ ppm: 0.24–0.30 (m, 2H), 0.60 (d, 2H, J = 8.4 Hz), 0.86 (s, 3H), 0.88 (s, 1H), 0.98–1.02 (m, 1H), 1.09 (d, 3H, J = 7.2 Hz), 1.14 (s, 3H), 1.66–1.72 (m, 1H), 1.80–1.84 (m, 1H), 1.93–1.96 (m, 1H), 2.00–2.04 (m, 1H), 2.33–2.37 (m, 1H), 2.40–2.47 (m, 1H), 2.82–2.88 (m, 1H), 2.93–2.98 (m, 1H), 3.43–3.48 (m, 1H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 0.97, 1.24, 4.44, 17.77, 20.29, 24.31, 29.12, 30.28, 35.60, 38.05, 38.28, 44.61, 48.38, 54.00; ESI-MS: calculated for $C_{14}H_{25}N$ ($M + H^+$): 208.35, found: 208.2.

5.1.9. (1R,2R,3R,5S)-N-Cyclohexyl-2,6,6-trimethylbicyclo[3.1.1] heptan-3-amine hydrochloride (**7e**)

Method A; white powder; yield: 30%; ^{1}HNMR (400 MHz, D_2O) δ ppm: 0.88 (s, 4H), 1.08 (d, 4H, J = 7.2 Hz), 1.15 (s, 3H), 1.21–1.32 (m, 4H), 1.59 (d, 1H, J = 12 Hz), 1.70–1.84 (m, 4H), 1.96–2.00 (m, 3H),

2.11 (br s, 1H), 2.35–2.48 (m, 2H), 3.15 (br s, 1H), 3.52–3.58 (m, 1H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 19.86, 22.64, 23.95, 24.01, 24.52, 26.68, 28.50, 29.75, 31.49, 32.70, 37.99, 40.49, 40.89, 47.00, 52.99, 54.62; ESI-MS: calculated for $C_{16}H_{29}N$ ($M+H^+$): 236.41, found: 236.2.

*5.1.10. 4-((((1R,2R,3R,5S)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl) amino)methyl)-phenol hydrochloride (**7f**)*

Method A; white powder; yield: 62%; ^{1}HNMR (400 MHz, DMSO-d_6) δ ppm: 0.84 (s, 3H), 1.06 (d, 3H, $J=7.2$ Hz), 1.19 (s, 3H), 1.38 (d, 1H, $J=9.6$ Hz), 1.77 (t, 1H, $J=4.8$ Hz), 1.94–1.98 (m, 2H), 2.14 (t, 1H, $J=6$ Hz), 2.27–2.32 (m, 2H), 3.23 (br d, 1H), 3.96 (d, 1H, $J=12.8$ Hz), 4.08 (d, 1H, $J=8.8$ Hz), 6.80 (d, 2H, $J=8.4$ Hz), 7.40 (d, 2H, $J=8.4$ Hz), 9.00 (br s, 1H), 9.43 (br s, 1H), 9.75 (br s, 1H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 19.88, 22.64, 26.62, 31.38, 32.69, 37.96, 40.46, 40.68, 46.93, 48.65, 55.90, 115.93, 122.59, 131.62, 156.54; ESI-MS: calculated for $C_{17}H_{25}NO$ ($M+H^+$): 260.39, found: 260.2.

*5.1.11. (1R,2R,3R,5S)-N-(4-Fluorobenzyl)-2,6,6-trimethylbicyclo [3.1.1]heptan-3-amine hydrochloride (**7g**)*

Method A; white powder; yield: 67.1%; ^{1}HNMR (400 MHz, D_2O) δ ppm: 0.84 (s, 3H), 0.88 (d, 1H, $J=10.4$ Hz), 1.05 (d, 3H, $J=7.2$ Hz), 1.15 (s, 3H), 1.78–1.85 (m, 2H), 1.96–1.99 (m, 2H), 2.00–2.10 (m, 2H), 2.34–2.38 (m, 1H), 2.40–2.54 (m,1H), 3.41–3.47 (m, 1H), 4.15 (q, 2H, $J=13.2$ Hz), 7.12–7.17 (m, 2H), 7.42–7.46 (m, 2H); ^{13}CNMR (125 MHz, D_2O) δ ppm: 19.94, 22.65, 26.61, 31.41, 32.66, 37.97, 40.45, 40.64, 46.94, 48.49, 56,37, 115.99, 116.16, 126.84, 131.91 (d, $J_{C-F}=8.9$); ESI-MS: calculated for $C_{17}H_{24}NF$ ($M+H^+$): 262.38, found: 262.1.

*5.1.12. (1R,2R,3R,5S,E)-N-Benzylidene-2,6,6-trimethylbicyclo[3.1.1] heptan-3-amine (**8a**)*

Method B; yellow oil; yield: 64%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 1.08 (d, 3H, $J=7.2$ Hz), 1.16 (s, 3H), 1.34 (s, 3H), 1.37 (d, 1H, $J=9.6$ Hz), 1.94–1.97 (m, 1H), 2.02–2.08 (m, 2H), 2.21–2.25 (m, 1H), 2.33–2.36 (m, 1H), 2.47–2.50 (m, 1H), 3.55–3.59 (m, 1H), 7.43–7.46 (m, 3H), 7.82–7.84 (m, 2H), 8.23 (s, 1H); ^{13}CNMR (100 MHz, $CDCl_3$) δ ppm: 19.99, 23.66, 28.17, 33.94, 36.06, 38.97, 41.88, 43.58, 47.78, 70.39, 128.20, 128.53, 130.21, 136.81, 157.60; ESI-MS: calculated for $C_{17}H_{23}N$ ($M+H^+$): 242.37, found: 242.2.

*5.1.13. (1R,2R,3R,5S,E)-N-(4-Fluorobenzylidene)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-amine (**8b**)*

Method B; yellow oil; yield: 78%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 1.02 (d, 3H, $J=7.2$ Hz), 1.10 (s, 3H), 1.28 (s, 3H), 1.31 (s, 1H), 1.88–2.02 (m, 3H), 2.13–2.16 (m, 1H), 2.27–2.30 (m, 1H), 2.41–2.44 (m, 1H), 3.47–3.52 (m, 1H), 7.07–7.11 (m, 2H), 7.74–7.78 (m, 2H), 8.14 (s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.85, 23.55, 28.02, 33.85, 35.92, 38.86, 41.70, 43.46, 47.56, 70.20, 115.40 (d, $J_{C-F}=21.6$ Hz), 129.87 (d, $J_{C-F}=8.6$ Hz), 132.90 (d, $J_{C-F}=2.6$ Hz), 156.17, 163.03, 165.02; ESI-MS: calculated for $C_{17}H_{22}NF$ ($M+H^+$): 260.36, found: 260.1.

*5.1.14. (1R,2R,3R,5S,E)-2,6,6-Trimethyl-N-(4-methylbenzylidene) bicyclo[3.1.1]heptan-3-amine (**8c**)*

Method B; yellow oil; yield: 64%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 1.04 (d, 3H, $J=7.2$ Hz), 1.14 (s, 3H), 1.31 (s, 3H), 1.33 (d, 1H, $J=9.6$ Hz), 1.91–2.05 (m, 3H), 2.17–2.20 (m, 1H), 2.30(t, 1H, $J=10.4$ Hz), 2.33 (s, 3H), 2.35–2.47 (m, 1H), 3.49–3.54 (m, 1H), 7.23 (d, 2H, $J=8$ Hz), 7.68 (d, 2H, $J=8$ Hz), 8.18 (s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.91, 21.48, 23.61, 28.11, 33.95, 36.00, 38.93, 41.78, 43.52, 47.64, 70.38, 128.11, 129.22, 134.06, 140.30, 157.65; ESI-MS: calculated for $C_{18}H_{25}N$ ($M+H^+$): 256.40, found: 256.2.

*5.1.15. (1R,2R,3R,5S,E)-N-(4-(Dimethylamino)benzylidene)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-amine (**8d**)*

Method B; white powder; yield: 84%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 0.99 (d, 3H, $J=7.2$ Hz), 1.08 (s, 3H), 1.26 (s, 3H), 1.28 (s, 1H), 1.84–1.99 (m, 3H), 2.12 (t, 1H, $J=7.6$ Hz), 2.27–2.28 (m, 1H), 2.38–2.41 (m, 1H), 3.00 (s, 6H), 3.41–3.44 (m, 1H), 6.71 (d, 2H, $J=9.2$ Hz), 7.61 (d, 2H, $J=8.8$ Hz), 8.06 (s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.87, 23.56, 28.11 34.06, 36.20, 38.92, 40.28, 41.83, 43,68, 47.70, 70.36, 111.71, 124.92, 129.37, 151.82, 157.84; ESI-MS: calculated for $C_{19}H_{28}N_2$ ($M+H^+$): 285.44, found: 285.2.

*5.1.16. 4-((E)-(((1R,2R,3R,5S)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl)imino)methyl)phenol (**8e**)*

Method B; yellow powder; yield: 90%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 1.00 (d, 3H, $J=7.2$ Hz), 1.04 (s, 3H), 1.25 (s, 3H), 1.28 (t, 1H, $J=3.6$ Hz), 1.83 (t, 1H, $J=4.8$ Hz), 1.95–2.00 (m, 2H), 2.16–2.19 (m, 1H), 2.28–2.38 (m, 1H), 3.55–3.61 (m, 1H), 6.61 (d, 2H, $J=8.8$ Hz), 7.46 (d, 2H, $J=8.4$ Hz), 8.11 (s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.77, 23.58, 28.09, 34.01, 35.45, 38.98, 41.66, 42.93, 47.50, 70.47, 116.12, 125.40, 130.48, 160.63, 161.22; ESI-MS: calculated for $C_{17}H_{23}NO$ ($M+H^+$): 258.37, found: 258.2.

*5.1.17. 2-((E)-(((1R,2R,3R,5S)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl)imino)methyl)phenol (**8f**)*

Method B; yellow oil; yield: 90%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 1.06 (d, 3H, $J=7.2$ Hz), 1.08 (s, 3H), 1.17 (d, 1H, $J=10.0$ Hz), 1.28 (s, 3H), 1.88–2.05 (m, 3H), 2.14–2.17 (m, 1H), 2.37–2.48 (m, 2H), 3.46–3.51 (m, 1H), 6.86–6.90 (m, 1H), 6.97 (d, 1H, $J=8.0$ Hz), 7.24–7.32 (m, 2H), 8.25 (s, 1H), 13.70 (br s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.78, 23.40, 27.79, 34.02, 36.34, 38.50, 41.40, 44.34, 47.29, 68.60, 116.80, 118.32, 118.67, 130.93, 131.74, 161.14, 161.61; ESI-MS: calculated for $C_{17}H_{23}NO$ ($M+H^+$): 258.37, found: 258.2.

*5.1.18. 3-((E)-(((1R,2R,3R,5S)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl)imino)methyl)phenol (**8g**)*

Method B; yellow powder; yield: 72%; ^{1}HNMR (400 MHz, $CDCl_3$) δ ppm: 0.99 (d, 3H, $J=7.2$ Hz), 1.07 (s, 3H), 1.22 (s, 1H), 1.26 (s, 3H), 1.86 (t, 1H, $J=4$ Hz), 1.94–1.98 (m, 2H), 2.15 (t, 1H, $J=6.8$ Hz), 2.25–2.38 (m, 2H), 3.51–3.57 (m, 1H), 6.86 (q, 1H, $J=3.2$ Hz), 7.19 (q, 2H, $J=3.2$ Hz), 7.27 (s, 1H), 8.12 (s, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ ppm: 19.78, 23.57, 28.06, 33.92, 35.56, 38.95, 41.68, 43.02, 47.54, 70.58, 114.67, 118.46, 120.85, 129.78, 137.06, 156.67, 159.67; ESI-MS: calculated for $C_{17}H_{23}NO$ ($M+H^+$): 258.37, found: 258.2.

5.2. 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–HCl or synthesized compounds in the presence of 1 μg/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 A/Hong Kong/8/68 (H3N2) virus was then added to the plates. After incubation in 5% CO_2 at 37 °C for 72 h, 10 μL of CCK-8 reagent was added to each well, and the mixture was incubated for 3 h. The A450 was then measured with an UVstar-Microplates Synergy HT. Data were analyzed using GraphPad Prism Demo.

5.3. Plaque reduction assay

A monolayer of MDCK cells was infected with 0.01 MOI A/Hong Kong/8/68 virus for 1 h at 37 °C. The inoculums were then removed, and the cells were washed twice with phosphate-buffered saline (PBS). The cells were then overlaid with 1% agar DMEM-containing amantadine or the synthesized compounds in

the presence of 2 μg/mL trypsin and 0.3% BSA. Two to three days after infection, the monolayers were fixed and stained with 0.1% crystal violet solution.

References

[1] J. Cohen, M. Enserink, Science 324 (2009) 1496–1497.
[2] K.F. To, P.K. Chan, K.F. Chan, W.K. Lee, W.Y. Lam, K.F. Wong, N.L. Tang, D.N. Tsang, R.Y. Sung, T.A. Buckley, J.S. Tam, A.F. Cheng, J. Med. Virol. 63 (2001) 242–246.
[3] E.D. Clercq, Nat. Rev. Drug Discov. 5 (2006) 1015–1025.
[4] P.E. Aldrich, E.C. Hermann, W.E. Meier, M. Paulshock, W.W. Prichard, J.A. Snyder, J.C. Watts, Antiviral agents. 2, J. Med. Chem. 14 (1971) 535–543.
[5] N. Kolocouris, G.B. Foscolos, A. Kolocouris, P. Marakos, N. Pouli, G. Fytas, S. Ikeda, E.D. Clercq, J. Med. Chem. 37 (1994) 2896–2902.
[6] N. Kolocouris, A. Kolocouris, G.B. Foscolos, G. Fytas, J. Neyts, E. Padalko, J. Balzarini, R. Snoeck, G. Andrei, E.D. Clercq, J. Med. Chem. 39 (1996) 3307–3318.
[7] A. Kolocouris, D. Tataridis, G. Fytas, G. Mavromoustakos, G.B. Foscolos, N. Kolocouris, E.D. Clercq, Bioorg. Med. Chem. Lett. 9 (1999) 3465–3470.
[8] G. Zoidis, C. Fytas, I. Papanastasiou, G.B. Foscolos, G. Fytas, E. Padalko, E.D. Clercq, L. Naesens, J. Neyts, N. Kolocouris, Bioorg. Med. Chem. 14 (2006) 3341–3348.
[9] G. Zoidis, N. Kolocouris, L. Naesens, E.D. Clercq, Bioorg. Med. Chem. 17 (2009) 1534–1541.
[10] G.S. Gandhi, K. Shuck, J.D. Lear, G.R. Dieckmann, W.F. DeGrado, R.A. Lamb, L.H. Pinto, J. Biol. Chem. 274 (1999) 5474–5482.
[11] J. Wang, S.D. Cady, V. Balannik, L.H. Pinto, W.F. DeGrado, M. Hong, J. Am. Chem. Soc. 131 (2009) 8066–8076.
[12] V. Balannik, J. Wang, Y. Ohigashi, X. Jing, E. Magavern, R.A. Lamb, W.F. DeGrado, L.H. Pinto, Biochemistry 48 (2009) 11872–11882.
[13] W. Hu, S. Zeng, C. Li, Y. Jie, Z. Li, L. Chen, J. Med. Chem. 53 (2010) 3831–3834.
[14] A.K. Judd, A. Sanchez, D.J. Bucher, J.H. Huffman, K. Bailey, R.W. Sidwell, Antimicrob. Agents Chemother. 41 (1997) 687–692.
[15] R.W. Sidwell, D.F. Smee, Antiviral Res. 48 (2000) 1–16.
[16] D. Stead, P. O'Brien, A. Sanderson, Org. Lett. 10 (2008) 1409–1412.
[17] S. Li, G. Chiu, V.L. Pulito, J. Liu, P.J. Connolly, S.A. Middleton, Bioorg. Med. Chem. Lett. 17 (2007) 1646–1650.
[18] H. Yu, L. Shao, J. Fang, J. Organomet. Chem. 692 (2007) 991–996.
[19] A.L. Stouffer, R. Acharya, D. Salom, A.S. Levine, L.D. Costanzo, C.S. Soto, V. Tereshko, V. Nanda, S. Stayrook, W.F. DeGrado, Nature 451 (2008) 596–599.
[20] J.R. Schnell, J.J. Chou, Nature 451 (2008) 591–595.