Heterodimers of Histidine and Amantadine as Inhibitors for Wild Type and Mutant M2 Channels of Influenza A

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Inhibitors bearing the imidazole, adamantane and related structures were synthesized and tested against WT, S31N and S31N-L26I mutant M2 channels. Although amantadine (**1**) only inhibited WT M2 channel, compound **6** containing the imidazole and adamantane groups showed good inhibitory activity to WT and mutant M2 channels. The stereochemistry and basic pK_a of α-amine are important for the activity of inhibitors and our data showed that derivatives of natural histidine are more active for M2 channels than those of unnatural histidine. The significance of our present results is that we have established a prospective strategy of drug discovery of WT and mutant M2 channels against influenza A.

Keywords M2 ion channel, imidazole, histidine, amantadine, inhibitor, amination

Introduction

Serious concern to avian flu H5N1, which has the potential to infect human beings to bring out a life-threatening pandemic of influenza A, promotes international actions like worldwide surveillance of the widespread disease and global cooperative development of vaccines and drugs. Although several potential drug targets could be used for drug discovery and development to control influenza A virus, current effective drugs against flu A in clinic are only targeting neuraminidase (NA) and M2 ion channel.

M2 proton-selective channel is one of the major proteins that are essential for the life cycle of flu virus A and exists as disulfide-linked homotetramers anchored in the viral lipid envelope. As an integral membrane protein containing 97-amino-acids, a small N-terminal ectodomain, a single transmembrane (TM) domain, and C-terminal cytoplasmic tail, M2 ion channel opens to permit proton permeation from its N-terminal (the endosome) to C-terminal when pH is lower than 6.2.[1] The function of M2 ion channel is associated with two cascade events:[2,3] bound at the H^+ associative site by its "ligand" H^+ and then making the channel responsive sites open for H^+ entering by its conformation changes. The H^+ associative site is for anchoring and relay of protons and the channel responsive sites are for delivering the conformation change of the channel. The influx of H^+ ions triggers the fusion of viral and endosomal membranes and then to release the viral genes into the cytoplasm. Amantadine (Symmetrel, **1**) and Rimantadine (Flumadine, **2**) (Figure 1) are good uncoating blockers of M2 channel used for the prophylaxis and therapy of influenza A infections in clinic for decades.[4] General assumption for their inhibition[3,5] is that entering **1** or bound **1** catches influent proton and inhibits the proton relay function of imidazole group of His37 or/and the gating function of indole group of Trp41.

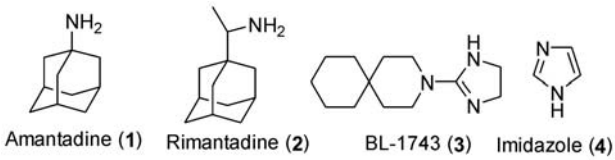

Figure 1 Structures of M2 channel effectors.

In addition to the occurrence of central nervous system (CNS) side effects of **1** and **2**, the urgent and serious challenge for **1** and **2** in clinical use is the rapid emergence of drug resistance and the ready transmissibility of drug-resistant viruses, especially in H3N2 (higher than 90% worldwide)[6,7] and H5N1 (higher than 90% in Vietnam and Thailand).[8] The mutations of S31N and L26I-S31N are the most common mutations of M2

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channel, which can make these mutant viruses 3000-fold less responsive to **1** and **2** than M2 wild type (WT) virus.[7,9,10] These drawbacks make **1** and **2** almost useless as single agent in the treatment of avian or human influenza A virus infections. It is imperative to develop alternative anti-flu drugs targeting WT and mutant M2 channels for the prophylaxis and treatment of seasonal or pandemic influenza in the future.

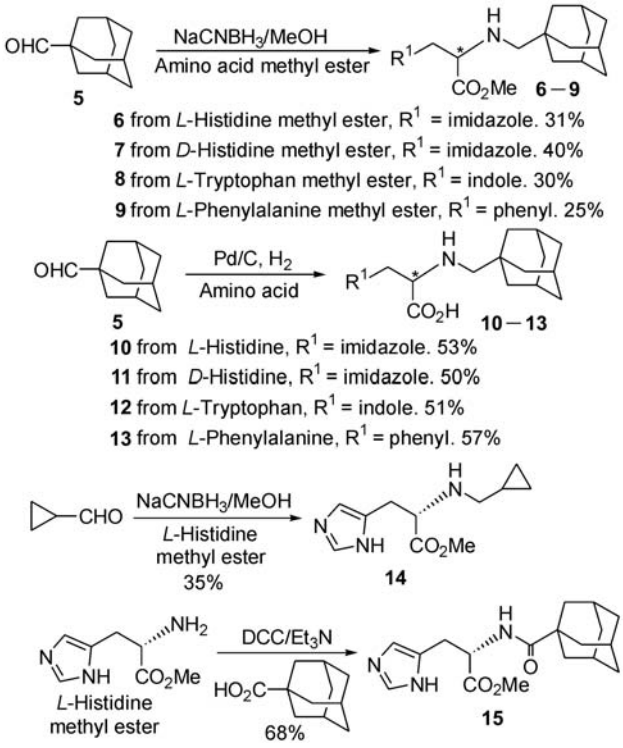
The recent data[11-13] showed that the binding sites of **1** in the co-crystal and NMR structures are different. Although we cannot conclude right now that two or more molecules of **1** bind at different binding sites of an intact M2 channel at the same time, it is true that there are two or more potential recognition sites for the interaction of **1** with M2 channel. Considering these, we proposed that it could have a chance to inhibit WT and also mutant M2 channels by tuning of the basic structure of the known M2 effectors to make simultaneous bindings of two or more recognition sites that are the common features of M2 channels.

As the first try of our medicinal chemistry efforts to drug discovery of M2 channel inhibitors, we designed and synthesized some compounds containing two or more potential binding groups so that they can interact with at least two recognition sites of M2 channels at the same time. Because **1**, **2** and their analogues are the active inhibitors for WT M2 channel,[14] the adamantine group of **1** and **2** was logically chosen as one class of potential blocking moiety for the first series of inhibitors against WT and mutant M2 channels. On the other hand, imidazole (**4**, Figure 1) was used as another class of potential M2 channel blocking moiety because it could be accommodated to interact with M2 channel according to the observation that imidazole can rescue fully or partly the M2 channels' proton-selective activity of single mutations of H37 (H37G, H37S, H37T) and double mutations of H37/W41 (H37G/W41Y, H37G/W41A).[15] It is expected that these compounds with adamantane, imidazole or their related structural units can inhibit WT and mutant M2 channels by the interaction with M2 channels' common properties.

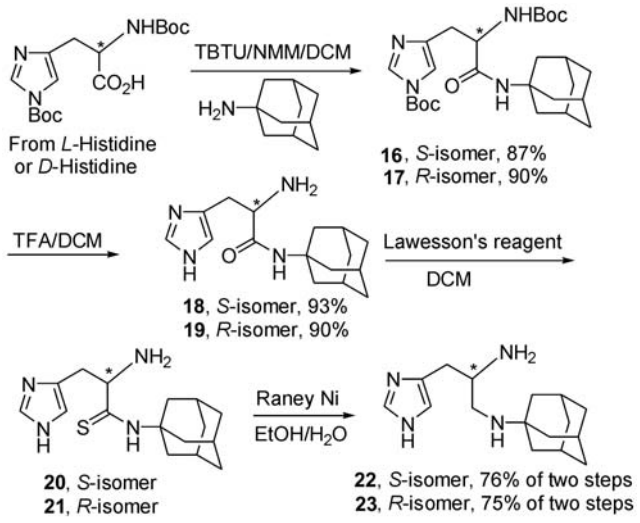
Results and discussion

The synthetic routes of the compounds are shown in Schemes 1 and 2. The reductive amination of adamantane aldehyde (**5**)[16] or cyclopropyl aldehyde with *L*- or *D*-amino acid methyl esters produced compounds **6**—**9**[17] and **14** by $NaCNBH_3$, and with *L*- or *D*-amino acid gave acid compounds **10**—**13** through the hydrogenation catalyzed by Pd/C. Amide **15** was formed by the condensation of *L*-histidine methyl ester with 1-adamantanecarboxylic acid by the general method. Amides **16** and **17** were prepared from Boc-protected *L*-histidine and *D*-histidine by the similar method used for the preparation of **15**. After the deprotection of **16** and **17**, and the treatment of **18** and **19** with Lawesson's reagent, thioamides **20** and **21** were proceeded to reduce by Raney Ni and generate amines **22** and **23**, respectively.

Scheme 1 Synthesis of the compounds **6**—**15**



Scheme 2 Synthesis of the compounds **16**—**23**



As expected that **1** is a positive compound to WT M2 channel, **1** inhibited WT M2 channel but did not show obvious inhibitory activity to S31N and S31N-L26I mutant M2 channels up to 1.0 mmol/L (Figure 2a). Although **1** can not block mutant M2 channels, compounds **6** and **7** bearing adamantane and imidazole groups showed good inhibitory activity to mutant and WT M2 channels (Figure 2b). The significant activity difference between the active compounds **6** and **7** and the inactive compounds **8** and **9** demonstrated that the imidazole group is a necessary moiety for constructing the block-

ing activity to WT and mutant M2 channels. Both of histidine enantiomers were used for the study and natural histidine derivative **6** possesses 3 times better inhibitory activity to WT M2 channel and 10-fold better inhibitory activity to mutant M2 channel than unnatural histidine derivative **7**; the activity difference of enantiomers **18** and **19** follows to that of **6** and **7**. These results demonstrated that M2 channels can distinguish the enantiomers and the stereo-configurations of these inhibitors are important for their recognition and inhibition to M2 channels, especially more distinct for mutant M2 channels with a larger residue of Asn31 than WT M2 channel with a smaller residue of Ser31. Contrary to the esters **6** and **7**, their corresponding acids **10** and **11** exhibited almost no activity to M2 channels; this may be due to their zwitterion forms that can obstruct **10** and **11** from entering into relatively hydrophobic M2 channels.

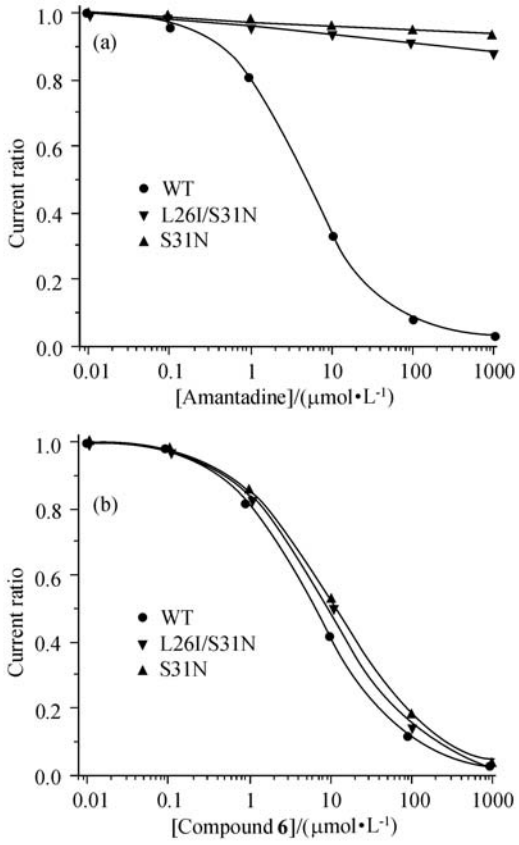


Figure 2 The inhibition of M2 ion channels by amantadine **1** (a) and compound **6** (b). Membrane currents of 293T-rex cells that expressed M2-WT, S31N and L26I-S31N with extracellular solution pH changing from 6.8 to 5.5, holding potential at −40 mV. The potency of the compound was estimated by constructing a dose-response relationship based on electrophysiological recordings (n=4—6). In all cases, the compounds were pre-applied for >30 s before being co-applied with the agonist (pH 5.5). A logistic curve fit to the data yielded an IC_{50} which indicated at Table 1.

The fact that compound **14** expressed only a weak inhibition to WT and mutant M2 channels showed that the imidazole group conjugated with very small moiety of a cyclopropane skeleton was not effective for the binding to WT and mutant M2 channels but it is possible to find potent WT and mutant M2 inhibitors by modifications with relatively bulky skeletons at similar position(s). Comparison of **14** (a conjugate of an imidazole group with a cyclopropane group) with **8** and **9** (conjugates of an adamantane group with a phenyl group and an indole group, respectively), it seems that the imidazole group is relatively more important and efficient than the adamantane group for the binding to M2 channels. The inhibitory activity of compound **18** is more obvious to WT M2 channel than to mutant M2 channels. The inhibitory difference between active amine **6**, less active compound **18** and inactive amide **15** implies that a basic pK_a and a bulky linkage of exact nitrogen configuration are essential for interacting with M2 channels. In order to know if more amine groups at the amantadine terminus are helpful to binding, we explored to synthesize compounds **22** and **23** to compare with their amide forms of **18** and **19**. Even though the orientation of secondary amines with the adamantane group in **6**, **19** and **23** is same while that in **7**, **18** and **22** is same, the data showed that only amide **18** with same orientation of protonable nitrogen as **6** has weak activity while amide **19**, diamines **22** and **23** are totally inactive. One reason is that two protonable amine groups in diamines **22** and **23** possibly made complicated recognition with M2 channels and produced too weak entry ability to relatively hydrophobic M2 channels. In the other aspects, the suitable bulkiness of the amine and the ideal distance between the imidazole group and the bulky amine moiety are indispensable for an inhibitor's binding activity to M2 channels.

Conclusions

In summary, by the tuning of essential molecular moieties which can potentially interact with different binding sites in an M2 channel, the first generation of inhibitors active for WT and mutant M2 channels were synthesized and identified. From current preliminary structure-activity relationship (SAR) data, the compounds derived from natural histidine have better activity against M2 ion channels. Many other factors, including suitable blocking moieties and their suitable distance, feasibly protonated amine, and good entry ability to M2 channels, may also affect the recognition and inhibition of inhibitors to M2 channels. This new line of inhibitors could act in a different mechanism from amantadine (**1**) in that they possibly interact with at least two recognition sites of WT and mutant M2 channels. Although the activity of current inhibitors is not potent enough yet, the success in the discovery of active heterodimeric structures of **6** and **7** with the imidazole group and the adamantane group paved a potential way for drug discovery of WT and mutant M2 channels. Discovering more potent inhibitors is possible

after more detailed understanding of the structure-activity relationship (SAR) is acquired. We are currently pursuing more compounds for M2 inhibitors bearing imidazole, adamantane, and other bulky skeletons like "cage-like" scaffold moiety[14] including the skeleton of BL-1743 (Figure 1) and related structures.

Experimental

Materials and instruments

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer at 400 and 100 MHz. Coupling constants (J) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million units relative to the solvent. The low resolution of EIMS and high resolution of FABMS were recorded on a VG ZAB-HS mass spectrometer and a MAT 95XP (Thermo) mass spectrometer, respectively. The low resolution of ESIMS was recorded on an Agilent 1200 HPLC-MSD mass spectrometer. All the reagents were of analytical reagent grade.

General procedures of the reductive amination of an aldehyde and amino acid methyl esters by $NaCNBH_3$

After the mixture of 0.53 mmol of methyl ester of an amino acid and 0.65 mmol of an aldehyde in 5 mL of MeOH was stirred at room temperature for 1.5 h, 0.07 g (1.11 mmol) of $NaCNBH_3$ was added to the mixture at 0 ℃. The reaction progress was checked by TLC and the completed reaction was treated with sat. $NaHCO_3$, extracted with CH_2Cl_2 and then dried over anhydrous $MgSO_4$. After evaporated in vacuum and purified by silica gel column chromatography (MeOH in CH_2Cl_2, 0% to 2% in volume), the title compound was prepared.

***N*-(1-Adamantylmethyl)-*L*-histidine methyl ester (6)** Yield 31%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.50 (s, 1H), 6.78 (s, 1H), 3.65 (s, 3H), 3.37—3.41 (m, 1H), 2.96 (dd, J=5.8, 15.0 Hz, 1H), 2.82 (dd, J=8.4, 6.4 Hz, 1H), 2.24—2.28 (m, 1H), 2.03—2.07 (m, 1H), 1.90—1.97 (m, 3H), 1.55—1.70 (m, 6H), 1.40—1.50 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.9, 134.6, 131.6, 120.2, 62.4, 60.84, 51.8, 40.7 (3C), 37.1 (3C), 33.4, 29.5, 28.4 (3C); HRFABMS calcd for $C_{18}H_{28}O_2N_3$ 318.2176, found 318.2166 $[M+H]^+$ (100).

***N*-(1-Adamantylmethyl)-*D*-histidine methyl ester (7)** Yield 40%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.53 (s, 1H), 6.82 (s, 1H), 3.71 (s, 3H), 3.39 (dd, J=4.0, 4.4 Hz, 1H), 3.01 (dd, J=4.0, 10.8 Hz, 1H), 2.80 (dd, J=6.8, 8.4 Hz, 1H), 2.32 (d, J=11.2 Hz, 1H), 2.10 (d, J=11.2 Hz, 1H), 1.95—1.99 (m, 3H), 1.65—1.75 (m, 3H), 1.62—1.68 (m, 3H), 1.49—1.60 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.6, 134.4, 130.3, 122.1, 62.1, 60.9, 52.0, 40.8 (3C), 37.1 (3C), 33.4, 28.7, 28.4 (3C); HREIMS calcd for $C_{18}H_{27}O_2N_3$ 317.2098, found 317.2098 $[M]^+$ (100).

***N*-(1-Adamantylmethyl)-*L*-tryptophan methyl ester (8)** Yield 30%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.32 (brs, 1H), 7.64 (d, J=7.6 Hz, 1H), 7.33 (d, J=8.0 Hz, 1H), 7.16—7.20 (m, 1H), 7.10—7.14 (m, 1H), 7.04 (s, 1H), 3.63 (s, 3H), 3.56 (t, J=6.4 Hz, 1H), 3.10—3.20 (m, 1H), 2.31 (d, J=11.2 Hz, 1H), 2.07—2.18 (m, 1H), 1.88—1.95 (m, 3H), 1.59—1.74 (m, 6H), 1.43—1.48 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 175.8, 136.2, 127.6, 122.8, 121.9, 119.3, 118.9, 111.6, 111.1, 63.4, 61.0, 51.5, 40.7 (3C), 37.2 (3C), 33.6, 29.2, 28.5 (3C); ESIMS m/z (%): 367.0 ($[M+H]^+$, 100).

***N*-(1-Adamantylmethyl)-*L*-phenylalanine methyl ester[17] (9)** Yield 25%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.21—7.32 (m, 5H), 3.66 (s, 3H), 3.44 (t, J=6.8 Hz, 1H), 2.90—2.98 (m, 2H), 2.29 (d, J=11.2 Hz, 1H), 2.07 (d, J=11.2 Hz, 1H), 1.92—1.98 (m, 3H), 1.60—1.74 (m, 6H), 1.44—1.49 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 175.4, 137.7, 129.2 (2C), 128.3 (2C), 126.6, 64.2, 60.8, 51.5, 40.6 (3C), 39.6, 37.2 (3C), 33.55, 28.4 (3C); ESIMS m/z (%): 328.2 ($[M+H]^+$, 100).

General procedures of the reductive amination of aldehyde 5 and amino acids by Pd/C hydrogenation

The mixture of 0.5 mmol of amino acid hydrochloric salt and 0.6 mmol of 1-adamantadine aldehyde **5**[16] in 5 mL of MeOH was treated with 1.0 equiv. of Et_3N and then stirred at r.t. for 5 h. After the careful evacuation of air and the addition of catalytic amount of Pd/C, the reaction mixture was charged with H_2 at one atmosphere. The reaction progress was checked by TLC and the reaction was generally completed after stirred at room temperature for 8 h. After filtered and evaporated in vacuum, the residue was purified by silica gel column chromatography (MeOH in DCM, 5% to 15% in volume) to give the title compound.

***N*-(1-Adamantylmethyl)-*L*-histidine (10)** Yield 53%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.66 (s, 1H), 7.00 (s, 1H), 3.59—3.63 (m, 1H), 3.20—3.30 (m, 1H), 2.29—3.07 (m, 1H), 2.83 (d, J=12.4 Hz, 1H), 2.62 (d, J=12.4 Hz, 1H), 1.95—2.06 (m, 3H), 1.67—1.75 (m, 9H); ^{13}C NMR (CD_3OD, 100 MHz) δ: 171.3, 135.7, 134.9, 114.2, 64.4, 58.6, 39.3 (3C), 36.2 (3C), 32.1, 28.1 (3C), 26.5; ESIMS m/z (%): 304.0 ($[M+H]^+$, 100).

***N*-(1-Adamantylmethyl)-*D*-histidine (11)** Yield 50%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.68 (s, 1H), 7.02 (s, 1H), 3.60—3.66 (m, 1H), 2.29 (dd, J=3.6, 12.0 Hz, 1H), 3.05 (dd, J=6.0, 9.6 Hz, 1H), 2.86 (d, J=12.4 Hz, 1H), 2.65 (d, J=12.4 Hz, 1H), 2.06—2.10 (m, 3H), 1.78—1.93 (m, 6H), 1.69—1.73 (m, 6H); ^{13}C NMR (CD_3OD, 100 MHz) δ: 171.2, 135.7, 134.9, 114.1, 64.4, 58.6, 39.3 (3C), 36.2 (3C), 32.1, 28.1 (3C), 26.5; ESIMS m/z (%): 304.0 ($[M+H]^+$, 100).

***N*-(1-Adamantylmethyl)-*L*-tryptophan (12)** Yield 51%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.73 (d, J=8.0 Hz, 1H), 7.43 (d, J=8.0 Hz, 1H), 7.29 (s, 1H), 7.17—7.21 (m, 1H), 7.10—7.14 (m, 1H), 3.81—3.85 (m, 1H), 3.63 (dd, J=5.2, 10.0 Hz, 1H), 3.27 (dd, J=6.0, 9.2 Hz, 1H), 2.67 (d, J=12.4 Hz, 1H), 2.42 (d, J=12.4 Hz, 1H), 1.92—1.96 (m, 3H), 1.72—1.77 (m, 3H), 1.58—1.65 (m, 3H), 1.39—1.414 (m, 6H); ^{13}C NMR ($CD_3OD+CDCl_3$, 100 MHz) δ: 171.2, 136.8, 126.6,

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124.1, 122.2, 119.6, 117.9, 111.7, 108.0, 64.2, 59.2, 39.0 (3C), 36.1 (3C), 32.0, 27.6 (3C), 26.2; ESIMS *m*/*z* (%): 353.2 ([M+H]$^+$, 100).

***N*-(1-Adamantylmethyl)-*L*-phenylalanine (13)** Yield 57%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.28—7.38 (m, 5H), 3.71 (t, J=7.2 Hz, 1H), 3.34—3.38 (m, 1H), 3.12 (dd, J=6.8, 7.4 Hz, 1H), 2.58 (dd, J=12.4, 27.2 Hz, 2H), 1.98—2.05 (m, 3H), 1.68—1.86 (m, 6H), 1.53—1.60 (m, 6H); ^{13}C NMR (CD_3OD+$CDCl_3$, 100 MHz) δ: 170.7, 135.9, 129.1 (2C), 129.0 (2C), 65.2, 59.4, 39.2 (3C), 36.2 (3C), 35.7, 32.2, 27.7 (3C); ESIMS *m*/*z* (%): 314.2 ([M+H]$^+$, 100).

***N*-(1-Cyclopropylmethyl)-*L*-histidine methyl ester (14)** **14** was got by similar procedure as the preparation of compounds **6**—**9**: Yield 35%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.54 (s, 1H), 6.83 (s, 1H), 3.71 (s, 3H), 3.53—3.56 (m, 1H), 3.02 (dd, J=4.4, 10.8 Hz, 1H), 2.84 (dd, J=6.8, 8.4 Hz, 1H), 2.53 (dd, J=5.6, 6.4 Hz, 1H), 2.36 (dd, J=4.4, 7.2 Hz, 1H), 0.89—0.95 (m, 1H), 0.47—0.50 (m, 2H), 0.11—0.16 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 174.7, 134.7, 131.6, 119.9, 61.1, 53.2, 51.9, 29.7, 11.1, 3.52, 3.38; HRFABMS calcd for $C_{11}H_{18}O_2N_3$ 224.1394, found 224.1392 [M]$^+$ (100).

Coupling reaction for the preparation of 15, 16 and 17

To the suspension of 8.54 mmol of hydrochloride salt of an amine in 100 mL of dichloromethylene (DCM) with ice-water cooling bath, 3.0 mL of trimethylamine, 8.45 mmol of an acid, 2.72 g (8.47 mmol) of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 1.0 mL of *N*-methyl morpholine (NMM) were added orderly. After the addition, the reaction was stirred at room temperature for 18 h and then treated with sat. $NaHCO_3$, extracted by CH_2Cl_2, dried over Na_2SO_4, evaporated in vacuum and silica gel column chromatographed (MeOH in CH_2Cl_2, 1% to 3% plus 1% concentrated NH_4OH in volume for **15**, MeOH in CH_2Cl_2, 3.3% in volume for **16** or **17**) to give the compound **15**, **16** or **17**.

***N*-Adamantylcarboxy-*L*-histidine methyl ester (15)** Yield 68%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.49 (s, 1H), 6.72 (s, 1H), 4.62—4.65 (m, 1H), 3.56 (s, 3H), 2.96—3.10 (m, 2H), 1.93—2.00 (m, 3H), 1.72—1.82 (m, 6H), 1.58—1.68 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 178.7, 172.2, 135.4, 134.6, 115.3, 52.5, 52.1, 40.5, 38.9 (3C), 36.4 (3C), 29.0, 28.0 (3C); ESIMS *m*/*z* (%): 332.0 ([M+H]$^+$, 100).

***N*,*N*′-Di(*tert*-butylcarbamate)-adamantylamino-*L*-histidine amide (16)** Yield 87%; ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 8.00 (s, 1H), 7.15 (s, 1H), 6.25 (s, 1H), 5.71 (s, 1H), 4.18 (s, 1H), 2.92—3.00 (m, 1H), 2.72—2.82 (m, 1H), 2.65—2.70 (m, 1H), 1.92—1.97 (m, 3H), 1.78—1.85 (m, 6H), 1.50—1.60 (m, 6H), 1.40 (s, 9H).

***N*,*N*′-Di(*tert*-butylcarbamate)-adamantylamino-*D*-histidine amide (17)** Yield 90%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.02 (s, 1H), 7.16 (s, 1H), 6.21 (s, 1H), 5.83 (s, 1H), 4.27 (s, 1H), 2.99—3.05 (m, 1H), 2.87 (dd, J=6.0, 8.8 Hz, 1H), 1.97—2.05 (m, 3H), 1.85—1.89 (m, 6H), 1.58—1.60 (m, 6H), 1.43 (s, 9H).

Deprotection of 16 and 17

The solution of 0.9 g (1.84 mmol) of compound **16** or **17** in 20 mL of DCM was treated with 1.0 mL of trifluoroacetic acid (TFA) from 0 ℃ to room temperature for 5 h. After the removal of volatile components, the residue was purified by silica gel column chromatography (MeOH in DCM, 6.3% in volume with 1% concentrated NH_4OH) to provide the compound **18** or **19**.

***N*-(1-Adamantyl)-*L*-histidine amide (18)** Yield 93%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.57 (s, 1H), 6.82 (s, 1H), 3.43 (dd, J=1.6, 5.6 Hz, 1H), 2.88 (dd, J=5.6, 8.8 Hz, 1H), 2.71 (dd, J=6.8, 7.6 Hz, 1H), 1.90—1.98 (m, 3H), 1.85—1.94 (m, 6H), 1.58—1.68 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ: 174.2, 134.9, 133.4, 117.8, 55.3, 51.1, 43.5, 40.9, 36.1, 35.7, 32.7, 29.5; HRFABMS calcd for $C_{16}H_{25}O_1N_4$ 289.2023, found 289.2014 [M]$^+$ (100).

***N*-(1-Adamantyl)-*D*-histidine amide (19)** Yield 90%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.54 (s, 1H), 7.21 (s, 1H), 6.80 (s, 1H), 4.66 (brs, 1H), 3.48—3.55 (m, 1H), 2.97 (dd, J=4.4, 10.4 Hz, 1H), 2.84 (dd, J=6.8, 7.6 Hz, 1H), 1.98—2.06 (m, 3H), 1.90—1.95 (m, 6H), 1.58—1.65 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 173.7, 135.2, 132.4, 119.5, 55.4, 51.3, 41.4 (3C), 36.3 (3C), 32.0, 29.4 (3C); ESIMS *m*/*z* (%): 289.4 ([M+H]$^+$, 100).

Formation of thio-amides 20 and 21 by Lawesson's reagent

0.1 g (0.35 mmol) of compound **18** or **19** and 0.35 g (0.87 mmol) of Lawesson's reagent were dissolved in 20 mL of dried benzene. Compound **18** or **19** was almost converted to thio-amide **20** or **21** after the reaction mixture was refluxed for 20 h. The reaction mixture was evaporated and purified by silica gel column chromatography to yield the mixture of compounds **18** and **20** or **19** and **21**.

Reduction of thio-amides 20 and 21

To the solution of the mixture **18** and **20** or **19** and **21** from the last step (about 0.35 mmol) in 20 mL of 80% *tert*-butanol, 1.0 g of Raney Ni was added and then the reaction was refluxed for 8 h. After filtered and evaporated, the residue was silica gel column chromatographed (MeOH in DCM, 20% in volume) to provide the compound **22** or **23**.

(2*S*)-1-[*N*-(1-Adamantyl)]-3-[(1*H*-imidazol-4-yl)-propane-1,2-diamine (22) Yield 76%; ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.56 (s, 1H), 6.83 (s, 1H), 2.92—2.99 (m, 1H), 2.53—2.70 (m, 3H), 2.35—2.42 (m, 1H), 1.96—2.06 (m, 3H), 1.57—1.70 (m, 12H); ^{13}C NMR (CD_3OD, 100 MHz) δ: 134.8, 133.6, 117.8, 51.4, 50.3, 45.6, 41.7 (3C), 36.4 (3C), 32.9, 29.62 (3C); HRFABMS calcd for $C_{16}H_{27}N_4$ 275.2230, found 275.2213 [M]$^+$ (100).

(2*R*)-1-[*N*-(1-Adamantyl)]-3-[(1*H*-imidazol-4-yl)-propane-1,2-diamine (23) Yield 75%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.49 (s, 1H), 6.76 (s, 1H), 3.66 (brs, 2H), 2.95—3.06 (m, 1H), 2.75 (dd, J=4.8, 10.0 Hz, 1H), 2.65 (dd, J=4.4, 7.2 Hz, 1H), 2.52—2.57 (m, 1H), 2.40—2.45 (m, 1H), 2.00—2.07 (m, 3H), 1.55—1.66 (m, 12H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 134.8, 133.1, 119.4, 51.9, 50.6, 46.4, 42.6 (3C), 36.6 (3C), 32.9, 29.5 (3C); ESIMS *m*/*z* (%): 275 ([M]$^+$, 100).

Electrophysiological recordings by patch clamp

The stable cell lines of transformed 293T-rex expressed WT, S31N-L26I and S31N mutant M2 channels of avian H5N1 were successfully established according to the literature.[18] M2-transformed 293T-rex cells were used 24—48 h after induction with 1 μg/mL tetracycline. Perforated whole-cell voltage-clamp recordings were carried out at room temperature (23—25 ℃) using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) as described previously[19] (Li *et al.*, 2004). Drug applications and extracellular pH changes were performed using a commercially available automated fast solution exchange system (RSC-200 Rapid solution changer). All data are reported for *n* number of cells. Differences in the compound's inhibition were determined from statistical tests using IC_{50} value and a comparison between two groups was made using Student's *t* test (Table 1).

Cytotoxicity of the compounds to the three transformed cell lines

The cytotoxicity (CC_{50}) of these compounds to three transformed cell lines was detected by the general MTT method to quantify the number of viable cells without the induction 3 d after the addition of the compound or the carrier. Briefly, 1000 cells/well were seeded in 96-well plates and allowed to attach for 20 h before the treatment with a compound in 0.1% DMSO or the same volume of the carrier. Every concentration of each compound or the carrier is in triplicate. After incubated for 72 h, cells were treated with MTT reagents and relative cell viability of each well was calculated. The data of CC_{50} are shown in Table 1.

Table 1 The IC_{50} (μmol/L) for compounds estimated on patch-clamp recording the 293T-rex cells expressed M2 WT, mutants of S31N and L26I/S31N and the CC_{50} (μmol/L) for compounds detected by general MTT method

Compd.	WT-M2		S31N-M2		S31N/L26I-M2	
	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}	CC_{50}
1	4.18	＞200	NA[a]	＞200	NA	＞200
6	5.84	332.5	10.96	300.2	9.77	400.4
7	18.9	463.1	ND[b]	399.7	112.1	＞500
8	NA	63.9	ND	62.6	ND	59.3
9	NA	419.0	ND	372.2	ND	＞500
10	NA	＞500	ND	＞500	ND	＞500
11	NA	＞500	ND	＞500	ND	＞500
12	NA	336.1	ND	371.1	ND	482.5
13	NA	＞500	ND	96.1	ND	＞500
14	319.0	＞500	465.0	＞500	399.0	＞500
15	NA	70.3	ND	80.6	ND	64.5
18	137.2	428.1	ND	352.0	NA	＞500
19	NA	＞500	ND	＞500	ND	＞500
22	NA	＞500	NA	＞500	NA	＞500
23	NA	＞500	NA	＞500	ND	＞500

[a] NA means "IC_{50}＞500 μmol/L"; [b] ND means "not determined yet".

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