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Inhibition of influenza virus infection and hemagglutinin cleavage by the protease inhibitor HAI-2

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ABSTRACT

Influenza virus remains a significant concern to public health, with the continued potential for a high fatality pandemic. Vaccination and antiviral therapeutics are effective measures to circumvent influenza virus infection, however, multiple strains have emerged that are resistant to the antiviral therapeutics currently on the market. With this considered, investigation of alternative antiviral therapeutics is being conducted. One such approach is to inhibit cleavage activation of the influenza virus hemagglutinin (HA), which is an essential step in the viral replication cycle that permits viral-endosome fusion. Therefore, targeting trypsin-like, host proteases responsible for HA cleavage *in vivo* may prove to be an effective therapeutic. Hepatocyte growth factor activator inhibitor 2 (HAI-2) is naturally expressed in the respiratory tract and is a potent inhibitor of trypsin-like serine proteases, some of which have been determined to cleave HA. In this study, we demonstrate that HAI-2 is an effective inhibitor of cleavage of HA from the human-adapted H1 and H3 subtypes. HAI-2 inhibited influenza virus H1N1 infection in cell culture, and HAI-2 administration showed protection in a mouse model of influenza. HAI-2 has the potential to be an effective, alternative antiviral therapeutic for influenza.

1. Introduction

Influenza virus is both an important public health concern as well as a significant economic burden [1]. Influenza virus is divided into A, B, and C types, and influenza A virus is further categorized by the viral surface proteins, resulting in 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes [2]. While many of these viral subtypes remain in the aquatic bird reservoir, certain subtypes have transmitted to humans, with the H1N1 and H3N2 subtypes currently causing seasonal outbreaks. Both vaccination and antiviral treatments are utilized to circumvent seasonal and pandemic outbreaks. Vaccination is an effective measure to prevent influenza outbreaks, but difficulty lies in the rapid production of sufficient amounts of vaccine when antigenic shift or drift of the viral HA and NA has resulted in the emergence of an antigenically distinct virus. In addition to vaccination, M2 ion channel blockers (Symmetrel® and Flumadine®) and neuraminidase inhibitors (Tamiflu® and Relenza®) have been utilized to inhibit influenza infection. While these antivirals can be effective therapeutics, multiple strains of both H1N1 and H3N2 influenza have emerged that are resistant to each of these antivirals [3]. In light of this, alternative inhibitors of influenza replication are being explored. One such target for the development of novel anti-influenza therapeutics is the inhibition of HA cleavage activation by host proteases.

The viral HA is synthesized as a fusion-inactive precursor (HA0) that must be cleaved by host cell proteases in order to fuse with the endosomal membrane during virus entry [4]. For low pathogenicity influenza viruses, such as the human-adapted strains, cleavage occurs at the C-terminal end of an arginine residue (Arg343 in H1 numbering), producing the HA1 and HA2 subunits that remain associated by disulfide bonds [5,6]. Cleavage of the HA precursor into the HA1 and HA2 subunits primes the HA molecule for fusion, where the N-terminal residue of HA2 is the first residue of the fusion peptide. HA cleavage is most likely driven by extracellular or membrane bound, trypsin-like serine proteases. Trypsin is commonly used as model protease in studies of HA cleavage-activation and trypsin-like, host cell proteases such as tryptase Clara, matriptase, plasmin, and some members of the kallikrein and transmembrane serine protease (TMPRSS) families have been found to cleave HA *in vitro and in vivo* [7–14]. HA cleavage activation by these proteases, and potentially others, is thought to be a viable target in the development of anti-influenza therapeutics [15]. The protease inhibitor aprotinin has been explored as an inhibitor of influenza replication, where it appears to be an

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effective antiviral [15,16]. However, it is likely to be somewhat specific for a sub-set of proteases, and with the apparent redundancy of HA cleavage *in vivo*, investigation of additional serine protease inhibitors is needed to expand therapeutic options.

Hepatocyte growth factor activator inhibitor-1 and -2 (HAI-1 and HAI-2) are encoded by the SPINT1 and SPINT2 genes, and are serine protease inhibitors that reside on the plasma membrane of a number of tissues, including the respiratory tract [17]. Both HAI-1 and HAI-2 contain two kunitz-type inhibitor domains that have been found to be potent inhibitors of a number of trypsin-like, serine proteases, including hepatocyte growth factor activator [18–20]. HAI-1 is mainly found complexed with the trypsin-like serine protease matriptase on the plasma membrane, with crystal structures of a complex of HAI1 and matriptase recently determined [21]. Uncertainty still remains in the biological role of HAI-2, but it appears to be similar to HAI-1 with regards to the inhibition of both extracellular and transmembrane proteases [20]. With respect to influenza virus, there is a striking correlation between the proteases found to cleave and activate the viral HA and the proteases inhibited by HAI-2. However, HAI-2 has not been investigated for the potential to inhibit influenza replication as an antiviral treatment.

In this study, we show that HAI-2 has the ability to inhibit both HA cleavage and influenza virus infection, and confirmed its role as a potential therapeutic in a mouse model of influenza.

2. Materials and methods

2.1. Cells, plasmids, viruses, and proteins

293T and MDCK cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Cellgro), and 10 U/ml streptomycin (Cellgro). The plasmid encoding A/PR/8/34 (H1N1) HA was generated by following the methods described in Sun et al. [22]. The plasmid encoding the A/Aichi/2/68 (H3N2) HA was provided by David Steinhauer. Influenza virus, A/PR/8/34 was propagated in eggs and used to produce non-cleaved virus by a single replication round in 293T cells. TPCK-treated trypsin was purchased from Pierce.

2.2. Cloning, expression, and purification of HAI-2

The gene encoding for mouse HAI-2 (MGC-6479) was obtained from the mammalian gene collection [23]. The HAI-2 specific primers (5′-forward-gccagccgagaactagacgtccac; 5′-reverse-tcattttaggcccggggtcagg) were used to amplify the ectodomain of HAI-2, which was cloned into a modified pSUMO vector (provided by Holger Sondermann), and named HAI2-pSUMO. HAI2-pSUMO was transformed into *Escherichia coli*, RIL(DE3) (ArcticExpress) cells and grown in 1 liter of Luria Broth containing 35 μg/ml kanamycin. The culture was induced with 0.2 mM IPTG and incubated overnight at 20 °C. Cells were collected by centrifugation and resuspended in buffer A (50 mM TRIS, 300 mM NaCl, 10 mM imidazole, pH 7.8). The cells were then lysed by sonication and cell debris removed by centrifugation. The bacterial lysate was loaded onto a 7 mL bed volume, Ni^{2+} NTA agarose resin column pre-equilibrated with buffer A, and subsequently washed with buffer B (25 mM TRIS, 150 mM NaCl, 10 mM imidazole, pH 7.8). HAI-2 was eluted with buffer C (25 mM TRIS, 150 mM NaCl, 200 mM imidazole, pH 7.8), and incubated with thrombin overnight at 4 °C in buffer A. HAI-2 was then loaded onto a size-exclusion column equilibrated with GFB buffer (50 mM Tris, 150 mM NaCl). The protein concentration was determined [24], and resulted in a yield of 0.4 mg/L. For studies in mice, purified HAI-2 was then treated to remove bacterial endotoxins using a Detoxi-Gel Endotoxin Removing Column (Thermo Scientific) according to the manufacturer's recommendations.

2.3. HA peptide cleavage inhibition assay

Peptides were designed to mimic the consensus cleavage site region of the HA of the H1 (IPSIQSRGLF) and H3 (VPEKQTRGLF) subtypes. As a FRET pair, MCA (7-methoxycoumarin-4-yl acetyl) was added to the N-terminus, and DNP (N-2,4-dinitrophenyl) was added to the C-terminus (RS Synthesis). HAI-2, trypsin and each peptide were diluted in buffer B, producing a final concentration of 0–1 μM HAI-2, 0.8 nM trypsin, and 100 μM peptide. The reaction was immediately carried out at 37 °C and monitored for cleavage by the change in fluorescence at 390 nm (SpectraMax GeminiXS, Molecular Devices). The IC_{50} value denotes the HAI-2 concentration at which trypsin was inhibited by 50%, as compared to the uninhibited control. The initial rate at each HAI-2 concentration was both plotted and fitted using Origin software (OriginLab Corp.) to determine the IC_{50} values.

2.4. HA cleavage inhibition by Western blot

293T cells were transfected with 1 μg of each HA-expressing plasmid using Lipofectamine 2000 (Invitrogen) for 12 h at 37 °C. 3 μg/ml trypsin along with a concentration range of HAI-2 (0–1 μM) were pre-incubated in buffer A for 10 min at 37 °C, the cells washed with phosphate buffered saline (PBS) and treated with each protease-inhibitor mixture for 45 min at 37 °C. The cells were then processed by cell surface biotinylation in preparation for western blot analysis [22]. Inhibition of HA cleavage was assessed by western blot using anti-A/PR/8/34 (H1) and anti-A/Hong Kong/1/68 (H3) antibodies (NIAID Biodefense & Emerging Infections Research Resource Repository). Western blot images were taken by FujiFilm LAS-3000. The pixel intensity of individual band was measured by Image J, and relative cleavage efficiencies were calculated by the following equation: $(HA_2/HA_0 + HA_2) \times 100\%$.

2.5. Inhibition of viral infection in cell culture

To measure influenza infection of individual cells, non-cleaved A/PR8/34 virus was generated by infection 293T cells. 293T cells were chosen due to their lack of endogenous proteases capable of cleaving HA. The non-cleaved virus was incubated with the inhibitor-protease mixture described above for 20 min at 37 °C. Cleavage of HA was terminated by the addition of 5 μg of trypsin inhibitor (EMD Chemicals). MDCK cells were grown in 24-well plates containing glass cover slips and infected with virus at each HAI-2 concentration and incubated for 5 h at 37 °C. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, washed with PBS and assessed for infection by immunofluorescence using a polyclonal anti-nucleoprotein antibody coupled followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody. The cell nuclei were stained with Hoechst 33258. Cells were imaged on a Nikon E600 epifluorescence microscope using a 20X objective.

To measure influenza replication and spread, egg-derived A/PR8/34 virus (containing cleaved HA) was used to infect MDCK cells at a low multiplicity of infection (approximately 0.01 PFU/cell) in 24-well plates. Trypsin was included in the media to allow virus spread, and samples were treated with each HAI-2 concentration, or were untreated. Media was collected at 48 h, remaining trypsin activity was blocked by the addition of 5 μg of trypsin inhibitor, and supernatant containing virus was transferred to a 96 well plates for virus quantification by hemagglutination assay.

For VSV control experiments, MDCK cells were grown in 24 well plates containing glass cover slips and infected with vesicu-

lar stomatitis virus (VSV, strain Orsay) at each HAI-2 concentration and incubated for 5 h at 37 °C. For HPIV1 experiments, LLC-MK1 cells were grown in 24 well plates containing glass cover slips and infected with human parainfluenza virus type 1 (HPIV-1, strain C35) at each HAI-2 concentration and incubated for 7 days at 37 °C. Cells were incubated in the presence of 1 μg/ml trypsin, with trypsin replenished every 48 h. For analysis, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, washed with PBS and assessed for infection by immunofluorescence using a monoclonal anti-VSV G antibody (P5D4) followed by an Alexa Fluor 488-conjugated anti-mouse secondary antibody, or with a mouse anti-HPIV-I antibody (Millipore) conjugated with FITC. Cell nuclei were stained with DAPI. Cells were imaged on a Nikon E600 epifluorescence microscope using a 20X objective.

2.6. In vivo characterization of HAI-2 in a mouse model of influenza

4-week old, female BALB/c mice were inoculated under mild anesthesia (isoflurane). The inoculums were administered intranasally, with half of total volume per nostril. Infection was performed at time zero with 50 μl of a solution containing 2×10^5 PFU of influenza virus (A/PR/8/34) diluted in PBS. HAI-2 was administered post-influenza inoculation, with the first HAI-2 administration (0.75 mg/kg HAI-2 diluted in 50 μl PBS) at 2 h post infection. Subsequent HAI-2 administrations (0.75 mg/kg HAI-2 diluted in 50 μl PBS) were performed at 12 h intervals during the course of 3 days (total of 6 doses).

The experiment was performed twice with 3 animals per treatment group, with animals housed in different cages depending on the treatment. Mice were monitored twice daily for clinical signs and body weight loss. Animals showing weight loss higher than 20% of initial weight were euthanized. Lung tissue was removed post-mortem and utilized for viral titer determination by plaque assay. Data were analyzed by SPSS software (Version 15.0). All p-values were determined by one-way ANOVA. All work with animals was carried out according to the Cornell University Animal Care and Use program under Animal Welfare Assurance A3347-01, and complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

3. Results

3.1. HAI-2-mediated inhibition of peptide mimics of H1N1 and H3N2 influenza

Inhibition of peptide mimics of the HA cleavage site by HAI-2 was first investigated, using the established activity of trypsin as a surrogate of *in vivo* trypsin-like proteases [25]. HAI-2 activity was competed with HA cleavage site peptide mimics of both the human-adapted H1 and H3 subtypes (A/PR/8/34 and A/Aichi/2/68 respectively). The peptide sequences were designed based on the consensus amino acid sequence of cleavage site region of each subtype. Competition of HAI-2 and each peptide with trypsin resulted in an IC_{50} value of 22.4+/−10 nM for the H1 peptide mimic and 89.9+/−6 nM for the H3 peptide mimic (Fig. 1A and B). While HAI-2 was slightly more active against cleavage of the H1 subtype peptide mimic, potent inhibition was observed against both peptide substrates.

3.2. Inhibition of influenza H1N1 and H3N2 HA cleavage by HAI-2

To determine whether HAI-2 has the ability to inhibit HA cleavage in cell culture, HAI-2 at a concentration range of 0–1 μM was incubated with trypsin, and subsequently incubated with HA-expressing cells. Inhibition of HA cleavage was assessed by western blot analysis, where HA2 bands are not detected well with the anti-sera used, which is selective for the HA1 subunit. Effective inhibition of HA cleavage was observed for both the H1 and H3 subtypes (A/PR/8/34 and A/Aichi/2/68 respectively), where cleavage was virtually abolished at an HAI-2 concentration of 150 nM, and greatly reduced at 50 nM HAI-2 (Fig. 1C and D). Thus, cleavage of the HA from both of the currently circulating human-adapted subtypes is potently inhibited by HAI-2.

3.3. Inhibition of influenza virus infection by HAI-2

As an additional measure of inhibition in cell culture, HAI-2 activity on infectious virus particles was determined, both directly at the level of virus entry, as well as at the level of virus spread. To examine virus entry directly, non-cleaved, inactive influenza A/PR/8/34 (H1N1) was generated by a single round of replication in mammalian cells that are incapable of cleaving HA. Inhibition of viral activation was assessed by treatment of the virus with trypsin and various concentrations of HAI-2, followed by immunofluorescence staining of the viral nucleoprotein in infected cells. Similar to inhibition of HA cleavage observed by western blot analysis, effective inhibition of infection of influenza A/PR/8/34 infection was observed by treatment with HAI-2 (Fig. 2A).

To examine the effects of HAI-2 on influenza virus propagation and spread, influenza A/PR/8/34 (H1N1) was produced in embryonated chicken eggs, yielding virions with cleaved and active HA. These viruses were used to infect MDCK cells at a low multiplicity of infection, and cells were incubated for 48 h to allow virus replication and spread. Media was then harvested and virus titer determined by hemagglutination assay (Fig. 2B). Potent inhibition of influenza A/PR/8/34 infection, in a dose-dependent manner, was observed by treatment with HAI-2. Overall, HAI-2 effectively inhibited influenza virus infection in cell culture.

3.4. Effect of HAI-2 on other enveloped viruses

As a control, we assessed the effect of HAI-2 on the entry of an unrelated virus (vesicular stomatitis virus, VSV) that contained a glycoprotein (G) that is not activated by proteolytic cleavage. HAI-2 treatment had no effect on VSV entry (Fig. 3A). We also assessed the effect of HAI-2 on the entry of a related virus (human parainfluenza virus type 1, HPIV-1) that contains a fusion protein (F) that is believed to be activated by similar proteases to influenza HA. As with influenza infection, HAI-2 treatment inhibited HPIV1 infection in a dose-dependent manner (Fig. 3B).

3.5. Mouse model of inhibition of influenza infection by HAI-2

To characterize the action of HAI-2 *in vivo*, and to further investigate its effectiveness as a therapeutic, we used a mouse model of influenza. To first examine any potential toxicology problems with HAI-2, BALB/c mice were exposed intranasally to SPINT2 at doses of 2.5 mg/kg, 0.75 mg/kg, and 0.25 mg/kg, and body weight and clinical signs monitored for 7 days. Mice were then euthanized and examined by gross and clinical pathology. At the highest dose (2.5 mg/kg), mice lost some weight initially, but recovered and did not show any long-term effects of inhibitor treatment. At the lower doses (0.75 mg/kg, and 0.25 mg/kg) there was no weight loss or other clinical signs of toxicity. For all doses, gross pathology and clinical pathology at 7 days post-exposure were unremarkable There was no adverse effect on the respiratory tract, and both BUN and ATL levels were within normal range, indicating no adverse effect on renal or hepatic function (not shown).

The mouse-adapted influenza virus A/PR8/34 (H1N1) was inoculated intranasally into BALB/c mice, along with 0.75 mg/kg

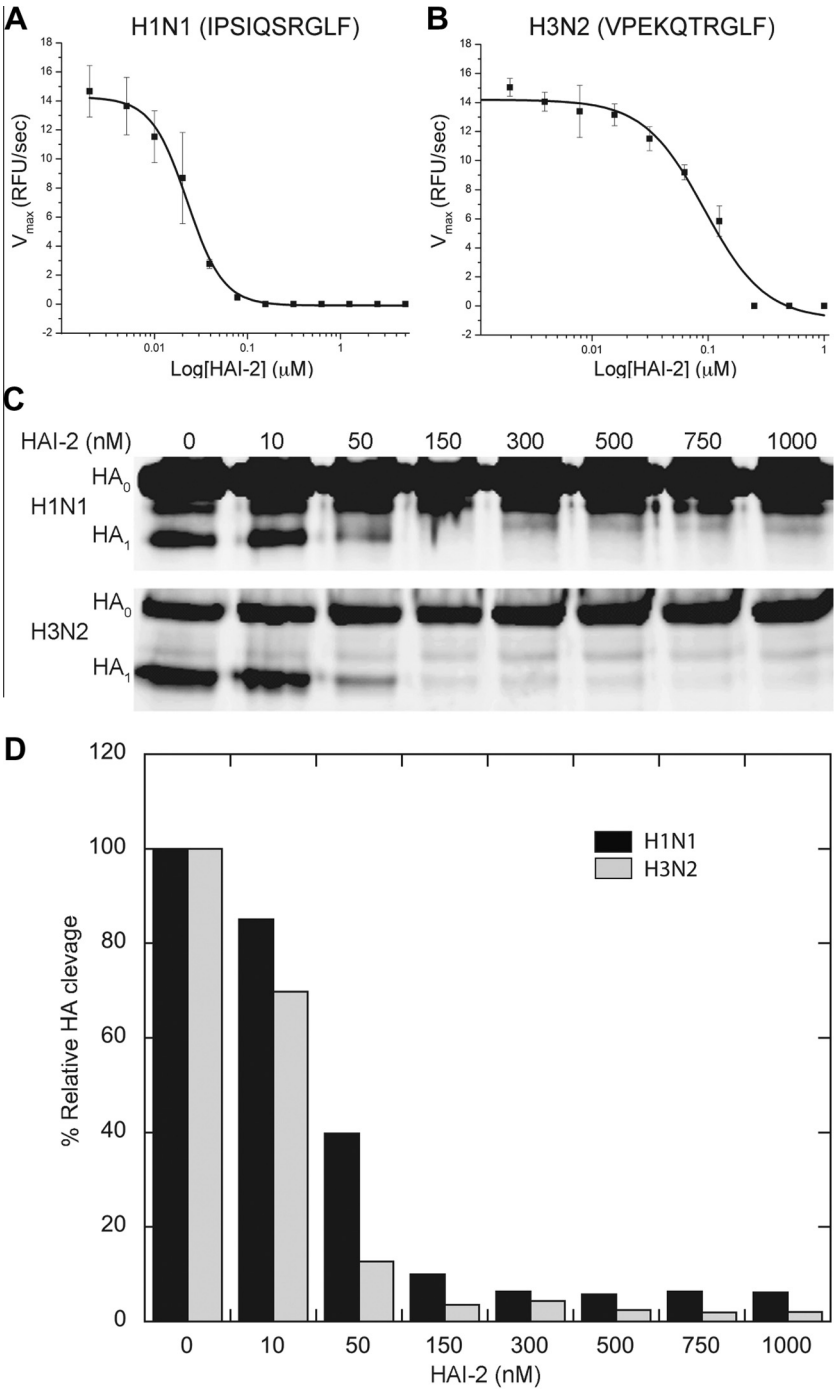

Fig. 1. Inhibition of HA cleavage from the human-adapted subtypes by HAI-2. (A and B) Graph of HAI-2 inhibition of trypsin cleavage of (A) the H1 subtype cleavage site peptide mimic, and (B) the H3 subtype cleavage site peptide mimic. Peptide sequences are indicated. (C) Western blot analysis of HAI-2 inhibition of H1N1 HA and H3N2 HA cleavage by trypsin. (D) Quantification of the percent HA cleavage from (C) using densitometry.

HAI-2. Mice were then monitored twice daily for changes in body weight and survival. In influenza-infected mice HAI-2 treatment showed a significant increase in mouse survival (ANOVA test, p value = 0.03) (Fig. 4) compared to untreated mice, with an approximately one-log drop in virus titer in the mouse lung following HAI-2 treatment (not shown).

4. Discussion

Despite vaccination, influenza remains as both an economic burden, and a significant concern for public health. Antiviral therapeutics have proven to be successful, but are becoming increasingly insensitive due to the emergence of drug-resistant influenza strains. Therefore, alternative therapeutics are currently being explored to combat these newly emerging strains. One such approach is to target host functions, such as proteases, involved in influenza activation. Targeting host functions rather than viral proteins may prove to be more effective, due to the decreased likelihood for the emergence of resistant influenza strains. Broad-spectrum protease inhibitors naturally expressed in the human respiratory tract may prove to be excellent therapeutics due to the ability to inhibit the inbuilt redundancy of HA-cleaving

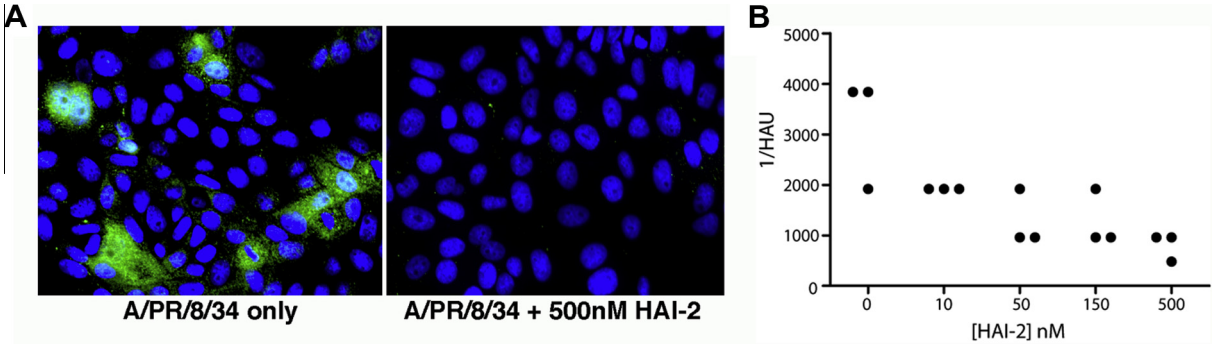

Fig. 2. Inhibition of influenza infection by HAI-2. (A) Immunofluorescence staining of the viral nucleoprotein (NP) of infected cells after trypsin treatment with 500 nM HAI-2, and no protease treatment (mock). The viral NP was labeled with Alexa fluor 488 (green) and the nucleus was stained with DAPI (blue). (B) MDCK cells were infected with a low MOI of influenza virus, and infection was allowed to spread for 48 h in the presence of representative amounts of HAI-2. Virus yield was measured by hemagglutination (HA) assay in triplicate, and individual HA units (HAU) in each condition plotted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

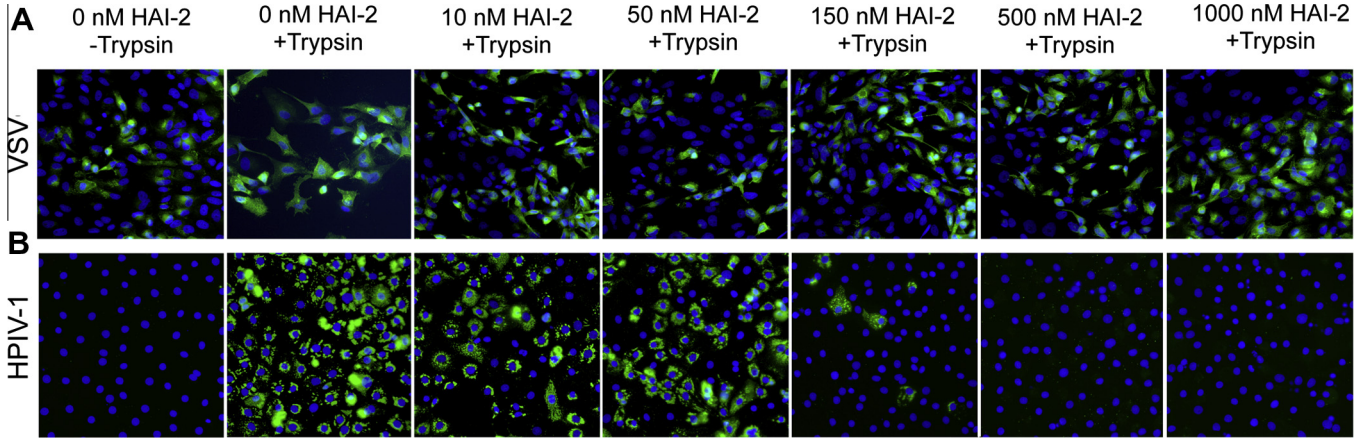

Fig. 3. Effects of HAI-2 on other enveloped viruses. (A) Immunofluorescence staining of the VSV glycoprotein (G) in infected cells after trypsin treatment with representative amounts of HAI-2, and no protease treatment (mock). The viral G protein was labeled with Alexa fluor 488 (green) and the nucleus was stained with DAPI (blue). (B) Immunofluorescence staining of the HPIV-1-infected cells after trypsin treatment with representative amounts of HAI-2, and no protease treatment (mock). HPIV1 is labeled with FITC (green) and the nucleus was stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteases, without the adverse side effects that may be seen by other approaches [26,27].

The serine protease inhibitor, HAI-2 is a highly attractive anti-influenza candidate, due to the high correlation between the proteases that HAI-2 inhibits and the proteases that have been shown to activate HA. To date, the majority of proteases found to cleave HA are of the trypsin-like, serine protease family, and separate studies have determined that a number of these HA-cleaving proteases are inhibited by HAI-2 [20]. Therefore, HA cleavage activation by these host proteases may also be potently inhibited by HAI-2, resulting in inhibition of influenza infection. We therefore investigated whether HAI-2 has the ability to inhibit HA cleavage, using trypsin as an established model protease for influenza infection in cell culture. HAI-2 offered potent inhibition of trypsin cleavage *in vitro,* as determined by nM IC_{50} values. Furthermore, HAI-2 effectively inhibited HA cleavage and viral activation in cell culture models, without affecting infection of an unrelated virus (VSV). HAI-2 treatment showed very limited toxicity when delivered intranasally to mice, and a lethal mouse model of influenza showed significantly increased survival of influenza virus-infected mice. Thus, HAI-2 has the potential to be a viable treatment to circumvent influenza infection. Its effectiveness in a mouse model means that its action is not limited to trypsin, and shows that HAI-2 can act on the trypsin-like proteases present in the respiratory tract and responsible for influenza infection *in vivo*. While a comprehensive evaluation

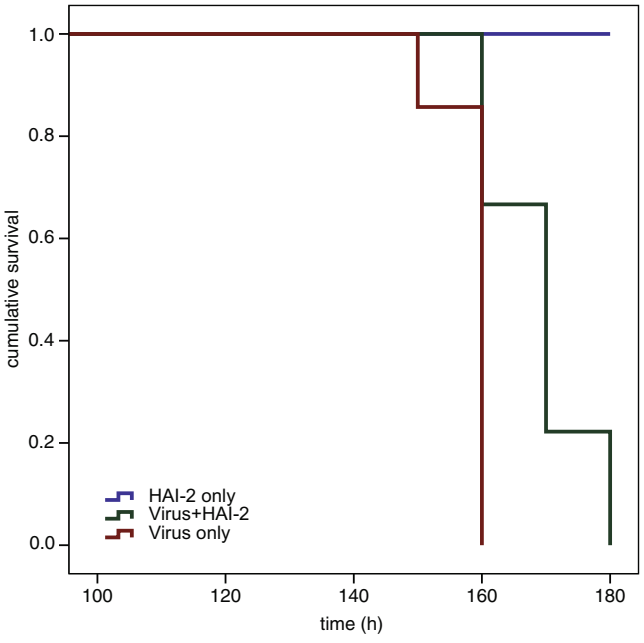

Fig. 4. Inhibition of influenza virus infection by HAI-2 in a mouse model. Mice were treated with HAI-2 alone (0.75 mg/kg), influenza virus A/PR/8/34 + HAI-2 (0.75 mg/kg), or influenza virus A/PR/8/34 alone. Cumulative survival of mice is shown.

of the toxicity and bioavailability of HAI-2 in experimental models of relevance to humans has not yet been carried out, our findings suggest that HAI-2 is a viable candidate for further drug development. Its action against HPIV-1 also suggests it can be broadly acting, and target additional respiratory viruses.

In summary, we have determined that HAI-2 is an inhibitor of HA cleavage during influenza infection. Based on the potent inhibition shown here in biochemical and cell culture assays, we consider that HAI-2 may also be a effective inhibitor of host trypsin-like proteases that cleave HA in the human respiratory tract, and the determination of this is a future aim of this work.

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