PNAS
www.pnas.org

Supplementary Information for

A molecularly engineered antiviral banana lectin inhibits fusion and is efficacious against influenza virus infection in vivo

Evelyn M. Covés-Datson, Steven R. King, Maureen Legendre, Auroni Gupta, Susana M. Chan, Emily Gitlin, Vikram V. Kulkarni, Jezreel Pantaleón García, Donald F. Smee, Elke Lipka, Scott E. Evans, E. Bart Tarbet, Akira Ono, David M. Markovitz

David M. Markovitz
Email: dmarkov@med.umich.edu

This PDF file includes:

Materials and Methods

H84T and D133G BanLec purification. Briefly, to produce non-His-tagged proteins, cleared lysates were added to a Sephadex G-75 column that had been equilibrated with PBS. The column was then washed with PBS until the OD of the flow-through at 280 nm was less than 0.02. Elution of the protein was then performed using 0.2 M methyl α-D-glucopyranoside. For all lectins, endotoxin testing was performed using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific). Endotoxin removal was performed by adding glucose to a 1 M concentration and passing pooled eluates containing protein through Mustang E filters (Pall). Following endotoxin removal to < 0.1 endotoxin units/mg of protein, the Vivaspin 20 centrifugal unit with 3K MWCO was used to remove glucose and concentrate the lectin in water. Protein and endotoxin concentrations were then measured by BCA and LAL assays, respectively.

Cells, viruses, and anti-influenza antibodies. *Viruses:* Influenza A Virus, A/Perth/16/2009 (H3N2), FR-370; Influenza A Virus, A/Florida/3/2006 (H1N1), FR-364; Influenza A Virus, A/Louisiana/08/2013 (H1N1)pdm09, FR-1440; Influenza A Virus, A/Texas/12/2007 (H3N2), FR-1442; and Influenza B Virus, B/Brisbane/60/2008 (Victoria Lineage), FR-177, were obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA and propagated one to two times on MDCK cells, except for when used in lectin blot experiments. Virus titers were determined by $TCID_{50}$ assay and converted to estimated plaque-forming units (PFU) by the conversion $TCID_{50} \approx 0.7$ PFU. *Anti-influenza antibodies:* Mouse Monoclonal Antibody to Recombinant H1 HA from Influenza A/Solomon Islands/3/2006 (H1N1), Clone AT170.119.5, FR-503; Mouse Monoclonal Antibody to Recombinant H3 HA from Influenza A/Perth/16/2009 (H3N2), Clone AT250.656.170, FR-557; and Mouse Monoclonal Antibody to Recombinant HA1 Domain from Influenza B/Brisbane/60/2008 (Victoria Lineage), Clone ATCC 008 3F2, FR-1334, were obtained through the International Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. All antibodies recognize the HA1 subunit of HA.

ELISA. All steps were performed at room temperature. Recombinant H1 HA with Histidine Tag, from Influenza A/California/07/2009 (H1N1)pdm09, FR-559, and recombinant H3 HA with Histidine Tag, from Influenza A/Perth/16/2009 (H3N2), FR-472, were obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. To determine whether H84T binds HA, following overnight incubation with HA, ELISA plates were washed with PBS 5 times before being blocked for 2 h in PBS containing 2% bovine serum albumin. After 5 washes in PBS, H84T or D133G BanLec (0.0001-10 μM) was added to the wells in dilution buffer (PBS containing 0.05% Tween-20 and 2% fetal bovine serum) and incubated for 2 h. Plates were then washed before the addition of rabbit polyclonal anti-BanLec antibody diluted 1:4000 in dilution buffer. Following a 45 min incubation, plates were washed and biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) added to wells at 1:200,000 in dilution buffer for 45 min. Plates were washed again before HRP-conjugated streptavidin was added at 1:300 in dilution buffer for 45 min. After a final set of washes, prepared TMB (BioRad) was added to wells for colorimetric analysis and absorbance measured at 450 nm. To assess whether carbohydrates could inhibit the interaction between H84T BanLec and influenza HA, a fixed concentration of lectin was used (0.1 μM) and was pre-incubated with methyl α-D-mannopyranoside (0.0001-0.1 μM), galactose (0.0001-0.1 μM), or yeast mannan (approximately 0.00001-0.01 μM) before being added to the HA-coated wells. To determine whether human sera contain antibodies against H84T, steps were followed as described above, except that plates were coated with H84T, dilutions of sera were used as primary antibody in place of rabbit polyclonal anti-BanLec antibody, and biotinylated goat anti-human antibody (Jackson ImmunoResearch Laboratories) was used as the secondary antibody. Samples were deidentified prior to use in the study.

Lectin blot analysis. A/Florida/2006 (H1N1), A/Perth/2009 (H3N2), and B/Brisbane/60/2008 virus particles were lysed in RIPA buffer and proteins extracted following manufacturer's instructions. Where indicated, prior to downstream use, proteins were incubated overnight with 10-15 units PNGase F (Roche). Proteins were then prepared in Laemmli buffer (BioRad) containing 5% β-mercaptoethanol and resolved by 4-20% tris-glycine SDS-PAGE. Proteins were transferred to PVDF membranes for downstream processing or stained with GelCode Blue Stain Reagent (Thermo Fisher Scientific) or silver stain according to manufacturer's instructions. PDVF membranes with blotted proteins were blocked in 10% milk for 1 h at room temperature and then incubated in 100 nM H84T or D133G in 5% BSA for 1 h at 4ºC. Three washes in PBS-T were performed before incubation with affinity-purified rabbit α-BanLec primary antibody at 4ºC overnight. Further washes in PBS-T were performed, followed by incubation with goat α-rabbit secondary antibody (Pierce) for 1 h at room temperature. A final set of washes was performed and the blot developed with ECL reagents (Millipore) as described by the manufacturer. The amount of binding of H84T to the PNGase F- versus mock-treated HA1 for each strain was quantitated by comparing the lectin blot band intensity using ImageJ, with mock-treated set at 100%. For the experiments depicted in Fig. 3A-B and Fig. S11, BCA assay (BioRad) was performed according to manufacturer's instructions and equal amounts of protein were loaded into gels; for the experiments depicted in Fig. 3C-D, equal volumes but not amounts of protein were used.

Virus infection and immunofluorescence staining. For immunofluorescence assays, after fixation cells were blocked in 3% BSA/PBS, permeabilized in 0.1% Triton X-100, and incubated with mouse anti-M1 matrix antibody (Takara), rabbit anti-LAMP1 antibody (Abcam), pooled mouse anti-influenza A protein antibodies, or pooled mouse anti-influenza B protein antibodies for 1 hour. Mouse Monoclonal Antibody Influenza Type A (Pool), FR-51 and Mouse Monoclonal Antibody Influenza Type B (Pool), FR-829, were obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. Following 1 wash in 3% BSA/PBS and 2 washes in PBS, cells were incubated with the appropriate secondary antibody, goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 594, for 30 minutes. Slides were mounted using Prolong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). Immunofluorescence was visualized by confocal microscopy on a Nikon A-1 instrument.

Neutral red assay for assessment of anti-influenza B activity. Monolayers of MDCK cells in 96-well plates were treated with 0.1, 1.0, 10, or 100 μg/ml of H84T in MEM medium supplemented with 10 mg/mL EDTA and 1 IU/mL trypsin. Three wells were infected with <100 50% cell culture infectious dose ($CCID_{50}$) virus and two remained uninfected for CC_{50} measurements. Plates were incubated for 3 days at 37ºC with 5% CO_2, then stained with 0.011% neutral red, a vital stain. The incorporated dye was eluted with 50% Sorensen's citrate buffer/50% ethanol and the plate read on a spectrophotometer at 540 nm. ODs were converted to percent cytopathic effect compared with untreated cell controls and normalized to virus controls. The EC_{50} and CC_{50} were then calculated by regression analysis.

In vitro cytotoxicity. For the MTT assay, briefly, cells were incubated for 4 h at 37ºC with 10 μl MTT labeling reagent and resulting formazan salt crystals solubilized with 100 μl 10% SDS in 0.01 M HCl. After overnight incubation at 37ºC to fully solubilize the crystals, absorbance was measured at 595 nm.

Cell-associated virus assay. Primers to influenza A *M1* (forward primer: 5′ AAG ACC AAT CCT GTC ACC TCT GA 3′, reverse primer: 5′ CAA AGC GTC TAC GCT GCA GTC C 3′) and to *GAPDH* (forward primer: 5′ ACA TCG CTC AGA CAC CAT G 3′, reverse primer: 5′ TGT AGT TGA GGT CAA TGA AGG G 3′) were used.

Hemagglutination inhibition assay. Three-fold serial dilutions of H84T were pre-incubated with virus, with turkey red blood cells (RBCs), or with both RBCs and virus before the H84T-virus mixture was

added to RBCs, H84T-RBC mixture was added to virus, or the H84T-virus and H84T-RBC mixtures were added together in 96-well V-bottom plates. Three dilutions of A/WSN/1933 (H1N1) virus were used (1:8, 1:16, and 1:32). Plates were gently tapped to mix and then incubated for 30 min at room temperature. Hemagglutination was assessed visually.

Virus-cell fusion assay. Viral fusion to the cell membrane was triggered at 37ºC using fusion buffer (150 mM NaCl solution with 25 mM HEPES, Corning) at pH ~5.25. Fluorescence dequenching of R18 was monitored on a microplate fluorometer with excitation and emission wavelengths of 560 and 590 nm, respectively. Maximal dequenching was achieved with 1% Triton X-100 set at 100%.

Pharmacokinetics of single dose intraperitoneal (IP) H84T. *Plasma and lung sample processing:* Blood samples (0.5-1 ml) were collected via cardiac puncture tubes and deposited into K3 EDTA tubes and mice euthanized at 0, 5, 15, and 30 minutes and 1, 2, 4, 8, 24, 48, and 72 hours post-dose (n = 4 per time point) for collection of liver and lungs. Blood samples were placed on ice and centrifuged at 2500 rpm for 10 minutes under refrigerated conditions, after which plasma was collected and frozen at -80ºC within 1 hour of collection. Liver and lungs were rinsed with 0.9% sodium chloride, blotted dry, and weighed. Mouse lung tissue was weighed (5 mg) and homogenized in 1mL of PBS with 1M glucose and complete EDTA free protease inhibitor cocktail. The homogenized tissue was incubated on ice for 1 h to allow greater extraction of H84T and centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to clean 1.5 mL vials and cleared by centrifuging again at 15,000 rpm for 10 minutes at 4ºC. *ELISA:* Standard curves were prepared in duplicate in mouse plasma or lung homogenate and quality control samples analyzed in addition to unknown samples. 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific) were coated with 100 ng of capture antigen, HIV gp120, and sealed and incubated overnight at 4ºC. The capture antigen was aspirated and discarded, and the wells were washed 3 times with wash buffer (0.05% Tween-20 in PBS). The wells were blotted dry, filled with blocking buffer (3% BSA in PBS), and incubated at room temperature (RT) for 2 h. After 3 more washes, 100 µl mouse plasma or processed mouse lung tissue supernatant were added to each well and the plate was incubated at RT for 1 h on a mini blot rocker. The wells were washed with wash buffer 6 times and the primary antibody (rabbit polyclonal anti-BanLec at 1:25,000) in blocking buffer was added and incubated at RT for 1 h on a mini blot rocker. The wells were then washed with wash buffer 8 times and secondary antibody (biotinylated goat anti-rabbit at 2 µg/ml) in blocking buffer was added and incubated for 30 min at RT on a mini blot rocker. The wells were washed with wash buffer 8 times and detection antibody (high sensitivity HRP-conjugated streptavidin, 1:200 for plasma and 1:400 for lung) was added and incubated at room temperature for another 30 min on a mini blot rocker. After a final set of 8 washes, the substrate-chromogen TMB was added and incubated at room temperature for 10-20 min in the dark. The reaction was stopped by the addition of stopping solution (0.5M H_2SO_4) and read at 450 nm on a plate reader. The following PK parameters were calculated using the Phoenix WinNonLin Non-compartmental Analysis program, version 6.4.0.768: maximum concentration, C_{max} (ng/ml or ng/g); time at which maximum concentration occurs, T_{max} (hrs); area under the curve, AUC (ng*hrs/ml); half-life, $t_{1/2}$ (hrs).

Distribution of single dose intraperitoneal (IP) H84T. Mice were culled at 4 time points post injection (6h, 12h, 24h, and 48h) and lungs, liver, kidneys, heart, spleen, and brain harvested and snap frozen. Frozen organs were homogenized on dry ice by mortar and pestle and protein extraction performed in the presence of protease inhibitors. Protein extracts were separated by 4-20% SDS-PAGE and immunoblotted using rabbit anti-BanLec polyclonal antibody and HRP-conjugated anti-rabbit secondary antibody.

Therapeutic efficacy of systemic H84T against influenza in BALB/c mice. *Animals:* Female 18-20 g BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). *Virus:* Recombinant influenza A/WSN/33 (H1N1) virus containing the HA gene of influenza A/New Caledonia/20/99 (H1N1) was generated by reverse genetics. The Asp225Gly mutation was introduced in the HA gene encoding the HA1 subunit by PCR based site-directed mutagenesis by Dr. Larissa Gubareva (CDC, Atlanta, GA). The

recombinant virus was designated influenza A/WSN/33 HAnc-Asp225Gly (H1N1) and became lethal after 7 serial passages in the lungs of animals. Virus was plaque purified and a virus stock was prepared by growth on MDCK cells. In each study, five non-treated normal control mice were maintained for weight comparison. For influenza virus challenge, mice were anesthetized by IP injection of ketamine/xylazine (50 mg/kg//5 mg/kg) prior to challenge by the intranasal route with approximately 5 x 10^3 (2 x LD_{50}) cell culture infectious doses ($CCID_{50}$) (IP efficacy) or 1 x 10^3 $CCID_{50}$ (SC efficacy) of virus per mouse in a 90 µl inoculum volume. Mice were weighed prior to treatment and then every other day thereafter to assess the effects of treatment on ameliorating weight loss due to virus infection. All mouse groups were observed for morbidity and mortality through day 21 post-infection.

Therapeutic efficacy of intranasal H84T against H3N2 influenza virus. Mouse-adapted influenza A/Hong Kong/8/1968 (H3N2) virus was kindly provided by Dr. Brian E. Gilbert, Baylor College of Medicine. Frozen stock (2.8 x 10^7 50% tissue culture infective doses [$TCID_{50}$]/ml) of virus was diluted 1:250 in 0.05% gelatin in Eagle's minimal essential medium and delivered by aerosolization for 20 min to achieve the 90% lethal dose (LD_{90}) to LD_{100} (~100 $TCID_{50}$ per mouse) in wild type six-week-old C57BL/6J mice of a single sex. Animals were examined and weighed daily and sacrificed if they met euthanasia criteria, including signs of distress or loss of 30% preinfection body weight. Mice were treated daily for 5 days beginning 4 hours post-infection with 40 µL intranasal PBS (sham) or H84T BanLec (0.1 mg/kg) without anesthesia.

Animal care and use. The IP and SC efficacy studies were conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University dated September 2, 2016 (expires Sept. 1, 2019). The work was done in the AAALAC-accredited Laboratory Animal Research Center of Utah State University. The U.S. Government (National Institutes of Health) approval was renewed April 7, 2013 (Animal Welfare Assurance no. A3801-01) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revision; 2011). The in vivo safety and distribution studies were conducted in accordance with the approval of the Institutional Animal Care & Use Committee of the University of Michigan.

Quantitation of immunofluorescence staining. The number of punctae and percent overlap were calculated using ImageJ software first by converting individual color channel images to grayscale, then by converting them to binary (black and white only) images. The Analyze Particles function was used to calculate the number of particles in each individual color channel, followed by the Image Calculator function to calculate the number of particles with overlap between color channels. The percent overlap was calculated by dividing the number of overlapping particles by the number of particles in a particular color channel as described in the relevant figure legends. The diameter of punctae was calculated by using the Measure tool to assess pixel length. At least three micrographs per group were used for each quantitation.

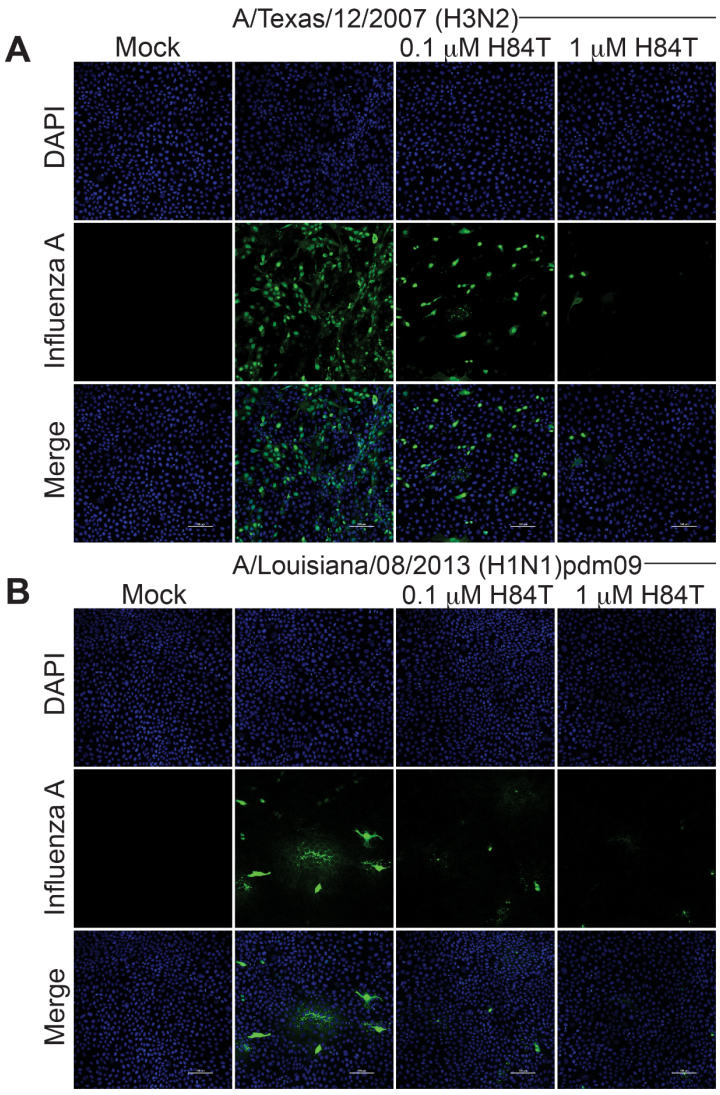

Fig. S1. H84T decreases spreading infection of oseltamivir-resistant influenza strains.

Representative immunofluorescent micrographs of influenza A antigen staining (green) and nuclei (blue) 16-18 h post-infection. MDCK cells were pre-treated for 1 h with 0.1 or 1 μM H84T and infected with the oseltamivir-resistant strains A/Texas/12/2007 (H3N2) (A) or A/Louisiana/08/2013 (H1N1)pdm09 (B) at MOI = 0.05 for 16-18 h. Data are representative of two independent experiments.

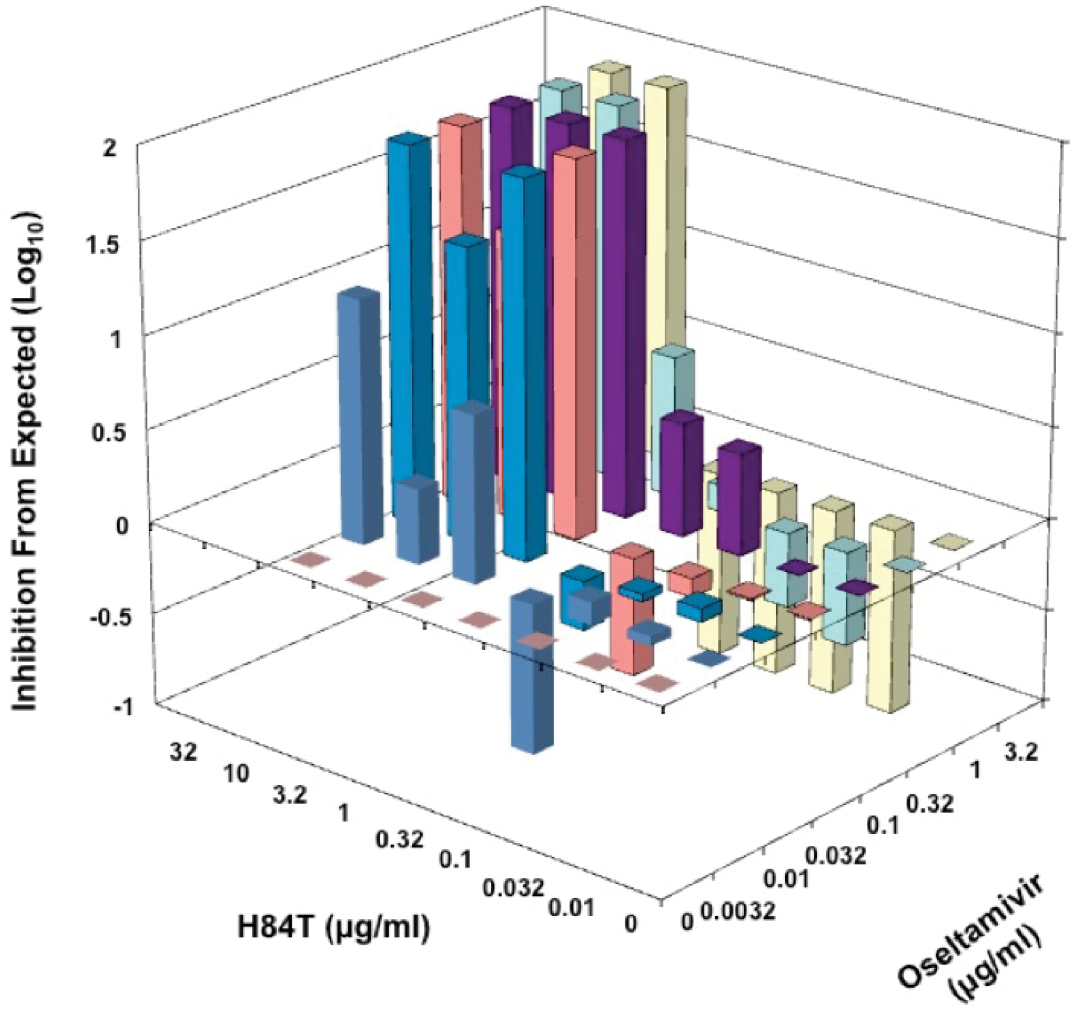

Fig. S2. H84T and oseltamivir exhibit minor synergy against A/California/07/2009 (H1N1)pdm09 virus. Three-dimensional MacSynergy™ II plot showing dual drug interactions of H84T and oseltamivir carboxylate. MDCK cells were infected with influenza A/California/07/2009 (H1N1)pdm09 virus and treated with dual drug combinations. The X- and Y-axis are the concentrations of H84T and oseltamivir, respectively. The Z-axis is the calculated drug-drug interaction based on Bliss independence and expressed as inhibition from expected. The null reference plane represents no interactions whereas bars above, or below the null reference plane represent significant interactions. Synergy volume was 36.32 $\mu M^2\%$, indicative of minor but significant synergy.

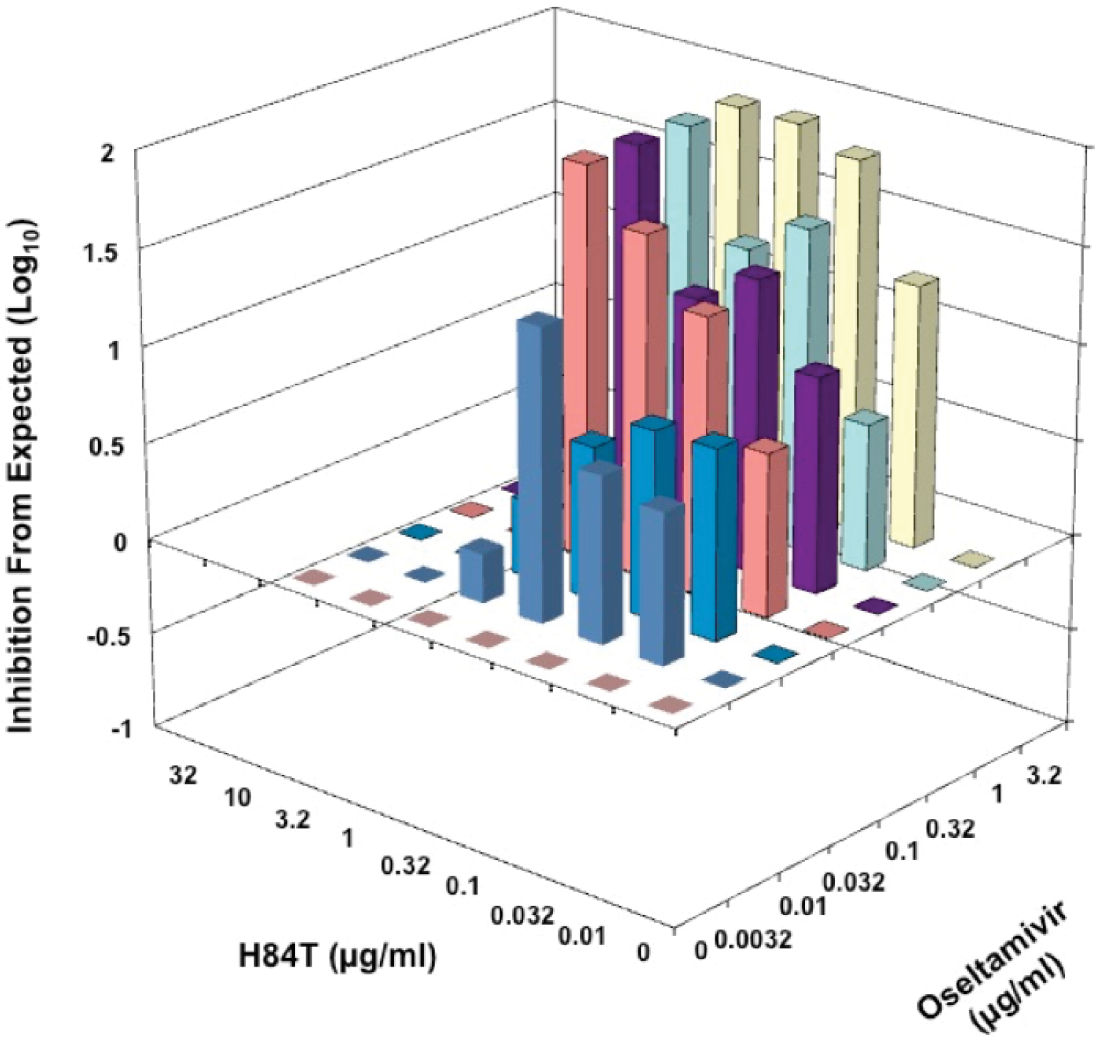

Fig. S3. H84T and oseltamivir exhibit minor synergy against A/Perth/16/2009 (H3N2) virus. Three-dimensional MacSynergy^{TM} II plot showing dual drug interactions of H84T and oseltamivir carboxylate. MDCK cells were infected with influenza A/Perth/16/2009 (H3N2) virus and treated with dual drug combinations. The X- and Y-axis are the concentrations of H84T and oseltamivir, respectively. The Z-axis is the calculated drug-drug interaction based on Bliss independence and expressed as inhibition from expected. The null reference plane represents no interactions whereas bars above, or below the null reference plane represent significant interactions. Synergy volume was 29.54 $\mu M^2\%$, indicative of minor but significant synergy.

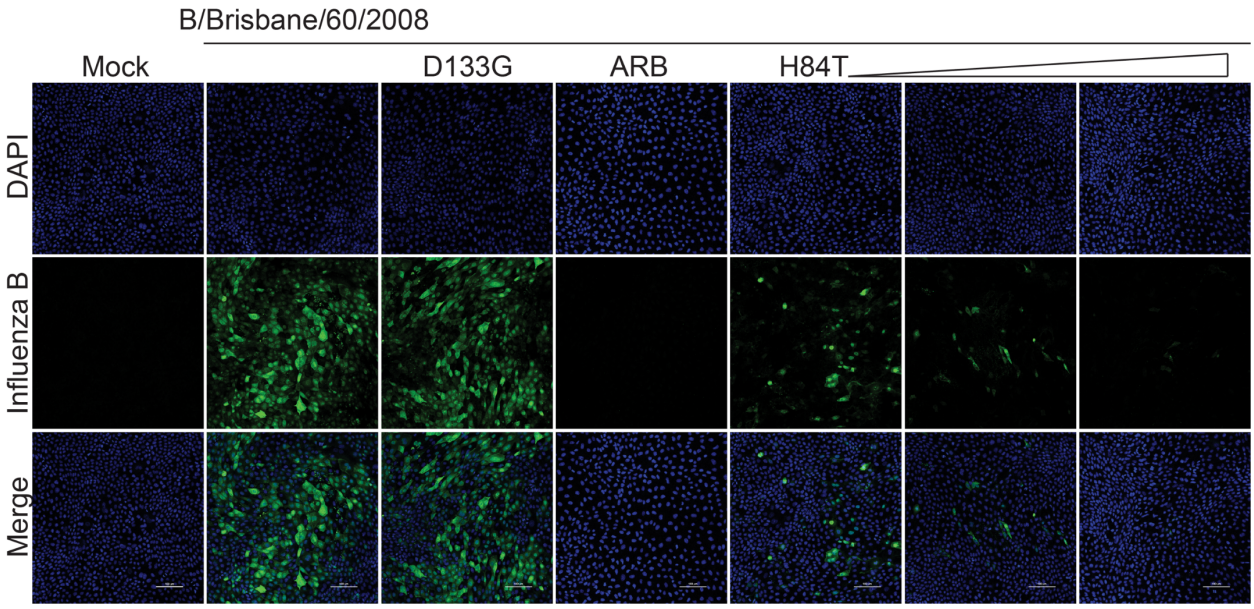

Fig. S4. H84T decreases spreading infection of influenza B virus. Representative immunofluorescent micrographs of influenza B antigen staining (green) and nuclei (blue) 16-18 h post-infection. MDCK cells were pre-treated for 1 h with 0.1, 1, or 10 μM H84T, or 40 μM of the fusion inhibitor arbidol (ARB) and infected with B/Brisbane/60/2008 at MOI = 0.05 for 16-18 h. Data are representative of two independent experiments.

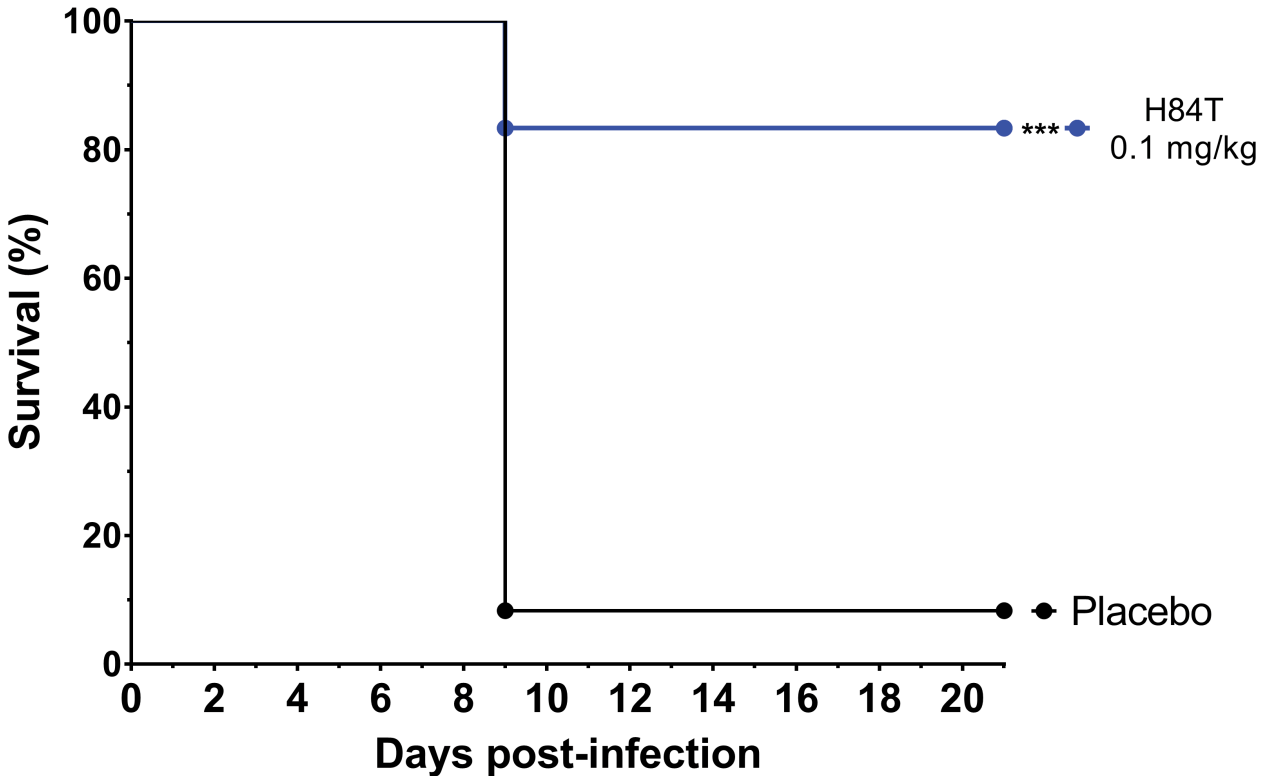

Fig. S5. Intranasal H84T is efficacious against lethal H3N2 influenza virus infection.

Survival of C57BL/6J mice challenged by aerosolization with 90% to 100% mouse lethal dose of mouse-adapted A/Hong Kong/8/1968 (H3N2) following intranasal treatment with H84T (0.1 mg/kg) or PBS (placebo) once daily for 5 days beginning 4 h post-challenge. ***$P < 0.001$. The Kaplan-Meier survival curve was compared by the log-rank (Mantel-Cox) test followed by pairwise comparison using the Gehan-Breslow-Wilcoxon test.

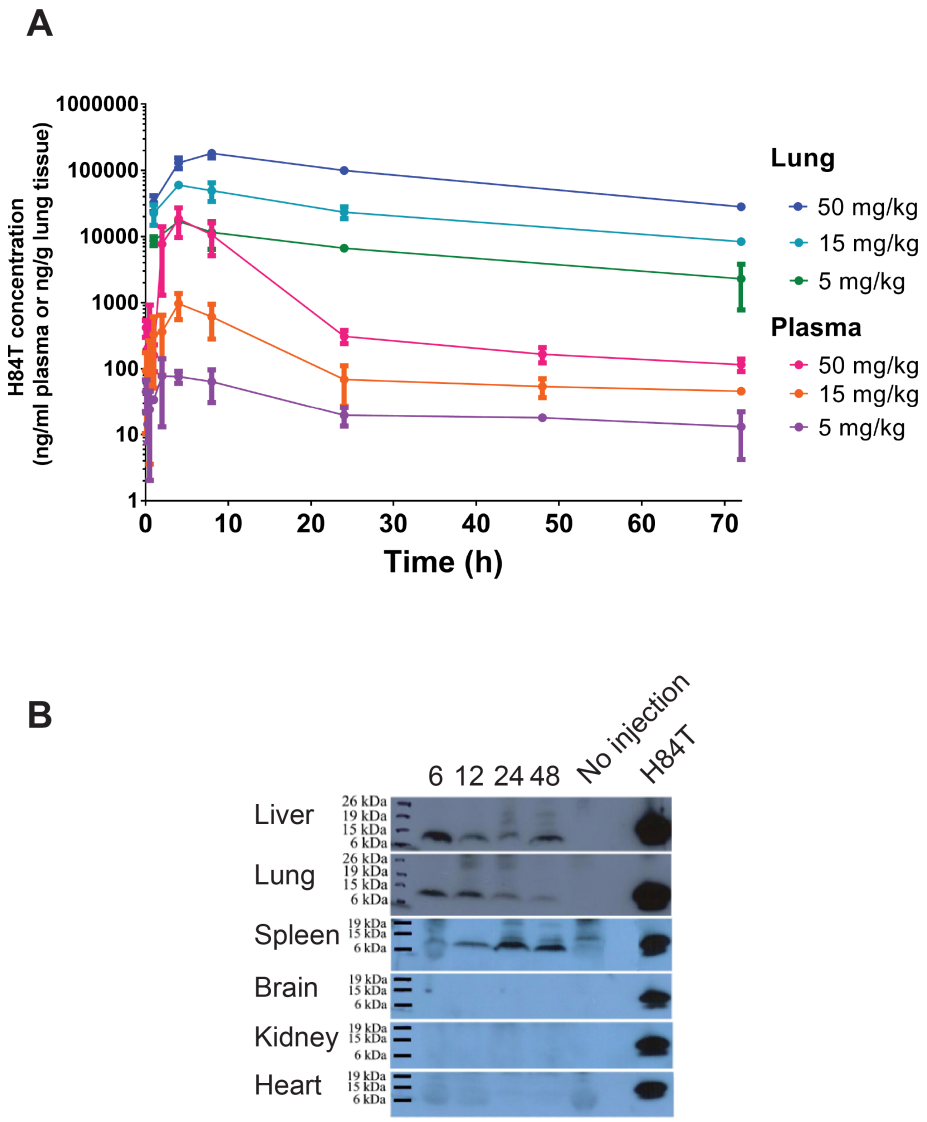

Fig. S6. H84T distributes to the lung and has a long serum and tissue half-life. (**A**) Concentration of H84T in the lung and plasma of male CFW mice following intraperitoneal administration of 5, 15, or 50 mg/kg H84T. Plasma and lungs were collected at 11 time points and the concentration of H84T determined by ELISA. Data points represent mean ± standard deviation ($n = 4$ per group). (**B**) Immunoblots of H84T distribution to liver, lung, spleen, brain, kidney, and heart. 15-week-old female 129svev/B6 mice were injected with 50 mg/kg H84T IP. Mice were culled at 4 time points post-injection (6 h, 12 h, 24 h, and 48 h) and organs harvested for protein extraction and immunoblotting.

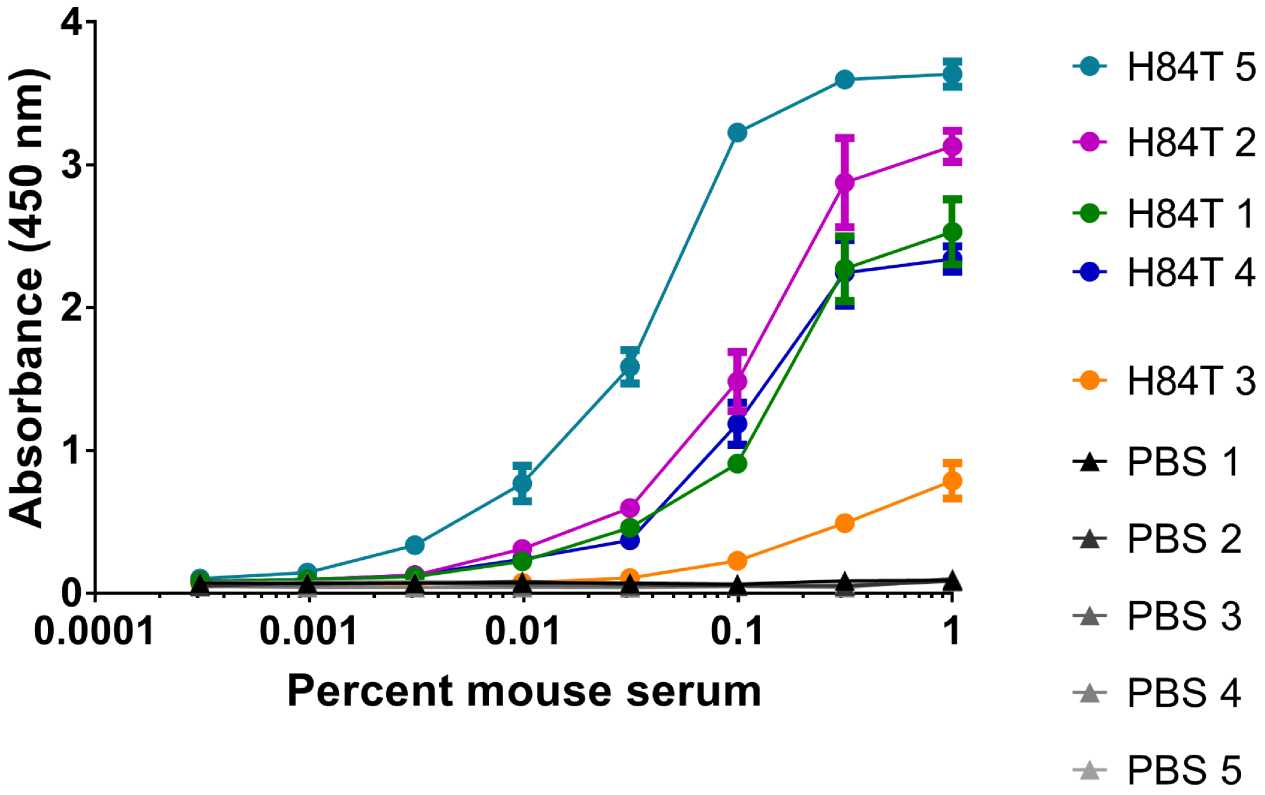

Fig. S7. Mice develop antibodies against H84T. BALB/c mice were injected intraperitoneally (IP) with two 50 mg/kg doses of BanLec (V66D/H84T/A100D, a variant of H84T engineered to increase solubility for biochemical assays) or with PBS administered 21 days apart (n = 5 per group). Mouse sera were collected two days after the second injection for detection of anti-BanLec antibodies by ELISA. A fixed amount (100 ng) of H84T was used to coat plates, and serially diluted sera used as the primary antibody. Error bars denote the standard error of the mean of technical triplicate values.

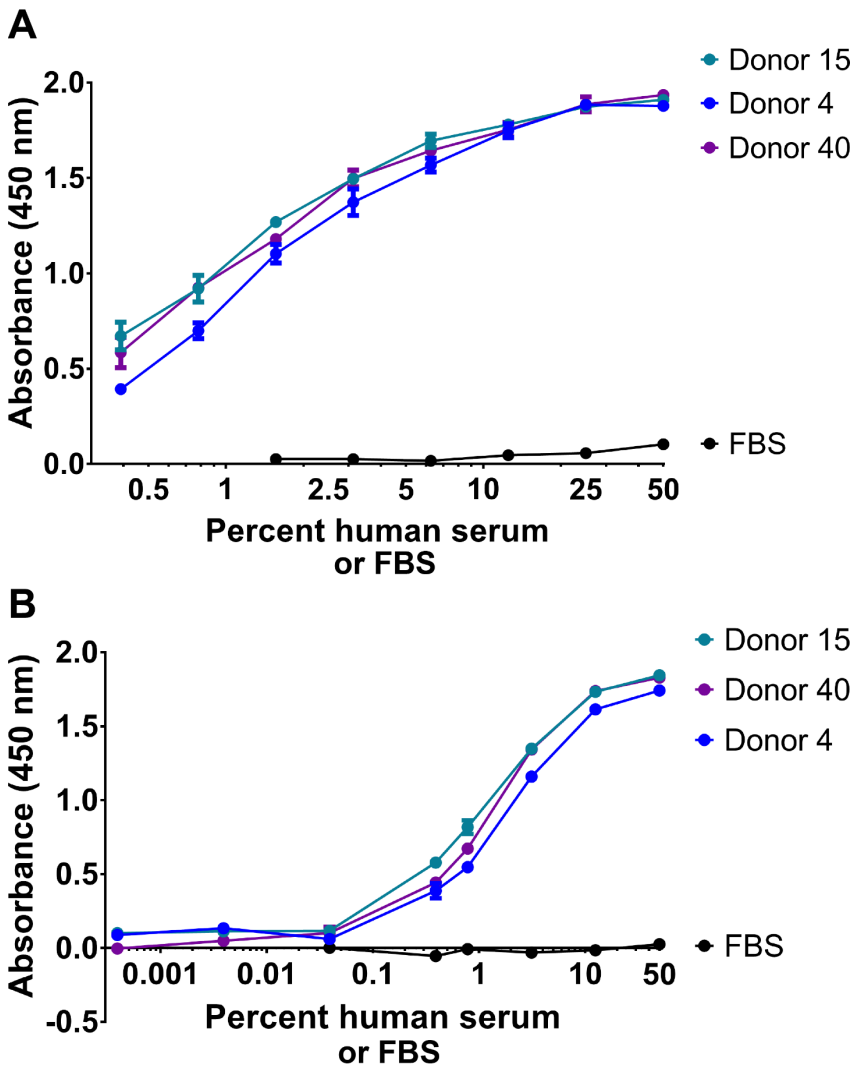

Fig. S8. Human sera contain antibodies against H84T. (**A** and **B**) Human sera from three healthy donors were analyzed for the presence of anti-H84T antibodies by ELISA. A fixed amount (100 ng) of H84T was used to coat plates, and serially diluted sera (with different dilution ranges in A versus B) used as the primary antibody. FBS, used in place of human serum, serves as a negative control. Error bars denote the standard error of the mean of technical triplicate values. Data are representative of five independent experiments.

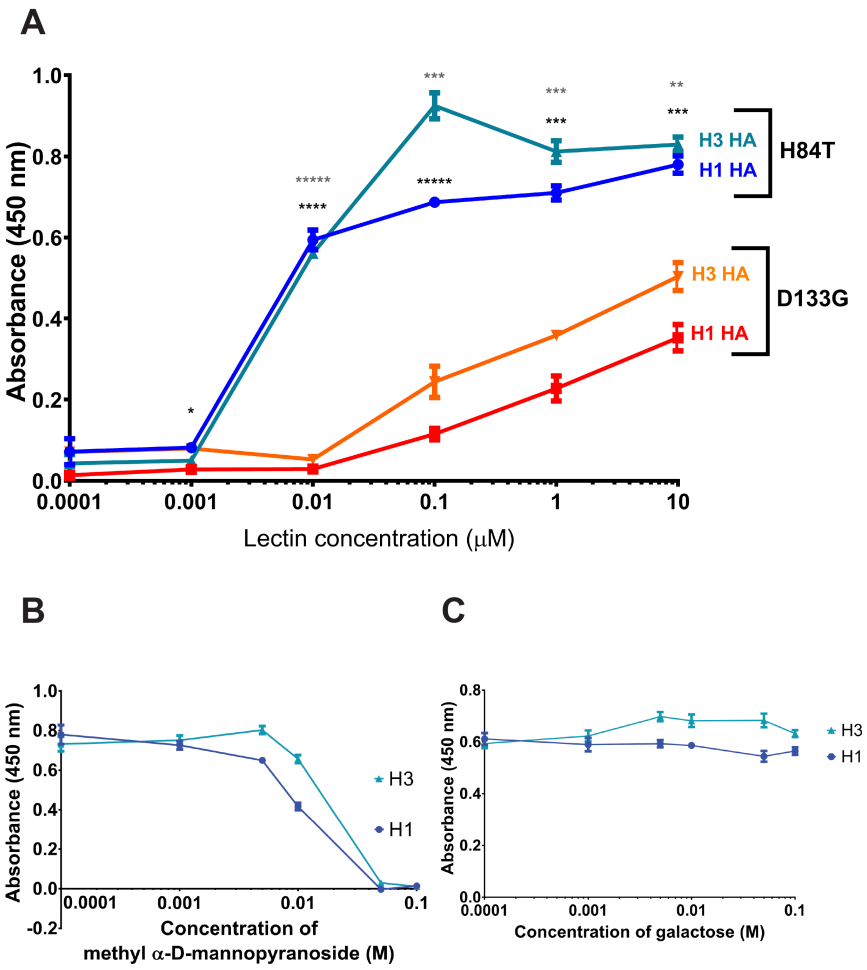

Fig. S9. H84T specifically binds to influenza HA. (**A**) H84T and D133G binding to HA as measured by ELISA. A fixed amount (100 ng) of recombinant glycosylated H1 or H3 HA proteins from A/California/07/2009 (H1N1)pdm09 and A/Perth/16/2009 (H3N2), respectively, was used to coat plates. Concentrations of H84T and D133G ranged from 0.0001 to 10 μM. Data are representative of three independent experiments. (**B** and **C**) Competition ELISA for H84T binding to HA. A fixed amount (0.1 μM) of H84T was used, while the concentrations of the known BanLec ligand methyl α-D-mannopyranoside in (B) or galactose, not a BanLec ligand, in (C) were varied (0.0001-0.1 μM). Data are representative of three independent experiments. Error bars denote the standard error of the mean of technical triplicate values. Statistical analyses

were performed by *t* test using the Holm-Sidak method. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, and *****$P < 0.00001$, comparing the H84T to the D133G groups for each HA type (gray asterisks = H3, black asterisks = H1).

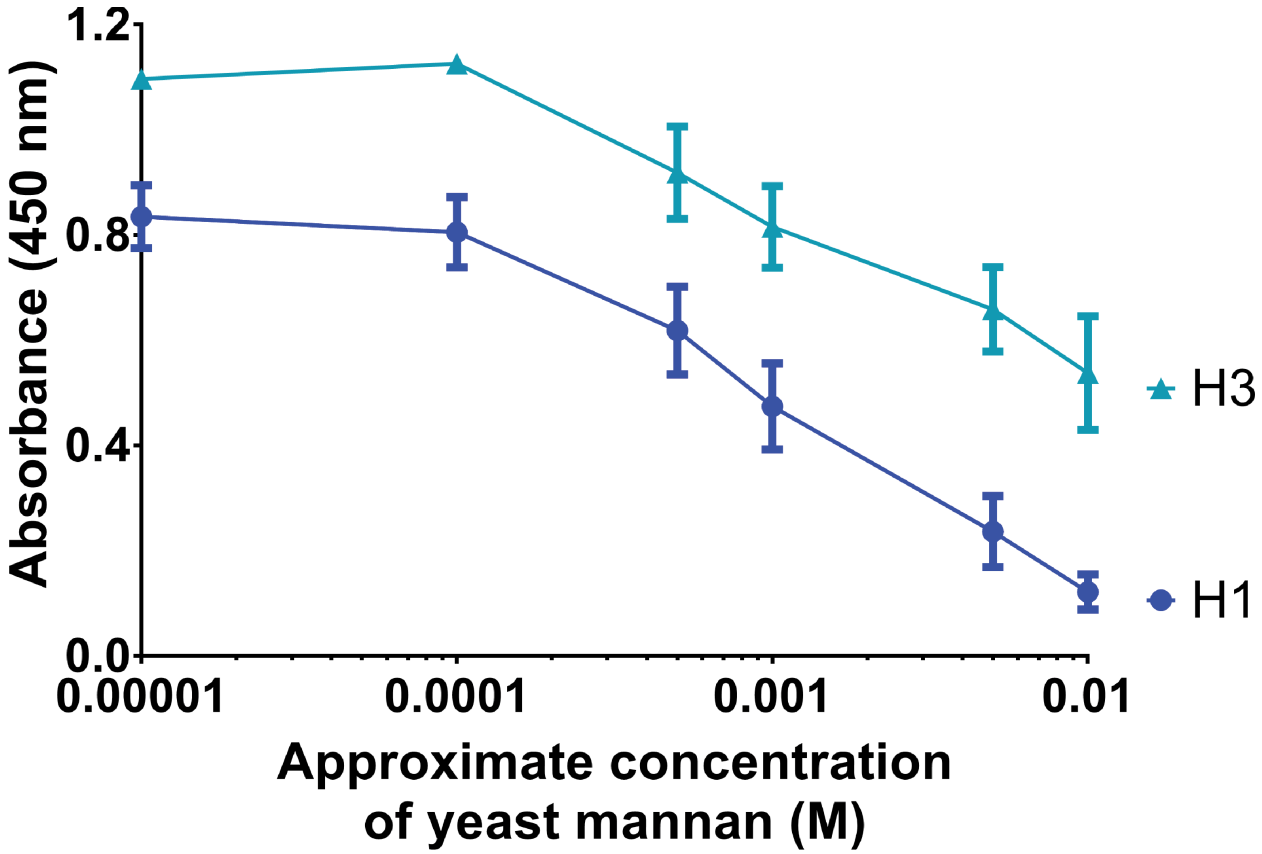

Fig. S10. Yeast mannan inhibits H84T binding to HA. Competition ELISA for H84T binding to HA. A fixed amount (0.1 μM) of H84T was used, while the concentrations of yeast mannan, a mannose-rich predicted BanLec ligand, were varied (0.00001-0.01 μM). Concentrations are approximate because yeast mannan is a heterogeneous mixture of molecules with different numbers of mannose residues. Error bars denote the standard error of the mean of technical triplicate values. Data are representative of three independent experiments.

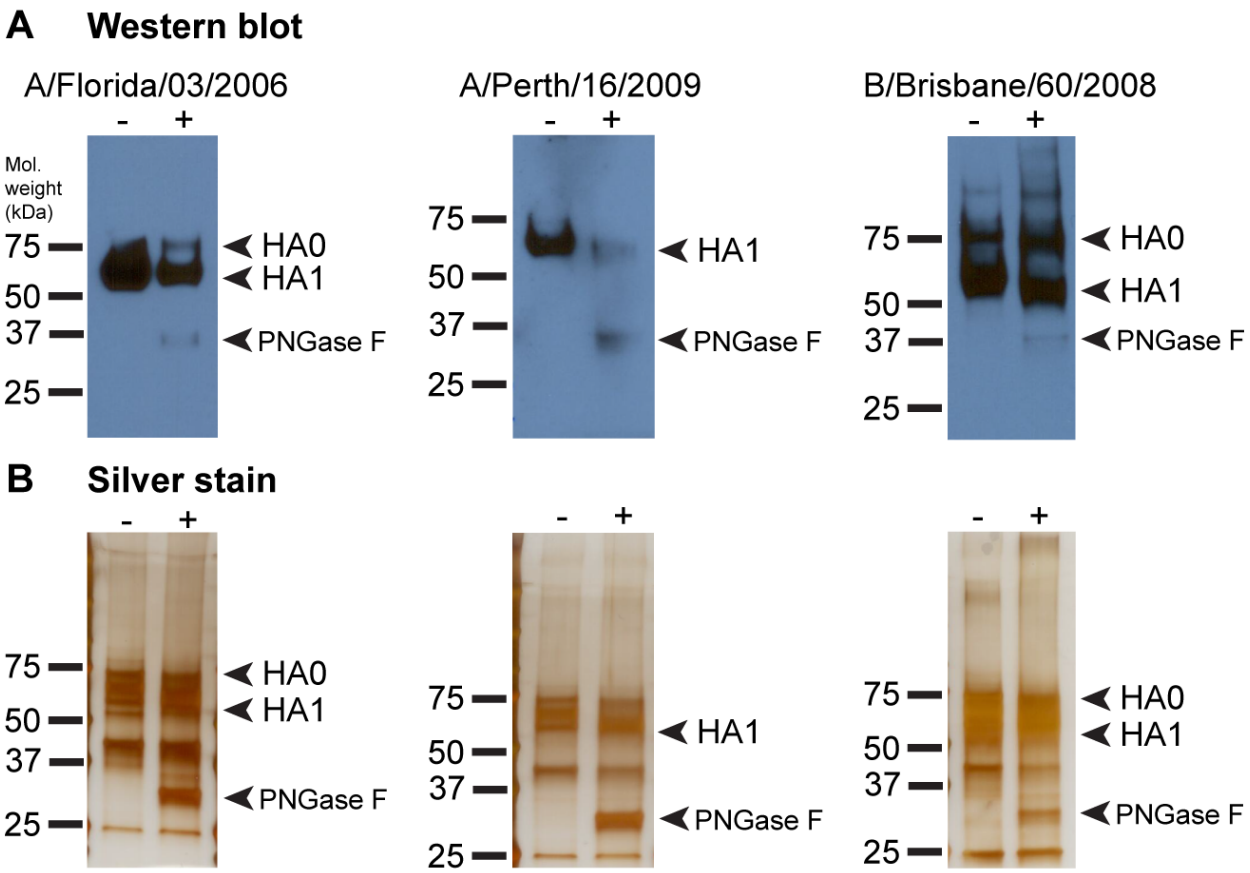

Fig. S11. PNGaseF treatment deglycosylates HA. (**A**) Western blots performed on PNGaseF- and mock-treated viral lysates from the indicated influenza strains, executed in parallel with and using the same amount of protein as in Fig. 3A. PNGase F-treated HA proteins migrate faster due to deglycosylation. Note that the antibodies used to detect HA were raised against glycosylated proteins and thus do not recognize HA in PNGaseF-treated samples as well as that in mock-treated samples. + PNGaseF-treated, - mock-treated. (**B**) Silver stains performed in parallel with the western blots in A.

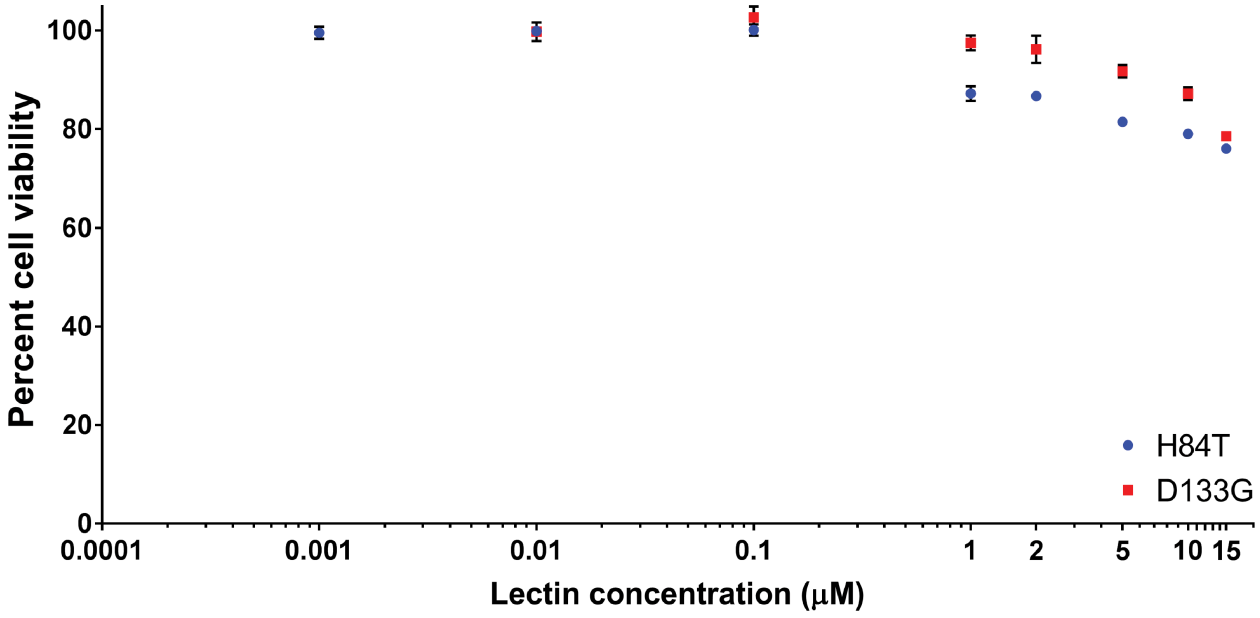

Fig. S12. H84T is not cytotoxic to MDCK cells at IC_{50} concentrations. Triplicate wells of MDCK cells were treated for 24 h with H84T or D133G (0.001-15 μM). Cytotoxicity was assessed by MTT assay and normalized to untreated cells. Error bars denote the standard error of the mean. Data are representative of three independent experiments.

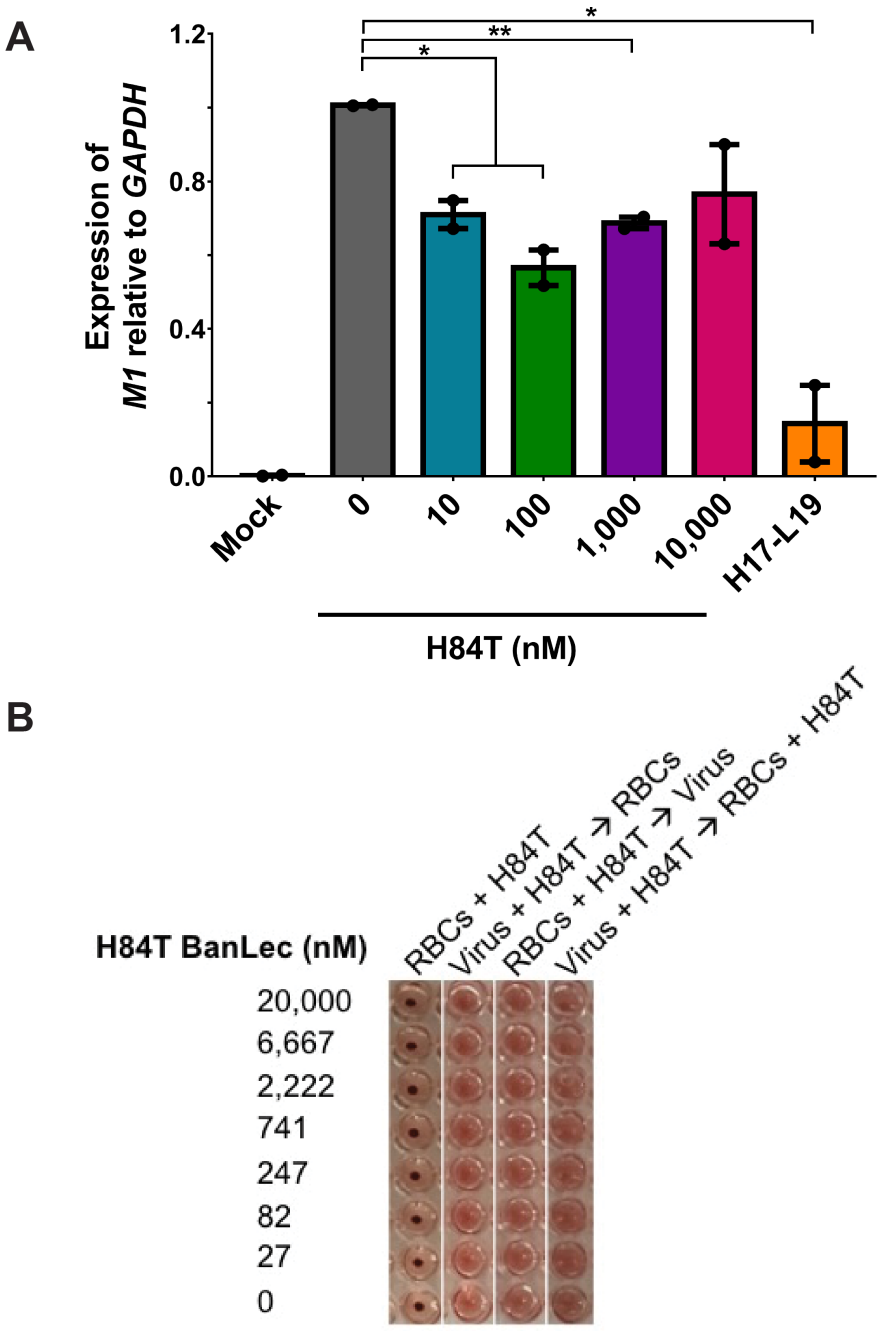

Fig. S13. H84T minorly inhibits influenza virus attachment. (**A**) A/WSN/1933 was incubated with 0-10,000 nM H84T for 30 min at 25ºC and the virus-H84T mixture incubated with MDCK cells for 1 h at 4ºC. Excess virus was removed and cells were collected in Trizol for RNA extraction. The relative amount of cell-associated virus was assessed by qRT-PCR using primers

specific to influenza *M1*, normalized to *GAPDH* expression. The positive control for attachment inhibition is the H17-L19 monoclonal antibody against A/WSN/1933. The negative control for *M1* amplification by qRT-PCR is mock infection. Bars represent the mean values two independent experiments performed in technical duplicate, and error bars represent the standard error of the mean. Statistical significance was determined by *t* test. $*P < 0.05$ and $**P < 0.01$, as compared to the infected, untreated group. (**B**) Binding of A/WSN/33 to cells was monitored by its ability to hemagglutinate turkey red blood cells (RBCs). H84T was pre-incubated with virus (virus + H84T → RBCs), with cells (RBCs + H84T → virus), or with both cells and virus (virus + H84T → RBCs + H84T) before hemagglutination. Data are representative of two independent experiments.

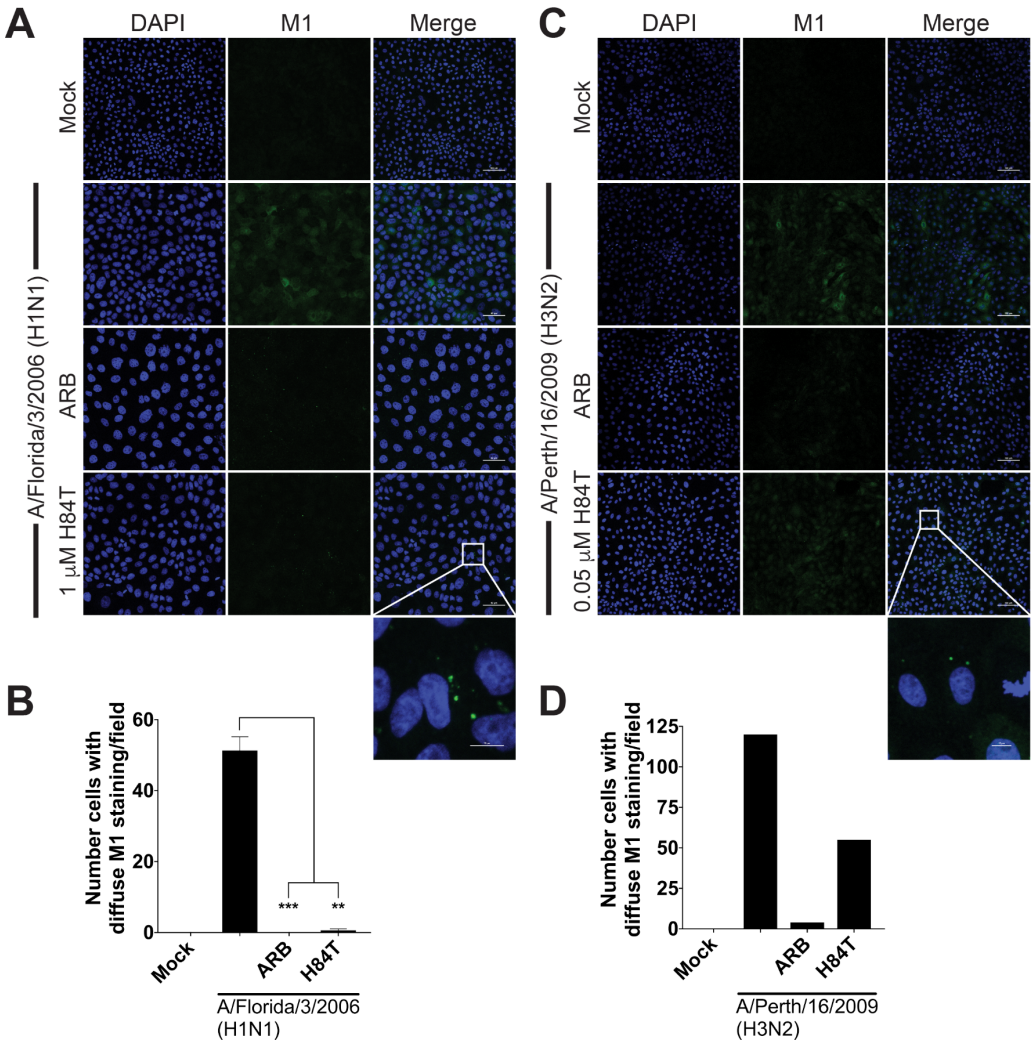

Fig. S14. H84T reduces uncoating of multiple diverse strains of influenza. (**A** and **B**) MDCK cells were pre-treated for 1 h with 1 (A) or 0.05 (B) μM H84T or 40 μM of the fusion inhibitor arbidol (ARB) and incubated with A/Florida/3/2006 (H1N1) (A) or A/Perth/16/2009 (H3N2) (B) (MOI 0.5) for 2.5 h at 37ºC. Immunofluorescence was visualized by confocal microscopy. (A and B) Representative immunofluorescent micrographs of M1 antigen staining (green) and nuclei (blue) 2.5 h post-infection. Data for (A) and (B) are representative of three independent experiments each. (**C** and **D**) Quantitation of the number of cells with diffuse cytoplasmic M1 staining per field. Statistical analyses were performed by *t* test. $^{**}P < 0.01$, and $^{***}P < 0.001$, as compared to the infected, untreated group. Error bars represent the standard error of the mean.

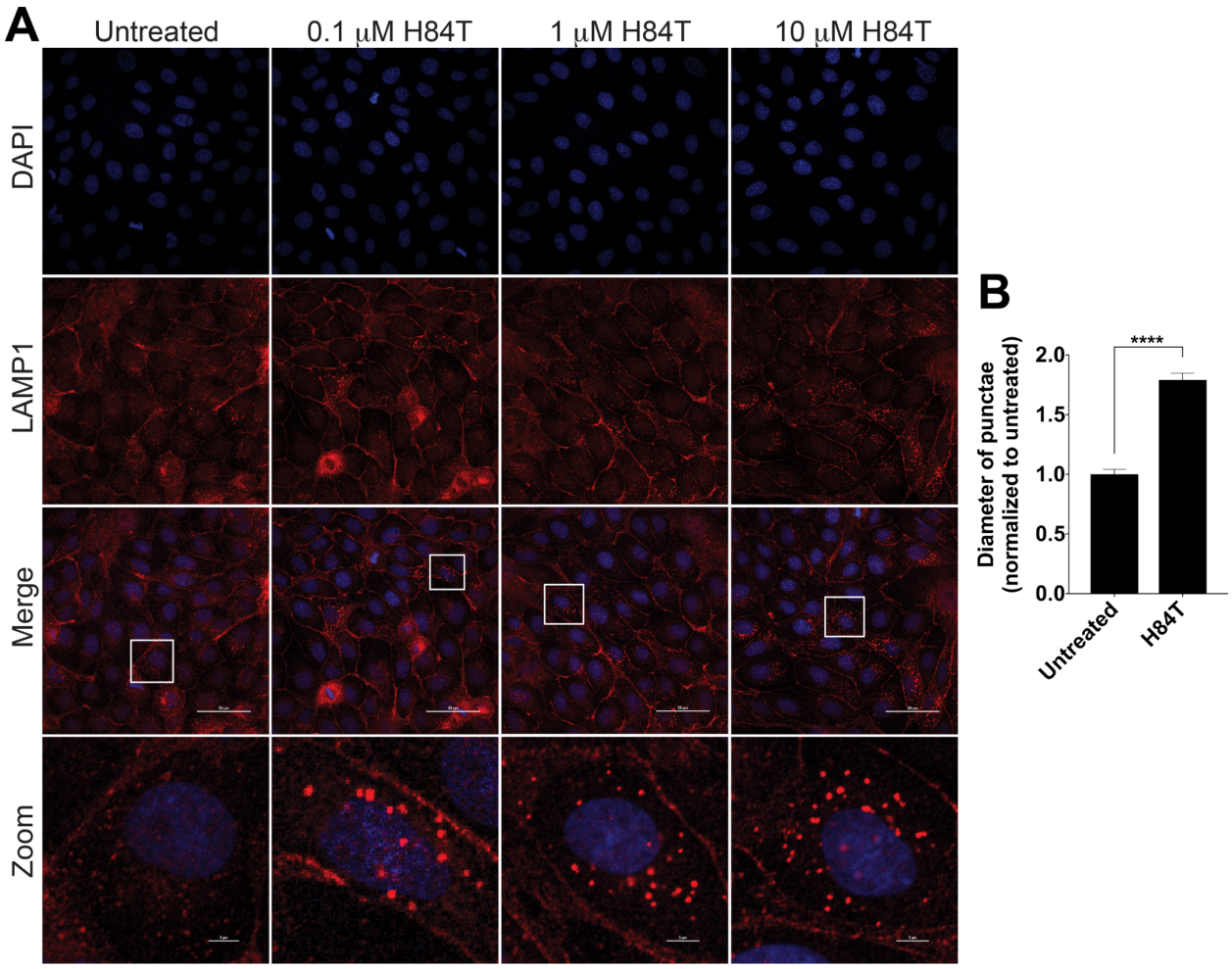

Fig. S15. H84T treatment alone increases the number of large LAMP1-positive punctae. (**A**) Representative immunofluorescent micrographs of LAMP1 antigen staining (red) and nuclei (blue) in MDCK cells 4 h post-treatment with 0.1, 1, or 10 μM H84T. No influenza virus was added. Immunofluorescence was performed to detect LAMP1 antigen. The bottom row (zoom) shows micrographs depicting single cells from the merged images, indicated by the boxes. Data are representative of two independent experiments. (**B**) Quantitation of the size (diameter) of punctae in the untreated versus 10 μM H84T-treated cells. Statistical analyses were performed by *t* test. ****$P < 0.0001$, as compared to the untreated group. Error bars represent the standard error of the mean.

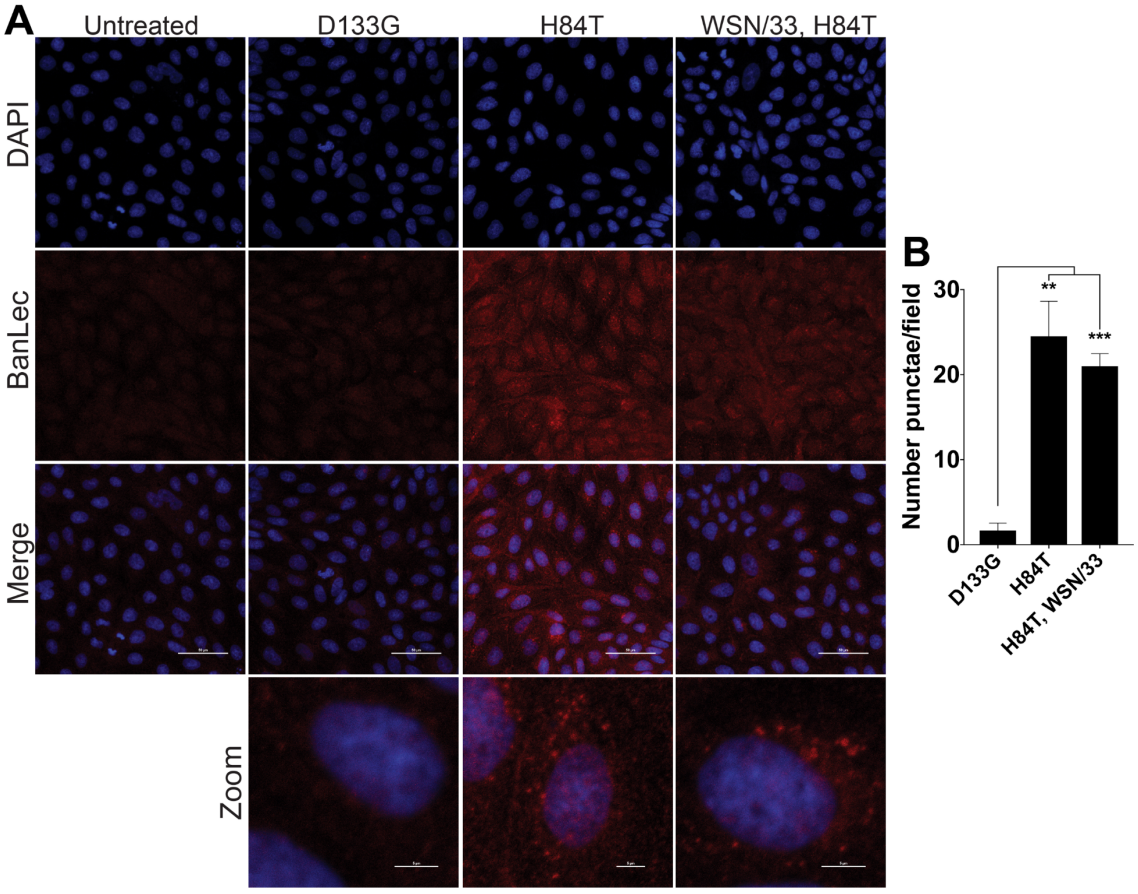

Fig. S16. H84T but not D133G is internalized into cells. (**A**) Representative immunofluorescent micrographs of BanLec antigen staining (red) and nuclei (blue). Following 1 h pre-treatment with D133G or H84T (10 μM), MDCK cells were incubated or mock incubated with A/WSN/1933 (H1N1) at 4ºC for 1 h to allow only for attachment, excess virus was removed, treatment-containing medium was replaced, and the cells were incubated at 37ºC for an additional 3 h. The bottom row (zoom) shows micrographs depicting single cells from the merged images, indicated by the boxes. Data are representative of two independent experiments. (**B**) Quantitation of the number of BanLec (D133G or H84T)-positive punctae per field. Statistical analyses were performed by *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$, as compared to the D133G-treated group. Error bars represent the standard error of the mean.

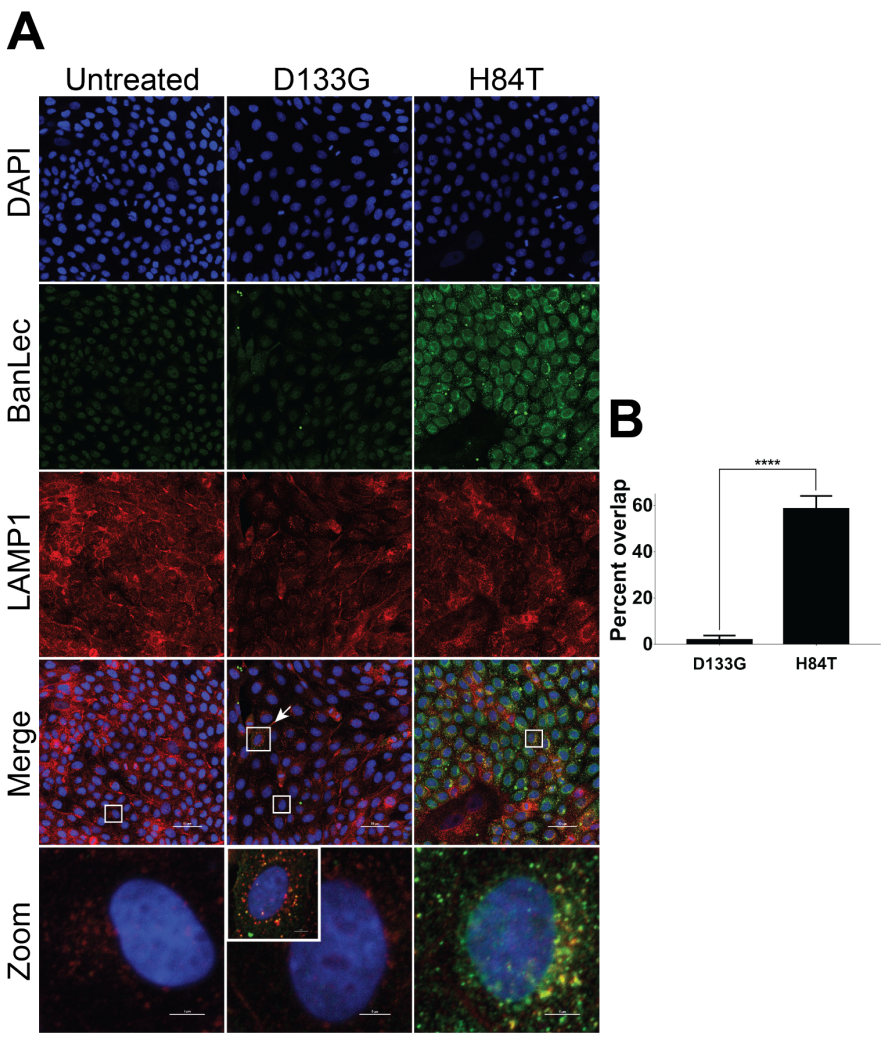

Fig. S17. H84T is internalized into the late endosome/lysosome. (**A**) Representative immunofluorescent micrographs of BanLec antigen staining (green), LAMP1 staining (red), and nuclei (blue). MDCK cells were incubated or mock incubated at 37ºC for 4 h with D133G or H84T (10 μM). The bottom row (zoom) shows micrographs depicting single cells from the merged images, indicated by the boxes. The cell depicted in the inset is indicated by an arrowhead. Data are representative of four independent experiments. (**B**) Quantitation of the percent of BanLec (D133G or H84T)-positive punctae that are also LAMP1-positive (percent overlap). Statistical analyses were performed by *t* test. ****$P < 0.0001$, as compared to the D133G-treated group. Error bars represent the standard error of the mean.

Table S1. Effect of Oseltamivir Carboxylate and H84T BanLec combination on an influenza A/California/07/2009 (H1N1)pdm09 virus infection in MDCK cells. Mean ± standard deviation from two studies.

Oseltamivir Carboxylate (µg/ml)	**Virus Titer (log_{10} $CCID_{50}$/ml) H84T BanLec (µg/ml)**								
	32	**10**	**3.2**	**1.0**	**0.32**	**0.1**	**0.032**	**0.01**	**0**
3.2	0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	5.3 ± 0.4	5.5 ± 0.3	5.2 ± 0.2	5.7 ± 0.9	4.2 ± 0.2
0.32	0	0	0	0	4.5 ± 0.7	5.1 ± 0.8	5.7 ± 0.9	5.8 ± 0.4	5.3 ± 0.4
0.1	0	2.6 ± 0.1	3.8 ± 0.4	3.4 ± 0.5	4.3 ± 0.4	5.9 ± 0.6	6.2 ± 0.2	6.7 ± 0.5	6.8 ± 0.4
0.032	0	2.2 ± 0.2	3.4 ± 0.5	5.3 ± 0.4	4.9 ± 0.2	7.3 ± 0.4	7 ± 0.4	6.9 ± 0.2	7 ± 0.4
0.01	0	2.4 ± 0.1	4.2 ± 0.2	5.4 ± 0.1	5 ± 0	7 ± 0.4	7.2 ± 0.2	7 ± 0.4	7.4 ± 0.1
0.0032	1.7 ± 0	3.8 ± 0.4	5.8 ± 0.4	6.7 ± 0.9	6.4 ± 0.5	7.6 ± 0.1	7.3 ± 0.4	7.2 ± 0.2	7.8 ± 0.4
0	1.9 ± 0.2	4.6 ± 0.1	7.2 ± 0.2	7.2 ± 0.2	7.4 ± 0.1	6.9 ± 0.2	7.6 ± 0.1	7.4 ± 0.5	7.9 ± 0.2

Oseltamivir carboxylate EC_{90} = 0.06 µg/ml

H84T BanLec EC_{90} = 3.8 µg/ml

The shaded area indicates a possible region of synergy defined as a one-log (or greater) decrease in virus titer when compared to both individual drugs alone.

Table S2. Effect of Oseltamivir Carboxylate and H84T BanLec combination on an influenza A/Perth/16/2009 (H3N2) virus infection in MDCK cells. Mean ± standard deviation from two studies.

Oseltamivir Carboxylate (µg/ml)	**Virus Titer (log_{10} $CCID_{50}$/ml) H84T BanLec (µg/ml)**								
	32	**10**	**3.2**	**1.0**	**0.32**	**0.1**	**0.032**	**0.01**	**0**
3.2	0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	0	0	1.9 ± 0.2	2.4 ± 0.1	3.8 ± 0.4
0.32	0	0	0	0	0	2.8 ± 0.4	2.6 ± 0.1	3.5 ± 0.7	4.3 ± 0.4
0.1	0	0	0	0	0	2.9 ± 0.6	2.8 ± 0.4	3.2 ± 0.2	4.3 ± 0.4
0.032	0	0	0	0	0	3.4 ± 0.5	4.2 ± 0.2	4.8 ± 0.4	5.6 ± 0.1
0.01	0	0	0	0	3.8 ± 0.4	4.4 ± 0.5	4.8 ± 0.4	4.8 ± 0.4	5.8 ± 0.4
0.0032	0	0	0	0	3.9 ± 0.6	3.8 ± 0.4	5.6 ± 0.1	5.8 ± 0.4	6.8 ± 0.4
0	1.9 ± 0.2	0	0	0	4.2 ± 0.2	5.3 ± 0.4	6.8 ± 0.4	6.9 ± 0.6	7.5 ± 0.7

Oseltamivir carboxylate EC_{90} = 0.005 µg/ml

H84T BanLec EC_{90} = 0.04 µg/ml

The shaded area indicates a possible region of synergy defined as a one-log (or greater) decrease in virus titer when compared to both individual drugs alone.

Table S3. Effect of Oseltamivir Carboxylate and H84T BanLec combination on an influenza A/Duck/Minnesota/1525/81 (H5N1) virus infection in MDCK cells. Mean ± standard deviation from two studies.

Oseltamivir Carboxylate (µg/ml)	**Virus Titer (log_{10} $CCID_{50}$/ml) H84T BanLec (µg/ml)**								
	32	**10**	**3.2**	**1.0**	**0.32**	**0.1**	**0.032**	**0.01**	**0**
3.2	0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1.0	0	2.0 ± 0.4	2.3 ± 0.4	2.8 ± 0.4	3 ± 0.7	3.1 ± 0.6	2.8 ± 0.7	2.6 ± 0.1	2.3 ± 0.4
0.32	0	2.9 ± 0.6	2.8 ± 0.4	4 ± 0.4	3.8 ± 0.4	4 ± 0.7	4.6 ± 0.1	4.4 ± 0.1	3.9 ± 0.6
0.1	3.0 ± 0.7	3.8 ± 0.4	5.9 ± 0.6	6.2 ± 0.2	6.4 ± 0.1	6.2 ± 0.2	5.8 ± 0.4	6 ± 0.4	5.8 ± 0.4
0.032	4.8 ± 0.4	5.3 ± 0.4	7.3 ± 0.4	7.5 ± 0.3	6.9 ± 0.2	7.1 ± 0.6	6.9 ± 0.8	7.6 ± 0.1	7.3 ± 0.4
0.01	4.9 ± 0.6	5.3 ± 0.6	7.2 ± 0.2	6.6 ± 0.1	7.2 ± 0.2	7 ± 0.4	7.2 ± 0.2	7.3 ± 0.4	7.8 ± 0.4
0.0032	5.8 ± 0.4	4.9 ± 0.6	7.6 ± 0.1	6.7 ± 0.5	7.1 ± 0.6	7.3 ± 0.4	7.3 ± 0.4	7.2 ± 0.7	7.3 ± 0.4
0	5.9 ± 0.6	6.4 ± 0.1	7.3 ± 0.4	7.6 ± 0.1	7.4 ± 0.1	7.3 ± 0.4	7.2 ± 0.2	7.1 ± 0.6	7.6 ± 0.1

Oseltamivir carboxylate EC_{90} = 0.17 µg/ml

H84T BanLec EC_{90} = 7.8 µg/ml

The shaded area indicates a possible region of synergy defined as a one-log (or greater) decrease in virus titer when compared to both individual drugs alone.

Table S4. Effect of Oseltamivir Carboxylate and H84T BanLec combination on an influenza B/Brisbane/60/2008 virus infection in MDCK cells. Mean ± standard deviation from two studies.

Oseltamivir Carboxylate (µg/ml)	Virus Titer (log_{10} $CCID_{50}$/ml) H84T BanLec (µg/ml)								
	32	**10**	**3.2**	**1.0**	**0.32**	**0.1**	**0.032**	**0.01**	**0**
3.2	0	4.6 ± 0.1	4.5 ± 0.7	4.5 ± 0.7	4.0 ± 0.7	4.5 ± 0.7	6.0 ± 0.7	5.6 ± 0.1	5.4 ± 0.5
1.0	0	4.2 ± 0.2	4.5 ± 0.7	4.8 ± 0.4	5.8 ± 0.4	5.0 ± 0.7	6.4 ± 0.5	5.5 ± 0.7	6.3 ± 0.4
0.32	3.2 ± 0.2	4.0 ± 0.4	4.9 ± 0.6	5.0 ± 0.7	6.0 ± 0.4	6.5 ± 0.7	6.8 ± 0.4	6.6 ± 0.1	7.3 ± 0.4
0.1	3.8 ± 0.4	4.3 ± 0.4	5.9 ± 0.6	6.3 ± 0.4	6.1 ± 0.6	7.0 ± 0.4	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.1
0.032	4.0 ± 0.4	4.5 ± 0.7	6.0 ± 0.7	6.1 ± 0.6	6.8 ± 0.4	7.4 ± 0.1	7.3 ± 0.4	7.3 ± 0.4	7.6 ± 0.1
0.01	5.3 ± 0.4	4.8 ± 0.4	6.3 ± 0.4	6.2 ± 0.2	6.5 ± 0.7	7.1 ± 0.6	7.0 ± 0.4	7.2 ± 0.2	7.3 ± 0.4
0.0032	5.2 ± 0.2	4.9 ± 0.6	6.2 ± 0.2	6.4 ± 0.1	6.7 ± 0.5	7.2 ± 0.7	7.1 ± 0.6	7.0 ± 0.4	7.2 ± 0.2
0	5.6 ± 0.1	6.0 ± 0.7	6.4 ± 0.1	6.5 ± 0.3	6.6 ± 0.1	6.8 ± 0.4	6.8 ± 0.4	6.9 ± 0.6	7.4 ± 0.1

Oseltamivir carboxylate EC_{90} = 0.90 µg/ml

H84T BanLec EC_{90} = 2.1 µg/ml

The shaded area indicates a possible region of synergy defined as a one-log (or greater) decrease in virus titer when compared to both individual drugs alone.

Table S5. Plasma pharmacokinetics of H84T.

Dose (mg/kg)	**C_{max} (ng/ml)**	**T_{max} (hr)**	**AUC_{last} (ng*hr/ml)**	**$t_{1/2}$ (h)**	**AUC/ Dose**
5.0	110	4.5	2011	93	402
15.0	1065	4.3	14558	238	971
50.0	20435	3.5	184715	33.5	3694

Maximum concentration, C_{max} (ng/ml or ng/g)
Time at which maximum concentration occurs, T_{max} (hrs)
Area under the curve, AUC, total exposure levels (ng*hrs/ml)
Half-life, $t_{1/2}$ (hrs)

Table S6. Lung tissue pharmacokinetics of H84T.

Dose (mg/kg)	**C_{max} (ng/g)**	**T_{max} (hr)**	**AUC_{last} (ng*hr/g)**	**$t_{1/2}$ (h)**	**AUC/ Dose**
5.0	17160	5	460345	32	92069
15.0	60492	5	1691649	29	112777
50.0	180638	8	6183249	25	123665

Maximum concentration, C_{max} (ng/ml or ng/g)
Time at which maximum concentration occurs, T_{max} (hrs)
Area under the curve, AUC, total exposure levels (ng*hrs/ml)
Half-life, $t_{1/2}$ (hrs)