New Small Molecule Entry Inhibitors Targeting
Hemagglutinin-mediated Influenza A Virus Fusion

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ABSTRACT

Influenza viruses are a major public health threat worldwide and options for antiviral therapy are limited by the emergence of drug-resistant virus strains. The influenza glycoprotein hemagglutinin (HA) plays critical roles in the early stage of virus infection, including receptor binding and membrane fusion making it a potential target for the development of anti-influenza drugs. Using pseudotype virus based high throughput screens, we have identified several new small molecules capable of inhibiting influenza virus entry. We prioritized two novel inhibitors, MBX2329 and MBX2546, with aminoalkyl phenol ether and sulfonamide scaffolds respectively, that specifically inhibit HA-mediated viral entry. The two compounds (a) are potent ($IC_{50} = 0.3–5.9 \mu M$), (b) are selective ($CC_{50} > 100 \mu M$) with selectivity index (SI) values >20-200 for different influenza strains, (c) inhibit a wide spectrum of influenza A virus that includes the 2009 pandemic influenza A/H1N1/2009, highly pathogenic avian influenza (HPAI) A/H5N1, and oseltamivir resistant A/H1N1 strains, (d) exhibit large volumes of synergy with oseltamivir (36-331 $\mu M^2$ with 95% confidence) and (e) have chemically tractable structures. Mechanism of action studies suggest that both MBX2329 and MBX2546 bind to HA in a non-overlapping manner. Additional results from HA-mediated hemolysis of chicken red blood cells (cRBCs), competition assay with MAb C179 and mutational analysis suggest that the compounds bind in the stem region of the HA trimer and inhibit HA mediated fusion. Therefore, MBX2329 and MBX2546 represent new starting points for chemical optimization and have the potential to provide valuable future therapeutic options and research tools to study the HA mediated entry process.
INTRODUCTION

Influenza A viruses are members of the Orthomyxoviridae family of negative-strand RNA viruses, and are the etiological agents of influenza, a contagious, acute, and febrile respiratory disease (43, 54, 60). Influenza A viruses are responsible for seasonal epidemics and have caused the three pandemics in the 20th century (1918, 1957 and 1968) as well as the 2009 H1N1 pandemic. Wild aquatic birds are the natural reservoir of Influenza A viruses. Pandemics occur when a “new influenza virus” emerges, due to antigenic ‘shift’, to which the human population is immunologically naive (4, 18, 43, 51, 54, 60). Vaccination is the primary strategy for the prevention and control of seasonal influenza. Both inactivated vaccines and the live attenuated vaccine are effective in preventing influenza A virus infections (18); however, the vaccine efficacy can vary depending upon several factors, including the genetic relatedness among viruses used for the vaccine and circulating strains.

Currently, there are two classes of FDA-approved drugs for treatment or chemoprophylaxis of the influenza – matrix protein 2 [M2] inhibitors, amantadine and rimantadine, and the neuraminidase (NA) inhibitors (NAIs), oseltamivir and zanamivir (10-12). The M2 inhibitors block the activity of the ion channel formed by M2, and thereby prevent release of viral genome segments into the cytoplasm (10-12). However, M2 ion channel inhibitors are limited in their clinical utility for treatment of influenza A viruses since all currently circulating influenza A virus strains (including the 2009 pandemic A/H1N1 and the seasonal A/H3N2) are resistant to M2 inhibitors (55). NAIs, such as oseltamivir, bind the NA protein and inhibit its enzymatic activity, thereby inhibiting the efficient release of newly synthesized viruses from infected cells (49, 54). Recently, however, significant levels of oseltamivir-resistant seasonal influenza A(H1) viruses have also been encountered; the resistance has been
associated with a single amino acid change in the viral neuraminidase (H274Y) (3). In 2008, the
CDC reported that the majority of seasonal H1N1 isolates were oseltamivir resistant (19-22).

Although the majority of 2009 H1N1 pandemic isolates remain susceptible to NAIs, the
possibility that the H274Y mutation could appear in the pandemic H1N1 strain and result in an
oseltamivir-resistant virus is a major health concern (6, 27, 48). Therefore new antiviral
strategies, including a focus on different viral targets, or cellular factors or immune-modulating
drugs, are needed. For example, T-705 (favipiravir), an inhibitor of influenza RNA polymerase,
has been identified as a potent anti-influenza agent from in vitro and in vivo preclinical studies
with activity against a range of influenza strains including H5N1 (29, 47).

Viral entry is the first essential step in the viral replication cycle; consequently, blocking
viral entry into the target cell will lead to suppression of viral infectivity and is an attractive
antiviral strategy. In addition, the acute nature of influenza virus infection and the accompanying
cytokine storm (35) makes blocking the viral entry process particularly attractive since it inhibits
influenza-induced cytokine pulmonary immune pathology. Influenza virus protein hemagglutinin
(HA) plays a key role in viral entry. HA is responsible for binding of the virus to host cells and
subsequent membrane fusion within the late endosome (49). It also plays an important role in
host immune responses by harboring the major antigenic sites responsible for the generation of
neutralizing antibodies. Mature HA is a homotrimer, and each monomer is composed of two
disulfide-linked polypeptides, HA1 and HA2, generated by proteolytic cleavage of the primary
translation product HA0 and modification by multiple glycosylations. Most of the HA1 subunit
forms the head region of HA, while the HA2 subunit is the primary component of the stem
region. Following binding, the virus is internalized by endocytosis. Within the low pH (5.0 to 5.5)
environment of the endosome, HA undergoes conformational rearrangements resulting in
exposure of the fusion peptide, which inserts into the endosomal target membrane of the host
cell. After fusion of the viral and endosomal membranes, the viral ribonucleoproteins (RNPs) are released into the cytosol and transported into the nucleus, where replication occurs (49, 50, 54).

To identify potential influenza virus entry inhibitors, we used a high throughput screening assay to screen a chemical compound library composed of over 100,000 unique small molecules, and two novel compounds, MBX2329 and MBX2546, were selected based on their potency and their mechanism of action was characterized. These entry inhibitors may be developed as viable broad-spectrum therapeutic options for influenza virus infection.
MATERIALS AND METHODS

2.1. Cell lines, viruses, and plasmids. 293T, A549, MDCK and BHK cell lines were procured from ATCC. The cell lines were maintained in either DMEM or MEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (50 units/ml).

Influenza A viruses subtypes A/PR/8/34 (H1N1), A/California/10/2009 (H1N1), A/Florida/21/2008 (H1N1-H275Y) [osc-res], A/HongKong (H5N1), A/Texas/12/2007(H3N2), and B/Florida/4/2006 were used in this study. Virus stocks were prepared either in 10-day-old embryonated eggs or in a Madin-Darby canine kidney (MDCK) cell culture.

The work with HPAI A/HongKong (H5N1) strain was carried at the ABSL-3+ select-agent facility at Utah State University. The ABSL-3+ select-agent facility has been inspected and approved by USDA/CDC. Possession and use of Select Agents is monitored by the responsible university biosafety officer. Select Agents are governed by standard laboratory procedures approved by the Institutional Biosafety Committee and access to ABSL-3+ laboratory and Select Agents are restricted to employees that have FBI and USDA clearance and appropriate ABSL3+ training.

Plasmid vectors expressing the hemagglutinin (HA) [H5] gene from a highly pathogenic H5N1 avian influenza virus (Goose/Qinghai/59/05) (16) and the envelope proteins of EBOV Zaire (GenBank accession number L11365) [EBOV-GP] (25), vesicular stomatitis virus (VSV-G) (1), Lassa virus (LASV) (39, 40) were described earlier.
2.2. Mutagenesis of the H5 HA gene. The alanine substitution mutations were introduced between HA1 residues 35-64, and between HA2 residues 1-20, and 75–127 since the variations in these regions appeared to be the basis for dividing HA into two groups as reported earlier (42). All alanine substitution mutations in the H5 HA gene were generated by site-directed mutagenesis with the Quick-Change mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the supplier's protocols. All mutations were confirmed by DNA sequencing of flanking regions. The full length of the HA gene was confirmed by DNA sequencing for those mutants that displayed defective phenotypes.

2.3. Compound library. The chemical library screened represents a broad and well-balanced collection of ~106,000 compounds accumulated over a number of years from a variety of distinct sources. The library achieves broad coverage across property space involving the following chemical descriptors: calculated logarithm of n-octanol/water partition coefficient (ClogP), polar surface area (PSA), globularity (three dimensional structure) and molecular weight (average: 394.5 Da) (42).

2.4. Pseudotyping. Avian influenza pseudotype viruses expressing H5 HA [HIV/HA(H5)] were produced by co-transfecting 12 μg of HA(H5) with 12 μg replication defective HIV vector (pNL4-3-Luc-R–E–) into 293T cells (90% confluent) in 10 cm plates with Lipofectamine 2000 (Invitrogen) as previously described (41). The supernatants containing the pseudotype viruses were collected 48h post-transfection, pooled, clarified from floating cells and cell debris by low-speed centrifugation, and filtered through a 0.45 μm pore-size filter (Nalgene). The culture supernatants containing HIV/HA(H5) were either used immediately or flash frozen in aliquots and stored at -80°C until use. Pseudotype viruses bearing VSV envelope protein (HIV/VSV-G), LASV envelope protein (HIV/LASV-GP), and EBOV envelope protein (HIV/EBOV-GP) were also
prepared in a similar fashion, using the same Env-deficient HIV vector as previously described (2).

2.5. High-throughput screening (HTS) of chemical libraries. HTS of chemical libraries using pseudotype virus was performed essentially as described earlier in 96 well plates (2). The final concentration of test compound was 25 µM while the final concentration of DMSO was maintained at 1% in all wells. Low passage A549 cell monolayers were infected with 100 µl of p24 normalized HIV/HA(H5) virus containing 8µg/ml polybrene in the presence of test compounds. After 2h, the inoculum was removed, the cells were washed briefly, fresh medium was added, and the plates were incubated for 72h. Prior to each screening, each batch of the viral preparation was titrated to determine the amount of virus required to infect the target cells, so that a relatively high luciferase activity could be recorded while still remaining in a linear response range ($10^5$-$10^6$ RLU). Infection was quantified from the luciferase activity of the infected A549 cells using the Britelite Plus™ assay system (Perkin Elmer) in a Wallac EnVision 2102 Multilabel Reader (Perkin Elmer, MA). In the absence of compound, the assay showed an average luciferase signal of $1.2 \times 10^6 \pm 0.6 \times 10^6$ RLU, a signal to background ratio of $>10^3$, and a calculated screening window coefficient (Z’ factor) (63), of $>0.5 \pm 0.2$. The luciferase signal standard error was ± 50%, and greater than 90% inhibition of luciferase activity at 25 µM concentration was used as the criterion for designating a compound as a “hit”. Test compounds were in DMSO solutions with 80 compounds per plate. Controls were also included in each plate; 8 wells for 0% inhibition (DMSO only, maximum signal = positive control) and 8 wells for 100% inhibition (e.g., bafilomycin for HIV/HA(H5), minimum signal = negative control). The percent inhibition was calculated as: $100 \times \left[1 - \frac{\text{Relative Luciferase Unit (RLU) in the presence of compound} - \text{RLU of negative control}}{\text{RLU of positive control (without any inhibitor) - RLU of negative control}}\right]$. 

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2.6. Antiviral assay: Antiviral activity of the influenza inhibitors was evaluated by two methods: enzyme-linked immunosorbent assay (ELISA) and plaque reduction. For the ELISA assay, MDCK cells were plated to be 80% confluent on 96-well plates. Cells were then infected with influenza virus at a virus dilution that would result in 90% CPE after 3 days (MOI=1) from a seed stock of $1 \times 10^6$ pfu/ml in the presence of compounds. The viral replication was determined by measuring the levels of influenza nucleoprotein (NP) using Influenza A NCP ELISA Kit (Photometric) [Virusys Corp]. Percentage of protection was calculated as 

$$1 - \frac{\text{mean of } OD_{450\text{ compound}} - \text{mean of } OD_{450\text{ media}}}{\text{mean of } OD_{450\text{ DMSO}} - \text{mean of } OD_{450\text{ media}}} \times 100\%,$$

where mean of $OD_{450\text{ compound}}$, mean of $OD_{450\text{ media}}$, and mean of $OD_{450\text{ DMSO}}$ are the absorbance of compound-virus-containing samples; the absorbance of no virus control samples; the absorbance of DMSO-virus-containing samples, respectively. $IC_{50}$ is defined as the compound concentration that generates 50% reduction in NP concentration.

The plaque reduction assay was performed following standard protocol (36, 56). Confluent monolayer of MDCK cells were infected with 100 pfu of virus (alone or in presence of compounds). After 1 h of virus adsorption at 37 °C, viral inoculums was replaced by a 50:50 mix of 1% seaplaque agarose (in de-ionized water) and 2× MEM containing 2.5 µg/ml trypsin and compounds at desired concentrations. Plaques were counted after 3-5 days of incubation at 37 °C by visual examination. The $IC_{50}$ was calculated as the compound concentration required to reduce virus plaque numbers by 50%.

2.7. Hemagglutination assay. The hemagglutination assay (HA assay) was performed as previously described (57). 4 mL of influenza A/PR/8/34 (H1N1) virus particles were
concentrated over a 30% Sucrose cushion. The samples were spun at 55,000 rpm for 1 h in a Beckman SW55 rotor at 4°C. Virus pellets were resuspended in 400 µl of PBS. Two-fold serial dilutions were mixed with an equal volume of 0.5% animal erythrocyte suspension (CRBCs; Lampere Biological Laboratories) in a U-bottomed 96-well plate in presence or absence of the compounds (Final concentration 10µM). In addition, we used antiserum to influenza virus H1 HA (ATCC cat# V-301-501-552)” as control. HA titers were recorded after 2h incubation at 4°C. HA assay experiments were repeated at least three times.

2.8. Hemolysis inhibition assay. The procedure to determine the inhibitory effects of the compounds on virus-induced hemolysis at low pH was slightly adapted from Luo et al. (24). cRBCs were washed twice with PBS and re-suspended to make a 2% (v/v) suspension in PBS that was stored at 4°C until use. 100 µl of compound diluted in PBS was mixed with an equal volume of influenza virus A/PR/8/34 (H1N1) strain (10^8 pfu/ml) in a 96-well plate. After incubating virus-compound mixture at room temperature for 30 min, 200 µl of 2% chicken erythrocytes pre-warmed at 37°C was added. The mixture was incubated at 37°C for another 30 minutes. To trigger hemolysis, 100 µl of sodium acetate (0.5 M, pH 5.2) was added and mixed well with erythrocyte suspension. The mixture was incubated at 37°C for 30 min for HA acidification and hemolysis. To separate non-lysed erythrocytes, plates were centrifuged at the end of incubation at 1,200 rpm for 6 min. 300 µl of supernatant was transferred to another flat bottom 96-well plate. OD_{540} was read on a microtiter plate reader. Percentage of protection was calculated as [1- (mean of OD_{540, compound} - mean of OD_{540, PBS})/(mean of OD_{540, DMSO} - mean of OD_{540, PBS})]×100%, where mean of OD_{540, compound}, mean of OD_{540, PBS}, and mean of OD_{540, DMSO} are the absorbance of compound-, virus-containing samples; the absorbance of no virus control samples; the absorbance of DMSO-, virus-containing samples, respectively. IC_{50} is defined as the compound concentration that generates 50% of the maximal protection.
2.9. Cytotoxicity assay. Cell viability was measured to determine the effect of compounds on cellular functions so that a 50% cytotoxicity concentration (CC$_{50}$) could be calculated; the ratio of this value to the IC$_{50}$ is referred to as the selectivity index (SI = CC$_{50}$/IC$_{50}$). Cell viability was determined by measuring the GAPDH level in cell lysate using an AlphaScreen® SureFire® GAPDH Assay kit (Perkin Elmer).

2.10. NMR Spectroscopy. Recombinant H5 HA and NA were obtained from BEI Resources. Monoclonal antibody C179 was obtained from Takara. All NMR experiments were performed on a Bruker 900 MHz spectrometer equipped with a cryogenic probe. WaterLOGSY experiments were performed as previously described (9, 34), with a relaxation delay of 2.5 s, mixing time of 2 s, and 1024 scans (or 5120 for the competition experiments). Saturation Transfer Difference (STD) experiments were performed as previously described (5, 26, 28), with a relaxation delay of 2.5 s, saturation time of 1 s, and 256 scans with "on" resonance saturation at -1.5 ppm and "off" resonance saturation at 30 ppm. Nuclear Overhauser Effect Spectroscopy (NOESY) experiments were performed as previously described (17) with mixing time of 1 s, and 24 scans. Spectra were processed by NMRPipe with a relaxation delay a 5 Hz line broadening and analyzed by NMRDraw (13).

2.11. Synergy Studies. Combined efficacy of the compounds used together with either oseltamivir carboxylate or amantadine was determined by methods reported previously (31-33). Briefly, combinations of drug dilutions were added to 96-well plates containing monolayers of MDCK cells, which were subsequently infected at a low multiplicity of infection. Cell viability was determined at 72 h following infection using Neutral Red. The efficacy of the two individual agents was used to calculate theoretical additive interactions. The calculated additive effects were then subtracted from the observed effects to reveal regions where greater than expected
inhibition was observed. Synergy plots represent the percent inhibition above or below expected (calculated additive inhibition), and are presented as the mean of four replicates at a level of 95% confidence, which eliminates insignificant deviations from the additive surface. The volume under the surface was calculated and used as a quantitative measure of synergy. Synergy volumes of >100 μM²% are generally considered to be significant (37, 38). Potential cytotoxicity was evaluated concurrently in uninfected MDCK cells with the same exposure to compounds to ensure that the antiviral effects and synergistic interactions were specific.
3.1. Identification of new influenza virus entry inhibitors. A chemically diverse small molecule library (106,440 compounds) was screened for inhibitors of the HA mediated entry process using influenza pseudotype virus [HIV/HA(H5)] following previously described methods (2, 57, 58). HIV/HA(H5) contains a ‘HIV core’ with a luciferase reporter gene (deletions in the HIV genome making it replication-deficient), and influenza H5 HA “envelope” protein (16). The inhibitory effect of the small molecules on virus entry was quantified by measuring the decrease of the mean luciferase activity in the presence of the test compounds. A total of 2,038 compounds (2.03% hit rate) produced ≥90% reduction of the mean luciferase activity of the positive control [uninhibited HIV/HA(H5)] at a test concentration of 25 µM. These “hit” compounds included both influenza entry inhibitors and off-target hit compounds that include (i) inhibitors of HIV replication, (ii) inhibitors of luciferase enzyme activity, and (iii) cytotoxic compounds. To specifically identify inhibitors of the HA mediated HIV/HA(H5) entry, we counter screened the “hit compounds” with HIV/VSV-G. HIV/VSV-G has the same HIV backbone but expresses a different envelop protein, VSV-G. The counter screen with HIV/VSV-G eliminated 1897 compounds as off-target hits (Table 1). From the remaining 141 hits, 36 compounds (0.035% hit rate) were chosen based on their low cytotoxicity (CC<sub>50</sub> ≥25 µM) and high antiviral selectivity index (SI) [CC<sub>50</sub>/IC<sub>90</sub> ≥ 10]. Two compounds, MBX2329 (molecular weight: 359.4 Da), an aminoalkyl phenol ether and MBX2546 (molecular weight: 394.4 Da), a sulfonamide, were prioritized based on their inhibitory potency (IC<sub>90</sub> ≤10 µM), and chemical tractability (synthetically accessible, stable structures with drug-like structures (Fig. 1, panel A). MBX2329 and MBX2546 displayed concentration dependent inhibitory activities with HIV/HA(H5) displaying IC<sub>90</sub> values of 8.6 µM and 5.7 µM respectively (Fig. 1, panels B and C).
To evaluate the spectrum of antiviral activity of MBX2329 and MBX2546, both compounds were investigated for inhibition of entry of Lassa virus (LASV) and Ebola virus (EBOV), both of which also bear type 1 envelope proteins similar to that of HA. The pseudotype platform was used because it provided a direct comparison of the activity of MBX2329 and MBX2546 against HIV/LASV-GP, HIV/EBOV-GP and HIV/HA(H5). Compounds MBX2329 and MBX2546 displayed little inhibitory activity against HIV/LASV-GP (IC\textsubscript{90} ~ 100 \mu M), HIV/EBOV-GP (IC\textsubscript{90} > 100 \mu M) or HIV/VSV-G (IC\textsubscript{90}=85->100 \mu M) [Table 2] suggesting that they specifically inhibit the entry of influenza viruses.

### 3.2. MBX2329 and MBX2546 are potent subtype specific inhibitors.

As shown in Table 3 and Figure 2, MBX2329, inhibited influenza A H1N1 virus strains, A/PR/8/34 (H1N1) (Fig. 2, panel A), A/Florida/21/2008 (H1N1-H275Y) [oseltamivir resistant strain] (Fig. 2, panel C), A/Washington/10/2008 (H1N1), and A/California/10/2009 (H1N1) [2009 pandemic strain] strains with IC\textsubscript{50} between 0.29 \mu M and 0.53 \mu M. Similarly, MBX2546 inhibited influenza A H1N1 virus strains A/PR/8/34 (H1N1) and A/Florida/21/2008 (H1N1-H275Y) with IC\textsubscript{50} of 0.3 \mu M and 5.8 \mu M (Fig. 2, panels B and D; Table 3) respectively. MBX2546 also inhibited other H1N1 strains, including A/California/10/2009/H1N1 (2009 pandemic strain) strains with IC\textsubscript{50} between 0.55 \mu M and 1.5 \mu M. Both MBX2329 and MBX2546 inhibited HPAI H5N1, A/Hong Kong/ H5N1 strain with an IC\textsubscript{50} of 5.9 \mu M and 3.6 \mu M, respectively (Table 3).

MBX2329 and MBX2546 displayed significantly less activity against influenza A H3N2 virus strain A/Texas/12/2007 (H3N2) and influenza B virus strain, B/Florida/4/2006 (Table 3). They did not inhibit influenza A H3N2 virus strains, A/Perth/16/2009 (H3N2), A/Victoria/3/75 (H3N2), A/Panama/2007/99 (H3N2), A/Sydney/05/97 (H3N2), A/California/7/04 (H3N2) and
A/Wyoming/03/2003 (H3N2), at the maximum concentration tested (data not shown). MBX2329 and MBX2546 also did not inhibit HIV/HA(H7) infection at 100 µM (Table 3). The 50% cytotoxic concentrations (CC_{50}) for both compounds against MDCK cells were higher than 100 µM. In summary, MBX2329 and MBX2546 specifically inhibit influenza viruses of group 1 HA (H1 and H5 subtype) and not group 2 HA (H3 and H7 subtype).

### 3.3. MBX2329 and MBX2546 bind to the group 1 HA specific conformational epitope in the HA stem region.

Specific inhibition of influenza viruses with group 1 HA by MBX2329 and MBX2546 suggests that they interact with group 1 HA. To verify that HA is the target of the compounds, we investigated the binding of MBX2329 and MBX2546 to recombinant H5 HA (a group 1 HA) using WaterLOGSY (Water Ligand Observed via Gradient Spectroscopy) NMR spectroscopy, which is designed to detect binding of small molecules to large molecular weight targets (9). Recombinant NA was used as the specificity control. In Figure 3, panel A, the upper spectrum corresponds to the 1D NMR spectrum of the downfield region of MBX2329 with the aromatic resonances of the compound denoted by red arrows. The second spectrum corresponds to the WaterLOGSY spectrum observed for MBX2329 in the absence of HA (i.e. a control experiment) and the third spectrum corresponds to the WaterLOGSY spectrum observed for MBX2329 in the presence of H5 HA. The relatively strong positively phased resonances of MBX2329 in the presence of H5 HA indicate that it is binding to HA. Conversely, the absence of the signals in the fourth spectrum, which corresponds to the WaterLOGSY experiment in the presence of NA, suggests that MBX2329 is not binding to NA.

We characterized the binding properties of MBX2546 in a similar manner (Fig. 3, panel B). The upper spectrum corresponds to the 1D NMR spectrum of the downfield region of MBX2546 with the aromatic resonances of the compound denoted by green arrows. The second
spectrum corresponds to the WaterLOGSY spectrum observed for MBX2546 in the absence of HA (i.e. a control experiment) and the third spectrum corresponds to the WaterLOGSY spectrum observed for MBX2546 in the presence of H5 HA. In the presence of H5 HA, the increased intensity of the positively phased resonances of MBX2546 clearly indicates that it is binding to HA. In contrast, in the presence of NA (the bottom spectrum of Fig. 3, panel B) the relatively weak signals, which resemble the no-protein control, suggest that MBX2546 is not binding to NA.

To further determine the region of HA binding of MBX2329 and MBX2546, a WaterLOGSY based competition assay was performed with the monoclonal antibody (C179). C179 was previously shown to bind to a group 1 HA conformational epitope in the stem region formed by amino acid positions 318-322 in HA1 subunit and 47-58 in HA2 (46). Overlap of binding sites would lead to a decrease in the signal of the compounds in the binding assay. As shown in panel C of Figure 3, the addition of an equivalent amount of MAb C179, with respect to HA, significantly decreases the binding of MBX2329. The average reduction of MBX2329 resonance intensities was 52% ±11% (n=8; p=0.001), suggesting that the antibody is displacing the compound. Likewise, an equivalent amount of MAb C179 similarly decreases the binding of MBX2546 to H5 HA observed by WaterLOGSY (Fig. 3, panel D) with the average reduction 81% ± 5% (n=8; p=0.0001) of the MBX2546 resonance intensities. The results once again suggest that the antibody is also displacing MBX2546. These results are consistent with the notion that both inhibitors inhibit influenza A viruses with group 1 HA by binding to a group 1 HA specific conformational epitope in the stem region of HA.

We next investigated whether MBX2329 and MBX2546 bind to overlapping sites on HA using a WaterLOGSY based competition assay. In this experiment, equal amounts of compound
were added in the WaterLOGSY experiment with the notion that overlapping binding sites would lead to a decrease in the signal of one or both compounds. Based on the absence of the change in signal intensity (Fig. 4, panel A), the compounds appear to be able to simultaneously bind to HA suggesting that they bind to different regions of HA. Furthermore, we used a NOESY experiment to examine the relative proximity of the compound binding sites. In this experiment the absence of intermolecular NOEs in the presence of H5 HA (Fig. 4, panel B) further suggests that MBX2329 and MBX2546 bind to sites that are >6 Å apart. We further examined the binding epitopes of MBX2329 and MBX2546 to H5 HA by an STD NMR experiment, which identifies 1H in closest proximity to the protein surface (5, 28). The relative STD for the interaction between MBX2329 and H5 HA is shown in Figure 4, panel C. In this representation, the red spheres and numbers represent the relative STD signal. The contact is relatively uniform with the aromatic ring of MBX2329 in closest contact. Similarly, Figure 4 panel D, shows the relative STD for the interaction between MBX2546 and H5 HA. In this representation, the green spheres and numbers represent the relative STD signal (the grey spheres represent STD signals that are too weak to be quantified). In this case, the contact is relatively non-uniform with the most important contacts in the center of the molecule.

The conformational antigenic epitope (amino acid positions 318-322 in HA1 subunit and 47-58 in HA2) recognized by MAb C179 is in the stem region and is specific for influenza virus with group 1 HA. To further explore the potential roles of amino acids in the group 1 HA specific region in binding to MBX2329 and MBX2546, we generated HIV/HA(H5) carrying single amino acid substitutions by alanine scanning mutagenesis and examined the sensitivity of these mutants to MBX2329 and MBX2546. As shown in Fig. 4, panels E, at 6.25 µM, the HIV/HA(H5) mutants bearing either a K51A mutation in HA1 or a G16A mutation in HA2 were less susceptible to inhibition by MBX2329 suggesting that MBX2329 interacts with the amino acid
residues K51 in HA1 and G16 in HA2. Interestingly none of the mutants was resistant to MBX2546 at the same concentration, further suggesting that they bind at different sites near the conformational epitope recognized by C179. Therefore, taken together, we conclude that (i) both MBX2329 and MBX2546 bind to HA near the group 1 HA specific conformational epitope in the HA stem region; and (ii) the binding sites are not overlapping in the stem region of trimeric HA. The results are consistent with the notion that these inhibitors block HA-mediated membrane fusion (see below).

3.4. MBX2329 and MBX2546 inhibit HA mediated fusion. Based on the results described above, both MBX2329 and MBX2546 bind to the HA stem region which is the target for group 1HA specific antibodies that disrupt the HA mediated membrane fusion process (7, 8, 15, 30, 46, 59). To investigate the role of these inhibitors in HA-mediated fusion, we performed hemagglutination and hemolysis assays.

The hemagglutination assay was performed to determine whether MBX2329 and MBX2546 prevented the binding of virus with cell surface receptors containing SA. Briefly, ten-fold serial dilutions of concentrated influenza A/PR/8/34 (H1N1) virus particles were mixed with chicken RBCs (cRBCs) using virus-only wells without inhibitor as positive controls and wells lacking both virus and inhibitor as the negative control. In addition, we used the antiserum to influenza virus H1 HA (ATCC cat# V-301-501-552)” at two different dilutions (1:10 and 1:25) as controls. The results of the hemagglutination experiment in the presence of either MBX2329 or MBX2546 were similar to the positive control (without any compound) as shown in Figure 5, panel A. Therefore, the results suggest that neither MBX2329 nor MBX2546 inhibits the binding of influenza virus to cRBCs.
The hemolysis assay was performed using the influenza A virus A/PR/8/34 (H1N1) to determine the effect of MBX2329 and MBX2546 on fusion. To trigger hemolysis, the virus-cell suspension was acidified (pH: 5.2) briefly to initiate HA conformational changes that lyse cRBCs to release hemoglobin. **Wells lacking the virus was used control to determine the effect of compounds on cRBCs.** Both MBX2329 and MBX2546 inhibited the acid-induced hemolysis in a dose dependent manner (Fig. 5, panel B) with IC$_{50}$ values of 2.1 µM and 1.56 µM for MBX2329 and MBX2546 respectively. Therefore taken together, the results indicate that MBX2329 and MBX2546 inhibit fusion of the virus with the endosomal membrane. Here also we used “antiserum to influenza virus H1 HA” as control (data not shown).

### 3.5. MBX2329 and MBX2546 exhibit strong synergy with oseltamivir.

Finally, the synergistic efficacy of MBX2329 and MBX2546 in combination with oseltamivir or amantadine was evaluated using influenza A(H1N1) virus strain A/California/10/2009 following previously described methods (32, 33). Both MBX2329 and MBX2546 in combination with oseltamivir displayed marked synergistic inhibition of influenza virus infection (331 ± 112 µM$^{2\%}$ for MBX2329 and 36 ± 2.8 µM$^{2\%}$ for MBX2546) as shown by plotting concentration vs. synergy (Figure 6, panels A and B). The large volumes of synergy produced by the combination were statistically significant as indicated by the values at the 95% confidence level (Table 4).

Cytotoxicity was also evaluated with the same experimental design used for the combined efficacy study to evaluate synergistic cytotoxicity. These studies used the same MDCK cell monolayers with the same drug exposures as were used for the antiviral studies. At the concentrations used in these studies, no significant cytotoxicity was observed with either oseltamivir, MBX2329 or MBX2546 (data not shown). Strikingly, the observed synergy was
restricted to the combination of the HA inhibitors and oseltamivir; no significant synergy was observed with the combination of HA inhibitors and amantadine (Table 4).
HA plays an important role in the early stages of viral infection by facilitating influenza virus entry into host cells by controlling two critical aspects of entry: receptor binding and membrane fusion. In this study, we describe two small molecules, MBX2329 and MBX2546, with aminoalkyl phenol ether and aminoacetamide sulfonamide scaffolds, respectively, that inhibit multiple influenza A viruses including the 2009 pandemic influenza A/H1N1, high pathogenic avian influenza (HPAI) A/H5N1, and oseltamivir resistant A/H1N1 strains, in a potent (IC\textsubscript{50} = 0.47–5.8 µM), and selective (CC\textsubscript{50} >100 µM) manner in vitro. Mechanistic studies indicate that these compounds bind to a conserved epitope in the HA stem region, which has been implicated in the HA-mediated membrane fusion process (Fig. 7). Furthermore, we have demonstrated that these inhibitors are highly synergistic with oseltamivir. Therefore, further optimization of these small molecule inhibitors as potential therapeutic agents, either individually or in combination with the existing anti-influenza virus treatments appears warranted.

MBX2329 and MBX2546 were selected as HA specific entry inhibitors from a library of >100,000 small molecules because they are (i) potent (IC\textsubscript{90} of 8.6 µM and 5.7 µM respectively), (ii) selective (CC\textsubscript{50} >100 µM), yielding selectivity index (SI) >20-200, and (ii) chemically tractable (synthetically accessible, stable structures with drug-like properties) (see Fig. 1, panels A, B and C). Considering the overall similarity between class I envelope proteins, we tested the activity of the two hit compounds against a series of prototypic RNA viruses bearing class I fusion proteins and influenza strains from different subtypes. In contrast to the potent inhibition of HIV/HA(H5), MBX2329 and MBX2546 were inactive against HIV/LASV-GP, and HIV/EBOV-GP [see Table 2] demonstrating the specificity of the two inhibitors for influenza virus. MBX2329 and MBX2546 strongly inhibited influenza viruses with H1, and H5 subtypes (see Table 3). Both of these subtypes are members of the group 1 HA, one of the two groups of the influenza A virus HA.
Both groups have very similar overall structures—the HA1 forming a membrane-distal domain that contains the receptor-binding subdomains and the HA2 polypeptide that forms the fusion subdomain, and the stem of the trimers (55). However, the rotation of the membrane-distal subdomains relative to the central stem varied between the different HAs and the variation is the basis for dividing HA into two groups. 

The broad activity of MBX2329 and MBX2546 against influenza viruses with group 1 HA concomitant with the lack of inhibition of influenza A viruses with group 2 HA (H3, and H7 subtypes) provided the first clue that the compounds interact with the conserved conformational epitope in the stem region of group 1 HA that is involved in fusion.

Several lines of evidence confirmed that MBX2329 and MBX2546 bind to the group 1 HA specific conserved epitope in the HA stem that is involved in fusion. First, the binding of MBX2329 and MBX2546 to H5 HA, and lack of apparent binding to N1 NA by WaterLOGSY NMR spectroscopy binding studies suggest that the compounds bind specifically to HA (see Fig 3, Panel A and B). Second, both MBX2329 and MBX2546 compete with MAb C179 (see Fig 3, Panel C). MAb C179 binds to the HA stem recognizing a unique group 1 HA epitope. The antibody interacts with residues from the N- and C-terminal regions of HA1 (residues 38, 40, 42, 291 to 293, and 318) and the N-terminal portion of HA2 (residues 18 to 21, 38, 41 to 43, 45, 46, 52, and 56), including helix A (14) as shown schematically in Figure 7. Therefore the competition suggests that both the compounds bind to this conserved epitope. An alternative explanation may be that binding of the MAb C179 antibody could result in a small conformational change that displaces the compound regardless of whether it binds in the region of the epitope. However, the structure of C179 in complex with a H2 HA, (belonging to group 1 HA like H5 HA), suggests that binding of C179 does not cause any conformational changes within HA (14). Therefore, the overlap between the compound and MAb C179 binding sites remains the most plausible explanation. Furthermore, the G16A mutation in HA2 rendered the
HIV/HA(H5) viruses resistant to MBX2329. The amino acid residue G16 of HA2 is located very close to the conformational antigenic epitope for MAb C179. Third, the compounds have no effect on HA mediated hemagglutination of cRBCs suggesting that they do not inhibit binding of HA to SA residues (see Fig. 5, panel A). Strikingly, as shown in panel B of Figure 5, both MBX2329 and MBX2546 blocked hemolysis in a low pH environment in a dose-dependent manner. Since virus absorption and HA conformational changes are two key events required for hemolysis, the results suggest that MBX2329 and MBX2546 interfere with the fusion step during viral entry. Interestingly, MAb C179 also has been shown to have no effect on virus attachment (46) but acts by blocking membrane fusion. Therefore, these results support the hypothesis that both MBX2329 and MBX2546 bind in the highly conserved epitope of group 1 HA that is involved in fusion.

The known structural features of HA also support the conclusion that MBX2329 and MBX2546 inhibit the HA mediated fusion by binding to this conserved epitope of HA. Structural differences among HA are mainly located in three domains. The first domain is the antigenic region on the surface of the HA1 globular heads. This domain is the most variable region and is primarily responsible for the antigenic differences among the HA subtypes, and binding to this domain does not affect virus entry. The second domain is the receptor binding domain that is involved in the interaction with cell surface SAs. Since MBX2329 and MBX2546 do not block receptor binding measured by the hemagglutination assay (see Fig. 5, panel A), they are unlikely to bind in the receptor binding domain. The third region is in the stem region near the hydrophobic pocket that contains the fusion peptide at the N terminus of the HA2. In addition to C179, several novel group 1 HA specific human antibodies, CR6261 and F10 (15, 30, 53, 59) have been discovered. These antibodies have very similar patterns of reactivity and neutralization when tested and they compete with C179. Like C179, CR6261 and F10 exhibit
broad activity against group 1 HAs, including the H1, H2, H5, and H9 subtypes. Crystal structures of C179 bound to H2 HA, CR6261 bound to H1 and H5 HA, and F10 bound to H5 HA revealed that these antibodies recognize conserved epitopes in the stem region of HA (7, 30). The epitope lies close to the virus membrane, and consists of an α-helix from HA2 and adjacent loops derived from HA1 (see Fig. 7). The binding of C179, CR6261 and F10 inhibit key conformational changes in the HA that drive the fusion of the viral and endosomal membranes.

Since both MBX2329 and MBX2546 compete with C179, we hypothesize that a similar interaction between the compounds and amino acid residues in this conserved epitope result in inhibition of key conformational changes in the HA that drive fusion. Clearly, further structural studies are needed to investigate these possibilities.

Several small molecule inhibitors that block the HA mediated fusion process have been identified (23, 24, 36, 44, 45, 56), and all of them display subtype-dependent activities. Importantly, both MBX2329 and MBX2546 exhibit increased potencies compared to these previously described antivirals. Like MBX2329 and MBX2546, these inhibitors appear to bind directly to HA and block the HA conformational change. These inhibitors include BMY 27709 (24), 180299 (a podocarpic acid derivative) (52), tert-butyl hydroquinone (TBHQ) (45), a series of N-substituted piperidine derivatives (36), \textit{N}-(1-thia-4-azaspiro[4.5]decan-4-yl)carboxamides (56) and stachyflin (61, 62). The \textit{N}-(1-thia-4-azaspiro[4.5]decan-4-yl)carboxamide compounds and TBHQ target influenza A/H3 viruses while 180299 displayed only strain specific inhibition of influenza A/H1 viruses. Interestingly, the piperidines exhibited higher potency against influenza A/H1 viruses with mutated M2 than with wild-type virus suggesting the possibility of a different mechanism of action, which would preclude them from being used clinically. Moreover, except for TBHQ, the binding sites of these inhibitors on HA are unknown, although drug resistance profiling and other studies suggest that these molecules bind to HA2 protein. These studies,
together with the results described in this report, demonstrate the feasibility and potential of using small molecules as entry inhibitors to block infection of influenza viruses.

Interestingly, competition studies showed that MBX2329 and MBX2546 do not compete with each other (see Fig. 4, panel A) and appear to simultaneously bind to different regions of HA. These results were further supported by the NOESY experiment where the absence of signal in the NOESY experiment suggested that the relative proximity of the binding sites for MBX2329 and MBX2546 on HA are >6 Å apart (see Fig. 4, panel B). It should be noted that while the absence of signals in the NOESY experiment does not prove that the compounds bind to distant sites; it is nevertheless consistent with that notion. This hypothesis is also supported by the studies on the resistant mutants generated in the current study. HIV/HA(H5) mutants with the K51A mutation in HA1 or the G16A mutation in HA2 rendered the viruses resistant to MBX2329 but not to MBX2546. This result is again consistent with the postulate that they bind at different sites. The STD NMR data provides further input on their binding interaction. The most important interactions of MBX2329 with H5 HA in the bound state occur through the aromatic ring (see Fig. 4, panel C). The smaller interactions of the aliphatic ring imply that this moiety could be modified somewhat (see Fig. 4, panel C). Interestingly, the most important contacts of MBX2546 with H5 HA were with the anilide aromatic ring in the center of the molecule (see Fig. 4, panel D). The STD NMR data also suggests that the two methyl groups at the 3-, and 5-positions of the second aromatic ring of MBX2546 are relatively distant from the HA(H5) protein surface and hence could be removed or modified to increase solubility or make new types of contacts.

The exact mechanism by which MBX2329/MBX2546 inhibits the low-pH-induced fusion of to the group 1 HA with endosomal membrane needs further investigation. Based on the
studies with MAb CR6261, most of the amino acid residues in this conserved epitope are solvent exposed in the prefusion state and accessible to antibodies. Interestingly many of the epitope amino acid residues are also accessible to interactions in the post-fusion state due to functional constraints on the protein sequence. We propose that both MBX2329 and MBX2546 interact with this highly conserved epitope in both the pre-fusion and post-fusion states, and inhibit key conformational changes in the HA that drive the fusion of the viral and endosomal membranes. This results in blocking of the viral RNA from the endosome and presumably the virus particle is degraded in the lysosome.

Another interesting aspect of the antiviral activity of MBX2329 and MBX2546 is the synergistic inhibition of viral replication in combination with oseltamivir (see Fig. 5 panels A and B). No significant synergy was observed with the combination of HA inhibitors and amantadine (see Table 4). To be viable, influenza virus strains must exhibit a balance between HA mediated entry process (early step) and NA mediated egress activity (late step). Therefore, observed synergy was likely due to the simultaneous disruption of NA mediated egress activities by oseltamivir (late step) and HA mediated entry activity by MBX2329/MBX2546 (early step). In contrast, both MBX2329/MBX2546 and amantadine act during the virus entry process and do not produce a synergistic increase in the antiviral activity. We are aware that the 2009 pandemic influenza A virus strain A/California/10/2009 (H1N1) is resistant to the amantadines. The synergistic effect is the increase in activity above the theoretical additive interactions calculated from the concentration-response curves of the single agent. Therefore, it is possible to calculate the synergy between MBX2329/MBX2546 and amantadine even though the strains are resistant to it. Moreover, previous reports (32) have shown that amantadine resistant influenza virus displayed synergy when amantadine was used in combination with oseltamivir and ribavirin. Further characterization of their combined efficacy with oseltamivir will be evaluated in
oseltamivir resistant strains of H1N1 influenza virus since these drug resistant strains sometimes represent the majority of all H1N1 isolates circulating in the population. For full coverage of influenza strains in the clinic, it may be necessary to combine two or more different types of small molecule inhibitors or antibodies against HA for a full coverage of the anti-influenza spectrum for potential clinical use.

In summary, we have identified two novel influenza inhibitors, MBX2329 and MBX2546, that could serve as starting points for the development of a therapeutic agent and can also be used as chemical tools for exploring the molecular mechanism of the low-pH induced HA conformational change.
ACKNOWLEDGMENTS

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Fig. 1. Inhibition of HIV/HA(H5) by MBX2329 and MBX2546. Panel A. Structure of MBX2329 and MBX2546. Chemical structures and molecular weights of MBX2329 and MBX2546 are shown. MBX2329 and MBX2546 with aminoalkyl phenol ether and aminoacetamide sulfonamide scaffolds respectively, were prioritized based on potency and selectivity against HIV/HA(H5). Panels B and C. The inhibitory effect of compounds MBX2329 (panel B) and MBX2546 (panel C) on HIV/HA(H5) infectivity was investigated using A549 cells as described in the Materials and Methods section. Three independent experiments were performed to determine the effect of compounds.

Fig. 2: Influenza virus inhibitory spectrum of MBX2329 and MBX2546. Panels A and B, The dose-dependent inhibitory effects of MBX2329 (Panels A) and MBX2546 (Panels B) on infection of influenza A virus vaccine strain, A/PR/8/34 (H1N1) and of MBX2329 (Panels C) and MBX2546 (Panels D) on infection of oseltamivir resistant influenza A (H1N1) virus strain A/Florida/21/2008 (H1N1-H275Y) on MDCK cells. MOI of 1.0 was used for infection. Three independent experiments were performed to determine the effect of compounds.

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Competition WaterLOGSY of MBX2329 (panel C) and MBX2546 (panel D) binding to H5 HA in the absence (upper spectrum) and the presence (lower spectrum) of MAb C179. For this set of experiments, the conditions were 20 µM MBX2329 or 10 µM MBX2546, +/- 0.2 µM HA, +/- 0.4 µM C179 in 50 mM PBS/pH 7.3 at 25°C using a 900 MHz spectrometer with a mixing time of 2s.

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Hemagglutination assay using cRBCS. Following incubation of MBX2546 or MBX2329 or antiserum to influenza virus H1 HA (ATCC cat# V-301-501-552) with anti-Influenza A virus strain A/PR8/34 (H1N1), a suspension of freshly prepared cRBCs was added and inhibition of hemagglutination was investigated as described in Materials and Methods. Three independent experiments were performed to determine the effect of compounds. Panel B. Inhibition of HA-mediated hemolysis of cRBCs by MBX2546 and MBX2329. Following incubation of influenza A virus A/PR/8/34 (H1N1) with MBX2546, or MBX2329, a suspension of freshly prepared chicken erythrocytes was added and the degree of hemolysis (y axis) was detected at pH 5.2 by measuring OD_{540} as described in Materials and Methods. Wells lacking the virus was used control to determine the effect of compounds on cRBCs.

Fig. 6. Synergy of double combinations of oseltamivir and MBX2329 or MBX2546 carboxylate against 2009 pandemic influenza A virus strain A/California/10/2009 (H1N1).
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**Fig. 7**
HIV/HA(H5) and HIV/VSV-G were generated by transfection of 293T cells with pNL4.3.Luc.R–E– as the HIV-I expression vector and with HA(H5) or VSV-G, respectively.

1897 primary hits inhibited HIV/VSV-G by more than 90% at 25µM, whereas the RLU values of the controls varied by ≤20%.

CC<sub>50</sub> values were determined by measuring the endogenous GAPDH in cellular lysates using “AlphaScreen® SureFire® GAPDH assay” (Perkin Elmer).

<table>
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<th>Compounds Screened</th>
<th>Number of</th>
<th>Specific Hits displaying SI&gt;10 and CC&lt;sub&gt;50&lt;/sub&gt; &gt; 25µM&lt;sup&gt;c&lt;/sup&gt;</th>
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<td></td>
<td>Primary hits (≥90% inhibition of HIV/HA(H5) in the HTS screen) [%]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Primary hits displaying ≥25% inhibition of HIV/VSV-G [%]&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>106,440</td>
<td>2038 (2.03)</td>
<td>1897 (1.84)</td>
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<sup>a</sup> HIV/HA(H5) and HIV/VSV-G were generated by transfection of 293T cells with pNL4.3.Luc.R–E– as the HIV-I expression vector and with HA(H5) or VSV-G, respectively.

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<sup>c</sup> CC<sub>50</sub> values were determined by measuring the endogenous GAPDH in cellular lysates using “AlphaScreen® SureFire® GAPDH assay” (Perkin Elmer).
Table 2: Specificity of MBX 2329 and MBX 2546

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<th>Cmpd#</th>
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<th>IC\textsubscript{90} (µM)</th>
<th>HIV/HA (H5)\textsuperscript{a, b}</th>
<th>HIV/ EBOV-GP\textsuperscript{a, b}</th>
<th>HIV/LASV-GP\textsuperscript{a, b}</th>
<th>HIV/VSV-G\textsuperscript{a, b}</th>
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<td>98.2</td>
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\textsuperscript{a} Pseudotype virus was generated by cotransfection of 293T cells with pNL4.3.Luc.R–E– and the respective envelope glycoproteins

\textsuperscript{b} Pseudotype virus host was 293T cells; cytotoxicity (CC\textsubscript{50}) for both compounds in 293T cells was >100 µM
Table 3: Activity of MBX 2329 and MBX2546 against different influenza virus subtypes\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Cmpd#</th>
<th>Structure</th>
<th>IC\textsubscript{50} (µM)</th>
<th>IC\textsubscript{90} (µM)</th>
<th>IC\textsubscript{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBX2329</td>
<td><img src="image" alt="Structure" /></td>
<td>0.45</td>
<td>0.29</td>
<td>0.3</td>
</tr>
<tr>
<td>MBX2546</td>
<td><img src="image" alt="Structure" /></td>
<td>0.30</td>
<td>1.5</td>
<td>5.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Infectious virus host was MDCK cells; pseudotype virus host was A549 cells.

\textsuperscript{b} Cytotoxicity (CC\textsubscript{50}) of all two compounds vs. MDCK and A549 cells was >100 µM in one or more of the four testing laboratories.
Table 4. Combined efficacy of HA inhibitors with oseltamivir or amantadine against Influenza A/California/10/2009

<table>
<thead>
<tr>
<th>Comp#</th>
<th>Structure</th>
<th>Synergy $^a$ (µM%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>oseltamivir (95%)</td>
</tr>
<tr>
<td>MBX 2329</td>
<td><img src="image" alt="Structure" /></td>
<td>331 ± 112</td>
</tr>
<tr>
<td>MBX 2546</td>
<td><img src="image" alt="Structure" /></td>
<td>36 ± 2.8</td>
</tr>
</tbody>
</table>

a. The efficacy of the compounds was determined in combination with oseltamivir or amantadine.
b. Volumes shown represent a minimal estimate of synergy at the 95% confidence level.