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Isocyanides as Influenza A Virus Subtype H5N1 Wild-Type M2 Channel Inhibitors

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Basic bulky amines such as amantadine are well-characterized M2 channel blockers, useful for treating influenza. Herein we report our surprising findings that charge-neutral, bulky isocyanides exhibit activities similar to—or even higher than—that of amantadine. We also demonstrate that these isocyanides have potent growth inhibitory activity against the H5N1 virus. The $-NH_2$ to $-N\equiv C$ group replacement within current anti-influenza drugs was found to give compounds with high activities at low-micromolar concentrations. For example, a tenfold improvement in potency was observed for 1-isocyanoadamantane (**27**), with an EC_{50} value of 0.487 μM against amantadine-sensitive H5N1 virus as determined by both MTT and plaque-reduction assays, without showing cytotoxicity. Furthermore, the isocyanide analogues synthesized in this study did not inhibit the V27A or S31N mutant M2 ion channels, according to electrophysiology experiments, and did not exhibit activity against amantadine-resistant virus strains.

Introduction

Influenza virus infections remain one of the largest global threats to both human health and the poultry industry.[1] Recently, influenza has become an important public health concern following the 2003–2009 potential avian influenza pandemic,[2] which was caused by a strain of highly pathogenic type A, subtype H5N1 influenza virus.[1a] Currently, anti-influenza drug research is focused on three major targets: hemagglutinin (HA), neuraminidase (NA), and the M2 proton channel.[3] Of the three, antiviral drugs targeting NA (oseltamivir and zanamivir) and M2 (amantadine and rimantadine) are currently on the market for influenza treatment.[3a]

Amantadine (**1**) was the first compound to be used for anti-influenza treatment in 1966,[4] and most research efforts have focused on cyclic or acyclic aliphatic amine structures in order to improve potency.[3a] Various amantadine derivatives have been studied, including spiro structures,[5] benzyl-substituted amines,[6] heterocyclic-linked amines,[7] or contracted rings.[8] Rimantadine (**5**), an amantadine analogue, was successfully introduced in 1994. Other amine-based compounds such as non-adamantane-based M2 inhibitors, spiropiperidine,[9] organosilane amines[10] and polycyclic amines[11] have also been investigated in attempts to inhibit the influenza virus. Replacement of the amino group with polar or basic groups, such as hydroxy, guanidine, amidine and hydroxylamine groups (Figure 1, **2**–**4**), led to the development of compounds with moderate viral inhibition relative to the original amine.[11b,12] These studies have confirmed that the amino group is the most important polar functional group in M2 inhibitors (Figure 2, **5**–**8**), taking into account both the potency and drug-like physiochemical properties.[11a,13] After the screening of a focused amine library, (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine (**7**) was

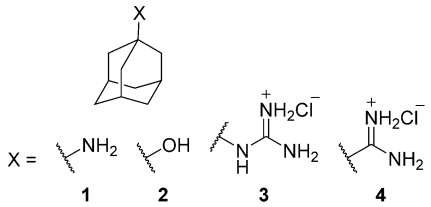

Figure 1. Adamantanes with various polar groups from past studies.

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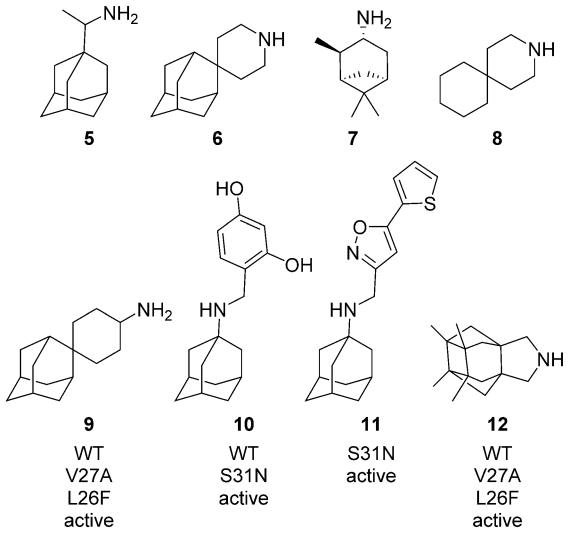


Figure 2. Typical A/M2 inhibitors with amine functional groups.

found to be active.[14] Additionally, spiropiperidine **8** was one of the amines without the adamantyl hydrophobic group that showed superior activity over amantadine.[12]

The ammonium group mimicking a water cluster might stabilize a diffusing hydronium ion; however, this mechanism needs to be confirmed with more examples.[15] The necessity of the amino group was also investigated during the development of wild-type (WT) and mutant M2 (V27A) dual inhibitors, such as the amantadine spiro derivative **9**.[15] To develop inhibitors against the mutant S31N M2 protein, the amine of the amantadine group was linked with a hydroxybenzyl or isoxazole moiety, yielding the adamantyl secondary amines **10** and **11**.[6,7] Polycyclic amine **12** was discovered to be active against the WT, V27A, and L26F mutant M2 channels.[11a] The amino group may play an essential role in the inhibition of the wild-type as well as the three M2 channel mutants.[15]

The isocyanide group is a widely used reactive reagent in multicomponent reactions and is frequently used in drug discovery research.[16] Isocyanides can be represented either in the zwitterionic or carbene form to illustrate their electronic structures. The carbene structure may explain the nucleophilic and electrophilic character of isocyanides, while the zwitterionic structure is consistent with their dipole character with the positive charge on the nitrogen and the negative charge on the carbon (Figure 3).[17] The advantages of isocyanides are their weak basic properties and increased hydrophobicity relative to the other nitrogen-containing functional groups, such as amines and amidines. Moreover, isocyanides appear to have

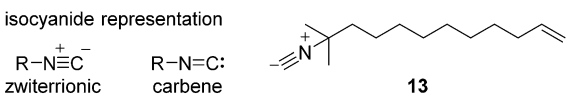


Figure 3. Typical isocyanide electronic representation (left) and an antifouling isocyanide **13** (right).

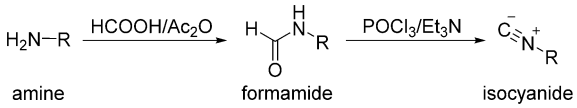
much better cell penetration, considering their lower clog*P*, and may not exhibit the neurotoxicity associated with amines. Isocyanide groups have also been discovered in some natural compounds; however, few of these have shown biological activity.[18] To the best of our knowledge, there is only one report of biological (antifouling) activity for the natural isocyanide compound **13** (Figure 3).[19]

The isocyanide group is not mentioned or listed in *Rapid Elimination of Swill* (REOS)[20] or other lists of inappropriate reactive functional groups.[21] Nevertheless, it is excluded from drug library design as a reactive functional group, similar to isocyanate.[22] In this study, the amino groups of six compounds were replaced by isocyanide functional groups, and the resulting compounds exhibit anti-H5N1 activity. Furthermore, the isocyanide group was not observed to have significant toxicity, which is consistent with previous studies.[23] This study aims to prove that the isocyanide functional group could be used in drug discovery research.

Results and Discussion

Chemistry

Formamides and isocyanides were synthesized according to known procedures (Scheme 1; see Procedures A and B in the Experimental Section below).[23] Several of the isocyanide compounds tested here were widely used in reactions, but their anti-influenza activities have not been reported. We prepared the corresponding isocyanides from three types of amines (Figure 4). Type I amines are highly active in inhibiting the influenza virus and consist of amantadine (**1**), rimantadine (**5**), (1*R*,2*R*,3*R*,5*S*)-(−)-isopinocampheylamine (**7**), 2-adamantylamine (**14**), and 2,4,4-trimethylpentan-2-amine (**15**), the latter of which has been proven to be the smallest type A M2 channel (A/M2) inhibitor.[10]



Scheme 1. Synthesis of formamides and isocyanides from amines.

Type II amines are aliphatic or aromatic amines, which were chosen according to the size of their hydrophobic regions relative to amantadine.[12] The amines used in this study included *tert*-butylamine **16**, 4-*tert*-butylcyclohexylamine **17**, 4-(*tert*-butyl)aniline **18**, 4-(*tert*-butyl)-2,6-dimethylaniline **19**, 3-amino-1-adamantanol **20**, and bornylamine **21**. Finally, type III amines are amino acid ester derivatives. Both the formamide and the corresponding isocyanide compounds were prepared for all three types of amines. To the best of our knowledge, no biological activity has been reported in previous studies for these isocyanides.

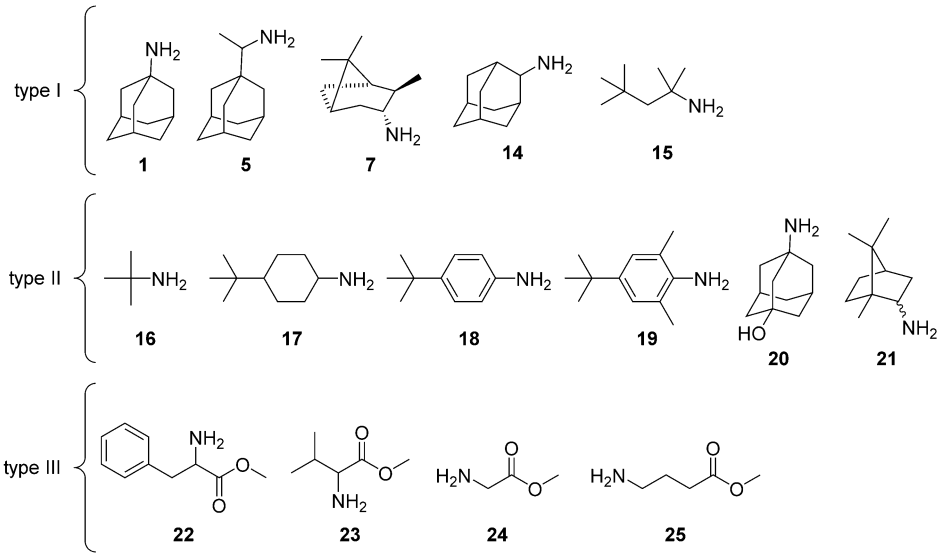


Figure 4. Three types of amine scaffolds were used in this study: type 1: known active M2 inhibitors, type II: analogues of compounds from type I, type III: amino acid esters.

Antiviral activity

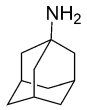
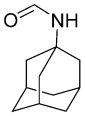
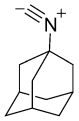
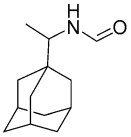
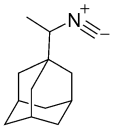
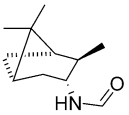
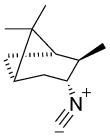
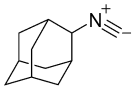
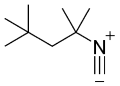
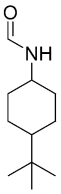
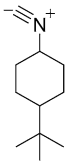
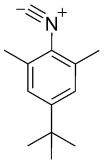
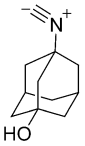
The activity against influenza A/chicken/Hubei/327/2004 (H5N1) virus and cytotoxicity of the compounds were tested using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays in MDCK cells (Table 1). In addition, the two-electrode voltage clamp (TEVC) assay was applied for test compound–M2 protein complexes to determine their pore blocking activity (Table 1). All tested compounds (including isocyanides) did not show cytotoxicity, with CC_{50} values greater than 100 μM. Amantadine formamide **26** and isocyanide **27** exhibited IC_{50} values of 0.38 and 0.48 μM, respectively, which are approximately tenfold lower than the IC_{50} value of 3.9 μM for amantadine. Amantadine formamide **26** was tested in an early structure–activity relationship (SAR) report and only exhibited very weak activity (AVI_{50}: 28 mg kg^{-1}) in the in vivo mouse test (relative to the amantadine AVI_{50} value of 4.6 mg kg^{-1}).[24] The weak in vivo inhibition could be attributed to poor bioavailability and/or rapid clearance of the formamide bond. Because the formamides and isocyanides were found to be well tolerated in cell-based assays, these functional groups were chosen as the amino group bioisosteres; 15 formamides and 15 isocyanides were synthesized from 15 amines (Figure 4), and a total of 12 compounds (four formamides and eight isocyanides) exhibited activity in our assay. All the data for the active compounds are listed in Table 1.

Rimantadine is another established and commercial drug, and its activity is superior to that of amantadine. The activity of rimantadine formamide was much lower than that of amantadine formamide, but the activity of rimantadine isocyanide (EC_{50}: 0.238 μM) was similar to the amantadine isocyanide. (1*R*,2*R*,3*R*,5*S*)-(−)-Isopinocampheylamine (**7**) was reported to be active against amantadine-sensitive viruses from a primary amine library screening;[14] the formamide **30** and isocyanide **31** derivatives of compound **7** exhibited respective EC_{50} values of 2.76 and 7.36 μM our MTT assays. 2-Adamantylamine **14** is known to bind to the M2 protein,[25] and its isocyanide derivative **32** showed an EC_{50} value of 9.8 μM. The amantadine derivatives *tert*-octylamine **15** and *tert*-butylamine **16** were also tested in the antiviral assay: in the A/M2 TEVC assay, *tert*-octylamine **15** exhibited activity similar to that of amantadine (87% inhibition at 100 μM).[12] Both the formamide and isocyanide analogues of octylamine were prepared, and the measured EC_{50} value of 3.8 μM for *tert*-octylisocyanide **33** was similar to that of amantadine.

On the other hand, the smaller fragment *tert*-butylisocyanide exhibited very low activity, with EC_{50} > 500 μM. Because amino groups were considered to be the most important pharmacophore in anti-influenza drug discovery, 70 amines with different linear and branched alkyl or aromatic groups were screened for activity. Five compounds turned out to be potent in the virus inhibition assays.[14] In addition to compound **7**, 4-*tert*-butylcyclohexylamine **17** (*cis*,*trans* mixture) was also active. However, the activities of the formamide **34** and isocyanide **35** derivatives of 4-*tert*-butylcyclohexylamine **17** were lower than their amine analogue. Although 4-(*tert*-butyl)aniline **18** and 4-(*tert*-butyl)-2,6-dimethylaniline **19** were not active against the influenza virus, their formamide and isocyanide derivatives were still tested. Only isocyanide of 4-(*tert*-butyl)-2,6-dimethylaniline compound **36** exhibited antiviral activity, and its activity was similar to that of amantadine. With an extra hydroxy group introduced in amantadine, the formamide and isocyanide analogues of 3-amino-1-adamantanol **20** proved to be inactive in our assays. Amongst the compounds of the type II group, isocyanide **36** exhibited activity even though its amine starting material was not active against the influenza virus. Neither formamides nor isocyanides derived from the type III amino esters **22**–**25** were active in our tests.

To determine if the target of the isocyanides and formamides is the M2 protein, the inhibitory activity of the compounds was tested on A/M2 channels expressed in *Xenopus* oocytes using the TEVC technique. All the inhibitors were tested at a concentration of 100 μM. None of the tested compounds proved to be more potent than amantadine in the TEVC test. The inhibitory activities of formamides **26** (78.6%) and **28** (81.8%) were stronger than their corresponding isocyanides **27** (68.2%) and **29** (42.1%); the activities were confirmed by the IC_{50} values of **26** and **27** (26 μM and 35 μM); the IC_{50} of **29** was over 100 μM. This result suggests that isocyanide **29** inhibits the virus through another mechanism than M2 blockade. Additionally, most of the other tested compounds exhibited modest inhibition with the exception of **33**, which appeared to

Table 1. H5N1 inhibitory activities of the active formamide and isocyanide compounds.

Compd		EC_{50} [μM][a]	A/M2 [%][b]
1		3.97 ± 1.32	91 ± 2.1
26		0.385 ± 0.212	78.6 ± 3.2
27		0.487 ± 0.158	68.2 ± 7.4
28		6.28 ± 2.62	81.8 ± 1.9
29		0.24 ± 0.07	42.1 ± 4.2
30		2.76 ± 1.36	34.3 ± 4.0
31		7.36 ± 2.55	37.6 ± 1.9
32		9.8 ± 3.23	ND
33		3.88 ± 2.37	6 ± 2.5
34		29.5 ± 8.52	32.6 ± 1.7
35		125 ± 42.6	32.9 ± 1.7
36		2.14 ± 0.64	35.0 ± 1.9
37		90.4 ± 28.5	ND

[a] Values are derived from the results of an MTT assay performed with MDCK cells infected with H5N1 virus (influenza strain A/chicken/Hubei/327/2004); data are the mean ± SEM of at least three independent experiments (CC_{50} values were also derived from these assays, and were all > 100 μM).[b] Percent inhibition of wild-type A/M2 at 100 μM.

have low blockade of the M2 channel; its activity could therefore also be attributed to a mechanism other than A/M2 inhibition. Coincidentally, a novel antiviral mechanism has recently been proposed for 2-aminoadamantanes.[26] None of the tested compounds exhibited strong blockade of the mutant S31N and V27A A/M2 proteins. Furthermore, the compounds were also not active against the amantadine-resistant A/WS/33 H1N1 virus strain according to the in vitro assays. Finally, a plaque-reduction assay was performed to confirm the activity of compound **29** using the H5N1 virus. Indeed, it caused a clear decrease in plaque size at a concentration of 1.2 μM

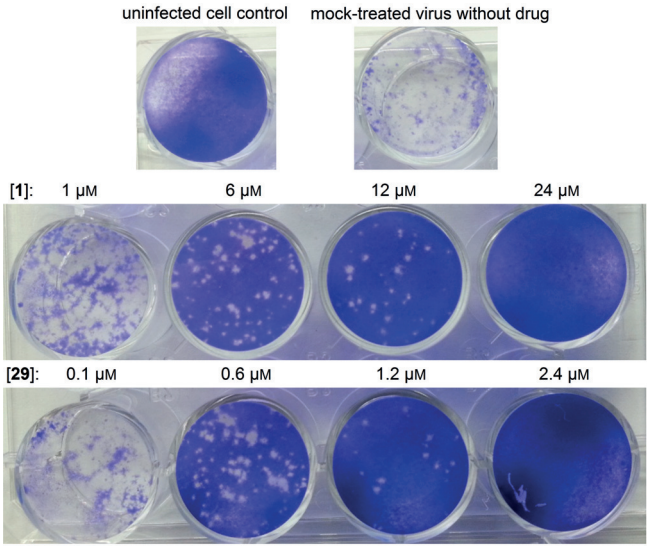


Figure 5. Plaque-reduction assay with the H5N1 virus. MDCK cells were infected with influenza virus (H5N1) in the presence of test compounds amantadine (**1**) and compound **29** at four concentrations as indicated. Plaques were visualized by crystal violet staining.

(Figure 5). The antiviral activity of the amine derivatives was maintained or improved upon replacement of the amino groups with formamide or isocyanide groups. Rimantadine isocyanide **29** was the most potent in the H5N1 inhibition assay, whereas rimantadine formamide **28** was one-sixth less active relative to amantadine formamide **26**. The amantadine and rimantadine isocyanides showed stronger activity than the other isocyanides. To understand how isocyanides may have a difference in M2 activity, isocyanides were compared with the reported active amines by molecular topology. The measured activities were consistent with a previous SAR study on aminoadamantane derivatives and are discussed below.

In an early pioneering SAR study of amantadines, researchers used animal models to assess antiviral activity.[24,27] However, SAR data were only listed without any rational explanations. Years after the biological target of amantadine was proposed to be the M2 protein based on analysis of mutations,[28] many new amantadine analogues were tested against various influenza virus strains. The most active derivatives, such as amantadine and rimantadine, were also found to be active against H2N2.[5d] From that in vivo activity data, we could conclude that the amino group can only tolerate a two-bond distance from the carbon atom of adamantane (a bulky hydrophobic group), whereas amines with a distance of over four bond lengths connecting the adamantane appear to have decreased activity.[4,5c,d] Furthermore, the size of amantadine was also investigated by SAR studies. Whereas 3-methyladamantan-1-amine, with a hydrophobic group radius of 3.6 Å was active (the radius was calculated based on the van der Waals radius of the atoms and the bond length using MarvinSpace Marvin 14.9.22, 2014, ChemAxon), 3,5,7-trimethyladamantan-1-amine, with a radius of 4.2 Å, was not active.[24] According to the reported SAR data and the size of the M2 channel, drug activity is thus dependent on the size of the hydrophobic moiety and the length of the linker that connects the moiety with the amine. This conclusion is continuously confirmed by the recently reported active amines.[8,29]

The channel size limitation was also demonstrated by compound **38**; its M2 blocking activity in TEVC experiments has been confirmed;[8] however, larger azapropellanes did not block the A/M2 proton channel even though the compounds exhibited activity against the H1N1 strain.[29] Thus, the M2 activity seems to be very sensitive to the size of the hydrophobic moiety.

The pore binding site was considered to be a pharmacologically relevant drug binding site supported by X-ray crystal (PDB codes: 3C9J, 2LJC) and solid-state NMR (PDB: 2KQT) structures.[30] In the determined wild-type structures, the amine points to the C terminus of the channel. For amine **38**, the amino group is oriented toward the walls of the channel and not in the top (N-terminal) or bottom (C-terminal) directions according to the molecular dynamics (MD) results.[8] This result can be explained by the size of the scaffold, as it has a perpendicular dimension of 4.0 Å and a horizontal length of 7.1 Å, which allows the molecule to fit in the channel in the perpendicular direction. For comparison, azapropellane amine **39** did not inhibit the A/M2 channel well, despite the fact that moderate activity against H1N1 was reported.[29] Azapropellane amine **39** has respective dimensions of 4.9 and 5.8 Å in the horizontal and perpendicular directions, and the size of both sides are longer than 4.2 Å (the radius of M2 channel) (Figure 6). The possibility that the amine may point to the N terminus is not ruled out when the amine is attached to other substituents.[6,7] For isoxazole analogue **11**, the adamantane moiety of the compound is bound to the spacious pore between Asn31 and Gly34. When blocking S31N, the aromatic group could face up toward the N terminus based on the solution NMR structure data and modeling analysis.[31]

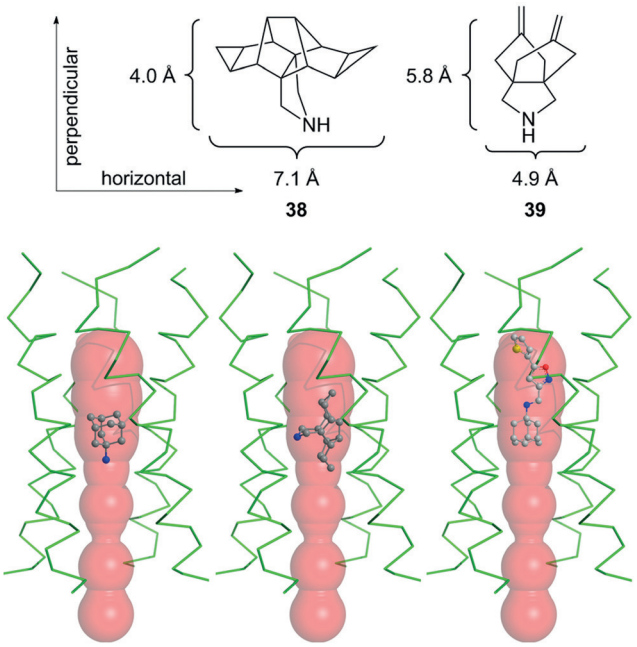


Figure 6. Three orientations of amine compounds have been proposed in the literature: the N atom of the amine is illustrated as a blue sphere, and the pink shadow represents the M2 channel. The amantadine amine **1** (left) points downward to the C terminus, whereas the polycyclic amine **38** is directed toward the side (middle),[8] and the adamantyl secondary amine **11** (right) points toward the N terminus (with S31N M2 channel). Image produced by Mole2 and PyMOL.[31b]

Activities of isocyanide compounds were also studied based on structural information. Considering the structural information from 2KQT, a large space remains between the amine nitrogen atom and the pseudo-center of the four His37 residues. The distance between these constituents is 7.6 Å. From the published SAR results, the M2 channel can only tolerate amino groups with limited lengths. The radius analysis showed that there is an extra channel of ~5 Å for the amantadine amine before the radius of the channel is significantly reduced ("extra space" shown in Figure 7). In many studies, water molecules are present in the top space of the His37 tetramer network. Therefore, a remaining length of ~3 Å is present if one water molecule is included, which is sufficient space for an extra bond or atom. The water molecules were precisely determined to be located on the His47 tetramers by high-resolution X-ray analysis (PDB code: 3LBW).[32] The location of the water molecules on the His47 tetramers could increase the antiviral activity of the isocyanide relative to the corresponding amine by al-

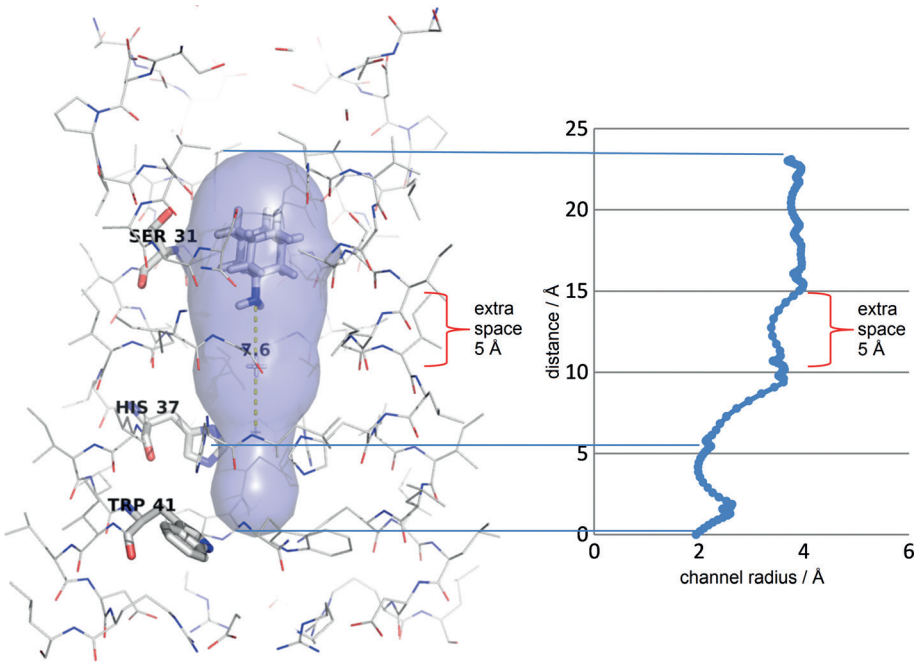


Figure 7. Channel radius distribution of pores in the protein–inhibitor complex; red brackets refer to the extra space for potential inhibitors.

lowing the isocyanide to fit in the channel space when the amine is directed toward the C terminus. No structural information is available from previous studies regarding the interactions between isocyanide or formamide analogues and the M2 protein. Therefore, the mechanism that allows isocyanides to block the channel is similar to the mechanism of amantadine. The activities of the various isocyanide derivatives are discussed based on molecular modeling and docking analysis. Molecular docking was performed to determine the binding details of the channel–compound complex (Supporting Information). The formamide group of **26** and isocyanide group of **27** are oriented toward the viral interior, fitting at the portion of the channel. Compounds **26** and **27** can fit in the channel well, but **29** could not be placed in the M2 channel adequately, with the N≡C group pointing toward the C terminus. This could explain the lack of M2 activity of compound **29**.

Replacement of an isocyanide group with other inactive amine-containing moieties such as branched aliphatic amines, aromatic amines, or amino acid esters, did not promote antiviral activity. Therefore, the conclusion that the isocyanide group does not exhibit antiviral activity on its own can be drawn.

Conclusions

Replacement of the amino group with functional groups such as formamides and isocyanides have shown activity in cell-based viral inhibition and TEVC assays. The present work clearly demonstrates that the isocyanide group can be used to replace the amino group in anti-influenza compounds without causing cytotoxicity. Additionally, amantadine isocyanide **27** exhibited stronger activity than its parent amine. In one case, isocyanide **36** was active against the H5N1 influenza virus, whereas its corresponding amine **19** was not. Our results suggest that isocyanides can be used as a novel replacement in the design of proton channel inhibitors.

Experimental Section

Chemistry

General: Unless stated otherwise, reagents and solvents used in the preparation of the following compounds were purchased from commercial sources (J&K, Aladdin, Adamas-Beta) and used as received. All NMR spectra were recorded at 400 MHz on a Bruker Instruments NMR unless otherwise stated. Chemical shifts are expressed as δ units using tetramethylsilane as the external standard (s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, br: broad peak). TLC analysis was performed using Aldrich 254 nm plates (60 Å, 250 μm) and visualized using UV, PMA, $KMnO_4$, and ninhydrin stains. The purities of test compounds are measured over 95% by qNMR protocol.

Procedure A. Synthesis of formamides: Formic acid (7.0 equiv) and acetic anhydride (6.0 equiv) were allowed to mix for 1 h at RT, after which the appropriate amine (1.0 equiv) was added dropwise to the mixture at 0 °C. The ice bath was removed, and the reaction was stirred at RT until the amine was consumed completely as determined by TLC. The solution was diluted with CH_2Cl_2 (100 mL), washed with water (100 mL), the aqueous phase was then extracted with CH_2Cl_2 (2×100 mL), and the combined organic phases were separated, dried (Na_2SO_4), filtered, and concentrated to give the desired product without purification.

Procedure B. Synthesis of isocyanides: A stirred solution of formamide (1.0 equiv) in CH_2Cl_2 (50 mL) at 0 °C was treated with Et_3N (5.0 equiv), followed by $POCl_3$ (1.0 equiv) dropwise over 15 min. The reaction was allowed to warm to RT while stirring. After 10 min, the reaction was stopped by the addition of water (50 mL), and the organic layer was extracted with CH_2Cl_2 (2×50 mL). The combined organic extracts were dried (Na_2SO_4), filtered and concentrated to give the crude product. Purification by flash column chromatography (PE/EtOAc) gave the desired isocyanide.

***N*-((3*S*,5*S*,7*S*)-Adamantan-1-yl)formamide (26)**: Following procedure A, (3*S*,5*S*,7*S*)-adamantan-1-amine (1 g, 6.61 mmol) gave **26** as an off-white solid (1.12 g, 95%): ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.82 (d, J = 1.8 Hz, 1H), 2.01 (d, J = 11.3 Hz, 6H), 1.91 (s, 6H), 1.75 ppm (d, J = 1.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, one major rotamer): δ = 163.9, 52.1, 47.7, 46.5, 46.2, 39.3, 38.4, 35.2, 28.0, 23.3, 20.7 ppm; GC–MS (EI): m/z = 179.1 $[M]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{17}NO$: 180.1388, found: 180.1376; Purity: >98%.

1-Isocyanoadamantane (27): Following procedure B, *N*-((3*S*,5*S*,7*S*)-adamantan-1-yl)formamide (3 g, 16.73 mmol) gave **27** as an off-

white solid (2.02 g, 78%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.09$ (s, 3H), 2.02 (s, 6H), 1.68 ppm (d, $J = 14.3$ Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 151.6$, 54.2, 43.5 (3C), 35.5 (3C), 28.7 (3C) ppm; MS (EI): $m/z = 161.1$ $[M]^+$; HRMS (ESI): m/z $[M+Na]^+$ calcd for $C_{11}H_{15}N$: 184.1102, found: 184.1124; Purity: >97%.

***N*-((3*S*,5*S*,7*S*)-Adamantan-1-yl)formamide (28)**: Following procedure A, *N*-(1-((3*R*,5*R*,7*R*)-adamantan-1-yl)ethyl) amine (3 g, 13.91 mmol) gave **28** as an off-white oil (1.02 g, 87%): ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.82$ (d, $J = 1.8$ Hz, 1H), 2.01 (d, $J = 11.3$ Hz, 6H), 1.91 (s, 6H), 1.75 ppm (d, $J = 1.7$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$, one major rotamer): $\delta = 160.8$, 51.8, 36.9 (3C), 36.8 (3C), 35.6, 28.2 (3C), 14.5 ppm; MS (EI): $m/z = 207.1$ $[M]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{13}H_{21}NO$: 208.1701, found: 208.1684; Purity: >99%.

(3*R*,5*R*,7*R*)-1-(1-Isocyanoethyl)adamantine (29): Following procedure B, *N*-((3*S*,5*S*,7*S*)-adamantan-1-yl)formamide (1.5 g, 7.24 mmol) gave **29** as a white solid (1.02 g, 74%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.29$–3.17 (m, 1H), 1.73 (d, $J = 12.3$ Hz, 4H), 1.68–1.58 (m, 7H), 1.55 (s, 4H), 1.33–1.22 ppm (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 154.6, 60.6, 37.9 (3C), 36.7 (3C), 35.0, 28.1 (3C), 15.1 ppm; MS (EI): $m/z = 189.10$ $[M]^+$; Purity: >97%.

***N*-((1*R*,2*S*,3*R*,5*S*)-2,6,6-Trimethylbicyclo[3.1.1]heptan-3-yl)formamide (30)**: Following procedure A, (1*R*,2*S*,3*R*,5*S*)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-amine (1 g, 6.52 mmol) gave **30** as a clear colorless oil (1.14 g, 96%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 8.08$ (dd, $J =$ 35.5, 23.6 Hz, 1H), 4.42–4.21 (m, 1H), 2.66–2.55 (m, 1H), 2.43 (dt, $J = 6.0$, 5.6 Hz, 1H), 2.04–1.92 (m, 2H), 1.77 (ddt, $J = 22.7$, 14.0, 7.2 Hz, 3H), 1.59–1.50 (m, 1H), 1.23 (t, $J = 4.3$ Hz, 4H), 1.03 (d, $J =$ 11.1 Hz, 4H), 0.86 ppm (dd, $J = 9.9$, 3.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, one major rotamer): $\delta = 163.9$, 52.1, 47.7, 46.5, 46.2, 38.4, 35.2, 30.0, 23.3, 20.7, 19.8 ppm; MS (EI): $m/z = 182.0$ $[M+H]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{19}NO$: 182.1545, found: 182.1530; Purity: >95%.

(1*R*,2*S*,3*R*,5*S*)-3-Isocyano-2,6,6-trimethylbicyclo[3.1.1]heptane (31): Following procedure B, *N*-((1*R*,2*S*,3*R*,5*S*)-2,6,6-trimethylbicyclo[3.1.1]heptan-3-yl)formamide (1 g, 5.52 mmol) gave **31** as a yellow oil (0.71 g, 74%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.82$ (ddd, $J = 8.2$, 4.6, 2.2 Hz, 1H), 2.63–2.54 (m, 1H), 2.52–2.41 (m, 1H), 2.32 (ddd, $J = 9.4$, 6.1, 2.4 Hz, 1H), 2.18 (s, 1H), 2.08 (dtd, $J = 14.1$, 5.7, 2.7 Hz, 1H), 1.99 (td, $J = 5.6$, 2.7 Hz, 1H), 1.88–1.81 (m, 1H), 1.26–1.17 (m, 6H), 0.98–0.88 ppm (m, 3H).^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 153.3, 47.1, 45.9, 40.9, 38.2, 36.3, 34.3, 27.8, 23.3, 20.5 ppm; GC–MS (CI): $m/z = 162.1$ $[M-H]^-$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{17}N$: 164.1439, found: 164.1506; Purity: >95%.

(1*R*,3*R*,5*R*,7*R*)-2-Isocyanoadamantane (32): Following procedure B, (adamantan-2-yl)formamide (1 g, 5.59 mmol) gave **32** as a white solid (0.54 g, 60%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 3.81$ (s, 1H), 2.22–2.08 (m, 4H), 1.95–1.82 (m, 4H), 1.80–1.57 ppm (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 154.1$, 59.1, 37.1 (2C), 32.7 (2C), 31.2 (3C), 26.6 (2C) ppm; MS (EI): $m/z = 161.1$ $[M]^+$; HRMS (ESI): m/z $[M+Na]^+$ calcd for $C_{11}H_{15}N$: 184.1102, found: 184.1104; Purity: >95%.

2-Isocyano-2,4,4-trimethylpentane (33): Following procedure B, *N*-(2,4,4-trimethylpentan-2-yl)formamide (1 g, 6.37 mmol) gave **33** as a yellow oil (0.556 g, 63%): ^{1}H NMR (400 MHz, DMSO): $\delta = 1.60$–1.57 (m, 2H), 1.46–1.41 (m, 6H), 1.04 ppm (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 152.9$, 56.9, 53.8, 31.8 (2C), 31.6 (3C), 31.0 ppm; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_9H_{18}N$: 140.1439, found: 140.1443; Purity: >96%.

***N*-(4-(*tert*-Butyl)cyclohexyl)formamide (34)**: Following procedure A, *N*-(4-(tert-butyl)cyclohexyl)formamide (5 g, 32.19 mmol) gave **34** as a yellow oil (5.78 g, 98%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.97$ (d, $J = 20.5$ Hz, 2H), 1.83 (dd, $J = 29.3$, 12.4 Hz, 2H), 1.65 (d, $J = 11.9$ Hz, 1H), 1.53 (t, $J = 11.8$ Hz, 1H), 1.10 (m, $J = 32.9$, 8.5 Hz, 4H), 0.85–0.86 ppm (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$, one major rotamer): $\delta = 160.6$, 47.6, 43.1, 33.4 (2C), 30.5 (3C), 27.5 (2C), 21.7 ppm; MS (EI): $m/z = 183.1$ $[M]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{21}NO$: 184.1701, found: 184.1686; Purity: >96%.

1-(*tert*-Butyl)-4-isocyanocyclohexane (35): Following procedure B, *N*-(4-(*tert*-butyl)cyclohexyl)formamide (2 g, 10.91 mmol) gave **35** as a yellow oil (0.97 g, 54%): ^{1}H NMR of 1:1 *trans*/*cis* mixture (400 MHz, $CDCl_3$): $\delta = 2.22$ (d, $J = 12.4$ Hz, 1H), 2.01 (d, $J = 10.9$ Hz, 1H), 1.81 (d, $J = 10.4$ Hz, 1H), 1.67 (d, $J = 10.7$ Hz, 1H), 1.53 (d, $J =$ 11.7 Hz, 1H), 1.44 (t, $J = 11.4$ Hz, 2H), 1.25 (s, 1H), 0.98 (d, $J =$ 10.2 Hz, 2H), 0.90–0.82 ppm (m, 9H, *cis*/*trans* = 1:1); ^{13}C NMR (100 MHz, $CDCl_3$, 1:1 *trans*/*cis* mixture): $\delta = 162.6$, 52.3 and 50.4 (*cis* and *trans* mixture), 47.4 and 46.6 (*cis* and *trans* mixture), 34.1, 32.5 and 32.3 (*cis* and *trans* mixture), 31.4, 27.4 (3C), 25.5, 21.2 (2C) ppm; MS (EI): $m/z = 164.7$ $[M]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{20}N$: 166.1596, found: 166.1586; Purity: >95%.

5-(*tert*-Butyl)-2-isocyano-1,3-dimethylbenzene (36): Following procedure B, *N*-(4-(tert-butyl)-2,6-dimethylphenyl)formamide (200 mg, 0.97 mmol) gave **36** as a white solid (90 mg, 34%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.02$ (s, 2H), 2.34 (s, 6H), 1.21 ppm (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 164.4$, 152.0, 134.3 (2C), 124.8 (3C), 34.6, 31.2 (3C), 19.2 (2C) ppm; MS (EI): $m/z = 205.1$ $[M+H_2O]^+$; HRMS (ESI) m/z $[M+H]^+$ calcd for $C_{13}H_{17}N$: 188.1439, found: 188.1448; Purity: >95%.

(1*R*,3*R*,5*R*,7*S*)-3-Isocyanoadamantan-1-ol (37): Following procedure B, *N*-((1*R*,3*S*,5*R*,7*S*)-3-hydroxyadamantan-1-yl)formamide (2 g, 10.25 mmol) gave **37** as a white solid (1.02 g, 56%): ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.96$ (s, 1H), 2.38 (d, $J = 28.9$ Hz, 4H), 2.14–1.90 (m, 8H), 1.72–1.50 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 159.8$, 79.8, 55.7, 46.8, 42.3 (2C), 39.7 (2C), 34.0, 30.0 (2C) ppm; MS (EI): $m/z = 177.1$ $[M]^+$; HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{11}H_{15}NO$: 178.1232, found: 178.1217; Purity: >96%.

Biological methods

Two-electrode voltage clamp analysis: A/Udorn/72 cDNA, encoding the A/M2 protein, was cloned into a pSUPER vector (a modified version of pGEM-HE). A/M2 V27A and A/M2 S31N single mutants were generated by QuicChange site-directed mutagenesis kit (Stratagene). cRNAs were transcribed in vitro using T7 RNA polymerase (Promega) as described previously.[33] Oocytes were prepared according to standard methods and injected with 50 mL water or 50 nL cRNA (0.8 g L^{-1}) and incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5 with NaOH 5 mM sodium pyruvate, 50 μg mL^{-1} gentamicin). Measurements were performed 2–5 days after injection. Currents were recorded by two-electrode voltage clamp, using the Gene-Clamp 500 amplifier (Axon Instruments) under control of pCLAMP7. Oocytes were perfused at room temperature at a rate of 2 mL min^{-1} in Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $NaNO_3$, 0.71 mM $CaCl_2$, 0.82 mM $MgCl_2$, and 15 mM HEPES for pH 8.5, or 15 mM MES for pH 5.5. Currents were recorded at −20 mV. Compounds were dissolved in DMSO (0.5%) and applied at pH 5.5 at 100 μM when the inward currents reach maximum. The compounds were applied for 2 min and the currents before and after application of drugs were compared. Data were analyzed using ORIGIN7.

Antiviral assay: Monolayers of MDCK cells were seeded in 12-well culture plates and incubated overnight. The cells were washed with phosphate-buffered saline (PBS) twice before being infected with 1 mL per well of a given dilution of the H5N1 virus (influenza strain A/chicken/Hubei/327/2004) in the maintenance medium without trypsin at a multiplicity of infection (MOI) of 0.01. After incubation at 37 °C in 5% CO_2 for 2 h, the medium was removed, and the plates were washed with PBS for three times, after which 1 mL per well of 1.5% agarose overlay (1 mL) containing Eagle's minimal essential medium (MEM), trypsin (2 mg mL^{-1}), and various concentrations of drugs or test compounds were added. Plates were incubated at 37 °C in 5% CO_2 for a further 3–4 days to check for plaque formation. The agars were then carefully removed, and the monolayer of cells was fixed with 10% formalin, stained with crystal violet (0.5%), and the plaques were counted. The percentage of plaque inhibition relative to the infected control (no drug) plates was determined for each drug concentration, and the 50% effective concentration (EC_{50}) was calculated by the Reed–Muench method. All data are obtained for at least three independent experiments.

Plaque-reduction assay: MDCK cells were infected with influenza virus strain A/chicken/Hubei/327/2004 (H5N1) at 0.01 MOI for 2 h at 37 °C. After viral adsorption, the cell monolayer was covered with overlay medium containing compounds and further cultured at 37 °C in 5% CO_2 for 48–72 h. The overlay medium was then removed, the cell monolayer was fixed with 10% formalin, stained with 0.5% crystal violet, and plaques were counted.

Molecular modeling

Tetrameric M2 transmembrane bundle of the influenza A virus (PDB code: 3LBW) was used as model structure. Molecular docking was done using AutoDock 3.0.5. The channel analysis was finished using Mole2 and PyMOL. The measurement of hydrophobic groups was carried out with ChemAxon.

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