Published in final edited form as:

ACS Infect Dis. 2017 May 12; 3(5): 330–335. doi:10.1021/acsinfecdis.6b00194.

Molecular Mechanism Underlying the Action of the Influenza A Virus Fusion Inhibitor MBX2546

Arnab Basu^{1,*}, Gloria Komazin Meredith¹, Courtney McCarthy¹, Aleksandar Antanasijevic², Steven C. Cardinale¹, Rama K. Mishra³, Dale L. Barnard⁴, Michael Caffrey², Lijun Rong⁵, and Terry L. Bowlin¹

¹Microbiotix Inc., Worcester, MA 01605, USA

²Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60612, USA

³Center for Molecular Innovation and Drug Discovery, Northwestern University, Evanston, IL 60208, USA

⁴Institute for Antiviral Research, Utah State University, Logan, UT 84322, USA

⁵Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA

Abstract

Influenza A virus envelop protein, Hemagglutinin (HA), plays important roles in viral entry. We previously have reported that MBX2546, a novel influenza A virus inhibitor, binds to HA and inhibits HA mediated membrane fusion. In this report, we show that (i) both binding and stabilization of HA by MBX2546 is required for inhibition of viral infection, and (ii) binding of HA by MBX2546 represses the low pH induced conformational change of the HA which is a prerequisite for membrane fusion. Mutations in MBX2546 resistant influenza A/PR/8/34 (H1N1) viruses are mapped in the HA stem region near the amino terminus of HA2. Finally, we have modeled the binding site of MBX2546 using molecular dynamics and find that the resulting structure is in good agreement with our results. Together these studies underscore the importance of the HA stem loop region as a potential target for therapeutic intervention.

Graphical Abstract

Keywords

Influenza A virus; inhibitor; endosome; hemagglutinin; fusion; virus entry

Influenza A virus contains 3 membrane proteins: hemagglutinin (HA), neuraminidase (NA), and the proton channel (M2). HA plays a critical role in influenza infection. HA mediated entry occurs through binding to receptor and conformational changes that result in fusion of the viral and target cell membranes. M2 transfers protons into the infecting virus in endosomes, and at acidic pH the matrix protein, M1, is dissociated from the genome–transcriptase complex, resulting in the release of the viral genome following fusion. At the end of infection, NA cleaves sialic acid residues from virus and cell glycoconjugates to ensure release of newly-made viruses from infected cells^{1–4}. Both M2 and NA are targets of anti-influenza drugs. However, NA inhibitors, zanamivir and oseltamivir are the only drugs currently recommended for clinical use by the CDC^{5–8}. Sporadic oseltamivir resistances are observed among currently circulating influenza A viruses^{9–11}. Taken together, these facts emphasize the need for new influenza-specific antiviral drugs with novel mechanisms of action.

The critical role of HA in influenza entry renders it an attractive target for therapeutics^{6,12–18}. Based on amino acid sequence, and structure, HA is classified into 2 phylogenetic groups, with H1 and H5 as examples of Group 1 HA, and H3 and H7 as examples of Group 2 HA^{1,4,16,19}. We have identified MBX2546, a group 1 (H1 and H5 subtypes) HA specific inhibitor of influenza A virus *in vitro*¹³. MBX2546 inhibits viral fusion with the endosomal membrane¹³. However, we do not know the mechanism by which

MBX2546 inhibits HA mediated fusion. This knowledge gap encouraged us to further study the mechanism of MBX2546 mediated inhibition of HA fusion.

To understand the difference in MBX2546 activity vs Group 1 and 2 HA, we first investigated the binding of MBX2546 to recombinant H5 HA (group 1 HA) and H3 HA (Group 2 HA) using WaterLOGSY (Water Ligand Observed via Gradient Spectroscopy) NMR as described previously^{12,13}. Previously, we used this technique to show binding of MBX2546 to recombinant H5 HA but not to recombinant NA¹³. The recombinant HA of H5 subtype was generated as previously described²⁰ and HA H3 subtype was obtained from Dr Ian A Wilson's laboratory, Scripps research Institute²¹. The first spectrum corresponds to the 1D NMR spectrum of the downfield region of MBX2546 with the aromatic resonances of the compound denoted by arrows while the second spectrum corresponds to the WaterLOGSY spectrum observed for MBX2546 in the absence of HA (i.e. a control experiment). The third and fourth spectrum corresponds to the WaterLOGSY spectrum observed for MBX2546 in the presence of H5 HA (group 1 HA) and H3 (group 2 HA) respectively (Figure 1, Panel A). The positively phased resonances of MBX2546 with respect to the control, suggest similar levels of binding for the two HAs under the conditions tested. The result is surprising given that MBX2546 does not inhibit influenza A virus with H3 HA¹³. As reported previously, we have observed that subtle chemical modification on either of the aromatic rings of MBX2546 result in inactive compounds¹³. Therefore, the specificity of the WaterLOGSY NMR assay was investigated using MBX2598, an inactive analog MBX2546. MBX2546 and MBX2598 have similar structure, where one of the benzene rings of MBX2546 is replaced with a pyridine ring (Table 1). We investigated binding of MBX2598 to H5 and H3 HA by NMR WaterLOGSY experiments. Our study showed that MBX2598 does not bind to either H5 HA or H3 HA (data not shown). Moreover, we have previously shown that MBX2546 binds specifically to h5 HA but not to recombinant NA¹³ by WaterLOGSY NMR under similar conditions¹³. NA was used as a control in this experiment too and MBX2546 did not bind to NA under similar experimental condition. Therefore, the possibility of non specific interaction was ruled out. Since MBX2546 does not inhibit influenza A virus of H3 HA¹³, the NMR WaterLOGSY experiments suggest that binding to the HA is, by itself, not sufficient for inhibition.

We next investigated the binding of MBX2546 with H1 and H3 HAs using a thermal shift assay at neutral pH following previously described methods¹⁶. The recombinant HA of H1 and H3 subtype were obtained Dr Ian A Wilson's laboratory, Scripps research Institute²¹. Stability curves and melting temperatures (T_m), were obtained with different concentrations of MBX2546 and H1 or H5 (both representative of group 1 HA) or H3 HA (group 2) (Figure 1, panels B and C) by tracking the fluorescence intensity of SYPRO Orange using a LightCycler® 480 System.. MBX2546 displayed a dose dependent shift in the T_m for H1 HA (Figure 1, panel B). An approximately 5°C upward shift in T_m for H1 HA was observed in the presence of 100 μ M of MBX2546 (Figure 1, panel B), demonstrating stabilization of H1 HA at neutral pH by MBX2546 binding. Likewise, ~5°C upward shift in T_m at 100 μ M of MBX2546 was observed with H5 HA (data not shown). In contrast, no detectable shift in T_m for H3 HA was observed in the presence of MBX2546 (Figure 2, panel C). An apparent dissociation constant (K_d) was calculated as 5.3 ± 0.2 μ M from the curves by plotting T_m against MBX2546 concentration (Figure 2, panel D). We again investigated the T_m of H1

HA in presence of MBX2598 by thermal shift assay. As expected, no shift in the T_m for H1 HA was observed, indicating that the compound did not stabilize the group 1 HA protein (Figure 2, panel E). The lack of thermal shift suggests that MBX2598 did not stabilize the H1 HA protein. The subtle modification in the aromatic ring of MBX2546 completely abolishes its activity on H1 and H5 HA. Therefore, taken together, the NMR WaterLOGSY binding assay and the thermal shift assay suggest that binding of MBX2546 to H3 HA is specific but it but lacks the necessary aspects of the interaction to needed to stabilize the prefusion form of H3 HA under the experimental conditions.

HA mediated membrane fusion is initiated by the transition of HA from the non fusogenic to the fusogenic state at low pH and can be blocked if this transition is inhibited^{1,22,23}. The non-fusogenic form of HA in the viral membrane is resistant to trypsin digestion. Once exposed to a low pH, the HA trimer refolds and becomes susceptible to trypsin^{14,16}. We performed a trypsin digestion assay to investigate whether MBX2546 blocks the low pH induced conformational change required for fusion following previously described methods¹⁷. Influenza virus A/PR/8/34 (H1N1) was used for the assay. The viruses were first incubated with 5 and 10 μ M of MBX2546 at 37°C for 15 min followed by an acid shock and incubation 10 mg/ml trypsin (Sigma) at 37°C for 1 h. The reaction was then terminated and samples run on a 10% Tris-glycine SDS-polyacrylamide gel and western blot using anti-HA1 antibody. H1 HA was protected against trypsin digest in the presence of 5 μ M and 10 μ M of MBX2546 (Figure. 2, panel A) suggesting that MBX2546 inhibited the low-pH-induced conformational change. To rule out the inhibitory activity of MBX2546 on trypsin, we investigated the activity of trypsin in presence of 10 μ M of MBX2546 using Trypsin Activity Assay Kit (Colorimetric) (Abcam). MBX2546 was found to have no effect on the activity of trypsin. Moreover, H3 HA was susceptible to trypsin digest when pre-incubated with 10 μ M of MBX2546 (data not shown). We do not know, however, whether MBX2546 prevents the native HA conformation from undergoing any change at low pH, or whether initial fusion intermediates of HA do form but are inhibited from undergoing the irreversible transition. Further elucidation of the structure stabilized by MBX2546 at low pH by using monoclonal antibodies specific for various forms of HA, X ray crystallography, or other techniques should provide insights into these questions. Interestingly, HA was susceptible to trypsin digest when pre incubated with 0.3 μ M of MBX2546 (data not shown). >16 fold higher concentrations of MBX2546 was required for inhibition of the low pH induced conformational change of HA in the trypsin susceptibility assay (see Figure 2, panel A), than the IC_{50} (0.3 ± 0.2 NM) of influenza A/PR8/34 (H1N1) virus in tissue culture. The exact reasons are not clear. One explanation may be that for membrane fusion, it has been estimated that at least three or four^{1,23–27} HA trimers are required for fusion pore formation and that even more may be needed for merging of smaller pores to a large one²⁷. Accordingly, only partial inhibition of the HA may be sufficient to block viral infection. The trypsin susceptibility assays examine each HA molecule separately, and thus total inhibition in these assays may require increased compound concentration.

To better understand where MBX2546 binds to the group 1 HA, we selected multiple MBX2546 resistant (MBX2546^r) viruses by *in vitro* serial passages in MDCK cells in the presence of increasing concentrations of MBX2546, starting with the IC_{50} concentration (0.3 μ M) as described previously¹⁷. Viral replication was monitored by observation of the

cytopathic effect (CPE) on MDCK cells. The virus was harvested after CPE reached 100 % and supernatant was used to infect a new flask at which time the drug concentration was doubled. This was repeated until drug concentration reached 2.4 μ M. The viruses were purified with two rounds of plaque purification in the presence of the compound. Drug sensitivity from each passage was determined by the plaque reduction assay with MDCK cells. Viral RNA was isolated using QIAmp Viral RNA Mini Kit (Qiagen). SuperScript III One Step RT-PCR kit (Invitrogen) was used to amplify HA using 5' - AGCAAAAGCAGGGGAAAATAAAAACAACC-3' and 5' - AGTAGAAACAAGGGTGTTCCTCATATC-3' primers and was sequenced at Sequegen. Four independent MBX2546^r virus clones were selected and then plaque purified and amplified in MDCK cells for biological characterization. Sequences comparison of the HA genes of WT and MBX2546^r A/PR8/34(H1N1) viruses identified four amino acid substitutions [N50D (HA2), S226P(HA1), T33A(HA2) and P294L(HA1)] in HA as shown in Figure 2, panel B. Interestingly, the N50D mutation was isolated from two different clonal populations. One clonal population contained the double mutation T33A and P294L. The resistant clones were designated as N50D, S226P, and T33A P294L. All the mutations (except the S226P) are in the stem region of HA (Figure 2, panel B). All three MBX2546^r clones were less susceptible to inhibition by MBX2546 suggesting that interactions with these amino acid residues are important for the ability of MBX2546 to inhibit fusion of HA (Figure 2, panel C). The IC₅₀ value MBX2546^r A/PR8/34 mutants were at least one log higher than the IC₅₀ value of wt influenza A/PR8/34(H1N1) virus ($\sim 0.3 \pm 0.2$ μ M) (Figure 2, panel C). The mutations in the MBX2546^r A/PR8/34 mutants support our earlier conclusion that MBX2546 binds to the HA stem region based on the competition of the MAbs C179¹³. MAb C179 exhibits broad activity against group 1 HAs, that includes the H1, H2, H5, and H9 subtypes. The two groups of HA have similar overall structures except in the stem region where the rotation of the membrane distal subdomains relative to the central stem varies between the two groups. Crystal structures of C179 bound to H2 HA, revealed that the antibody recognizes a conserved epitope in the stem region of HA that lies close to the virus membrane, and consists of an α helix from HA2 and adjacent loops derived from HA1^{28,29}. The binding of the MAb C179, to group I HA inhibits key conformational changes in the HA that drive the fusion of the viral and endosomal membranes. The trypsin protection assay (Figure 2, panel A) shows that MBX2546 inhibits conformational change from non fusogenic to fusogenic form and supports our hypothesis that MBX2546 binds at the similar position in the stem region and prevents change in HA conformation. The S226P is located in the top globular domain of HA (Figure 2, panel B). We do not know whether it affects the sensitivity to MBX2546. However, many of the epitopes from the non fusogenic state are also accessible to interactions in the post fusion state due to functional constraints on the protein sequence. Clearly, further structural studies are needed to investigate these possibilities. Interestingly, we found that MBX2546^r A/PR8/34 viruses retained their fitness of replication (Figure 2, panel D) in MDCK cells at an MOI of 1. Interestingly, the mutants grew at reduced rate during the first 24h of replication.

We next analyzed the simulated binding pose of MBX2546 with the H5 HA stem loop region obtained from 10 ns MDS. We considered the apo crystal structure of the H5 (PDB accession code:2FK0). The site identification module (SITE ID) of the Tripos molecular

modeling package¹² was used to identify the potential small molecule ligand binding sites in the crystal structure. Note that the docking followed by MDS was performed without any input from the mutagenesis data. To preposition the ligand MBX2546 in the ligand binding site, we used the FlexiDock docking package implemented in the SYBYL-X interface of Tripos software. FlexiDock is a restricted induced fit docking tool that works in a torsional space, keeping the bond lengths and the angles constant while allowing the amino acids interacting with the ligand to be flexible during the docking process. The FlexiDock engine generated 20 different poses of the binding ligands. The energetically most favorable pose of MBX2546 obtained from FlexiDock was considered for further modeling studies. The molecular dynamics simulations (MDS) in the Optimized Potentials for Liquid Simulations (OPLS2005) force field was used to carry out the MDS of the H5 and MBX2546 complex structures. All MDS computations were carried out in MacroModel9.8 implemented in Schrodinger software suite¹² in one NPT ensemble (constant pressure and temperature). In the MacroModel dynamics panel, stochastic dynamics were chosen as it includes random forces that stimulate the buffering of a system by solvent molecules. To constrain the bond lengths to the original values, the “SHAKE” option was selected. The simulation of the complex was carried out at 300 K with a time step of 1.5 fs and equilibrium time of 1 ps. The MD simulation was run for 1, 5, and 10 ns recording the energies and the trajectories of the system. The plot of the potential energy versus the time at 10 ns revealed that the system had attained an equilibrium condition. The MBX2546 ligand binding site is found to be in a location similar to that of the MBX2329 site within the stem loop¹² (Figure 3). The binding pose of MBX2546 in the HA stem region is shown in Figure 3. We believe that MBX2546 interacts with the side chains of HA2 N95, HA2 L98, and HA2 L99. However, further structural studies are needed to identify them. We are working to co-crystallize H1/H5 HA and MBX2546 to answer the question in collaboration with Dr. Michael Caffrey's Laboratory at the University of Illinois, Chicago.

In summary, we hypothesize that MBX2546 targets HA mediated viral host membrane fusion by directly binding to the stem region of the HA protein. MBX2546 stabilizes Group 1 HA to prevent the acid induced conformational change that is required for fusion. We believe that the interactions between MBX2546 and the HA alone is not sufficient to inhibit virus infection since MBX2546 to both H1 and H3 subtype of HA (Figure. 1, panels A). A positive shift in the melting temperature (T_m) suggests an enhanced ligand induced stabilization of H1 HA upon binding with MBX2546 (Figure. 1, panels B). Therefore, the most novel finding in this manuscript is that binding and inhibition can be separated for MBX2546. However, it is not exactly clear how MBX2546 prevents the native HA trimer from undergoing the low pH induced conformational change, which could occur through a number of mechanisms. Being subtype specific may reduce the chances of developing MBX2546 as therapeutic. However, subtype specificity appears to be a hallmark of small molecule fusion inhibitors that target the stem region of the HA protein^{12–17}. It is likely that future influenza therapies will use a combination of virus specific compounds to increase efficacy and decrease incidents of viral resistance, much like HAART for treatment of HIV infections. Therefore, it is possible that two subtype specific fusion inhibitors could be paired with a drug targeting a different stage of the viral life cycle, such as oseltamivir, to produce an effective triple therapy.

Acknowledgments

We thank Dr Ian A. Wilson from the Scripps Research Institute for providing the recombinant H1 and H3 HA protein. This research was supported by DHHS/NIH grants 2R44AI072861 and 1R21AI101676 01. We also thank Ms. Debra Mills and Peter Nash from Microbiotix for some virus titration assays.

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Figure 1. Specificity of binding to group 1 HA by MBX2546

Panel A. Detection of binding of MBX2546 to recombinant H5 HA and H3 HA by WaterLOGSY NMR. Arrows represent the aromatic peaks of MBX2546. For this set of experiments, the conditions were 50 μM MBX2546, $\pm 0.2 \mu\text{M}$ HA in 20 mM PBS/pH 7.2 at 25°C using a 900 MHz spectrometer with a mixing time of 2s. **Panels B and C.** Representative graph describing MBX2546 concentration dependent stabilization of the T_m of H1 HA (**Panel B**) and H3 HA (**panel C**). The change in RFU against temperature, demonstrating the thermostabilization of HA is plotted and the change in T_m in the presence and absence of ligand is calculated. The concentrations of the MBX2546 indicated in each graph. The data are the average of 2 separate acquisitions. **Panel D. T_m versus compound concentration standard curve.** The ligand bound T_m values for each compound concentration were plotted with respect to compound concentration. The binding affinity of MBX2546 for H1 and H3 HA was calculated from the curve. **Panel E.** Representative graph

describing the absence of concentration dependent stabilization effects of MBX2598 on the T_m of H1 HA. The data are the average of 2 separate acquisitions.

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Figure 2. MBX2546 binds to stem region of HA. Panel A. Inhibition of the HA susceptibility to trypsin digestion by MBX2546

The non fusogenic form of HA protein is resistant to trypsin digestion. Once exposed to a low pH, the HA trimer changes its conformation and becomes susceptible to trypsin. Influenza A/PR/8/34 (H1N1) virus was incubated at 37°C for 15 min in the presence of 5 μ M and 10 μ M of MBX2546, and the pH was lowered to 5.0. After neutralization, the mixtures were treated with trypsin as indicated by the “+” sign. The lysates were then run on a 10% SDS PAGE and western blot. **Panel B.** Location of MBX2546 escape mutants in the H5 HA structure. Sites of escape mutants are shown in the space filling representation in red. The K51 site has been presented previously¹³. **Panel C. Characterization of MBX2546-resistant viruses:** The dose dependent inhibitory effect of MBX2546 on the WT influenza A/PR/8/34 (H1N1) virus or its MBX2546^r mutants in MDCK cells. MOI of 1.0 was used for infection. Three independent experiments were performed to determine the susceptibility of WT and MBX2546^r viruses to MBX2546. **Panel D. Fitness of the MBX2546^r mutants.** MDCK cells were infected with WT and MBX2546^r A/PR/8/34/H1N1 viruses for comparison. Viral growth was calculated at different time points by CPE methods. Three independent experiments were performed to determine the viral growth.

Figure 3. Molecular dynamics model of the MBX2546 interaction with H5 HA
Panel A. MBX2546 is shown in a space filling representation in green. Note that only 1 molecule of MBX2546 is modeled to bind to the HA trimer.

Table 1

MBX2546 and its analogs

Compound	Structure	IC ₅₀ (μM) ^a	CC ₅₀ (μM) ^b	SI ^c
MBX2546		0.3	>100	>300
MBX2598		>25	>100	ND ^d

^a IC₅₀ was measured using influenza A virus (A/PR/8/34/H1N1) using MDCK cells by CPE assay^b Cytotoxicity (CC₅₀) of all compounds was measured using MDCK cells by MTS assay^c SI (selectivity index)= IC₅₀/CC₅₀^d ND=Not Determined