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Identification of Hits as Matrix-2 Protein Inhibitors through the Focused Screening of a Small Primary Amine Library

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Although amantadine derivatives are the only M2 drugs for influenza virus A, their use is limited in the U.S. because of drug resistance. Here we report the identification of multiple M2 inhibitors that were rapidly generated through focused screening of a small primary amine library that was designed using a scaffold-hopping strategy based on amantadine. These compounds are as active as amantadine and might be hits for further lead generation processes.

Introduction

There is currently an outbreak of H1N1 influenza (swine flu) around the world.[1,2] Although vaccination is the ideal way to prevent influenza virus infection, the preparation of a new vaccine requires more than 6 months.[3] Thus, antiviral drugs are the most effective for short-term defense against influenza. The only known anti-influenza A drugs are M2[a] (matrix-2 protein) inhibitors (amantadine and its derivative rimantadine) and NA (neuraminidase) inhibitors (zanamivir and oseltamivir).[4−6] Both **1** (amantadine) and **2** (rimantadine) (Figure 1) are limited in their use in the U.S. because of the rapid development of resistance. In addition, there is growing concern that antineuraminidase-resistant viruses may emerge if these drugs are widely used.[7] Thus, there is an urgent need to discover new types of M2 inhibitors for the development of new anti-influenza drugs. Although amantadine reached the market 40 years ago, all known M2 inhibitors to date are amantadine derivatives **3**−**5** (Figure 1)[8−13] with the exception of compound **6** (BL-1743).[14] Therefore, a vast area of chemical space remains to be explored.

Thus, we decided to design and screen a small primary amine library of scaffold-hops based on amantadine to generate new hits in the M2 inhibitor class. The mechanism of M2 inhibitors is to block the ion channel activity of the M2 protein of most influenza A viruses. This action inhibits viral replication by blocking proton flow. The amino group in amantadine is likely the pharmacophore and is necessary to block proton transport.[15] At the same time, the adamantyl group as the scaffold exerts an important steric effect.

As shown in Figure 2, the strategy for our library design was based on diversity and the structure−activity relationships of amantadine. The scaffold covers different molecular properties, with an emphasis on steric effect. This library contains linear, aromatic, monocylic, bicyclic, and tricyclic amines. For the diversity of primary amines, the smallest group is the methyl group and the largest group is the amantadyl group. We constructed the library (Supporting Information Table S1) by ordering 70 primary amines from commercial sources and used it directly for screening by multiple biological assays.

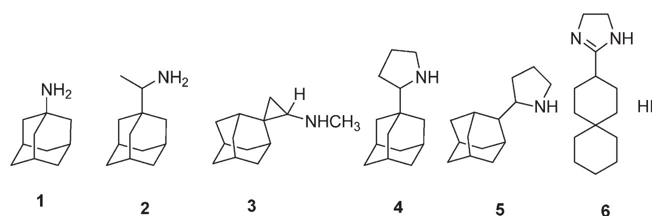

Figure 1. Reported M2 inhibitors: mainly amantadine derivatives.

To accurately measure the activity of these compounds, we employed three types of in vitro assays, including cell based assay, viral inhibition, and patch clamp analyses. The cell based assay was employed to measure the inhibitory activity by the survival of cells expressing M2, since M2 ion channel activity resulted in cell death.[16,17] Only the active compounds will be selected for further test by viral inhibition assay and patch clamp method. The antiviral activities was assessed by A/WS/33 (H1N1, amantadine resistant) and A/Hong Kong/8/68 (H3N2, amantadine sensitive) virus[18,19] described in Experimental Section. Finally, the active compounds were tested with the patch clamp technique to study the ion channel function. Patch clamp electrophysiology in mammalian cells is an assay for assessing functional activity. This approach enables ion channel and compound evaluation on a one-cell-at-a-time basis.

Herein, we focused on hit identification through focused screening. This study led to the identification of five new M2 inhibitors with the potential for hit-to-lead optimization.

Result and Discussion

The compound library was first screened by a cell viability based assay in which the inhibitory activity of the compounds was measured by the survival of cells expressing M2. The observation of M2 expression in 293Trex cells was adapted

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[a]Abbreviations: M2, matrix-2 protein; NA, neuraminidase; M2wt, matrix-2 wild type.

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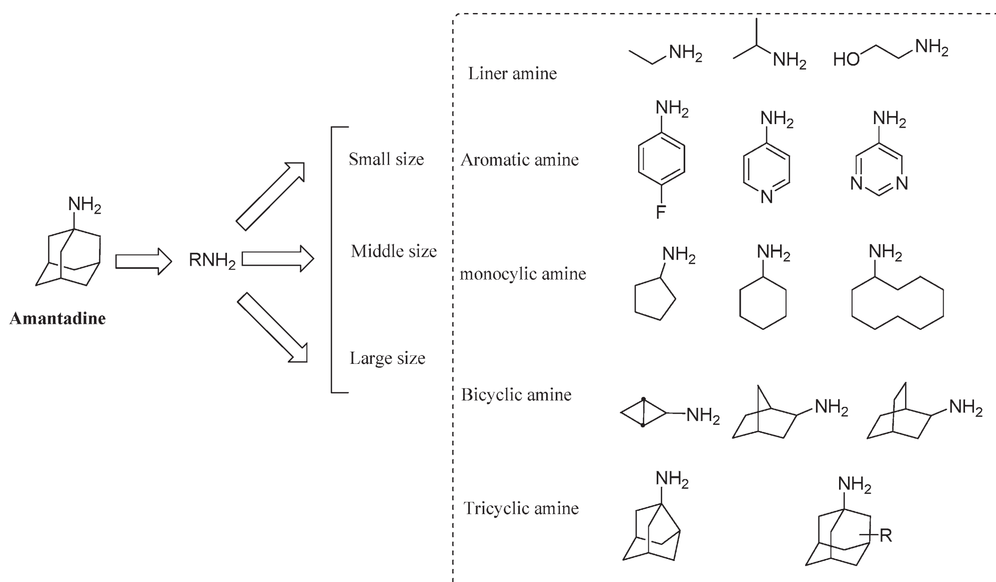

Figure 2. Design strategy used for the primary amine library.

Table 1. Compounds That Share the Inhibitory Activity of Amantadine Identified in the Small Library[a]

Compound No	1	7	8	9	10	11
Compound ID	Amantadine	ZSG-2-101B	LSR-2-007C	LSR-2-007D	ZSG-2-046C	ZSG-2-101E
Structure	NH_2	NH_2	NH_2	NH_2	NH_2	NH_2.HCl
Cell-based assay	3.525	3.37	22.98	5.960	25.47	18.97
Viral inhibition	5.960	33.49	6.018	1.363	2.304	38.21
Patch clamp method	8.8±2.7	4.8± 1.2	6.8±2.2	4.3±2.7	4.4±1.3	13.5± 4.1

[a] IC_{50} (mean ± SEM), μM.

into a screen format to screen for inhibitors of the M2 ion channel (Figures S1, S2, S5, S6).

Among these 70 compounds, 4-*tert*-butylcyclohexylamine **7** (ZSG-2-101B),[20] (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheylamine **8** (LSR-2-007C),[21] (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine **9** (LSR-2-007D),[21] (−)-*cis*-myrtanylamine **10** (ZSG-2-046C),[22] and 3-noradamantanamine hydrochloride **11** (ZSG-2-101E)[23] were identified to significantly inhibit the cell death and inhibit wild type M2 ion channels with different IC_{50} values (Table 1, Figure S3).

These five compounds were further tested by viral inhibition assay. A/WS/33 (H1N1, amantadine resistant) and A/Hong Kong/8/68 (H3N2, amantadine sensitive) viruses were tested. A potent inhibitor of the M2 ion channel will rescue the cell by inhibiting the replication of influenza virus. The results showed that these five compounds inhibit the amantadine sensitive virus replication with IC_{50} close to amantadine (Table 1, Figure S4). However, all the compounds have no inhibition on amantadine resistant virus.

All five active compounds were finally tested by patch clamp method. Patch clamp electrophysiology in mammalian cells has been overcomed by the implementation of multiwell plate format cell-based screening strategies for ion channels. To test the hypothesis that transmembrane domain of the M2 ion channel of influenza A virus mediates the low pH-induced activation of the channel, the wild type M2 protein was expressed in HEK 293 T cells and membrane currents were recorded. The M2 integral membrane protein of influenza A virus forms a proton-selective ion channel. We investigated the mechanism for proton transport of the M2 protein using the whole cell patch clamp technique. All five compounds were found to inhibit M2 ion channels as good as amantadine (Table 1 and Figure 3).

The five structures represent some extent of the middle steric effects. Compound **7** is a substituted cyclohexylamine. Compounds **8** and **9** are bicyclic compounds and entio-isomers configured in the *R*- and *S*-methyl groups. Compound **10** has the same scaffold as the previous two compounds with a methylamine as a functional group, whereas compound **11** is an exact amantadine-like analogue with one less methylene.

These structures suggest that the M2 wild type ion channel can accommodate a range of chemical space, but a minimal steric effect is required to block the channel. Whereas all linear, simple monocylic, and aromatic amines have no activity, substituted cyclohexylmaine and some bicyclic and tricyclic amines have inhibitory activities that mimic amantadine.

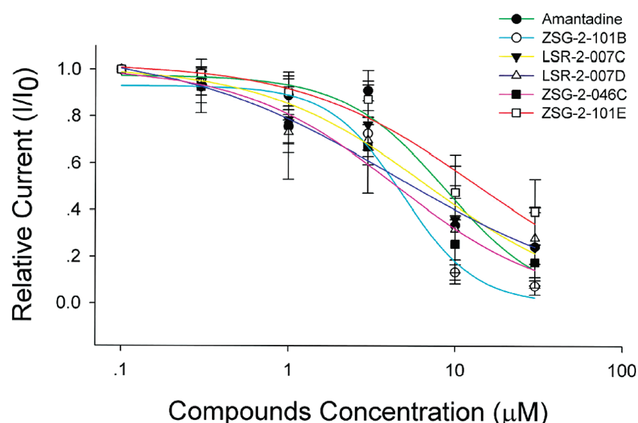

Figure 3. Inhibitory activity of compounds on M2 ion channel conductance detected by the patch clamp method.

Expansion of the size of amantadine by the addition of substituted groups to the ring, such as hydroxyl, diamino, and dimethyl groups (the last three compounds listed in Table S1) does not enhance inhibitory activity. Because of differences of the assay models and molecular properties, these compounds gave different activity trends. The data will be further analyzed in our later hit-to-lead optimization and SAR studies. At this stage, we disclosed here that these five compounds (**7**, **8**, **9**, **10**, and **11**) are inhibitors against M2wt protein and as active as amantadine. So these compounds along with the scaffold might be a basis to discover novel series of M2 inhibitors.

Considering that the mechanism of mutant M2 protein is not fully understood[24,25] and no mutant M2 inhibitor is reported to date, our work might be valuable for the mechanism study. In fact, we had probed wild type and mutant M2 ion channel with diverse amines, and yet none of the compounds is active against mutant M2. So the results may indicate that the mutant M2 ion channel not be narrowed compared to its wild type. Once the scientist can understand the mechanism of M2 mutation, it is highly possible for the chemist to develop mutant M2 inhibitors.

Conclusion

We reported the identification of M2 inhibitor hits (**7**, **8**, **9**, **10**, and **11**) that were generated through focused screening. The library was simply designed using a scaffold-hopping strategy based on amantadine and consists of only 70 commercially available primary amines. This study validates that focused screening is practical and efficient for hit identification. Also, we probed wild type and mutant M2 ion channel with diverse amines. This study may provide insights into the binding mode of the M2 ion channel.

Experimental Section

Chemicals. All the compounds in this library (Table S1) were purchased from Sigma-Aldrich, Alfa Aesar and Acros and used without further purification. The chemicals are all reagent grade with the purity of more than 95%.

Cell Viability Based Assay. M2-293Trex cells (1×10^4) were seeded into 96-well microtiter plates and grown in medium with or without 1 μg/mL of tetracycline for 24 h. After the medium was discarded, the cells were treated with pH 5.8 buffer (50 mmol/L MES, 25 mmol/L HEPES in PBS, with or without chemicals) for 3 h, followed by recovery in complete medium. Twenty-four hours later, 10 μL of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was added to each well for 3 h to measure the A450 by UVstar-Microplates Synergy HT. Compounds were judged to be positive in the assay if they restored cell viability. Data were analyzed using GraphPad Prism Demo.

Viral Inhibition Assays. 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 chemicals 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 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 measured with the UVstar-Microplates Synergy HT. Data were analyzed using GraphPad Prism Demo.

Electrophysiological Recording (Patch Clamp Method). M2-293Trex 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 °C) using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). Recording electrodes were pulled from 1.5 mM borosilicate pipettes (World Precision Instruments, Inc., Sarasota, FL) using a horizontal puller (model P-87; Sutter Instrument Company, Novato, CA). The extracellular solution consists of 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES. In most of the experiments, the solution was adjusted to pH 6.8 or 10 mM MES was adjusted to pH 5.5 or alternative pH values by the addition of NaOH or HCl. The patch electrode had a resistance of between 1.8 and 2.5 MΩ. The pipet tip was initially filled with amphotericin-free pipet solution containing 130 mM Cs methanesulfonate, 24 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 200 μg/mL amphotericin B. The pH of the intracellular solution was adjusted to 6.8 with CsOH. Salts and drugs were obtained from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. The currents were filtered at 10 kHz with 16-bit accuracy using Macintosh G4 computers (Apple Computer, Cupertino, CA) and ITC-16 analog-to-digital boards (Instrutech, Port Washington, NY), and external operations were compiled in IGOR Pro (Wavemetrics, Lake Oswego, OR). Drug application and changes in extracellular pH were carried out using a commercially available automated fast solution exchange system (RSC-200 rapid solution changer). All data are reported as the mean ± SEM for a number of cells. Differences in antagonist inhibition were determined from statistical tests using the IC_{50} and a comparison between two groups, which was accomplished using the Student's *t* test.

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Supporting Information Available: Details about compound library, sources of key compounds, biological assay procedures, and some primary activity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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