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Discovery of novel 1-phenyl-cycloalkane carbamides as potent and selective influenza fusion inhibitors

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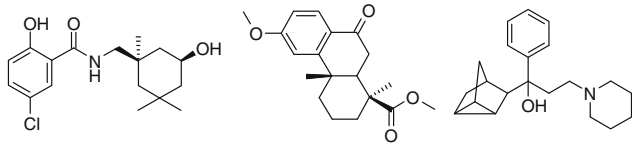
ABSTRACT

A novel class of HA inhibitors (**4a**) was identified based on ligand similarity search of known HA inhibitors. Parallel synthesis and further structural modifications resulted in 1-phenyl-cyclopentanecarboxylic acid (4-cyano-phenyl)-methyl-amide **4t** as a potent and selective inhibitor to phylogenetic H1 influenza viruses with an EC_{50} of 98 nM against H1N1 A/Weiss/43 strain and over 1000-fold selectivity against host MDCK cells.

Influenza or flu is a highly contagious respiratory illness caused by a group of enveloped RNA viruses, whose genomes are composed of seven or eight single-stranded RNA segments.[1] There are three types of influenza viruses, named A, B, and C, that cause seasonal flu and account for significant morbidity, mortality, and economic loss worldwide.[2] Among them, influenza A viruses infect many species including *Homo sapiens*, evolve rapidly through genetic shift and drift, and cause periodical epidemics and global pandemics.[3] Recently, a pandemic influenza A H1N1 swine flu strain emerged from Mexico in 2009, arguably as a result of the genetic reassortment of human, avian, and swine influenza viruses before overcoming transmission barriers and adapting fitness in human.[4] So far it has infected millions of people around the world and caused many human deaths. In the face of the persistent threat of human influenza infections and the potential of highly pathogenic avian influenza H5N1 to unleash another pandemic once inter-human transmission becomes possible, there is an urgent medical demand to come up with new and effective anti-influenza agents.[5]

Besides activities to develop anti-influenza antibodies and new vaccines, there have been significant efforts devoted to identifying small molecule drugs for the prophylaxis and treatment of influenza infections.[6] Of the 11 influenza A viral proteins, many are essential for viral replication and are potential targets for the development of anti-influenza drugs. Examples of anti-influenza small molecules that are in clinical use include oseltamivir and zanamivir, which target viral neuraminidase, and proton channel M2 blockers amantadine and rimantadine.[7] These four drugs are effective upon prompt administration after infection or through prophylactic use, but possess various degree of undesirable CNS side effects. More importantly, the drug-resistance issues have emerged in the clinic.[8]

The membrane fusion process is an early event in the influenza virus life cycle.[9] Hemagglutinin (HA) is a trimeric protein that anchors to the viral membrane and mediates host cell recognition, endocytosis, and the fusion of virus envelope with the host cell endosome membrane. Each HA monomer is expressed as a single polypeptide precursor HA0 and proteolytically cleaved during viral maturation to HA1 and HA2, which are linked by a disulfide bond. Highly conserved among all 16 HA subtypes, the HA2 section constitutes the core fusion machinery in the stem region which undergoes significant conformational rearrangements upon acidification in endosomal compartments. Several small molecule HA inhibitors such as **1**–**3** have been identified as potent anti-influenza agents that bind to HA and prevent low-pH triggered conformational changes (Fig. 1).[10–12] Recently, two independent studies reported



1 *2 (LY-180299)* *3 (Triperiden)*

Figure 1. The structures of HA inhibitors **1**–**3**.

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that HA2 stem-targeting antibodies can effectively inhibit influenza membrane fusion and neutralize the H5N1 avian virus as well as other seasonal flu strains.[13,14] It is anticipated that small molecule HA inhibitors that bind to the conserved stem region of HA2 would have a similar anti-influenza effect.

Besides compounds **1**–**3**, a number of structurally versatile HA inhibitors have been reported. However, many of these compounds turned out to be promiscuous and may not be true fusion inhibitors.[15] Herein we report the identification of a novel class of HA inhibitors through ligand similarity-based virtual screening. In this study, nine known HA inhibitors including **1**–**3** were selected as the basis for an MOS (maximum overlapping set)[16] similarity search against the Roche collection of about 1 million compounds. The top scored hits were identified, clustered, and filtered through in silico profiling and analyses. Nearly 2000 compounds arising from this search were evaluated for their prevention of viral cytopathic effect (CPE) to Madin–Darby canine kidney (MDCK) cells, which were infected with the influenza A/Weiss/43 strain.[17] A few compounds showed positive cell protection in the primary screening, and their anti-viral activities were confirmed by titrating multiple doses of the compounds in the CPE assays. The mechanism of action of the positive hits was further analyzed and confirmed using a red blood cell (RBC)-based hemolysis assay and an in vitro trypsin-catalyzed HA proteolysis assay.[18]

Based on the results of a primary single dose CPE screening and the secondary multiple dose confirmation assays, two closely related compounds **4a** and **4b** were found to effectively protect MDCK cells from cytopathic effects caused by influenza virus replication with an EC_{50} of 2.1 and 27.9 μM, respectively (Table 1). As a fact, they are among the many hits of MOS similarity search based on 3-(1-phenyl-cyclohexyl)-propionic acid, which was reported as an anti-influenza fusion inhibitor.[19] Compound **4a**, an amide of piperidine was over 10 times more potent than its morpholine analog **4b**. Notably, **4a** was not significantly cytotoxic toward MDCK cells (CC_{50} > 100 μM), indicating a good selectivity window between its anti-viral activity and general cytotoxicity.

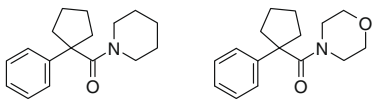
To confirm that these compounds indeed target influenza hemagglutinin protein and are able to block virus fusion as we expected, their inhibitory effects on the hemolysis of chicken red blood cells (RBCs) were tested. In this assay, fresh chicken RBCs were collected and incubated with influenza A/Weiss/43 viruses. After the binding of HA to host receptors on the surface of chicken RBC, a brief acidification (pH ~5) triggered an irreversible conformational change of HA protein, and resulted in pore formations on the RBC membrane and subsequent release of hemoglobin from RBCs to the supernatant. As summarized in Table 1, **4a** inhibited the hemolysis of RBCs with an IC_{50} of 2.2 μM, but did not have an effect on hemagglutination, in which only viral HA-receptor binding is involved (data not shown). This result suggested that **4a** negatively affected RBC hemolysis by binding to HA and interfering with crucial conformational changes of HA normally triggered by a low-pH environment. This assumption was further verified in an HA trypsin sensitivity assay. In this assay, HA protein was isolated from viral preparations after cleavage with bromelain. The purified HA, in the presence or absence of compounds, was subjected to a brief low-pH treatment followed by trypsin digestion.[20] It has been well-demonstrated that the low-pH triggered conformational changes of HA expose its internal trypsin cleavage sites and render HA sensitive to trypsin. Compound **4a**, like other known HA stabilizers, protected isolated HA from trypsin-induced degradation (Fig. 2, lane 8). Taken together, the CPE assay data and mechanism of action studies indicate that **4a** inhibits the virus fusion step by binding to HA and stabilizing the pre-fusion HA structure.

Due to its promising anti-influenza potency, compound **4a** was chosen for further medicinal chemistry optimizations. A fast parallel synthesis route via coupling of sterically hindered acyl chlorides with amines was used for structure–activity relationship (SAR) studies on scaffold **4** (Scheme 1). In a few instances, N-alkylation reaction was carried out for some of the compound synthesis.

Early modifications indicated that fusing additional rings onto the piperidine of **4a** was generally not tolerated. For instance, compound **4c** with the 1,2,3,4-tetrahydro-quinolinyl group was inactive in the CPE assay (Table 2). Attempts to replace the piperidine with other versatile primary amines also gave inactive compounds, such as anilide derivative **4d**. Surprisingly, when the amide NH was alkylated, two of the compounds (**4e** and **4f**) showed submicromolar potency in the CPE assay with an EC_{50} of 0.24 and 0.52 μM, respectively. While the exact role of the *N*-methyl group is not clear, *N*-methyl substitution was predicted to change the dihedral angle between the amide bond (O═C—N) and the aniline moiety (N—C═C) by about 26°, whereas in the case of **4d**, the amide and phenyl ring are almost coplanar. Additional changes on N-substitution with both hydrophobic and hydrophilic groups confirmed that N-substitution is important to control the orientation of the phenyl group. Among many N-substituted derivatives synthesized, the *N*-ethyl analog **4g** and the *N*-hydroxyethyl analog **4h** showed similar anti-viral activities with that of **4e**.

Given the preliminary SAR we obtained and the promising potency of **4e**, we decided to carry out systemic structural modifications to further improve the anti-viral potency and cytotoxicity profile of this class of compounds. In the studies, the R^1 phenyl

Table 1
Biological activities of compounds **1**, **2**, and **4a**–**b**



4a **4b**

Compound	EC_{50} (μM)/CPE[a]	CC_{50} (μM)[b]	IC_{50} (μM)/hemolysis[c]
1	0.06	>50	0.53
2	0.89	67	3.2
4a	2.1	>100	2.2
4b	27.9	>100	NA

[a] Compound concentration to inhibit 50% of cytopathic effect. Values are the mean of triplicate experiments.
[b] Compound concentration to inhibit 50% of MDCK cell growth relative to control.
[c] Half maximal inhibition concentration to prevent hemolysis of chicken RBCs.

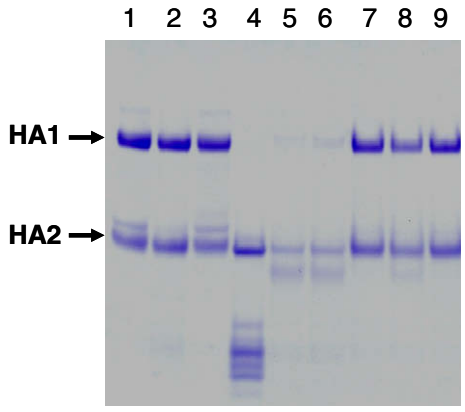
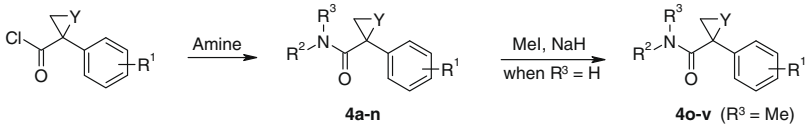
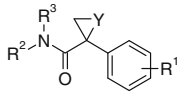


Figure 2. SDS–PAGE analysis of trypsin sensitivity assay showing **1**, **4a**, and **4t** protected purified HA from trypsin digestion. Lane 1, purified HA; lane 2, HA treated with trypsin without a prior acidification step; lane 3, acidified HA without trypin treatment; lane 4, trypsin only (overloaded); lane 5, DMSO control; lane 6, ribavirin (10 μM); lane 7, compound **1** (10 μM); lane 8, compound **4a** (10 μM); lane 9, compound **4t** (10 μM). Lanes 5–9 included all steps and components in a typical trypsin sensitivity assay. Positions of HA1 and HA2 are marked.



Scheme 1. A general synthetic route for HA inhibitors **4a**–**v**.

Table 2
Influenza inhibitory activities of compounds **4c**–**u** against A/Weiss/43 strain



4c-u

Compound	Y	R^1	R^2	R^3	EC_{50}[a] (μM)	CC_{50}[b] (μM)
4c	$-(CH_2)_3-$	H	1,2,3,4-Tetrahydroquinolinyl		>100	24
4d	$-(CH_2)_3-$	H	Ph	H	>100	>100
4e	$-(CH_2)_3-$	H	Ph	Me	0.24	52
4f	$-(CH_2)_3-$	H	*c*-Hexyl	Me	0.45	21
4g	$-(CH_2)_3-$	H	Ph	Et	0.26	56
4h	$-(CH_2)_3-$	H	Ph	$-(CH_2)_2OH$	0.58	79
4i	$-(CH_2)_3-$	3-CF_3	Ph	Me	2.26	20
4j	$-(CH_2)_3-$	4-Cl	Ph	Me	0.19	21
4k	$-(CH_2)_3-$	4-OMe	Ph	Me	0.75	69
4l	$-CH_2-$	4-Cl	Ph	Me	1.86	49
4m	$-(CH_2)_2-$	4-Cl	Ph	Me	0.27	24
4n	$-(CH_2)_4-$	4-Cl	Ph	Me	>10	7.4
4o	$-(CH_2)_3-$	H	2-Cl-Ph	Me	0.18	23
4p	$-(CH_2)_3-$	H	3-Cl-Ph	Me	0.46	17
4q	$-(CH_2)_3-$	H	3-CN-Ph	Me	0.73	56
4r	$-(CH_2)_3-$	H	3-Pyridinyl	Me	16.4	>100
4s	$-(CH_2)_3-$	H	4-Cl-Ph	Me	0.080	20
4t	$-(CH_2)_3-$	H	4-CN-Ph	Me	0.098	>100
4u	$-(CH_2)_3-$	H	4-CF_3-Ph	Me	2.12	21

[a] Values are mean of at least duplicate experiments.
[b] Cytotoxicity in MDCK cells.

substitution was generally not well tolerated except for small substituents at the *para*-position (**4j**–**k**). The cyclopentane group was found to be very important for anti-viral activity, as none of the ring-opened analogs we synthesized was active. We also looked into the relationship between the anti-viral activity and the size of aliphatic ring. Except for the cyclobutane derivative **4m**, which exhibited an EC_{50} equivalent to that of **4e**, all other ring expansion/contraction analogs were much less active in the CPE assay (**4l** and **4n** in Table 2). These results indicate that the orientation of the two phenyl rings and the cyclopentane moiety is crucial for effective interaction with the HA protein. To further improve the anti-viral potency, structural elaborations of **4e** on the aniline phenyl group were carried out. The SAR indicates that 2,4-substitution of the phenyl group is preferred for better activity in the CPE assay, while *meta*-substitution is detrimental to the activity. Structural modifications at the *para*-position yielded highly potent HA inhibitors, such as **4s** and **4t**[21], with an EC_{50} value below 100 nM against the A/Weiss/43 strain. In addition, viral specificity studies showed that **4s** and **4t** had similar anti-viral potency against another group 1 influenza strain A/PR/8/34 (H1N1) (data not shown), suggesting that they may be broadly active against other group 1 influenza viruses. The cytotoxicity studies showed that **4t** with a 4-cyano substitution had over 1000-fold selectivity (CC_{50}/EC_{50}) against MDCK cells. Overall, compound **4t** is about 20-fold more potent than the preliminary hit **4a** in the cell-based anti-viral assays. In the subsequent trypsin sensitivity test, **4t** was able to stabilize the HA protein and prevented trypsin mediated HA digestion more effectively than **4a**, which is constant with its improved anti-viral activity in the CPE assay (Fig. 2, lane 9). Efforts are ongoing to fully optimize the anti-viral activity and DMPK properties of this 1-phenyl-cycloalkane carbamide scaffold.

In line with other previously reported HA inhibitors including **1** and **2**, all compounds reported in Table 2 were specific for group 1 influenza virus based on our limited viral specificity tests. For example, they were inactive against influenza A/Hongkong/8/68 virus, a H3N2 strain that belongs to the group 2 influenza viruses. This selectivity between group 1 and 2 viruses is probably due to the structural difference at the putative binding site in the HA stem region between the two groups of viruses, as revealed by the co-crystal studies of *tert*-butyl hydroquinone (TBHQ) and H3 HA protein complex.[22] TBHQ inhibits the H3 and H14 strains specifically by binding to a site located at the stem region which is not found in the similar range of HAs of group 1 influenza viruses. It is clear that co-crystal structural studies of HA with the current leads will shed light on our understanding of the anti-flu specificity and benefit future structural optimizations that could broaden the activity of this series of compounds. Nevertheless, considering that both current pandemic H1N1 swine flu and highly pathogenic H5N1 avian influenza are group 1 influenza viruses and that the vast majority of influenza infections are caused by group 1 influenza viruses, highly potent and effective HA group 1 specific inhibitors like the ones described here should be a valuable addition to our arsenal in battling with the vicious influenza infections.

In summary, we report a ligand-based similarity approach to identify a novel class of anti-influenza inhibitors that bind to the HA protein and prevent its low-pH triggered conformational change and thus inhibit the membrane fusion process. Structural modifications of an initial hit resulted in a lead molecule, 1-phe-

nyl-cyclopentanecarboxylic acid (4-cyano-phenyl)-methyl-amide **4t**, that exhibits high activity against influenza A/Weiss/43 strain (H1N1) with an EC_{50} of 98 nM in the CPE assay and greater than 1000-fold selectivity against host MDCK cells. The identification of this series of molecules provides a good starting point for further optimization and development of influenza fusion inhibitors for treatment of flu.

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20. In a trypsin sensitivity assay, 6 μg of purified HA was incubated with compounds at 31 °C for 15 min. Then the pH of the solution was adjusted to ~5.0 to trigger HA conformational changes. After neutralization with Tris buffer, the solution was treated with 4 μg of trypsin at 37 °C for 30 min. The extent of trypsin digestion of HA was revealed on a 10% SDS–PAGE gel that was stained with Coomassie blue G-250.
21. As an example, synthesis of **4t**: To a solution of 1-phenyl-cyclopentanecarboxylic acid (190 mg, 1.0 mmol) in 5 mL of CH_2Cl_2 was added 0.5 mL of $SOCl_2$. The mixture was heated to reflux for 1 h before the solvent and excess $SOCl_2$ were removed under reduced pressure. The residue was dissolved in 10 mL of $ClCH_2CH_2Cl$. To this solution was added 4-aminobenzonitrile (130 mg, 1.1 mmol) and *N*-methylmorpholine (0.2 mL). After refluxed for 2 h, the reaction mixture was cooled down and washed with 10 mL of HCl (1 N) and saline. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was dissolved in 10 mL of DMF, to which was added NaH (60 mg, 60%, 1.5 mmol). The mixture was stirred at rt for 1 h before 125 μL of iodomethane (2.0 mmol) was added. After stirred at rt for 4 h, the reaction mixture was evaporated and worked up with water and ethyl acetate. From the concentrated organic phase, **4t** was isolated by preparative RP-HPLC (column: C18), using water and acetonitrile as eluents. ^{1}H NMR (MeOD, 400 MHz), 7.59 (d, 2H, *J* = 8.4 Hz), 7.32–7.22 (m, 3H), 7.12–7.06 (m, 4H), 3.08 (s, 3H), 2.44–2.37 (m, 2H), 1.98–1.96 (m, 2H), 1.77–1.62 (m, 4H).
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