EVALUATION OF A NOVEL COMPUTATIONALLY DESIGNED PROPHYLACTIC AGAINST PANDEMIC INFLUENZA VIRUS IN THE FERRET MODEL

by

Christopher Matthew Healy

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This thesis was presented

by

Christopher Matthew Healy

It was defended on

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and approved by

Thesis Advisor:
Kelly Stefano Cole, PhD
Associate Professor
Department of Immunology
School of Medicine
and
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Committee Members:

Robbie Mailliard, PhD
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Joshua T. Mattila, PhD
Assistant Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Kelly Stefano Cole, PhD

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ABSTRACT

The Influenza virus is responsible for hundreds of thousands of deaths and millions of infections worldwide each year. In spite of the annual seasonal influenza vaccine and various therapeutics that currently exist, influenza currently poses a major health hazard to the world's population. Vaccine efficacy is diminished due to the high mutation rate commonly exhibited by negative sense RNA viruses. This complicates the decision for which strains to include in the yearly vaccine, and often results in poorly matched vaccine and circulating strains. Antivirals are available to combat influenza infection, but are also becoming less effective due to the increasing development of antiviral resistance and the limited selection. The development of improved vaccine strategies and novel antivirals are of critical importance to the public's health and wellbeing.

Our research group has collaborated with researchers at the University of Washington in the evaluation of computationally designed influenza antiviral binder proteins. These proteins bind to the viral hemagglutinin surface protein and mimic antibodies that are naturally elicited in vivo during influenza infection, and prevent virus from binding and infecting cells by inhibiting the conformational change necessary for viral entry to host cells. Prior work evaluated viral protein binder HB36.6, demonstrating its efficacy against pandemic H1N1 in the ferret model. The present thesis was designed to evaluate an improved, smaller mini-binder that is less toxic

and more soluble in the ferret model. Studies were designed to compare intranasal and intratracheal administration of mini-binder A13r33 administered 24 hours prior to aerosol challenge with pandemic H1N1 in ferrets. Our working hypothesis was that the antiviral minibinder A13r33 would result in reduced infection as measured by improved clinical outcome and lower viral loads following virus challenge. Ferrets were monitored for 7 days post infection for clinical, viral and immunological parameters. Results from these studies are presented in this thesis and provide compelling data for further evaluation of broadly protective novel therapeutics against influenza viruses.

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1.0 INTRODUCTION

1.1 THE INFLUENZA VIRUS

1.1.1 Virus Structure and History

Influenza has likely afflicted mankind since early humans began domesticating animals. Historical documents from fifth century B.C.E. Greece, the Middle Ages, and the European Renaissance report of epidemics that shared symptoms with influenza (1). Though it is difficult to know with absolute certainty that influenza was the direct cause in all these cases, there remains strong evidence that this virus has plagued humanity since before its confirmed debut in 1899 with the Russian/ Asian Pandemic. The most famous influenza pandemic, the 1918 Spanish Flu was one of the worst disease outbreaks in human history, and is thought to have been responsible for somewhere around 500 million infections and between 50 and 100 million deaths (2). Since then numerous influenza pandemics, epidemics, and epizootic episodes have occurred. While none of these events have led to such widespread infection and death nonetheless influenza remains a dangerous infectious agent that continues to plague mankind.

Influenza is a negative-sense, single-stranded RNA virus of the *Orthomyxoviridae* family. There are three viral subspecies comprised of A, B, and C. Of the three subspecies only A and B are known to cause infection in humans. Regardless of subspecies all viruses possess eight

genetic segments encoding 11 or 12 known proteins. These genetic segments are packed together with a nucleocapsid protein, and together these protein-RNA complexes are packed into a capsid enclosed by a lipoprotein membrane. Influenza A viruses are further subdivided and classified by their glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are present on the viral envelope. There are 18 known hemagglutinin and 11 neuraminidase glycoproteins specific to influenza A, however only three HA and two NA subtypes have been responsible for human pandemics (3). Hemagglutinin is important for viral attachment to sialic acid residues found on the surface of target cells, allowing the virus entry into those cells (4, 5). Neuraminidase is an exosialidase, cleaving sialic acid residues on the cell surface. This allows newly budded daughter virions from reattaching to the surface of the cell they emerge from, preventing aggregation and increasing the infectivity of the virus (6).

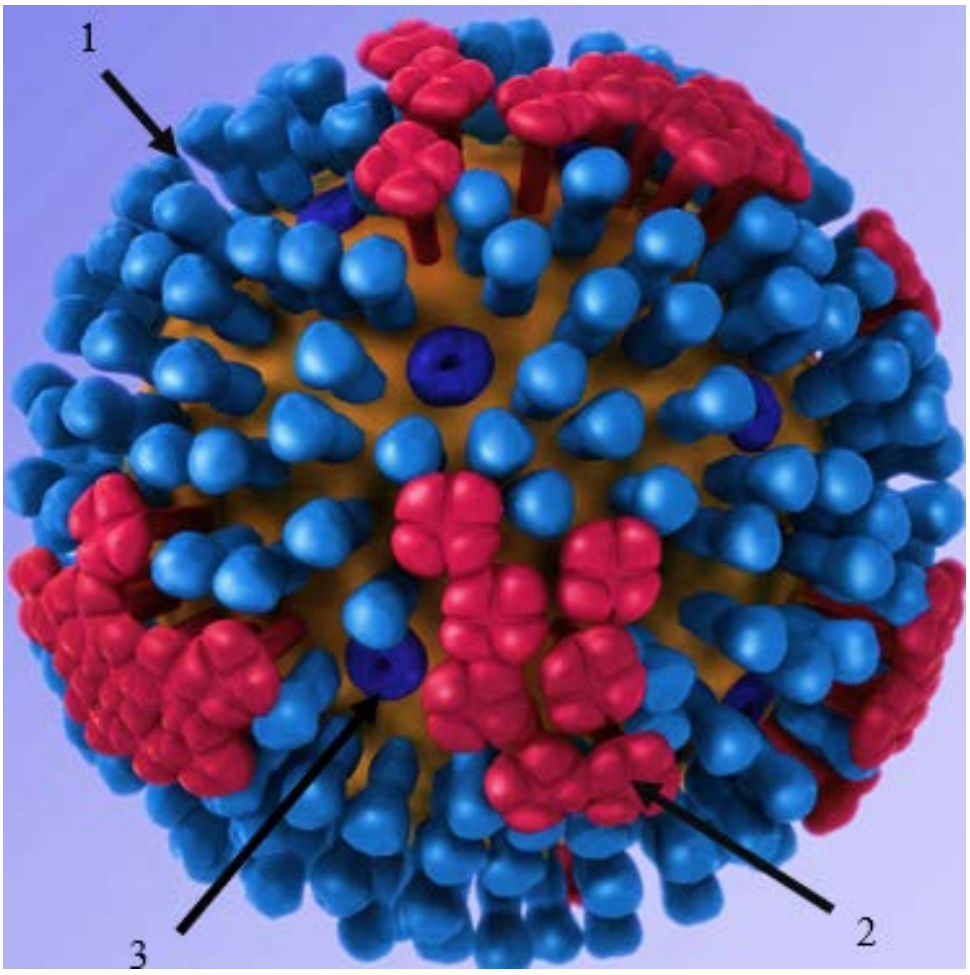


Figure 1. The Influenza Virion.

Three dimensional representation of the surface of an Influenza virion. 1. Hemagglutinin molecule. 2. Neuraminidase molecule, 3. M2 Protein channel. (CDC: https://www.cdc.gov/flu/images.htm).

The influenza virions specifically target the epithelial cells that line the upper and lower respiratory tract. As has been mentioned prior hemagglutinin molecules on the surface of the virus bind directly to sialic acid residues which is critical for viral entry (4, 5). Sialic acid residues are a diverse family of sugars. They are composed of a nine-carbon backbone and are ubiquitously distributed on the terminal ends of glycoproteins which coat cellular surfaces. Influenza hemagglutinin molecules display a preference for the sialic acid residues they bind. Avian strains bind to α-2, 3 linked sialic acids which are found intestinal epithelium of birds,

while human specific strains bind more to α-2, 6 linked sialic acids, which are found in respiratory epithelium (7). Following attachment the viral particle triggers endocytosis, via its hemagglutinin molecule, allowing for viral entry into the endosome. As the pH of the endosome drops a conformational change allows hemagglutinin to insert the fusion peptide into the endosome membrane and form a pore. The virus then releases its genome segments through this pore into the cell (8). Viral ribonucleoprotein must then travel to the nucleus in order for replication to occur. The NP, PA, PB1, and PB2 proteins which comprise the protein elements of influenza ribonucleoprotein contain nuclear localization signals, allowing this complex entry into the host cell nucleus. As the genome segments are negative sense RNA, the first step in viral replication requires positive sense RNA production. This allows for both the translation of viral protein as well as serves as a template for negative sense RNA. Viral RNA dependent RNA polymerase (comprised of PB1, PB2 and PA) generates viral messenger RNAs (mRNAs) (9). These mRNA possess a 5' methylated cap and a 3' poly A tail. The poly A tail is found in the viral ribonucleoprotein but the methylated cap is stolen by a process called "cap snatching." This involves the RNA dependent RNA polymerase's binding to the methylated cap of host mRNAs and cleaving it from the cellular mRNAs. This cap is then used to prime the nascent viral mRNA (10). This prevents viral RNA from being degraded prior to translation as well as interferes with the production of cellular proteins, limiting competition between host and viral protein production. Assembly of the protein and nucleoprotein elements then occurs, taking place at lipid rafts found on the inner wall of the cell membrane. These lipid rafts are sections of membrane that are cholesterol and sphingolipid enriched. Following assembly completion budding occurs. This results in a new daughter virion encased in a section of host membrane with the hemagglutinin and neuraminidase molecules functional domains extruding (11).

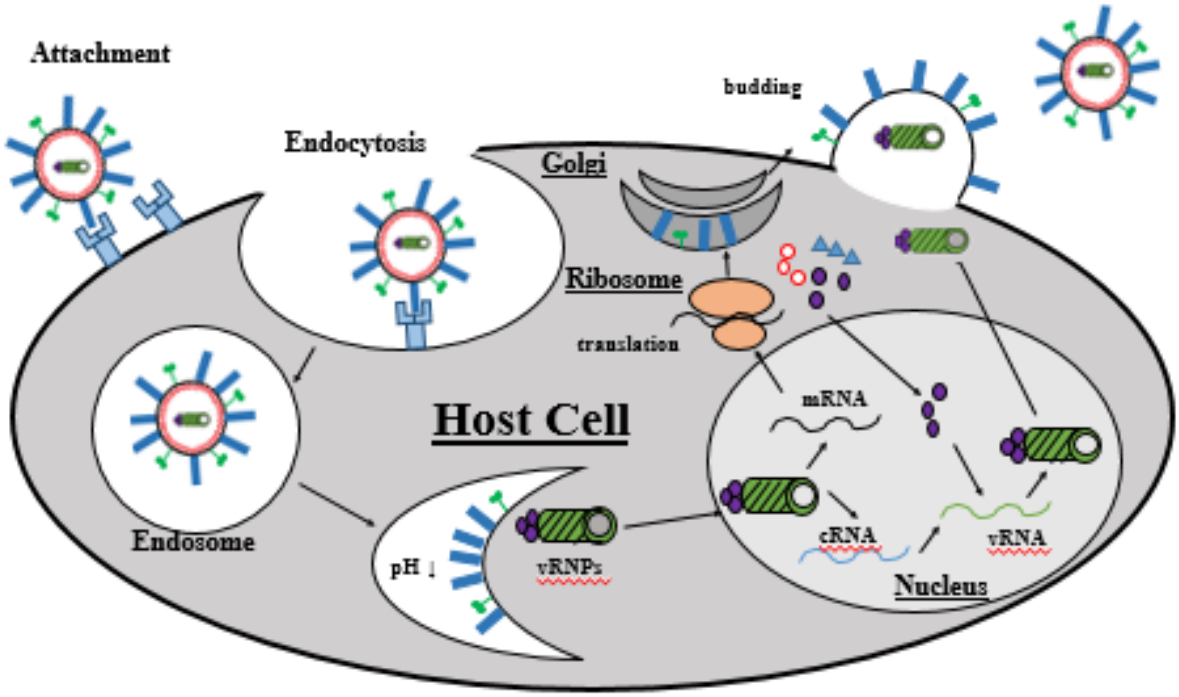


Figure 2. Influenza Virus Life Cycle.

1.1.2 Virus Epidemiology

Seasonal influenza strains are responsible for wide spread economic loss, morbidity, and death worldwide. In 2003 influenza was responsible for causing and around 22,000 to 25,000 deaths as well as estimated cost of over $87 billion in the United States alone (12, 13). Seasonal influenza can adversely affect a large portion of the entire population and is easily transmitted via coughing, sneezing, and droplets. Many of the infected will suffer from “flu like symptoms” such as fatigue, fever, muscle and joint pain, and sore/ running nose and throat. However children, the elderly, and the immune compromised are at risk of suffering from increased

disease severity (WHO Influenza Seasonal, http://www.who.int/mediacentre/factsheets/fs211/en/).

1.1.3 Influenza Pandemics

Influenza pandemics may have caused more deaths than died in both World Wars, combined. However it has been difficult without the advent of technologies like reverse transcription polymerase chain reaction (RT-PCR) to identify with certainty the pandemics for which influenza has been responsible. Other methods such as “seroarchaeology”, which analyzes antibodies taken from the elderly who lived through earlier pandemics, has also helped to identify pandemics caused by influenza (14). The earliest identified influenza pandemic occurred between 1899-1890, likely originating in Russia or Northern Asia and spread throughout Asia, Europe, and North America. Seroarchaeology concluded that this pandemic was likely caused by an H3 strain, which did not re-emerge as a pandemic strain until 70 years later as the 1968 Hong Kong Flu strain (14).

The next identifiable influenza pandemic is the 1918 H1N1 Spanish Flu epidemic which spread worldwide. It is likely that the virus adapted over several months or years into the highly pathogenic and infectious strain that killed an estimated 3 to 6% of the world’s population. Reports of smaller localized outbreaks prior to the 1918 debut are likely attribute to earlier, less lethal strains. It is unknown exactly where the strain originated, though some evidence suggests it either originated in China and was brought to the United States before spreading worldwide or originated in the US (2).

The Spanish Flu’s etymology arose from the wartime practice of censoring the news. Only Spain, which was neutral reported freely on the epidemic within their country, thus leading

to the pandemic's name (1). Following this outbreak influenza remained as a seasonal cause of localized epidemics, the next pandemic did not occur until 1957 with the H2N2 Asian Flu.

The H2N2 Asian Flu strain caught the world off guard as its hemagglutinin subtype was unlike that of previously identified human infectious strains (15). The 1918 strain primarily caused death by secondary or concomitant bacterial infections of the lungs which caused edema and lung consolidation, according to the American CDC and NIH (15) (https://www.nih.gov/news-events/news-releases/bacterial-pneumonia-caused-most-deaths-1918-influenza-pandemic). Most of those killed by the 1918 strain were previously healthy, from age groups usually that are less hard hit by the virus. Many who died from the 1957 strain however already suffered from chronic cardiac and/ or lung diseases. However deaths of individuals with no known health complications also occurred, and women in the later stages of pregnancy were known to vulnerable to the virus as well (16).

The next major influenza pandemic occurred in 1968 with the H3N2 Hong Kong flu outbreak. The outbreak occurred from 1968 to 1970, divided into two flu seasons. The United States and Canada suffered the most deaths in the 1968/1969 season, likely because there was reduced immunity to the neuraminidase subtype these populations. It is believed that the rest of the world had likely been immunized by the previous 1957 pandemic. Most of the deaths that occurred the 1969/1970 season occurred outside of North America, likely caused by antigenic drift of the neuraminidase (17).

In 1977 H1N1 returned as a pandemic strain, nearly 70 years after the deadly 1918 pandemic. Between 1918 and 1957 the H1N1 strain had remained as a seasonal strain causing local epidemics, which also immunized the survivors against this strain of influenza (1). However after 1957 this strain was supplanted by the H2 and H3 strains, and so primarily

children and young adults who had not been exposed to the H1 strains were the most susceptible. It is believed that the 1977 Russian strain pandemic did not naturally occur, but was either caused by an accidental release from a research lab or was kept frozen in the Russian permafrost or artic waters before being released (1, 18)

Most recently in 2009 H1N1 re-emerged again as a pandemic strain, however most of the world had prepared for the next outbreak of avian H5N1 and had prepared their response accordingly (CDC H1N1 Flu: https://www.cdc.gov/h1n1flu/cdcresponse.htm). By the time the WHO had declared the pandemic over in 2010 here had been 18,500 laboratory confirmed deaths, however models predicted that it was more likely that 150,000 to 575,000 had actually died as a direct cause of infection (19).

The 2009 pandemic flu, first isolated from humans in California and Mexico, was a reassortment of at least three different influenza virus parental strains. Six of this influenza strain's genes share sequence homology with H1N2 strains first isolated from swine in 1999 and 2000. The other two genes are from different Eurasian avian-like viruses. Its neuraminidase gene is closest to that of an H1N1 viruses isolated in Europe, while the matrix protein gene is similar to an H3N2 strain first isolated in Asia. Currently the exact sequence of events that led up to the creation of the pandemic 2009 strain are unknown. One hypothesis is that migrating birds and pigs that were a part of the international live swine trade intermingled, allowing these various viruses to reassort (20).

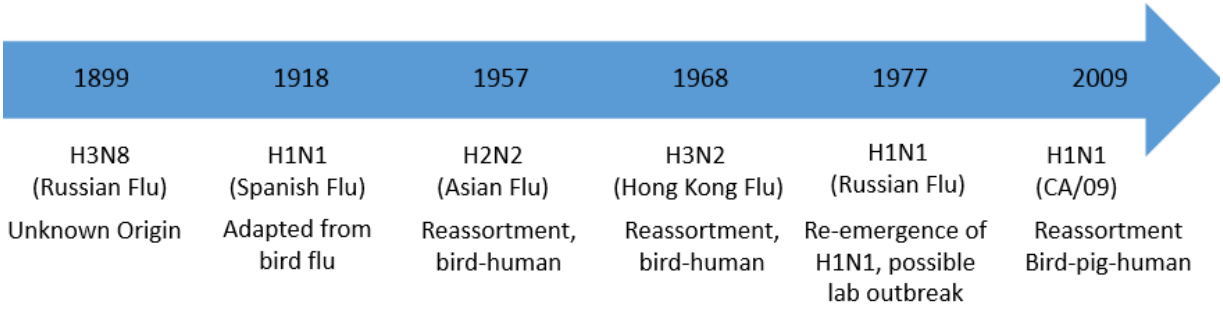


Figure 3. Timeline of Pandemic Strains of Influenza from 1899 to Modern Day.

1.2 INFLUENZA PREVENTION AND TREATMENT

Currently vaccination is considered by the CDC and WHO as the best method of preventing influenza infection as well as controlling the spread of the virus through the population. Unfortunately many individuals forgo vaccination which puts both them and the people they come into contact with at risk for infection, and potential hospitalization and death. Various antivirals such as M2 channel and Neuraminidase inhibitors can help reduce the severity of disease, but their efficacy is limited by host of factors including anti-antiviral resistant strains of influenza.

1.2.1 Vaccines

Currently vaccination is considered to be the most effective way of preventing the spread of influenza. However the high mutation rate of the virus necessitates the creation of new seasonal vaccines every year. Influenza's genome is RNA based and its RNA polymerase lacks the capacity to proofread the transcripts it produces, allowing for greater number of errors and mutations to occur than would with a DNA polymerase. This is propensity for small yet

significant mutations to accumulate over time is called antigenic drift. A second type of mutation is called antigenic shift and is specific to influenza A viruses only. Influenza's genome is segmented into eight different strands. Should the two viruses from different strains (such as H1N1 and H3N2) infect the same cell, segments of genome from the two different strains can intermingle, creating new viral strains (such as H1N2) (21).

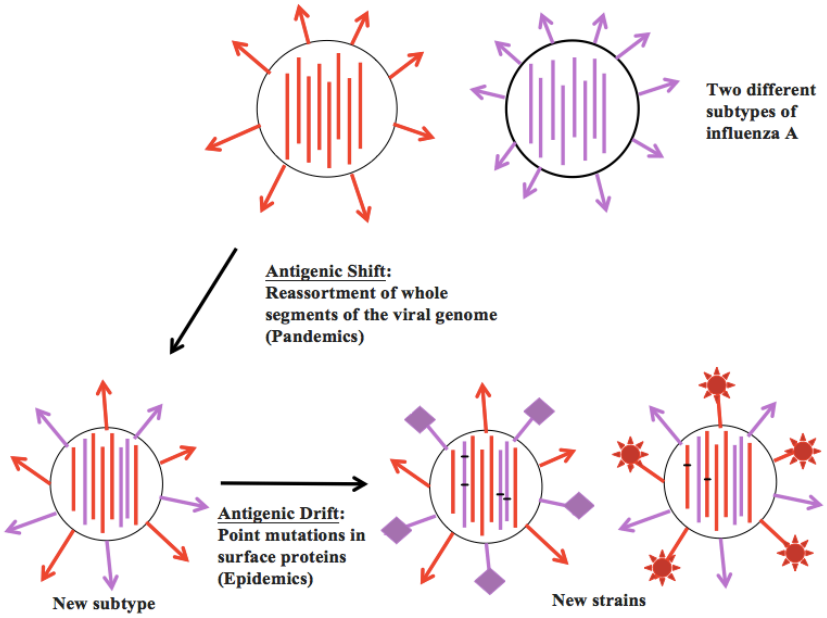


Figure 4. Antigenic Shift and Antigenic Drift.

Because of the high mutation rate of influenza the previous year's vaccine is reviewed and if deemed necessary updated in order to keep up with the strains that are predicted to be the most circulating. On a biannual basis the WHO organizes a meeting between representatives of national laboratories and research facilities to review surveillance data, clinical studies, and laboratory research in order to make recommendations to each country's public health body and vaccine production facilities regarding the formation of the influenza vaccines. Based on these meetings the WHO makes recommendations for the specific viruses to be included in the

vaccine. In the United States the Food and Drug Administration (FDA) has the final say as to which strains of influenza are to be included in the American influenza vaccine (CDC: https://www.cdc.gov/flu/about/season/vaccine-selection.htm).

Vaccines are then produced over a period of five to six months primarily from virus grown in fertilized chicken eggs. For those that are allergic to eggs or are unwilling to take a vaccine with component from egg grown viruses there are also a class of vaccines produced from cell culture grown viruses. According to the CDC there are a variety of trivalent (containing three different virus's components) and quadrivalent (four component) vaccines available to the public. The trivalent vaccines include standard and high dose intramuscular shots, as well as a vaccine made from cell culture grown virus, and a vaccine made with the adjuvant MF59. The quadrivalent vaccines include both intramuscular and intradermal vaccines, as well as a cell culture grown vaccine strains of influenza. (CDC: https://www.cdc.gov/flu/protect/keyfacts.htm). All of these vaccines fall into the category of inactivated influenza vaccines (IIV), which are vaccines made from viruses that have been chemically inactivated so that they are no longer infectious.

As of the time of publication there exist a variety of novel vaccine strategies currently under research. One such method uses recombinant influenza virus like particles (VLPs) that do not require chicken eggs or cells. VLPs share a similar structure to wildtype virus, except they lack genomic RNA fragments. VLP's can infect human and animal cells but cannot replicate making them safe to handle and an effective vaccine platform (CDC: https://www.cdc.gov/flu/protect/vaccine/how-fluvaccine-made.htm). The surface of influenza VLPs can be made to express viral surface glycoproteins such as hemagglutinin and neuraminidase. These can then be injected into a patient in the same manner as an IIV, whereby

the host's immune system will develop antibody dependent protective immunity with a diminished risk of infection or discomfort to the patient (22). VLPs can be grown in insect cells which has a twofold benefit, firstly it provides a vaccine option to those who are unwilling or unable to receive egg produced vaccines. Secondly certain strains of influenza, such as highly pathogenic H5N1 avian flu, will kill chicken eggs, precluding this system as an effective means to generate virus for vaccines (23).

Though vaccines are readily available to the public, vaccination efforts are hampered for a variety of reasons. Mutations within the viral genome necessitate the creation of new vaccines every year. The efficacy of each year's vaccine relies on accurately identifying the strains most likely to circulate widely. Yet these predictions are not always entirely accurate, leading to reduced vaccine efficacy. The 2014-2015 influenza season provides an excellent example. Total vaccine efficacy for that year was only 23%, likely as a result of the H3N2 virus circulating through the population being antigenically and genetically different from the A/Texas/50/2012 (H3N2) vaccine component (24). In order to address the shortcomings of the seasonal vaccine certain pharmaceutical therapeutics have been pursued.

Table 1 . Current Influenza vaccines available in the United States for 2016-2017 flu season.

Manufacturing Company	Vaccine Name	Vaccine Type
GlaxoSmithKline	Fluarix Quadrivalent	Quadrivalent, IIV, intramuscular, egg grown
Sanofi Pasteur	Fluzone Quadrivalent	Quadrivalent, IIV, intramuscular, egg grown
Sanofi Pasteur	Fluzone Intradermal Quadrivalent	Quadrivalent, IIV, intradermal, egg grown
Seqirus	Afluria Quadrivalent	Quadrivalent, IIV, intramuscular, egg grown
Seqirus	Flucelvax Quadrivalent	Quadrivalent, IIV, intramuscular, cell-culture grown
Seqirus	Afluria	Trivalent, IIV, intramuscular, egg grown
Seqirus	Fluvirin	Trivalent, IIV, intramuscular, with adjuvant, egg grown
Sanofi Pasteur	Fluzone High-Dose	Trivalent, IIV, intramuscular, high dose, egg grown
Protein Sciences	Flublok	Trivalent, Recombinant Influenza Vaccine, intramuscular
Medimmune	FluMist Quadrivalent*	Quadrivalent, Live Attenuated Influenza Vaccine (LAIV), intranasal spray

*Not recommended for 2016-2017 flu season

Based on data from the Centers for Disease Control for the 2016-2017 flu season.
https://www.cdc.gov/flu/protect/vaccine/vaccines.htm#modalIdString_CDCTable_0

1.2.2 Antivirals

Neuraminidase and M2 channel inhibitors are comprised of a number of pharmaceuticals that inhibit viral spread within a host. Neuraminidase is an influenza surface molecule which promotes daughter virus release from infected cells (25). Neuraminidase inhibitors like oseltamivir (Tamiflu) act as a sialic acid analogue and prevent neuraminidase from cleaving the bond between viral hemagglutinin and sialic acid residues on the infected cell's surface (26, 27).

This results in daughter virions aggregating on infected host cell surfaces and reduces viral release.

The Matrix-2 (M2) protein is an integral membrane proton channel that facilitates the dissociation of viral nucleoprotein from the matrix protein upon acidification of the endosome (28). This step begins the process by which the viral nucleoprotein dissociates with the matrix protein of the capsid, eventually resulting in viral genome unpacking (28). M2 channel inhibitors like Amantadine prevent the dissociation of the nucleoprotein from the matrix protein by inhibiting the ion channel activity of the M2 protein (29-31). M2 is also important in the formation of mature hemagglutinin molecules. Viral membrane proteins are translated and inserted into the endoplasmic reticulum for transport via the trans-Golgi network. M2 prevents over acidification of the Golgi's lumen, preventing premature conformational rearrangement of the nascent hemagglutinin and leading to its inactivation (29, 30, 32).

Both M2 channel and neuraminidase inhibitors efficacy has been proven clinically; reducing length of illness, morbidity, and mortality if taken quickly after the onset of symptoms (25, 33-36). However this reduction in symptom intensity and duration is slight (37). While the targets of both types of pharmaceuticals are conserved some influenza strains have developed resistance, limiting these drugs efficacy (31, 36, 38, 39). The incidence of drug resistance appears to be low, and when it does occur usually results in a reduction of infectivity, replicative ability, and pathogenicity for the mutant strain (25, 36, 40). Another concern is the lack of data indicating that oseltamivir or other antivirals reduces the transmission of virus (37). Clinical studies have demonstrated that patients on oseltamivir 24 hours post onset of symptoms can have relatively similar viral load counts to un-treated patients (41). These patients might experience

relief from their symptoms and feel well enough to return to the community, but remain infectious.

1.3 ANIMAL MODELS OF INFLUENZA

Various animal species can serve as useful models in understanding how infectious diseases infect, propagate, and spread through human populations. They serve as a bridge between the divide between in vitro and clinical research. However it is critical to select animals that best replicate human physiology, susceptibility, and clinical symptoms, while also taking into account practicality. Three such models be discussed, the murine, the nonhuman primate, and the musteline.

1.3.1 Murine Model

Mice (*Mus musculus*) have been and continue to be the most widely used model for influenza research mainly for reasons of practicality. Mice are small, easy to handle, and are relatively cheap to house, maintain, and breed when compared to other established models (3, 42). This allows researchers to perform studies with larger sample sizes than are practical for other non-murine models. Additionally the wide variety of genetically modified and mutant murine strains allows researchers to perform genetic and loss of function studies that other animal models would preclude. Additionally there exists a plethora of murine specific reagents readily available to researchers that are less plentiful for other animal models. (3). However the mouse model is not ideal for influenza research for a variety of reasons.

Mice are not a natural host for influenza, and their susceptibility to the virus is dependent upon both the strain of the mouse and influenza virus being used (3, 42). Inbred mouse strains used commonly in the laboratory setting may lack a functional Mx1 protein, a known antiviral factor. These Mx1 negative mice more susceptible to infection with influenza compared to wild type mice (3, 43). This can lead to higher infectivity and mortality rates amongst the lab strains than would be seen amongst wild type mice or in humans during an actual influenza outbreak.

Human strains of influenza are not able to replicate as efficiently in mice as they do in humans. It may be necessary then to adapt human specific influenza strains to mice by passaging the virus through murine cell lines in order to efficiently establish an infection in this model (44, 45). Additionally mice do not display the same clinical symptoms of influenza infection that is seen in humans (45). Mice commonly develop hypothermia during the course of infection, which is in direct contrast to the fever that humans commonly display (46). Finally mice have neither been known to display the common symptoms seen in humans of coughing or sneezing when infected with influenza (42).

Other issues regarding the use of mice is the lack of sialic acid residues with α-2, 6 linkage to galactose. Human specific strains of influenza preferentially bind to this complex, which is commonly found on the surface of epithelial cells of the human respiratory system. Mice do possess α-2, 6 sialic linked receptors, but they are confined to epithelial cells of the cecum, rectum, and blood vessels (47). Viral replication and viral induced damage occurs primarily in the lower lung of the mouse model, which generally occurs in the upper airways (48, 49). Finally mice do not easily transmit human specific strains between themselves, demonstrating unsuitability for research into viral spread (50). All of these factors diminish the suitability of using the murine in influenza research.

1.3.2 Nonhuman Primate Model

Nonhuman primates like Rhesus and Cynomolgus macaques are similar to humans in regards to physiology and genetics. This makes them an excellent model compared to genetically distant mammalian species like mice, Guinea pigs, and ferrets. Nonhuman primates are susceptible to a wide variety of unadapted influenza strains and exhibited similar common symptoms, such as fevers, malaise, and nasal discharge (42, 51). Highly pathogenic strains of influenza are thought to induce extreme morbidity and mortality through cytokine storms, in which an over expression of proinflammatory cytokines results in increased morbidity and death. Nonhuman primate are known to react similarly, overproducing cytokines mounting an abnormal and less effective immune response (51, 52).

While nonhuman primates are ideal for their susceptibility to human strains of influenza as well as their clinical similarities to humans post infection there are several issues that diminish their utility as effective models. Nonhuman primates have complex husbandry requirements, are expensive to maintain and house, and are not widely available for laboratory use (42). Additionally there are ethical considerations which make working with nonhuman primates a tricky and difficult proposition. Finally nonhuman primates carry a wide variety of zoonotic diseases that are transmittable to humans who work in close contact with them. Of particular concern is *Cercopithecine herpesvirus* 1 which is readily transmitted by bites, scratches, and mucosal contact with infected fluids. While the virus is relatively benign in nonhuman primates, zoonotic infectious of humans can results in fatal encephalomyelitis or severe neurological damage (53). These considerations make working with nonhuman primates less ideal regardless of their suitability as a model for influenza infection.

1.3.3 Musteline Model

Ferrets **(***Mustela putorius furo*) provide an excellent animal model for investigative research of the influenza virus, and have been used in this capacity since the 1930's. Other animal models including mice, cats, dogs, guinea pigs, and non-human primates have all been used in the past, but ferrets serve as a cost effective model that accurately simulates the course of infection observed in humans (3). Ferrets are small to medium sized, easily house, and cared for, are relatively inexpensive to study compared to non-human primates, more easily handled, and undergo a similar disease course as humans.

Ferrets are accepted as the best animal model for influenza research. While their larger size, limited availability from breeders, and paucity of ferret specific reagents available to the scientific community pose significant challenges, ferrets possess unique qualities that make them ideal models (50). Ferrets are readily susceptible to human strains of influenza without, removing the need to adapt viral strains to this model. Viral transmission and infection in the ferret model follows a similar progression as seen in humans. Ferrets present similar clinical symptoms as humans, including sneezing, coughing, nasal discharge, fever, and lethargy. The size of the ferret can also be preferable as well, bronchial alveolar lavage, blood sampling, and tissue collection at necropsy is more substantial (3). Finally and most importantly of all ferrets and humans share a similar distribution of α-,6 linkage sialic acid receptors in the respiratory tract, which helps explain both the susceptibility to human strains of influenza seen in ferrets (3).

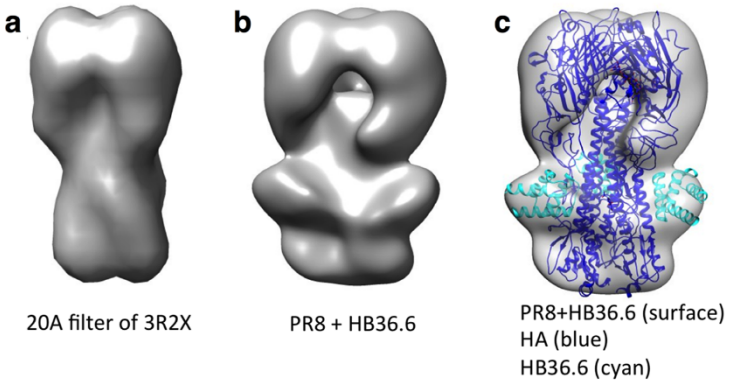
1.4 NOVEL PROTEIN BINDERS AS INFLUENZA ANTIVIRALS: HB36.6 AND A13R33

1.4.1 Protein Binder HB36.6

Prior work performed in Cole laboratory at the University of Pittsburgh's Regional Biocontainment Laboratory (RBL) evaluated the performance of the novel antiviral HB36.6. HB36.6 was initially developed by Drs. David Baker and Deborah Fuller at the University of Washington, using their computational modeling program Rosetta. HB36.6 was final product of several peptides initially designed to bind the highly conserved stem region of the influenza A hemagglutinin protein, and screened for improved affinity to a wide variety of influenza A hemagglutinin subtypes. This protein was a mimic of a naturally occurring neutralizing antibody whose binding would inhibit fusion of the virus with the host cell, thereby diminishing its infectivity and ultimately neutralizing the virus (54).

HB36.6 was generated by modification of its predecessor, HB36.5, where HB36.5 was subjected to random mutagenesis of its nucleotide sequence and a library of peptides was created. Each peptide's amino acid residues were individually mutated to all other possible amino acids, and the library of generated peptides were sequenced. Enrichment or depletion of each individual mutation during affinity maturation was evaluated and a new library was constructed consisting of the most optimal substations in the amino acid sequence. Yeast cells displaying hemagglutinin from Influenza A/South Carolina/1/1918 H1N1 were used in two rounds of sorting, and a peptide with nine substitutions, referred to as HB36.6, was selected. HB36.6 was confirmed to preferentially bind to the stem of hemagglutinin (Figure 5A-C), to

possess high affinity (Figure 5D), and to demonstrate comparable equilibrium binding constants (Figure 5E) against select hemagglutinin subtypes.



d

Influenza Virus	k_D (nM) HB36.5	HB36.6
A/South Carolina/1918 H1	1.5	1.5
A/California/2009 H1	17.4	3.1
A/Adachi/1957 H2	711.9	18.5
A/Vietnam/2004 H5	224.8	23.5
A/Wisconsin/1966 H9	82.8	13.2
A/Alberta/1976 H12	634	496

e

Influenza Virus	EC_{50} (µg/ml) HB36.6	FI6	Ribavirin
A/California/2009 (H1N1)	0.18	0.27	15
A/Puerto Rico/1934 (H1N1)	0.58	0.27	ND
A/New Caledonia/1999 (H1N1)	1.26	0.34	ND
A/Hong Kong/2003 (H5N1)	12	ND	16
A/Duck/MN/1981 (H5N1)	5.4	ND	18
A/Hong Kong/2009 (H9N2)	>320	210	ND
A/Brisbane/2007 (H3N2)	>320	0.12	ND
A/Nanchang/1995 (H3N2)	>320	28.8	ND

Figure 5. Characterization of HB36.6.

(A) Crystal structure of A/South Carolina/1/1918 (H1N1) hemagglutinin protein. (B) PR8 hemagglutinin bound to HB36.6. (C) Hemagglutinin protein from A/South Carolina/1/1918 (H1N1) (blue) with HB36.6 (cyan) docked into PR8 in B showing HB36.6 fits well into the stem region regardless of steric hindrance. (D) Equilibrium binding constants for HB36.5 and HB36.6 against various hemagglutinins demonstrated broad binding of group 1 subtypes. (E) EC50 (µg/ml) (compound concentration that reduces viral replication by 50%) of HB36.6, the monoclonal antibody FI6v3, and ribavirin against group 1 and group 2 viruses. ND = not determined (54).

Once HB36.6 was characterized in vitro, in vivo evaluations using BALB/c mice was performed. Mice receiving HB36.6 intranasally in doses ranging from 0.1 to 6mg/kg $\leq$ 48 hours prior to infection with a lethal dose of A/California/07/2009 pH1N1 adapted for mice demonstrated 100% survival. Additionally, treated mice exhibited less weight loss compared to mice that received no antiviral treatment (Figure 6A, B). Only 20% of mice that received the lowest dose of 0.01/kg $\leq$ 48 hours prior to infection survived (Figure 6B). Protection was also confirmed with two additional strains of influenza, A/Puerto Rico/8/1934 H1N1 and A/Duck/Minnesota/1525/1981 H5N1 following treatment with 3mg/kg of HB36.6 2 hours before infection. (Figure 6C) (54).

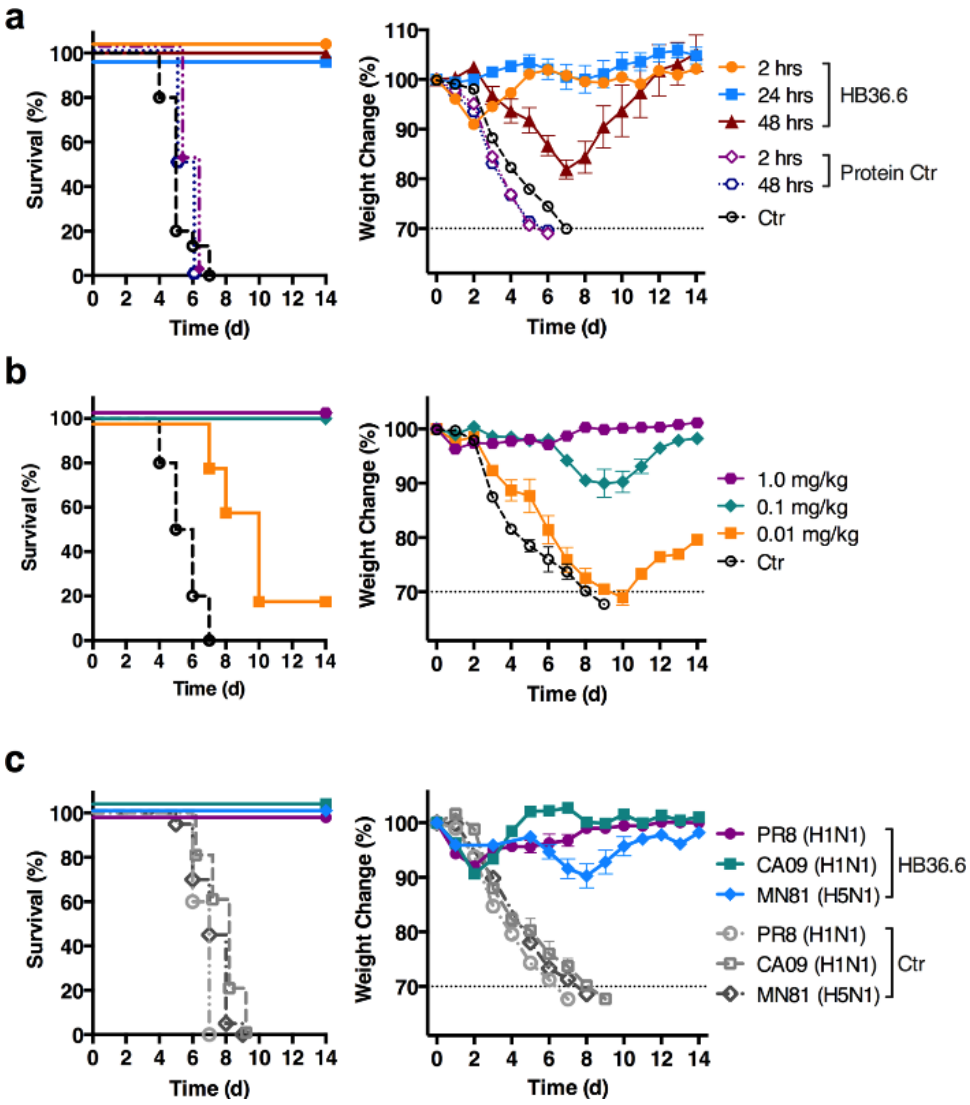


Figure 6. HB36.6 provides prophylactic protection against lethal influenza challenge in BALB/c mice. Survival curves and weight change in (A) BALB/c mice treated with 6mg/kg HB36.6 intranasally at 2, 24, and 48 hours before challenged with 10 times mouse lethal dose 50% (MLD_{50}) CA/09 H1N1. (B) BALB/c mice administered 0.01, 0.1, or 1.0 mg/kg dose of HB36.6 intranasally 2 hours prior to challenge with 10 MLD_{50} CA/09 H1N1. (C) BALB/c mice that received 3.0mg/kg HB36.6 intranasally 2 hours prior to infection with 10 MLD_{50} CA/09 H1N1, 6 MLD_{50} H1N1 A/PR/8/34 (PR8), or 3 MLD_{50} A/Duck/MN/1525/81 H5N1 (MN81). Note n=10 per group for Figures A and C, n=5 per group for Figure B (54).

1.4.2 HB36.6 Evaluation in Ferrets

Our laboratory group expanded upon the in vitro characterization and mouse studies by evaluating the efficacy of HB36.6's in the ferret model. Ferrets were chosen for these studies as they better recreate both the physiological conditions found in humans as well as the clinical symptoms post infection. The first study measured HB36.6's prophylactic efficacy based upon dose. Two sets of ferrets were administered HB36.6 2 hours prior to infection. The first group (n=4) received 2.5mg/kg of HB36.6 and the other group (n=4) received 10mg/kg. A third control group (n=4) did not receive any HB36.6 at all. Animals were challenged with A/California/07/2009 pH1N1 and the results of the experiment can be seen in Figure 7 below. Daily core temperatures in Celsius (Figure 7A) and weight change (Figure 7B) were monitored and recorded for all animals. Additionally a scoring system based on clinical symptoms and their severity was used to monitor the ferrets, with a higher score corresponding to a greater degree of illness (Figure 7C) (55). See Appendix A for clinical scoring sheets used to evaluate symptoms. Three days post infection all animals were euthanized and necropsied

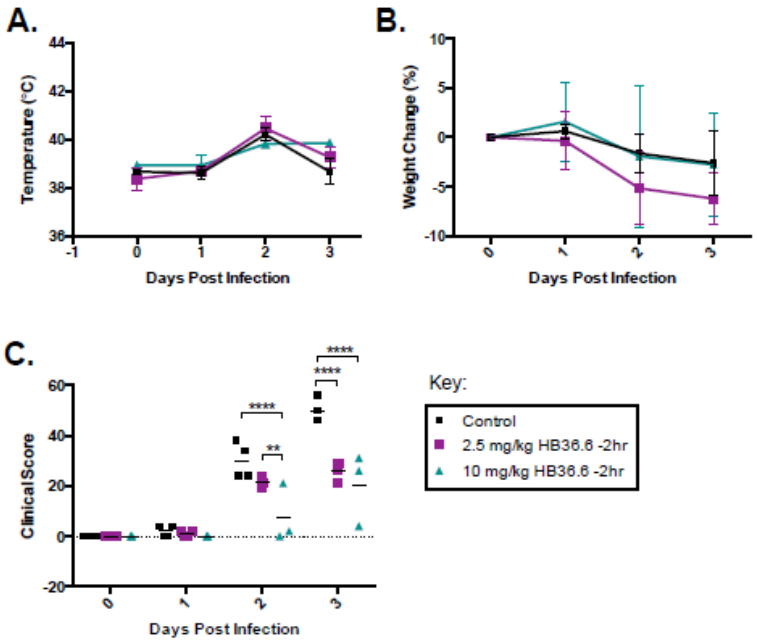


Figure 7. Clinical signs of disease in ferrets treated with low doses of HB36.6 and untreated control ferrets after aerosol exposure to A/California/07/09 (H1N1).

Three groups of ferrets (n=4, per group) were either administered 2.5m/kg of HB36.6, 10mg/kg of HB36.6, or nothing 2 hours before challenge with 2.6 x 10^7 $TCID_{50}$ A/California/07/09 2. (A) Temperature (°C) of treated and untreated control ferrets over the course of infection. (B) Percentage of weight change of treated and untreated control ferrets over the course of infection. (C) Individual ferret clinical scores for treated and untreated control ferrets over the course of infection. Graphs A and B are shown with mean and SD at each time point. Graph C is shown with mean at each time point. Statistical analysis was done using a two-way ANOVA for multiple comparisons where $\alpha = 0.05$ (55).

Ferrets treated with HB36.6 demonstrated no differences in temperature (Figure 6A). Treated ferrets on average lost more weight than the untreated controls, however, the weight loss was not significant (Figure 6B). Interestingly, clinical scoring among the three groups demonstrated significance (Figure 6C). Treated animals had fewer and less severe clinical signs of disease compared the untreated controls (Figure 6C). This suggested that HB36.6 was able to

reduce clinical signs of disease either by reducing the amount of virus replication or by reducing the immunological damage induced in vivo.

To address the quantitative measure of virus present in all three groups of ferrets following challenge, nasal washes were obtained daily and lung tissue harvested at necropsy. These samples were analyzed by quantitative polymerase chain reaction (qPCR) to determine the quantitative level of virus present in each sample. In order to calculate the titer of the virus, viral loads were to a viral standard curve generated from serially diluted RNA extracted from A/California/07/2009 pH1N1 RNA. Virus from daily nasal washes (Figure 7A) and lung tissues (Figure 7B) are shown below (55).

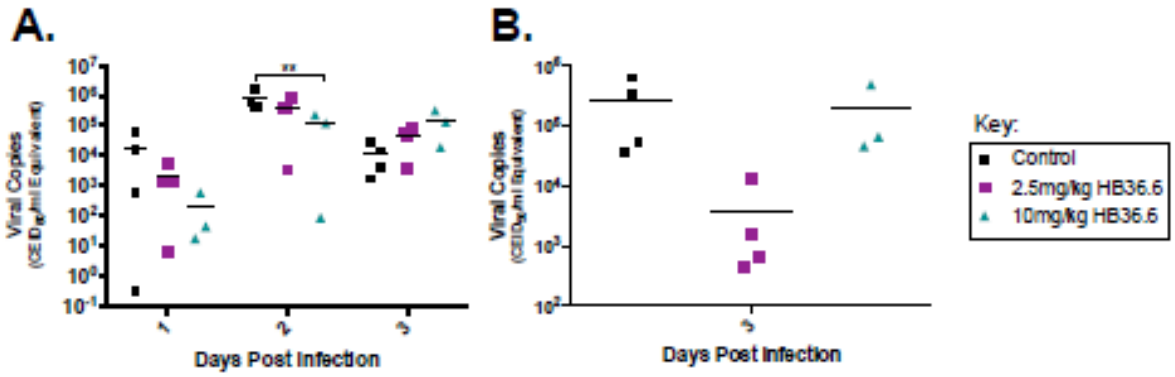


Figure 8. Viral loads from daily nasal washes and homogenized lungs in ferrets treated with low dose HB36.6 and untreated controls after aerosol exposure to A/California/07/09 (H1N1).

Three groups of ferrets (n=4, per group) were either administered 2.5m/kg of HB36.6, 10mg/kg of HB36.6, or nothing 2 hours before challenge with 2.6 x 10^7 $TCID_{50}$ A/California/07/09 2. Viral loads were measured by semi-quantitative reverse-transcriptase RT-PCR following aerosolized pH1N1 infection in (A) nasal wash, and (B) lung tissue at necropsy. All data is represented with the mean at each time point. Statistical analysis on nasal wash data was performed using a two-way ANOVA for multiple comparisons (55).

Viral loads for untreated controls were higher on days 1 and 2 post infection (Figure 8A). HB36.6 treated ferrets demonstrated reduced viral loads in a dose-dependent manner. Similarity

lung homogenates from HB36.6 treated ferrets showed reductions in viral load in a dose-dependent manner and compared to untreated controls (Figure 8B). These data suggested that HB36.6 was able to bind and inhibit virus infection in vivo in the ferret model. Given that the first study was only allowed to proceed for 3 days, corresponding to the approximate time of peak infection for this challenge virus in the ferret model (previous experience, unpublished data), additional studies following the ferrets clinically for up to 7 days post infection were performed. (55).

The second study was interested in evaluating longer time points post virus challenge and evaluating higher concentrations of HB36.6 pre-infection. A second study using higher doses of HB36.6 was performed. As before three groups of ferrets were used, however one group (n=4) received 20mg/kg of HB36.6 24 hours prior to infection while the second group (n=2) received the same dose 2 hours prior to infection. The third group (n=2) remained untreated and served as controls. Similar to data obtained for the first ferret study, temperatures, (Figure 8A), weights (Figure 8B), and clinical scores (Figure 8C) were monitored daily post influenza infection. Nasal washes collected daily (Figure 9A) and lungs harvested at necropsy (Figure 9B) were analyzed for viral loads (55).

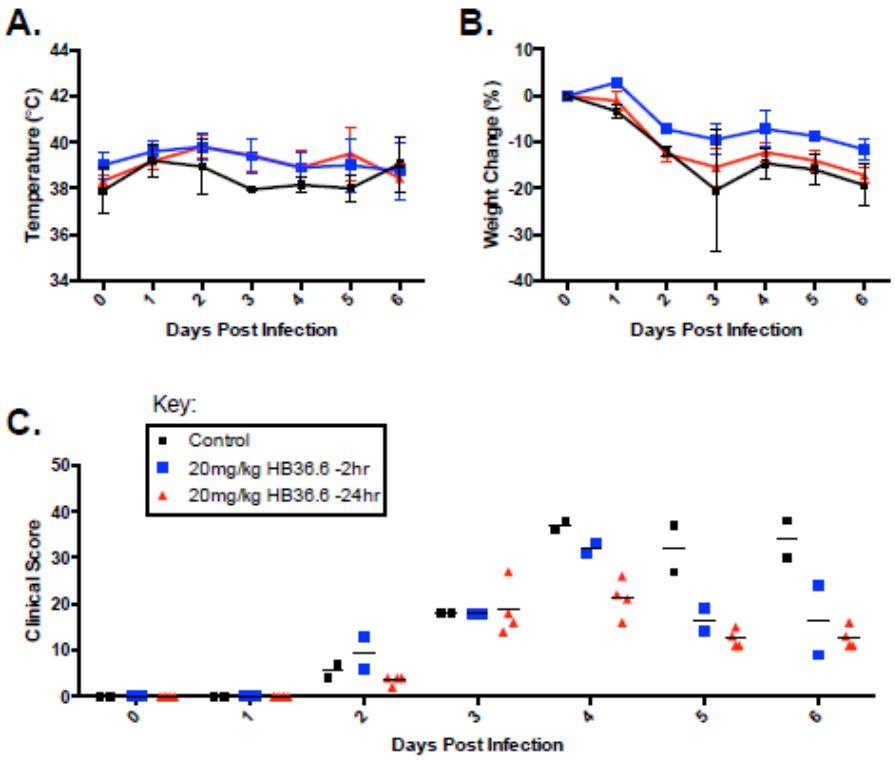


Figure 9. Clinical signs of disease in ferrets treated with high doses of HB36.6 and untreated control ferrets after aerosol exposure to A/California/07/09 (H1N1).

This study consisted of two untreated control ferrets, two ferrets treated with 20mg/kg HB36.6 two hours before infection with H1N1, and four ferrets treated with 20mg/kg HB36.6 24 hours before infection. Ferrets were challenged with 2.6 x 10^7 $TCID_{50}$ A/California/07/09. (A) Temperature (°C) of treated and untreated control ferrets over the course of infection. (B) Percentage of weight change of treated and untreated control ferrets over the course of infection. (C) Clinical scores of treated and untreated control ferrets over the course of infection. Graphs A and B are shown with mean and SD at each time point. Graph C is shown with mean at each time point (55).

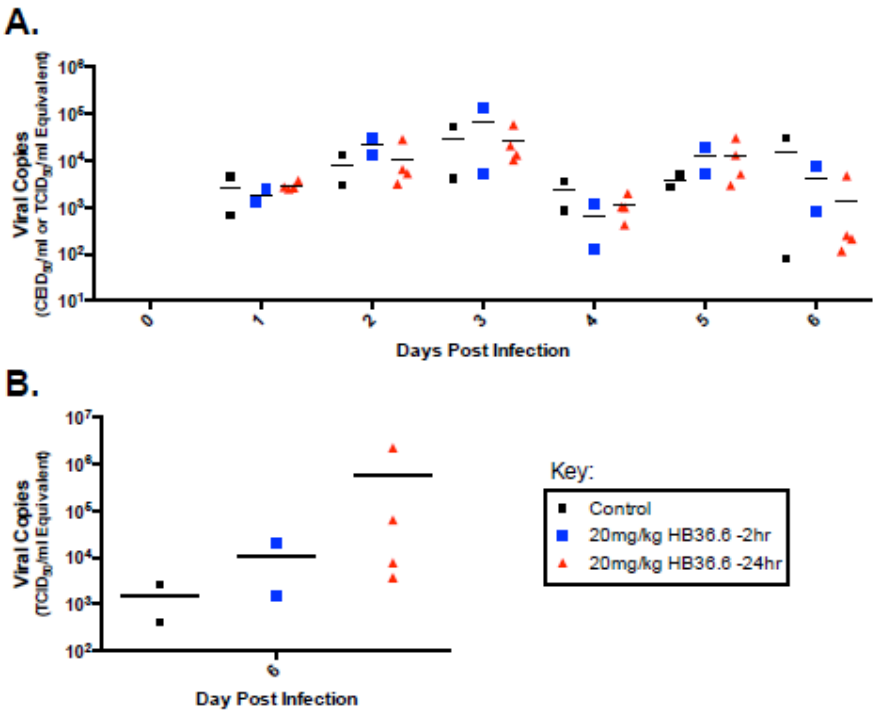


Figure 10. Viral loads from daily nasal washes and homogenized lungs in ferrets treated with high dose HB36.6 and untreated controls after aerosol exposure to A/California/07/09 (H1N1).

This study consisted of two untreated control ferrets, two ferrets treated with 20mg/kg HB36.6 two hours before infection with H1N1, and four ferrets treated with 20mg/kg HB36.6 24 hours before infection. Ferrets were challenged with 2.6 x 10^7 $TCID_{50}$ A/California/07/09. Viral loads were measured by semi-quantitative reverse-transcriptase RT-PCR following aerosolized pH1N1 infection in (A) nasal wash, and (B) lung tissue at necropsy. All data is represented with the mean at each time point. Statistics were not performed due to sample size of n=2 for some groups (55).

Ferrets treated with the higher dose of HB36.6 24 hours prior to infection lost almost as much weight as the untreated controls (Figure 8B). This is a marked contrast to the ferrets treated with HN36.6 2 hours prior to infection. Interestingly though both the treated and untreated animals showed a continuous downward trend in weight loss over the lengths of the study. While the clinical scores of the treated ferrets were much lower than the untreated controls, they remained high over the course of the study, especially when compared back to the clinical scores of the first study. Treated ferrets from the first study were never given a clinic score of 30, while

on day 4 of the second study the two ferrets given HB36.6 2 hours prior to infection scored over 30. Based on observations, we hypothesized that the higher dose of HB36.6 might be toxic to the ferrets and may have led to an increase in the clinical scores. Upon initial observation, it appeared that the viral load data conflicted the first study. However, this was not the case as the ending time point for measurement of viral loads in the lung tissue were different. For example, study 1 measured day 3 post infection compared to the second study which measured up till day 6. This explains the fall in the peak range of viral load after challenge (Days 3-4) and demonstrated a marked difference in treated (lower) vs. untreated (higher) ferrets. In contrast, when measuring viral loads at day 7 post challenge, the treated ferrets demonstrated higher viral loads while the untreated ferrets had lower viral loads. The latter is to be expected, as innate and adaptive immune responses generally begin to clear virus from the lungs over time. In the treated animals we hypothesized that the virus that was not completed reduced by the initial treatment with HB36.6 was lagging behind the initial challenge, and by day 7 had the opportunity to replicate in the lung. Thus, the day 7 data demonstrated a 'lag and catch up' of viral loads in the treated animals. A more detailed study of daily viral loads in multiple tissues will be warranted along with immune responses to further tease this apart. However, this general trend can be observed in the longitudinal nasal wash samples in this second study (55).

Together, these two studies demonstrated the efficacy of HB36.6 in blunting the early virus infection in the ferret model, supporting the in vitro and in vivo mouse data obtained by the University of Washington. Studies to minimize toxicity of the binder would be needed to move this product into clinical trials.

1.4.3 Minibinder A13r33

To further address the limitations on use of long peptide HB36.6, including but not limited to difficulty in production, cost, solubility, and potential toxicity in vivo, the Baker and Fuller labs began development of MiniBinders. These are smaller peptides than the antivirals (such as HB36.6) that were tested previously. These new peptides contain the biologically relevant portion of the original peptides. These mini-binders were shown to be more flexible in solution, more soluble, and would be less expensive to generate given their smaller size. Smaller size would also result in even less immunogenicity that the first generation binders like HB36.6.

To date animal studies have demonstrated that low doses (0.3mg/kg) of A13r33 given prophylactically are more effective in preventing weight loss and death in BALB/c mice compared to an equal dose of HB36.6 (Figure 10). A13r33 displays a higher binding constant (K_0) to A/Puerto Rico/8/34 H1N1 than both HB36.6 and monoclonal antibody F16. Furthermore a lower concentration of A13r33 is required to reduce viral replication by 50% (EC_{50}) when compared again to HB36.6 and F16 (Table 2). All of this suggests that A13r33 might be a more effective in controlling influenza infection than other antivirals tested in the past.

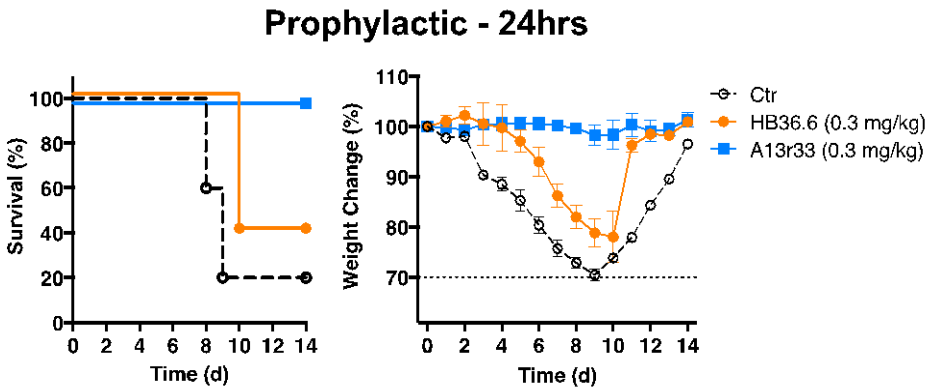


Figure 11. A13r33 provides greater prophylactic protection against lethal influenza challenge in BALB/c mice.

Survival curves and weight change comparison in BALB/c mice treated prophylactically with 0.3 mg/kg HB36.6, 0.3 mg/kg A13r33, or negative control.

Table 2. Comparison of HB36.6 to A13r33.

	Stability		K_D (nM)	EC_{50} (µg/ml)	
Binder	S-S	T_M (°C)	PR8 H1N1	PR8 H1N1	CA09 H1N1
HB36.6	0	65	0.2	0.56	1.8
A8	0	70	>30	>10	>10
A8r33	0	70	6	>10	>10
A13	2	80	>30	>10	>10
A13r33	2	>95	5	<0.003	<0.003
A18r33	3	>95	2	0.26	0.56
FI6 (antibody)	4	60	1	0.1	0.21

Equilibrium binding constants (K_0) for HB36.and A13r33 against various against A/PR/8/1934. EC50 (µg/ml) (compound concentration that reduces viral replication by 50%) of HB36.6, the monoclonal antibody FI6, and A13r33 against A/PR/8/1934 and A/CA/7/2009

2.0 STATEMENT OF PROJECT AND AIMS

During the 2015-2016 flu season the CDC estimated that over 300,000 people were hospitalized in the United States for flu related illness (CDC: https://www.cdc.gov/flu/about/qa/hospital.htm). As has been mentioned before influenza is responsible for the deaths of tens of thousands of Americans through both direct and indirect means, as well as costs tens of billions of dollars lost to hospitalizations, treatments, and lost work hours each year (12, 13). Currently vaccines are considered to be the best direct protection against the influenza virus. Accurately identifying the strains likely to circulate most widely is difficult and can lead to vaccines that provide little to no protection against more pathogenic strains. To combat this difficulty a variety of antivirals have been developed, but their success has been varied. Influenzas strains resistant to oseltamivir and other influenza antivirals has already been observed (31, 36, 38, 39). Worryingly some strains continue to exhibit resistance to antivirals after the selective pressure have been removed (56). Oseltamivir is known to only slightly decrease the time between onset and alleviation of symptoms, while providing moderate relief. Additionally viral loads in treated versus untreated patients do not differ much 24 hours post onset of symptoms, which calls into question the drug's efficacy (41). Finally oseltamivir is known to cause a plethora of side effects including nausea, headaches, and renal and psychiatric syndromes (37).

It is for these reasons that new antiviral drugs should be pursued for the sake of the public's health. This project, based upon prior work conducted both at the University of Washington as well as the University of Pittsburgh seeks to evaluate A13r33, a novel antiviral peptide. A13r33 is similar to a previously tested antiviral, HB36.6, in that both bind to viral hemagglutinin and interfere with virus-cell fusion. Unlike HB36.6 A13r33 binds with greater efficiency to two different strains of H1N1 and potentially neutralizes the virus at a smaller dose (Table 2). A13r33 also binds to influenza strains with a better efficiency than the F16 neutralizing antibody, which can bind to 16 hemagglutinin subtypes (57). Additionally we knew from prior work that animals treated with HB36.6 exhibited a reduction in the severity of clinical symptoms associated with infection (58). Based on this information we developed the following hypothesis: A13r33 would be effective at both reducing the viral loads and reducing the clinical signs of disease following aerosolized infection with pandemic H1N1. To test this hypothesis two aims were developed using ferrets infected with A/California/07/2009 p H1N1.

2.1 AIM 1

To demonstrate efficacy of the novel computationally designed antiviral minibinder A13r33 against influenza infection with A/California/07/2009 pH1N1 in the ferret model through analysis of clinical symptoms.

All ferrets used in these studies were monitored and scored daily with a detailed clinical scoring system developed by Dr. Kelly Cole and Dr. Deborah Fuller. Prior to infection all of the ferrets that were used in the study were weighed, their temperatures were taken, and clinical scores were collected. This data was used as a baseline of comparison allowing our research

group to compare the ferrets retrospectively between pre- and post-infection. Following infection with aerosolized virus weights, temperatures, and clinical scores were collected daily up till and including the day the animals were sacrificed.

2.2 AIM 2

To characterize the virological and immunological correlates of protection of A13r33 in the ferret model following exposure to aerosolized challenge with pandemic H1N1 influenza.

Viral titers were determined by extracting influenza genomic RNA from nasal washes and homogenized lung samples. To calculate viral loads from nasal washes, a one-step reverse transcription quantitate polymerase chain reaction (RT-qPCR) was employed to evaluate samples collected both pre-infection and one to seven days after infection. These were compared back to standard curve generated from RNA extracted directly from A/California/07/2009 pH1N1 grown in the lab with chicken eggs. Viral titers in the lung were calculated using a two-step RT-qPCR procedure. The first step generated cDNA from extracted RNA and the second analyzed the cDNA.

Viral titers are commonly associated with disease severity, and that high viral loads can lead to an overresponse of the host's immune system (59, 60). This overreaction further leads to hyper production of proinflammatory cytokines, inventively referred to as a 'cytokine storm.' Thusly this project sought to quantify the amount of virus to see if a) A13r33 treatment reduced viral loads and b) if viral loads corresponded to the severity of clinical scores.

2.3 SUPPORTING EXPERIMENTS

An additional experiment performed in a group of six male ferrets prior to work with A13r33. The purpose of this experiment was twofold. Firstly we sought to identify if an altered strain of A/California/04/2009 H1N1 (CA/09-NLuc) virus modified to express luciferase within the polymerase subunit (PA) was usable for our future experiments (61, 62). The virus was reported to image through the skin and tissues of animals using an In Vivo Imaging System (IVIS) from Perkin Elmer. This would allow us to view infection as it spread through the animal of the entire time course of infection without the need of sacrificing the animal. Our second goal was to identify which routes of challenge with virus, aerosol or intranasal, would lead to a representative model of influenza infection.

3.0 MATERIALS AND METHODS

3.1 BIOSAFETY

Influenza A H1N1 is classified as a biosafety level (BSL) 2 pathogen by the Centers for Disease Control (CDC). In accordance with established guidelines, work with the live virus was performed in a class II biosafety cabinet (BSC). Given the airborne transmissible nature of influenza, and H1N1 in particular, the University of Pittsburgh has established guidelines that require the additional use of Individual respiratory protection in the form of powered air purifying respirators (PAPRs) or N95 particulate respirator masks to be worn by all researchers and staff (63). Individuals working in a cell culture lab must don a liquid barrier gown, two pairs of gloves and eye protection prior to commencing work with virus in the BSC. When working with animals in the vivarium, individuals must don a full Tyvek, bouffant caps, disposable water-resistant shoe covers (double layered), double gloves and respiratory protection. To minimize aerosolized transmission of virus or cross-contamination among animal cohorts, all animals were triple housed in HEPA filtered isolator containment cages with locking doors (Allentown, PA). All equipment in the vivarium, including BSCs, surgical equipment, glass/ plastic-ware, pipettes, reagent bottles, etc. were decontaminated with a 1% Wescodyne® Germicidal Detergent (STERIS Corporation, cat # NJ138). All equipment in the BSL 2/ 2+ facilities where in vitro work was performed was decontaminated with a 10% bleach solution followed by 70% ethanol

solution. All work was performed in the laboratories and vivarium of the Center for Vaccine Research at the University of Pittsburgh. All facilities were inspected and monitored at least annually by the Department of Environmental Health and Safety and the Division of Laboratory Animal Resources.

3.2 VIRUS PROPAGATION

A stock of influenza virus, A/California/07/2009 H1N1, obtained from Biodefense and Emerging Infectious Research Resources Repository (BEI Resources) (BEI Resources cat #: NR13663) was used to infect specific pathogen free (SPF) fertilized chicken eggs obtained from Charles River Laborites in May of 2016. Eggs were incubated at 100°F and about 60% humidity for 11 days in a GQF Sportsman 1502 incubator. Eggs were candled in order to identify the main vein and the air sac of the egg. A mark was made on the shell on the side furthest from the vein and a hole was punched through the shell with a 1 inch 18 gauge blunt needle embedded in a rubber stopper. Virus was inserted via a tuberculin syringe with a 1 inch 23 gauge needle at approximately $2.3*10^8$ $TCID_{50}$/ml. Eggs were incubated for 40-48 hours prior to placing at 4°C overnight. Virus was then harvested from the allantoic fluid of each egg, which was clarified by centrifugation at 2000 rpm x 10 min before making 1 ml aliquots for storage at -80C. A virus titer of $2.3*10^8$ was confirmed by tissue culture infectious dose 50 ($TCID_{50}$/mL) using Madin-Darby Canine Kidney Epithelial cells (MDCK). See Appendix C for $TCID_{50}$ calculation method.

3.3 TISSUE CULTURE INFECTIOUS DOSE 50%

$TCID_{50}$ assays measure the amount of virus necessary to infect and kill 50% of the cells present in a given culture. $TCID_{50}$ assays are performed using tissue culture, however a similar assay using chicken eggs can be used in lieu. If eggs are used instead the assay is called a Chicken Egg Infectious Dose 50% assay ($CEID_{50}$). It is important to note that these two assays are only able to measure viral particles that are able to infect tissue cells or chicken eggs. They do not quantify all non-infectious virus particles that are present, which are still capable of causing an immune response. For these studies, $TCID_{50}$ assays were performed using Madin Darby Kidney (MDCK) cells, a cell type commonly used for in vitro influenza work.

Confluent T75 or T150 cell culture flasks containing MDC cells were exposed to 2 or 4mL of Trypsin respectively and the cells were enumerated using a dyeing agent (Trypan blue). The cells were plated at a concentration of $3 – 4 * 10^5$ cells/mL. 100µL /well of resuspended cells were pipetted into 96 well flat-bottom cell culture-treated plates. Cells were placed in an incubator at 37°C with 5% CO_2 overnight prior incubating with samples to be titered. The first dilution was added to the top row in quadruplicate at a 1:10 dilution, and serial 10-fold dilutions were performed down the plate. Negative controls (no virus) and positive controls (samples with known titer at a specific dilution as well as media/virus only) were included in each plate. Plates were incubated for 4 days then scored for cytopathic effect as determined by loss of monolayer in each well by light microscopy. The $TCID_{50}$ was calculated using the formula for Reed and Muench.

3.4 AEROSOL INOCULATION

All animals used in this study were exposed to aerosolized H1N1 influenza virus via a 3-jet Collison nebulizer (BGI, INC). The nebulizer was housed inside an airtight class III BSC (Baker Co.) maintained under negative pressure, and was controlled by a an AeroMP bio-aerosol exposure system (Biaera Technologies) (64). This device creates a uniform aerosol of particles 1 to 2 μm in size, allowing the ferrets to inhale these particles more deeply into their lungs and preventing the entirety of the virus from depositing onto the surfaces of the upper respiratory tract.

Ferrets were placed into exposure cages in the animal holding room and transported to the aerosol suite in HEPA filtered rodent transport boxes provided by the University of Pittsburgh's Division of Laboratory Animal Resources (DLAR). Ferrets were placed into the mobile transport cart which was attached to a Class III Glovebox containing the aerosol equipment. Once inside, the transport cart was sealed, the exposure cages with the ferrets inside were individually removed from their transport boxes and placed into the Class III Glovebox via a pass-through door. All ferrets were placed into the exposure chamber and exposed to aerosolized H1N1 for 10 minutes followed by a 10 min air wash to remove any residual virus that may be remaining on their fur. Following the aerosol exposure, ferrets were moved back into the transfer cart, placed back into their transport boxes and the boxes wiped with 1:128 solution of Vesphene (STERIS Corporation, cat#: 646108).The transport boxes were then removed from the aerosol line, and the ferrets returned to the vivarium. An all glass impinger (AGI) containing 10 mL of viral growth media was used to collect the influenza virus. The presented dose was calculated by using Guyton's Formula of $V_m = 2.1 \pm W_b^{0.75}$, where W_b is the weight of the animal, to find the respiratory minute volume (V_m). Multiplying V_m by the length

of exposure gives the total volume of experimental atmosphere inhaled (V_t). Multiplying V_t by the aerosol concentration (C_e) gives the presented dose. Three experiments were performed as part of this thesis:

1. Comparison of intranasal vs. aerosol pilot infection (infection date 5/2016)
2. Prophylactic study 1 (aerosol date 4/18/2017)
3. Prophylactic study 2 (aerosol date 5/5/2017)

For the first study conducted in May of 2016, modified A/California/04/2009 H1N1 (a generous gift from Dr. Stacey Schultz-Cherry of St. Jude Children's Research Hospital was used. Viral titers for this study were as follows, $10^{4.45}$ $TCID_{50}$/ mL for animals infected via aerosol or $10^{4.2}$ $TCID_{50}$/ mL infected via intranasal inoculation. See section 3.5.1 for further explanation of this experiment. For A13r33 study, the first group that was aerosolized on April 18th had an average presented dose of $3.9*10^6$ $TCID_{50}$/mL was calculated. The second group aerosolized on March 5 had an average presented dose of $4.05*10^5$ $TCID_{50}$/mL. See section 3.7 for further explanations of this portion of the study.

3.5 FERRETS

All ferrets were purchased from Triple F Farms through the University of Pittsburgh's Division of Laboratory Animal Resources (DLAR). Ferret sera were screened via hemagglutination inhibition assays and determined to be H1N1 sero-negative ferrets prior to acquisition (see Appendix A for how these assays are performed). DLAR monitored the clinical health of the animals, provided food, water, enrichment, and care. All ferrets were acclimated for 72 hours prior to any experimental manipulation per Institutional Animal Care and Use

Committee (IACUC) protocol. The University of Pittsburgh abides by all federal regulations governing the use of animals in research in the U.S. and is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited institution. All procedures were approved by the University of Pittsburgh's IACUC.

Ferrets were acclimated for a minimum of 48 hours prior to any manipulation by members of the Cole lab. All animals then were anesthetized for implantation of microchips, which were used to facilitate identification and temperature readings. Baseline weights and temperatures were obtained, as well as pre-bleeds and baseline nasal washes obtained prior to influenza infection. Twenty four hours prior to infection, selected ferrets received mini-binder via intranasal or intra-tracheal administration (5 mg/kg) as designated. Control animals received nothing. Following aerosol challenge with influenza, all ferrets were monitored daily: weights, temperatures, clinical scoring, and collection of nasal washes. The clinical assessment was developed by Drs. Cole and Fuller, and details the physical, respiratory, neurological and intake/output for each study ferret. A copy of the clinical assessment scoring sheet is shown in Appendix A.

i. **Sedation of Ferrets.** For blood draws and nasal washes, all ferrets were sedated with 2-5% isofluorane and 4-10% oxygen via anesthesia box or face mask. Total sedation was determined by observing a reduction of the animal's respiratory rate, lack of tone or tensing of the muscles in the neck and upper body, as well as by administering a toe pinch to the hind legs. Following procedures, all ferrets were monitored until fully awake and alert prior to placing back into their caging. As needed, ferrets were placed on a warming cloth to assist with recovery from sedation,

ii. **Implantation of IPTT-300 Microchip.** Following the 48 hour acclimation period a subcutaneous IPTT-300 microchip (BioMedical Data Systems, cat # 2145251) was inserted between the scapulae of the animals. The microchip was programmed to provide the ferret ID, and allowed for easy identification of the ferrets as well as measured the ferret's core temperature using a DAS-7006/7r handheld wireless scanner (BioMedical Data Systems, Inc.).

iii. **Blood Draw.**

iv. **Nasal Wash**. Ferrets were sedated with isofluorane prior to flushing 1 ml sterile 1X phosphate-buffered saline (PBS) (Gibco) through the nostrils to collect a nasal wash. For the procedure, ferrets were placed in a supine position with their head tilted down and slightly on an angle to one side. A pipette or syringe with the volume of PBS placed so the tip was situated at the nares of the ferret. The liquid was then flushed gently through one nostril as it was observed coming out of the other nostril. Liquid was recovered into a petri dish, stored into a 2.0 ml freezing vial and stored at -80°C. A notation was made with regard to how congested and full of mucous each sample/animal presented.

v. **Necropsy:** See Section 3.8

3.6 VIRAL INFECTION ROUTE STUDY

Prior to any work with A13r33 was performed, a small study using a modified strain of influenza gifted to the Cole lab by Dr. Stacey Schultz-Cherry of St. Jude Children's Research Hospital. A/California/04/2009 H1N1 virus was a modified strain of influenza using an eight

plasmid 293T/MDCK co-culture system to express a 19-kDa engineered luciferase within the polymerase subunit (61, 62). Male ferrets 10 -12 weeks of age were acquired and injected with subcutaneous temperature/ identification chips. Ferrets were divided into groups and were challenged with CA/09-NLuc either via aerosol or intranasal inoculation, with control animals (n=2) remaining uninfected. Infected animals (n=2) were given 10e4.45 TCID50/ mL via aerosol or 10e4.2 TCID50/ mL via intranasal inoculation (n=2). Nasal washes were collected daily following infection up till the time of necropsy. Additionally on days 1 and 3 IVIS imaging was performed using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer, part # 124262). On day 3 all animals were euthanized with 200mg/kg of Euthosol. Once death was confirmed, soft palate, nasal turbinates, trachea, right and left lung tissue samples were harvested. Fresh tissue samples were collected into RNA later for viral and immunological analyses, while samples for pathological studies were fixed in formalin. All work was performed at BSL 3 in the Regional Biocontainment Laboratory (RBL) due to the location of the IVIS.

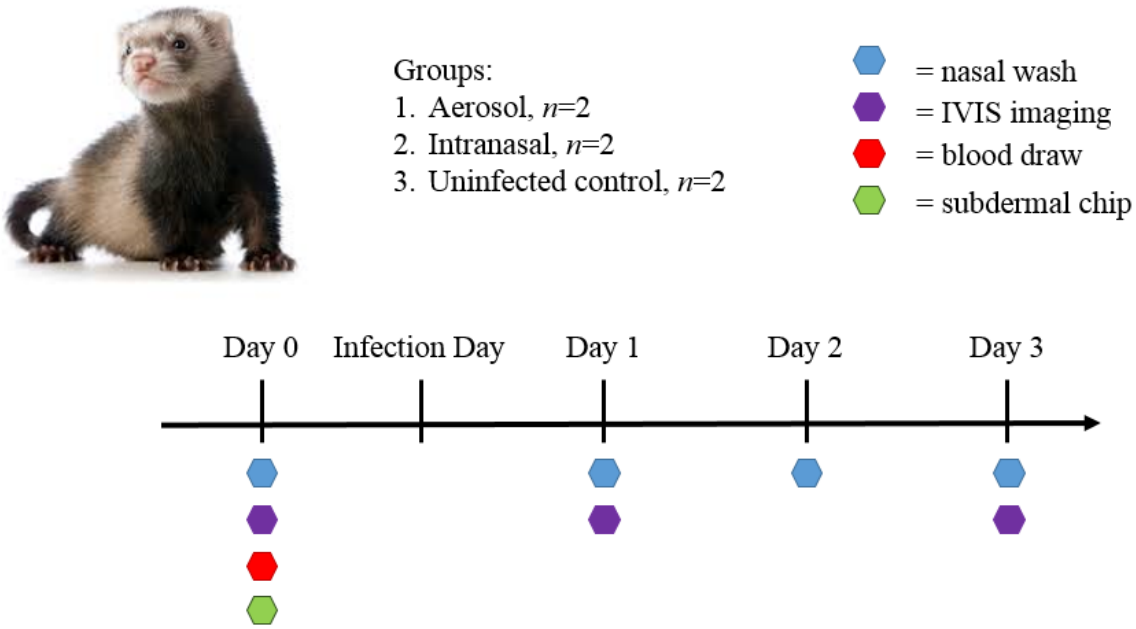


Figure 12. Timeline of viral challenge comparison between aerosolization versus intranasal injection studies with CA/09-NLuc in the ferret model.

Six ferrets were used in this stud. Animals in the infected group (n=4) were challenged with a modified strain of A/California/04/2009 H1N1 which expresses a luciferase within the viral polymerase subunit. Ferrets were challenged either with $10^{4.45}$ $TCID_{50}$/ mL of virus via aerosol (n=2), $10^{4.2}$ $TCID_{50}$/ mL via intranasal inoculation (n=2), or remained unchallenged as negative controls (n=2).

3.7 MINIBINDER FERRET STUDY

Two groups of nine ferrets each were received for this study, separated by a period of three weeks. The first group was aged 10 to 12 weeks and weighed an average of 350 grams. The second group were between 10 to 12 weeks of age and weighed on average 450 grams. One day prior to exposure to aerosolized H1N1 three animals from the group were given an intratracheal dose of 5 mg/kg of minibinder via a laryngoscope. A cannula was used to inoculate the carina,

which is the portion of the trachea directly above the bronchial branches of the lungs, with the minibinder. This procedure was performed by a DLAR veterinarian to ensure the safety and comfort of the animal. Three other ferrets in the same grouping were administered a dose of 5 mg/kg of minibinder intranasally via a micro-pipette. The last three animals of the group were kept as untreated controls and were administered nothing. All nine animals in each group were challenged with pandemic H1N1, 2.3 *10^8 $TCID_{50}$/mL and received an average aerosolized dose of 3.9*10^6 $TCID_{50}$/mL (first group) 4.05 *10^5 $TCID_{50}$/mL (second group) or of virus. Nasal washes and clinical scoring was performed for seven days following exposure. On the seventh day after the clinical scores and nasal washes were collected the animals were euthanized and necropsied.

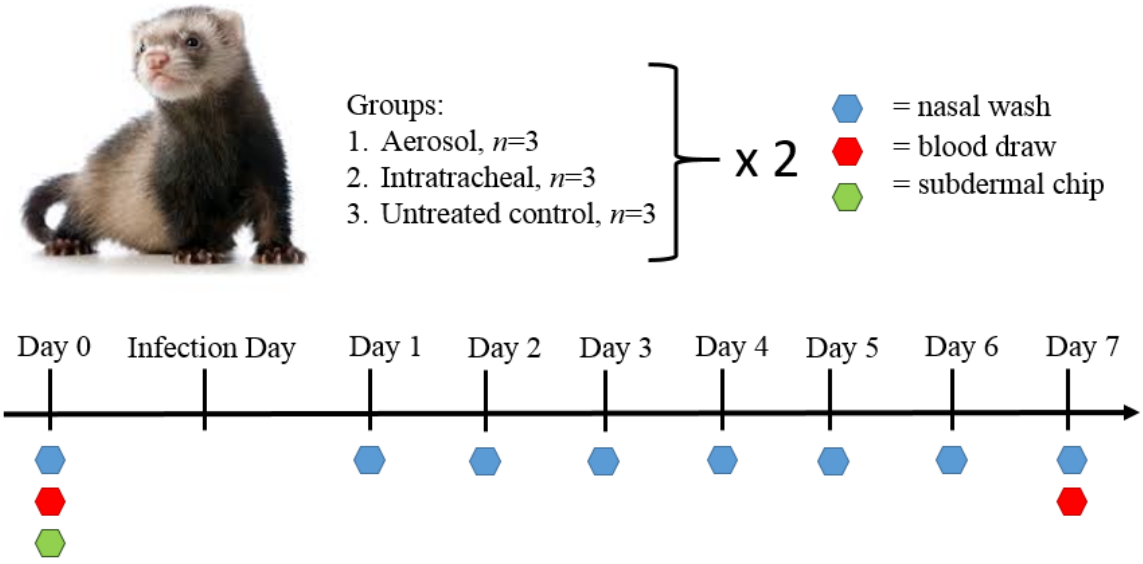


Figure 13. Timeline of A13r33 prophylactic study in the ferret model.

Nine ferrets were challenged with 2.3*10^8 $TCID_{50}$ pandemic A/California/07/2009 H1N1influenza virus. One day prior to infection three animals were given 5 mg/kg of minibinder intratracheally and three were given 5 mg/kg of the same drug intranasally. Three animals were left untreated as controls.

3.8 NECROPSY

Seven days after the ferrets were exposed to aerosolized pandemic H1N1 influenza virus, all animals were euthanized. The ferrets were first sedated with isoflurane, ranging from 2-5% depending upon previous experiences with the individual animals as well observations of breathing rate and muscle tone. The footpads of the hind legs were pinched hard, if a reaction was observed the animal was left under sedation. Nasal washes were collected quickly while the ferrets were still sedated. For the first set of nine animals a heart stick to collect blood was performed immediately after the nasal wash. A VanishPoint® 1mL syringe with a 27 gauge ½ inch needle was used to capture the blood samples. For the second group of animals it was decided that a heart stick would prove too difficult to accurately perform with the ferrets increased size. The method of blood collection used for the second group is detailed below. The ferrets were then put under sedation again until they no longer responded to a toe-pinch. The ferrets were then euthanized with an intraperitoneal injection of Euthasol at a concentration of 200mg/kg into the lower left quadrant of the abdomen. Following the injection the ferrets breathing and heart-rate were monitored until the ceased. Additionally cyanosis of the tissues, especially those of the mouth (palates, gums, and lips) was used to confirm death.

Once the ferrets were confirmed to have been euthanized, the carcass was moved into a class II BSC and placed onto an absorbent pad. For the first set of animals the nose was cut off using a pair of sharp surgical scissors. This was done to facilitate easy access to the nasal turbinates, which were removed with forceps. First the inside of the nasal cavity was scrapped with a single tong of the forceps in a vigorous manner. Then the forceps were used to pull tissue from the nasal cavity. Next a scalpel to make an incision from the clavicle to the lower abdomen and a pair of surgical scissors was used to cut the sternum of the ribs, exposing the lungs and

heart. These along with the trachea were carefully removed, and a section of trachea was taken. Pieces from the left and right lungs were taken as well and kept separate. Finally a bronchial alveolar lavage (BAL) was performed. 10mL of sterile 1x PBS was inserted into the lobes of the lungs via a 10mL syringe with a cannula attached to it. Once all lobes had been inflated, the syringe was then used to siphon the PBS as well as mucus and other debris out of the lungs. The BAL was then placed in a 5mL tube and flash frozen in a dry ice/ ethanol bath. The lungs and heart were then placed in a sealed plastic container containing a 10% formalin solution. The lung pieces, trachea section, and nasal turbinates were stored in 15 mL conical tubes containing around 3 mLs of RNAlater (Thermo Fischer Scientific). The BAL samples were later stored at -80°C, the lungs, nasal turbinates, and trachea were stored at room temperature for a week before being moved to a 4°C fridge. The lung and heart were transferred to new containers containing 10% formalin and left at room temperature.

The procedure for the second set of animals was similar to the first, with a few slight changes. After the nasal turbinates were collected and the chest and abdomen sliced open, the diaphragm was then cut. A 5 or 10mL syringe with an 18 gauge needle was then used to pierce the heart and collect the blood sample. The sample was then subdivided, with half of the blood injected into Vacutainers coated with 5.4mg EDTA (Becton, Dickson and Company). The vacutainers were then spun down in a centrifuge at 1200 rpm for 10 minutes, the serum was pipetted off and stored in cryovials at -80°C. The other half of the blood was injected into a PAXgene blood RNA tube (PreAnalytiX, cat #: 762165 (BD)). The PAXgene tubes were then stored directly at -80°C. Next the celiac artery that feeds blood into the spleen was nicked. A gauze pad was placed over this incision to absorb the blood, and the carcass was left alone for a few seconds to allow for most of the remaining blood to drain. The rest of the procedure

remained the same as that performed on the first set of animals except for the collection of the BAL. A 10mL syringe containing 10mL of 1x PBS was used to wash the inner surface of the lungs. Once the lobes were inflated the lungs were inverted over a petri dish and the BAL was allowed to flow into it. This was then collected and flash frozen via the method explained earlier in this section. Nasal turbinates, sections of lung from the right and left sides, and pieces of the trachea were also collected and stored in the same manner as described before.

3.9 TISSUE HOMOGENIZATION

Sections from the lobes of the right and left lung as well as trachea sections were homogenized in order to analyze viral load. Between 100 and 300mg of tissue were weighed in 50mL conical tubes. A volume of Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) equal to 10 times the mass of the tissue in milliliters was added to the tissue sample. The samples were then homogenized using an Omni TH-01 tissue homogenizer with an attached 7mm x 110mm tip set to the highest speed, around 35,000 rpm (Omni International). Cellular and tissue debris was then centrifuged out by spinning the homogenates at 1500 rpm for 10 minutes. Supernatants were transferred to cryovials, which were stored at -80°C.

3.10 RNA EXTRACTIONS

RNA was isolated from both nasal washes and tissue homogenates using a Trizol/ chloroform reaction followed by using either the QIAamp Viral RNA Mini Kit (Qiagen cat. #52904/ 52906) or the RNeasy Mini Kit (Qiagen cat. #74104), respectively. 100µL aliquots of nasal washes from days 0, 1, 3, 5, and 7 and 100µL aliquots of homogenized trachea were used in the extraction. Additionally 50µL aliquots of homogenized right and left lung fragments taken from the same ferret were combined into one aliquot, which was then likewise extracted.

3.11 RT-QPCR

3.11.1 RT-qPCR for Viral Titers

After the RNA extractions were performed a one-step Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) was performed using SuperScriptR III PlatinumR One-Step qRT-PCR Kit with ROX (Invitrogen, cat. #11745100). Amplification and analysis was performed on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher). Influenza A specific probes and primers used to detect the viral genome came from the BEI resources Influenza Virus Real-Time RT-PCR Assay Kit (BEI Resources NR-15592). See Appendix A Table 4 for primer and probe sequences.

A viral standard curve was used to compare the viral loads taken from the nasal washes and lung homogenates. A/California/07/2009 H1N1 virus grown in eggs and with a $TCID_{50}$ of $2.3*10^8$ was extracted using the QIAamp Viral RNA Mini Kit and serially diluted in ten-fold

increments from 10^{-1} to 10^{-8} in nuclease free water. The virus used to create the standard curve came from the same stock as that used to initially infect the ferrets. Each PCR run included samples of RNA extracted from animal tissues and nasal washes and the serially diluted viral RNA. R^2 values of ≥ 0.95 were used to calculate the amount of virus present in each sample.

A master mix of forward and reverse primers, Superscript Taq polymerase mix, 2X PCR mix, and nuclease free water was made for each run, see Appendix A Table 5 for master mix formula volumes per reaction. 20µl of master mix was pipetted into a MicroAmpR Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737). 5µl of sample taken from RNA extracted from homogenates/ nasal washes (experimental samples) or from virus (viral curve samples) was then pipetted into the appropriate wells and the plate sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971). The reaction plate was then placed into the QuantStudio 6 qPCR machine and run at the following parameters:

Step 1: Reverse Transcription, 50°C for 30 minutes
Step 2: Taq Inhibitor Activation, 95°C for 2 minutes
Step 3: PCR Amplification (40 cycles)
Denature, 95°C for 15 seconds
Anneal, 55°C for 30 seconds

3.11.2 RT-qPCR for Ferret Cytokines

RT-qPCR for the detection of cytokines in ferret tissue homogenates was performed in a two-step process. First cDNA was generated from extracted RNA with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher, cat # K1642) using a PTC-200 Thermocycler (MJ Research). 5X Reaction mix, Maxima Enzyme Mix, and

nuclease free water were mixed together into a master mix. See Table 6 in Appendix A for the formulation of this master mix. 15µl of master mix and 5µl of RNA extracted from homogenates were mixed together in 0.2mL flat cap PCR tubes. Note mass of template RNA was between 1pg and 5µg. Tubes were then loaded into the Thermocycler, which was run at the following parameters

Step 1: Incubation, 25°C for 10 minutes
Step 2: Amplification, 50°C for 15 minutes
Step 3: Reaction termination, 85°C for 5 minutes

Next quantitative PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher, cat # K1642) ≤ 500ng template cDNA. As with the aforementioned viral titer RT-qPCR, amplification and analysis of the cDNA product was performed on a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher). Ferret specific primers targeting cytokines of interested were manufactured by Integrated DNA Technologies, sequences for those primers can be found in Appendix A in Table 7. A master mix of forward and reverse primers, Maxima SYBR Green/ROX qPCR Master Mix, and nuclease free water was made for each run, see Appendix A Table 8 for master mix formula volumes per reaction. 20µl of master mix was pipetted into a MicroAmpR Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737). 5µl of sample taken from RNA extracted from homogenates was then pipetted into the appropriate wells and the plate sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971). The reaction plate was then placed into the QuantStudio 6 qPCR machine and run at the following parameters:

Step 1: UDG pre-treatment, 50°C for 2 minutes
Step 2: Initial denaturation, 95°C for 10 minutes
Step 3: PCR Amplification, (40 cycles)

Denature, 95°C for 15 seconds
Anneal, 60°C for 30 seconds
Extension, 72°C for 30 seconds

After cytokines were successfully detected expression levels of cytokines were normalized to the house keeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). ΔΔCt to determine fold change was used to determine the fold change in cytokine expression. First the QuantStudio 6 measures the rate of amplification in each well, and once a certain limit of fluoresce is detected (above background fluoresce provided by ROX) a Ct value is assigned. Subtracting GAPDH Ct values from experimental Ct values gives the change in threshold cycle. (ΔCt). Fold change is then calculated by subtracting the day 0 ΔCt from the subsequent days ΔCt values. Finally the formula $2^{-\Delta\Delta Ct}$ is used to obtain the absolute value for fold change (65).

4.0 RESULTS

4.1 PROJECT OVERVIEW

The goal of this project was to complement work performed by our collaborators at the University of Washington's on novel influenza antivirals. While their work was built around the mouse model of infection we sought to include the ferret model which better represents infection and disease progression in humans. This study sought to evaluate the efficacy of a novel protein minibinder (A13r33) to limit infection and disease following prophylactic treatment 24 h prior to virus challenge when administered either directly into the carina of the lungs or intranasally. Previous work from the Cole lab evaluated the efficacy of an earlier version of A13r33 (a larger protein binder, HB36.6) and demonstrated differences in clinical disease progression and viral loads following influenza infection when comparing treated and untreated ferrets. The present study was designed to further evaluate A13r33 mini-binder for its efficacy against influenza infection when administered prophylactically. The following sections will describe results from the preliminary ferret studies to compare intranasal and aerosolized influenza infection in ferrets, generation and characterization of the virus stock needed to perform these studies, and the pivotal study to evaluate A13r33 or its ability to protect against influenza in ferrets.

4.2 GENERATION OF REAGENTS NEEDED FOR STUDIES

4.2.1 Generation of Egg Grown Virus

One of the first critical steps in this project was to generate a large amount of high titer virus stock that could be used for all ferret and cell culture studies to insure minimal variation between each portion of the studies. To accomplish this, 30 day 10 SPF chicken embryos were inoculated with our seed stock of pH1N1 (from the BEI repository). Virus was allowed to propagate for 48 hours at 100°F, 60% humidity. At 48 hours, all eggs were placed in sealed egg cartons at 4°C overnight to stop the infection. The following day, the allantoic fluid containing the virus was harvested from each egg and combined. This solution was clarified by centrifugation and the virus stock was aliquoted for storage at -80C.

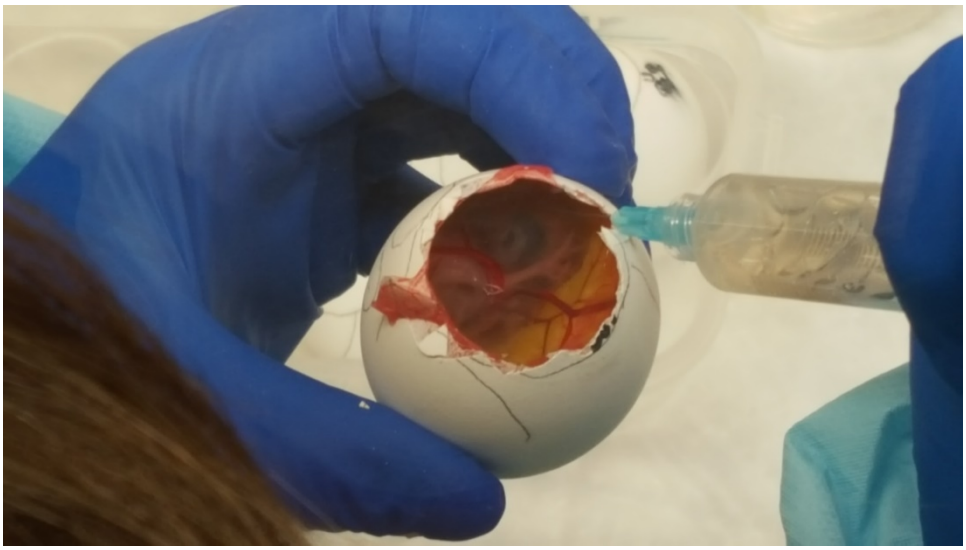


Figure 14. Day 13 Harvest of allantoic fluid from fertilized chicken eggs.
Allantoic fluid was harvested from fertilized chicken eggs 13 days after they had arrived in lab and 2 days after being inoculated with A/California/07/2009 pH1N1

4.2.2 $TCID_{50}$ to Determine Viral Titer

$TCID_{50}$ assays using MDCK cells were performed in order to quantify the titer of the virus that had been grown in the SPF chicken eggs. See Figure 21 in Appendix A for the layout of a standard $TCID_{50}$ plate. Cytopathic effect on the MDCK cells was observed and recorded, results are seen below in Table 3. For further information on how $TCID_{50}$ calculations are performed see Appendix C.

Table 3. Raw Data of Observed Cytopathic Effect used to determine $TCID_{50}$ of egg grown A/California/07/2009 pH1N1.

Dilution	Positive Wells	Negative Wells	% Death
10^{-1}	4 / 4	0 / 4	100%
10^{-2}	4 / 4	0 / 4	100%
10^{-3}	4 / 4	0 / 4	100%
10^{-4}	4 / 4	0 / 4	100%
10^{-5}	4 / 4	0 / 4	100%
10^{-6}	4/ 4	0 / 4	100%
10^{-7}	1/ 4	3 / 4	25%
10^{-8}	0 / 4	4 / 4	0%
10^{-9}	0 / 4	4 / 4	0%
10^{-10}	0 / 4	5 / 4	0%

4.3 PILOT FERRET STUDY TO COMPARE VIRUS CHALLENGE ROUTE

Six male ferrets (n=2 per group) were received for a pilot study to compare the early onset of infection following aerosol versus intranasal infection with a modified strain of A/California/04/2009 pH1N1 that encodes luciferase. Two ferrets were left as uninfected controls. The purpose of this pilot study was to compare the early infection and to the standard clinical, virological and immunological monitoring as well as the possibility of using IVIS (CT) to monitoring early events post infection.

4.3.1 Temperatures

Temperatures (°C) for infected ferrets were collected daily after challenge with influenza virus, as well as a few days prior to challenge (Figure 15). Temperatures for uninfected ferrets were not taken. Both groups of infected animals exhibited little change in temperature over the time course of the experiment, however this is likely due to the short duration of time between viral challenge and sacrifice.

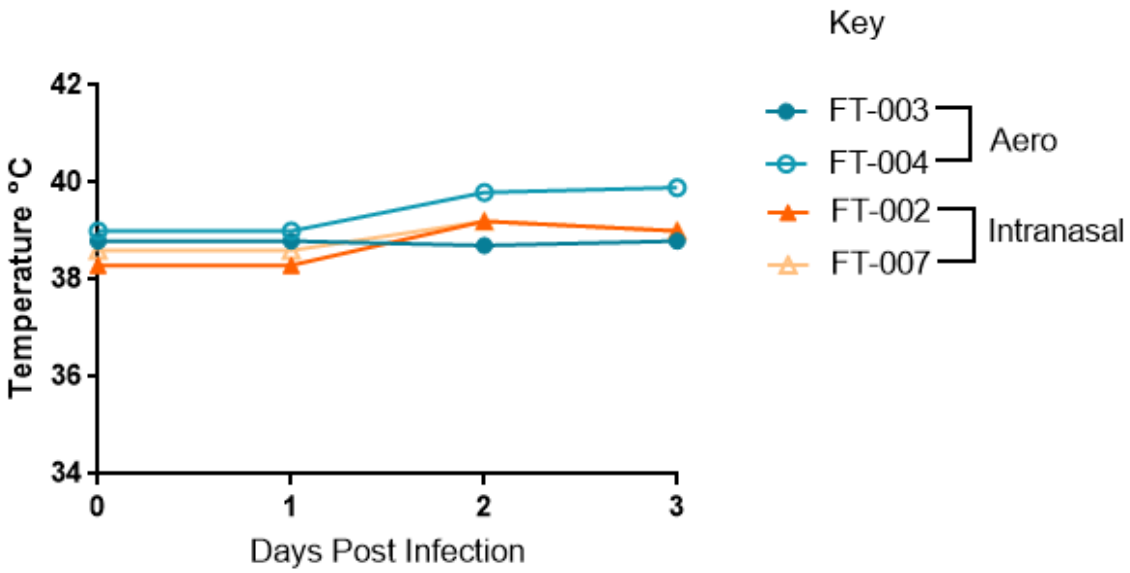


Figure 15. Temperature comparison between ferrets infected via aerosol or intranasal routes.
Two groups of ferrets (n=2/ group) were infected with a modified strain of A/California/04/2009 pH1N1 that produces luciferase in its polymerase protein. Ferrets challenged via aerosolized virus (circles) received $10^{4.45}$ $TCID_{50}$/ mL, ferrets challenged by intranasal injection (triangles) received a titer of $10^{4.2}$ $TCID_{50}$/ mL. Control animals (data not shown) were not un-infected Statistics not performed due to low sample size.

4.3.2 Weight Loss Percentage

A small amount of weight gain was observed for both the uninfected controls and the ferrets that were challenged intranasally with virus (Figure 16). Ferrets that were challenged via aerosol exhibited a consistent weight, until day 3. At this time point both ferret in this group exhibited some weight loss, however the results do not appear significant. It is unclear if this weight loss would have continued or been significant, due to the short duration of this study (3 days) and the small sample size of ferrets (n=2/ group).

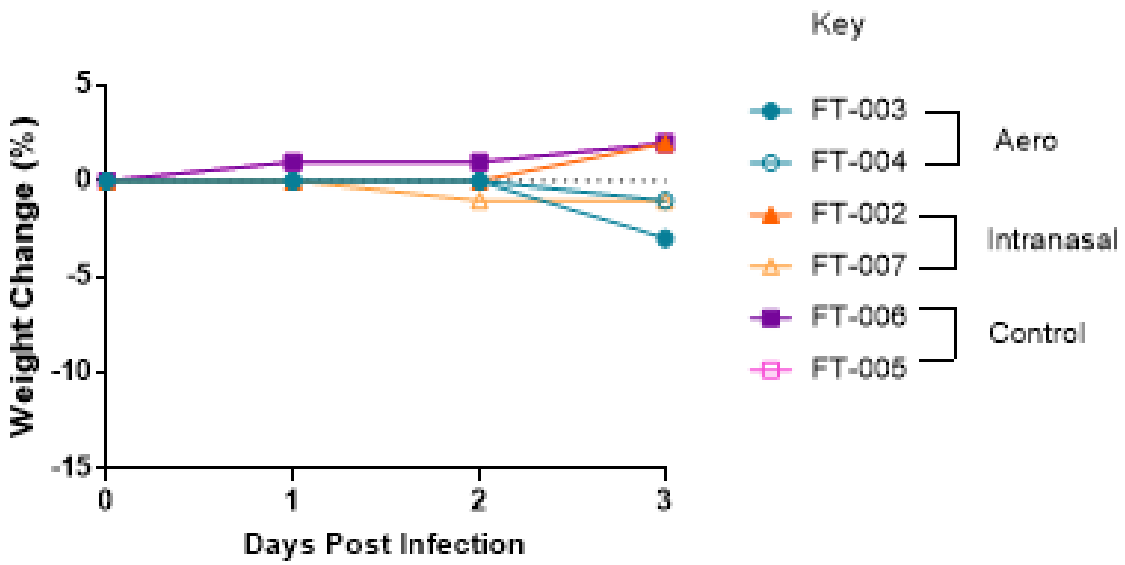


Figure 16. Calculated weight loss percentage of ferrets infected via aerosol or intranasal routes compared to uninfected controls.

Two groups of ferrets (n=2/ group) were infected with a modified strain of A/California/04/2009 pH1N1 that produces luciferase in its polymerase protein. Ferrets challenged via aerosolized virus (circles) received $10^{4.45}$ $TCID_{50}$/ mL, ferrets challenged by intranasal injection (triangles) received a titer of $10^{4.2}$ $TCID_{50}$/ mL. Control animals (squares) were not infected. Statistics not performed due to low sample size.

4.3.3 Clinical Scores

Clinical scores for all animals were observed over the duration of the study (Figure 17). Control animals did not see virus and as expected demonstrated no signs of disease. Ferrets infected with A/California/04/2009 pH1N1 via the intranasal route exhibited only minor signs of disease, including some nasal or ocular discharge, occasional coughing or sneezing, and were slightly less playful than they had been prior to infection. In contrast ferrets infected via the aerosol route exhibited higher clinical scores due to more severe signs of disease, including lethargy, reduced appetite and mucous in their stool, some dehydration, piloerection, coughing, sneezing, and ocular and nasal discharge. Since the study was only carried out to day 3, it is unclear whether the intranasally challenged ferrets were only delayed in their disease or whether

the overall disease course was different based on route of infection. Further studies are needed to tease this apart.

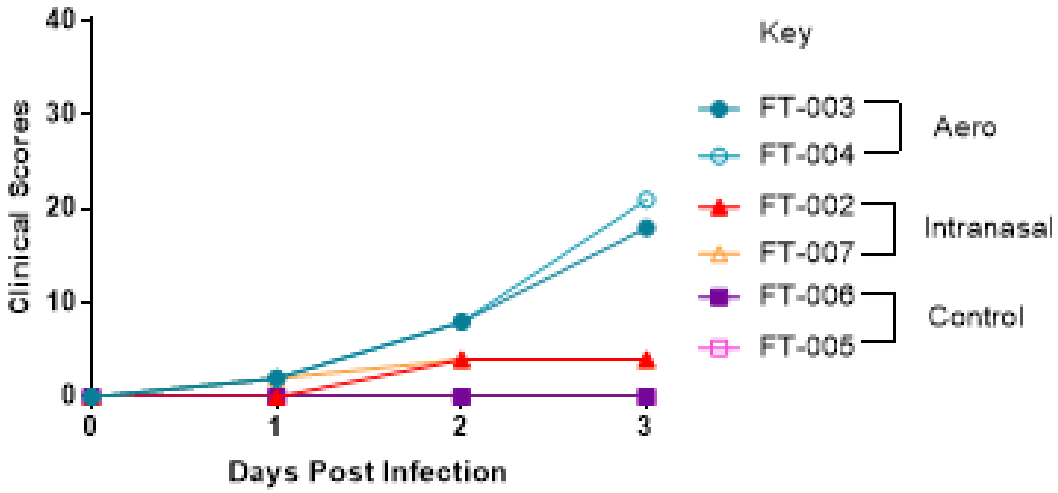


Figure 17. Clinical Scores of ferrets challenged either via aerosolized or intranasally instilled influenza compared to uninfected controls.

Two groups of ferrets (n=2/ group) were infected with a modified strain of A/California/04/2009 pH1N1 that produces luciferase in its polymerase protein. Ferrets challenged via aerosolized virus (circles) received $10^{4.45}$ $TCID_{50}$/ mL, ferrets challenged by intranasal injection (triangles) received a titer of $10^{4.2}$ $TCID_{50}$/ mL. Control animals (squares) were not infected. Statistics not performed due to low sample size.

4.3.4 Viral Titers in Nasal Washes and Select Tissues

Sections of the soft palate, the trachea, nasal turbinates (Figure 18A), and right and left lungs (Figure 18B) were taken from each animal at necropsy. Nasal washes were collected from animals on a daily basis by injecting 1mL of 1X sterile PBS into the nares of the ferret and allowing it run through the nasal cavity and out the other nostril (Figure 18C). $TCID_{50}$ assays were performed on MDCK cells to quantify the amount of virus in each sample. Interestingly, despite differences in clinical scores by day 3 post infection, there were little difference in the

titers of virus regardless of the route of viral entry into the ferrets. Nasal wash titers were also similar between the aerosol and intranasally challenged ferret groups (Figure 15C).

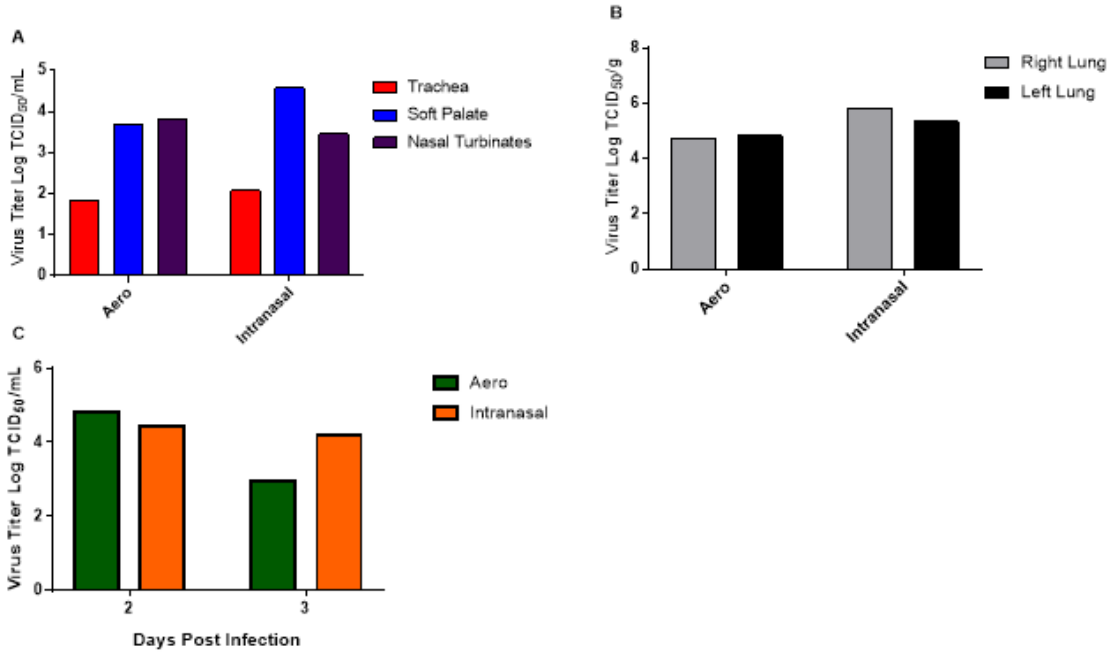


Figure 18. Viral Titers of A/California/04/2009 in various tissues and nasal washes measured by $TCID_{50}$.

Ferrets were infected via the indicated route and sacrificed 3 days post infection (DPI). From these animals the specified tissues were harvested. Nasal washes were also collected from each animal, sterile 1x PBS was injected via syringe into the nose and allowed to drain out. (A) Data is presented in $TCID_{50}$/mL. *n* = 2 ferrets/ group. (B) Data is presented in $TCID_{50}$/g. *n* = 2 ferrets/ group. (C) Nasal wash viral titers, data is presented in $TCID_{50}$/mL. *n* = 2 ferrets/ group. Graphs are shown as averages of the 2 animals $TCID_{50}$ values. Statistics not performed due to low sample size.

4.3.5 Summary of Study

In general both intranasal and aerosol H1N1 challenge of ferrets resulted in infection and disease in the ferret model. Interestingly, despite similar levels of virus within 3 days post challenge, there were marked differences in clinical signs of disease and the severity of

symptoms presented between the two infected cohorts. Further studies analyzing the immunological and pathological differences between animals infected by either route are necessary to understand the differences in observed symptoms. Additionally longer period of time between infection and sacrifice should also be considered. This will help confirm if the intranasally infected ferrets lagged behind the aerosol group in regards to the displayed symptoms or if this route of virus introduction is less efficient at establishing an infection.

4.4 A13R33 STUDY: AIM 1

For all figures in this section (Figure 16 through 18) ferrets that received the minibinder intranasally are represented by red symbols (n=6). Blue symbols represent those ferrets that received the minibinder via the intra-tracheal route (n=6). Control ferrets are represented by green symbols (n=6) and received no antiviral minibinder. All animals were challenged with A/California/07/2009 pH1N1 24 hours after A13r33 was administered. Ferrets represented by open figures (Study 1) were challenged with a viral load equal to $3.9*10^6$ $TCID_{50}$/mL. Ferrets represented by shaded figures (Study 2) were challenged with a viral load equal to $4.05*10^5$ $TCID_{50}$/mL.

Restatement of Aim 1: To demonstrate efficacy of the novel computationally designed antiviral minibinder A13r33 against influenza infection with A/California/07/2009 pH1N1 in the ferret model through analysis of clinical symptoms.

4.4.1 Temperature

Observations of animals were made in the morning over a seven day period following infection with pH1N1 Ca/09, as well as on a day prior to infection with the virus. Temperatures in degrees Celsius (°C) were collected via a subdermal transponder inserted between the scapulae of each animal. The transponder was injected on day 0[1], the same day that the first nasal wash, clinical score, weight, and temperature was collected. Twelve out of the eighteen ferrets used in this study were administered a single 5mg/kg dose of A13r33 minibinder 24 hours prior to infection with pandemic influenza virus. The antiviral minibinder was administered via either intranasal or intra-tracheal routes to the designated ferrets in the two treated groups. Figure 12 below details the observed results of this portion of the study. The black line represents the mean of all the ferrets within the specified groups: intranasal, intra-tracheal, and control. Open figures represent ferrets challenged with $3.9*10^6$ $TCID_{50}$/mL $TCID_{50}$/mL of virus, while shaded in figures represent ferrets challenged with $4.05*10^5$ $TCID_{50}$/mL $TCID_{50}$/mL. The data suggests that the titer of the virus used in the challenge had little impact on the temperature trends. All groups show an increase in temperature two days post infection, however the mean of the intranasally dosed ferrets did not spike as highly or as rapidly as the intra-tracheal or control ferrets. Ferrets that received A13r33 intranasally had their temperature return to baseline by Day 5 (Figure 19A). Ferrets in the intratracheal cohort appear to return to baseline by day 7 (Figure 19B). Interestingly the control group's average temperature plateaus between days 3 and 6, but then exhibited a day 7 increase, which was unseen in the two treated cohorts (Figure 19C).

[1] Note: Day 0 occurred at least three days after the ferrets arrived in the vivarium, allowing them time to settle and acclimatize to the new environment. Additionally Day 0 was at least 24 hours prior to the ferrets receiving their dose of A13r33. This spread out the number of invasive procedures performed on each animal and allowed for better observations of their health as it was affected by viral challenge and administration of the antiviral.

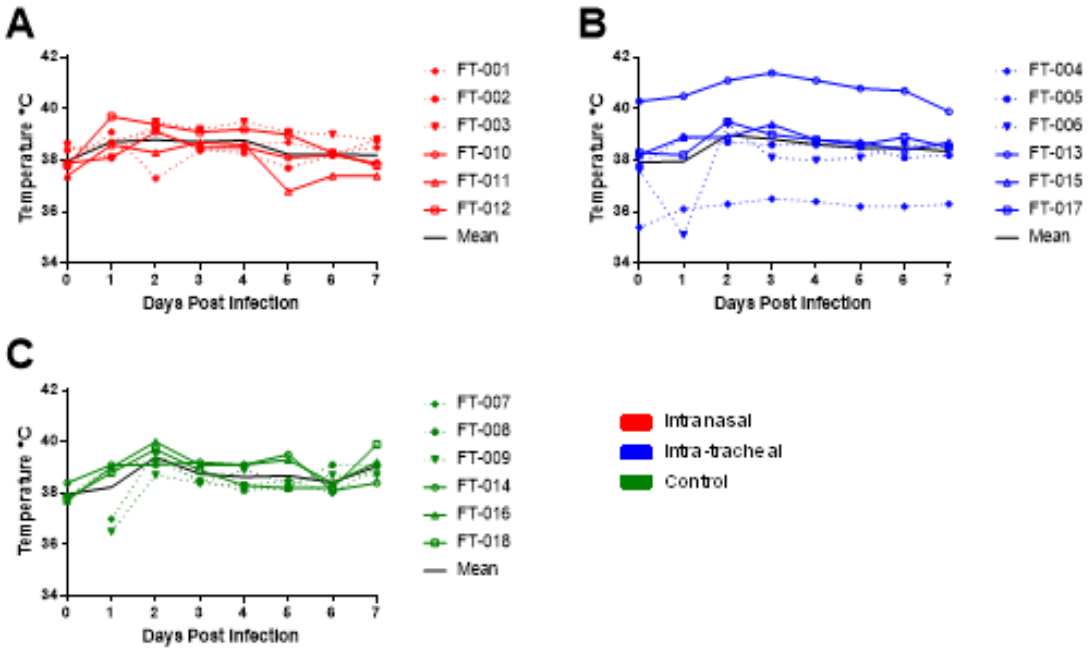


Figure 19. Temperatures of ferrets treated prophylactically with A13r33 compared to control untreated ferrets.

(A) Temperatures of ferrets that received A13r33 intranasally with either a micropipette or syringe. (B) Temperatures of ferrets that received an intra-tracheal dose of A13r33 via a cannula inserted through the mouth. (C) Temperatures of untreated control ferrets. Note that temperatures for F-007 and FT-009 were not taken for day 0 due to issues with the subdermal transponders. All graphs are shown with a black line denoting the mean for the respective group.

4.4.2 Weight Loss Percentage

Weight loss for ferrets in this study became noticeable within two days of aerosolized challenge with pandemic H1N1, and exhibited a general levelling off by day 3. Of all group in this study the ferrets that were administered A13r33 intranasally exhibited the most significant loss in weight as seen on day 3 of Figure 20A. Both intra-tracheal (Figure 20B) and control animals (Figure 20C) exhibited much less weight loss, with neither group losing more than 5% of their starting body weight. Another interesting trend is that the two treated groups (intra-

tracheal and intranasal demonstrated a general increase in body weight between day's 6 and 7. Control animals displayed a plateauing of their average weight over the same period of time.

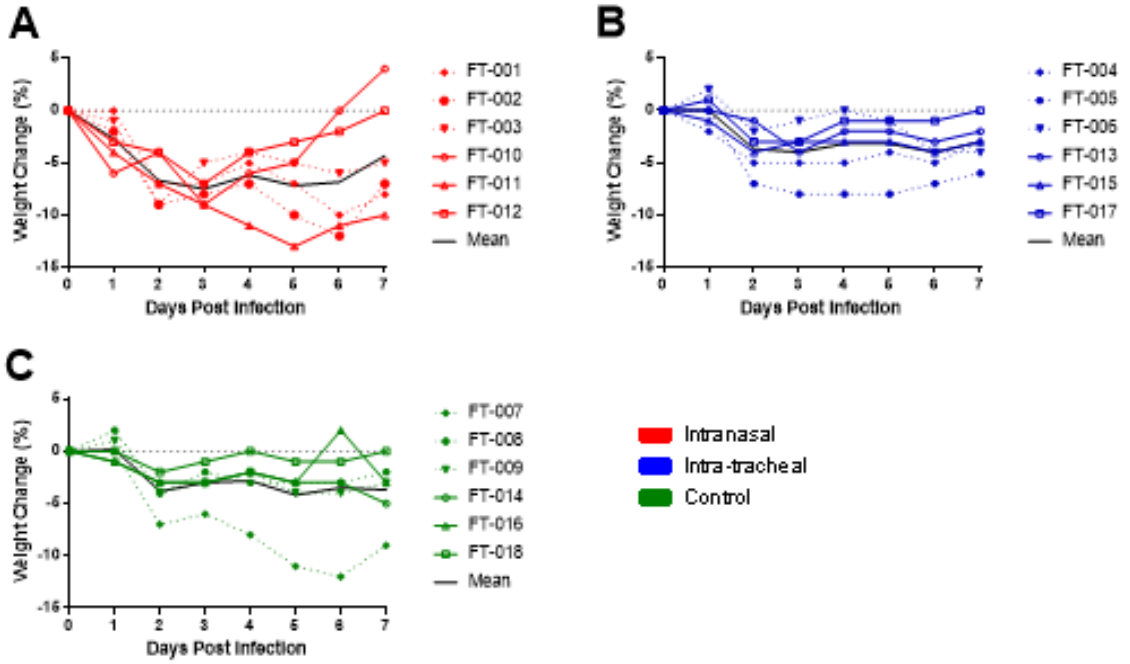


Figure 20. Calculated weight loss percentage of ferrets treated prophylactically with A13r33 compared to control untreated ferrets.

(A) Weight loss percentage of ferrets that received A13r33 intranasally with either a micropipette or syringe. (B) Weight loss percentage of ferrets that received an intra-tracheal dose of A13r33 via a cannula inserted through the mouth. (C) Weight loss percentage of untreated control ferrets. All graphs are shown with a black line denoting the mean for the respective group.

4.4.3 Clinical Scores

In order to quantify the clinical symptoms of infection a scoring system was developed prior to this study. Researchers at the Universities of Pittsburgh and Washington assigned numerical values of 2, 5, and 10 to a variety of symptoms (see Appendix A). Symptoms were recorded and the results tallied, giving a final score for the observed ferrets. Figure 14 below

details the results of this scoring system. There appears to be a correlation between the titer of virus received and the severity of illness exhibited. Ferrets challenged with a higher $TCID_{50}$ of pandemic H1N1 generally exhibited higher clinical scores. Control ferrets exhibited the highest clinical scores of all groups, and remained sicker for longer than the two treated groups (Figure 21C). Between days 1 and 2 after infection their average clinical score increased more rapidly when compared to the two treated cohorts. Interestingly the intranasal cohort exhibited two different peaks based on the titer of virus received. Animals challenged with a higher titer of virus peaked earlier on day 3 compared to the peak seen on day 5 for the cohort exposed to the lower titer of virus. By day 7 both the intranasal (Figure 21A) and intra-tracheal (Figure 21B) dosed ferret groups had returned to a score of 7 or 13 respectively (average score), compared to the controls which remained closer to 20 (again average score).

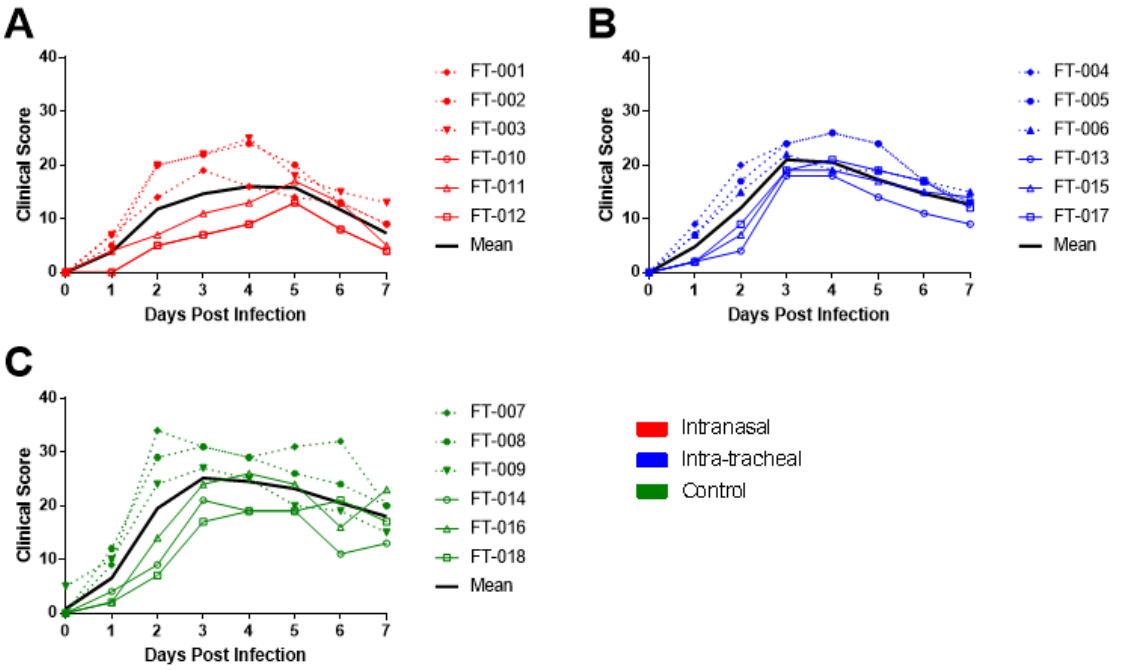


Figure 21. Clinical Scores of ferrets treated prophylactically with A13r33 compared to control untreated ferrets.

(A) Clinical scores of ferrets that received A13r33 intranasally with either a micropipette or syringe. (B) Clinical scores percentage of ferrets that received an intra-tracheal dose of A13r33 via a cannula inserted through the mouth. (C) Clinical scores percentage of untreated control ferrets. All graphs are shown with a black line denoting the mean for the respective group.

4.5 A13R33 STUDY: AIM 2

To quantitate the level of virus in ferrets treated with A13r33 with untreated controls, one-step RT-qPCR was performed on nasal washes and homogenized lung samples. Each RT-PCR reaction plate was run with serially diluted viral RNA extracted directly from the egg grown stock of A/California/07/2009 pH1N1. Using the equation for the standard curve generated by the QuantStudio 6^{TM}, the viral titers were calculated.

Restatement of Aim 2: To characterized the virological and immunological correlates of protection of A13r33 in the ferret model following exposure to aerosolized challenge with pandemic H1N1 influenza.

4.5.1 Viral Titers

This portion of the study was designed to evaluate A13r33's efficacy in reducing viral shedding by measuring the viral load present in daily nasal washes taken from all ferrets. Viral titers were highest in nasal washes taken 1 day after infection and interestingly the ferrets in the second group (Figure 22B) who received a lower titer of virus via aerosol when compared to the first group (Figure 22A) had higher titers of virus in their nasal washes. Lung homogenates were collected from sections taken from the right middle and left lower lobes of the ferrets' lungs. Viral loads for all lung homogenates were practically identical, regardless of the route of drug administration, the presence or absence of the antiviral minibinder, or the titer of virus received. Figures 22 E and F show viral loads taken from ferret tracheas (n=2/ group). Ferrets given A13r33 intratracheally appear to have a have on average a lower viral titer compared to the animals that were given the minibinder intranasally (Figure 22E). However the trachea samples from the second group show a higher viral load for the intratracheally treated versus the intranasally treated (Figure 22F). If both groups are combined it appears that the average of the viral titers for both treated groups are around the same, and lower than the viral titers for the untreated controls. Results appear to indicate that route of administration of antiviral played no major role in reducing the viral loads in the nasal washes or the lung and trachea samples.

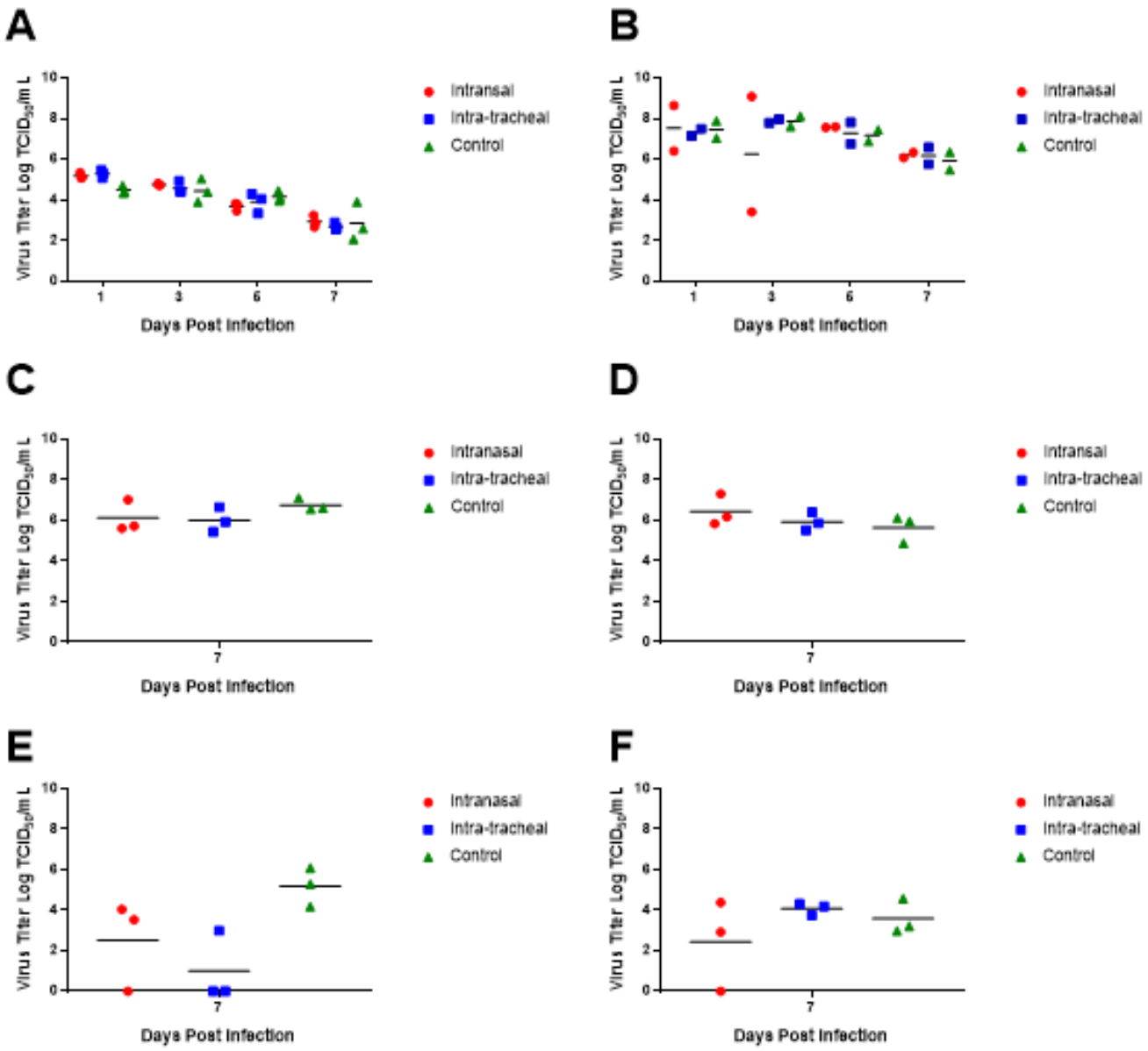


Figure 22. Quantification of viral loads in nasal washes and lungs in ferrets prophylactically treated with A13r33 after challenge with A/California/07/2009 pH1N1.

Ferrets in the prophylactic study were treated with 5 mg/kg A13r33 delivered either intranasally or intratracheally. Control animals were not administered A13r33 but were challenged with virus along with the ferrets that received the antiviral minibinder. Viral loads taken from homogenized lungs came from sections taken from the right middle and left lower lobes. (A) Viral loads in ferret group 1 after exposure to $3.9*10^6$ $TCID_{50}$/mL, n=3/ group. (B) Viral loads in ferret group 2 after exposure to $4.05*10^5$ $TCID_{50}$/mL, n=2/ group. (C) Viral loads taken from homogenized lung sections from group 1 after exposure to $3.9*10^6$ $TCID_{50}$/mL, n=3/ group. (D) Viral loads taken from homogenized lung sections from group 2 after

exposure to $4.05*10^5$ $TCID_{50}$/mL, n=3/ group. (E) Viral loads taken from homogenized trachea sections from group 1 after exposure to $3.9*10^6$ $TCID_{50}$/mL, n=2/ group. (D) Viral loads taken from homogenized trachea sections from group 2 after exposure to $4.05*10^5$ $TCID_{50}$/mL, n=2/ group.

5.0 RESULTS

The ferret studies performed using both HB36.6 (Allison Brichacek) and A13r33 (current thesis) support prior studies in the mouse model demonstrating the prophylactic efficacy in reducing clinical disease and blunting initial virus burden of initial infection. As had been demonstrated prophylactic administration of either peptide is not completely sterilizing in regards to high dose aerosol infection. This makes it unlikely that either peptide will provide sufficient protection against influenza mediated disease and infection when used alone. However, these studies are promising and warrant further evaluation. For example, if it is possible to effectively blunt the initial viral load so that patients do not succumb to severe disease, additional therapeutics or vaccines in combination with this treatment may be successful. Given the broad spectrum efficacy of the minibinder demonstrated in both mouse and ferret models, it is likely that this prophylactic will be of great interest as a long term product. The need for novel antivirals that are effective in controlling a wide array of influenza subtypes is extremely desirable compared to the need to generate a new vaccine each season. Future studies evaluating A13r33 need to include drug titrations, evaluation of timing for prophylactic administration pre-infection for efficacy, and the therapeutic efficacy of mini-binder post infection. This last study design is extremely important to validate A13r33's potential as a therapeutic to the public at large, and would allow how long post infection the peptide is

protective. Together these studies will provide critical insight into the efficacy of this novel peptide in protecting from pre- and post-exposure prophylaxis.

Viral titers in the nasal washes and lung homogenates appear the same between the various cohorts regardless of treatment with A13r33 route of minibinder treatment (Figure 19). Further analysis will be necessary however if this phenomenon is still observed across all dosages of A13r33 or is specific to only the dosage of 5mg/kg. Additional studies with larger sample sizes will also be necessary. A concern regarding the current influenza antivirals available to the public is that they do not appear to have much effect on the amount of virus shed by the infected (35, 66). This is a public health issue as certain antivirals, like oseltamivir, can relieve symptoms and make patients feel better more quickly after symptoms start. But these individuals who are seemingly recovered still shed virus and can infect others in the community without realizing it (41). In order to improve on this deficiency new antivirals need to not only control the clinical symptoms of infection but also control viral replication and shedding in the host. This group of mini-binders appears to possess both properties and should be further evaluated for its ability to reduce transmissibility post challenge.

Both HB36.6 and A13r33 demonstrated by the Fuller Lab as binding to and interfering with the replication of a wide variety of influenza A subtypes (Figure 5D-E, Table 2). This type of broadly effective antiviral is of great importance in the protection of the public from naturally arising seasonal and pandemic strains. Protection of troops from exposure by bioweapons and civilians targeted by bioterrorists is another potential application for broadly neutralizing like these two antivirals. An antiviral drug that does not require refrigeration and is stable at a wide range of temperatures, that can be aliquoted into a single dose for easy self- delivery such as through an oral or nasal spray, and that can be administered yet effective whether administered

just prior to or after the exposure would be the most ideal and desirable product. Given that weaponized influenza would be deployed in aerosol form, and the ease by which influenza can be acquired and modified to generate more pathogenic strains that do not necessarily exist in nature makes the possibility for a widespread influenza exposure a concern for both public health and national defense (67). Further development of a product that could blunt the initial exposure and reduce the overall disease burden would limit the number of critically ill patients and mortalities, reduce strain on hospitals and clinics, reduce the financial burden of care, and hopefully reduce the period of transmissibility in the infected post exposure. Each of these scenarios needs to be evaluated in the ferret model as proof of concept so that the mini-binder may be moved forward into clinical trials.

Given the differences in clinical scoring between treated and untreated cohorts (Figure 18), with insignificant differences in viral loads (Figure 19), further immunological analyses are warranted to determine the level to which the immune response (i.e., cytokine storm, pathology, etc.) may have contributed to the clinical signs of disease observed. Based on these results, we hypothesize that the intranasally administered A13r33 coated the upper respiratory tract of the ferrets, thereby limiting the foothold of the virus in this region of the respiratory system. Ferrets that had minibinder intratracheally administered more likely had higher concentrations of minibinder in the deeper portions of their lungs when compared to the intranasally administered ferrets. However ferrets like humans have the highest distribution of α-2, 6 sialic acid linked receptors in the upper respiratory tract, which coincidently the region that H1N1 replicates in (4, 5, 7). This would explain why the intratracheally administered animals displayed greater clinical signs of infection than the intranasal cohort, but less severe symptoms compared to the control animals. It is also possible that the intratracheal route of administration may have caused

irritation or distress to the ferrets. The lag in clinical scores for the intranasal cohort is of some interest as well, the peak in clinical signs of infection for this cohort did not occur until day 5. At this period of time the other two cohorts appeared to be recovering. One hypothesis is that the drug had been reabsorbed by the ferrets at this time, allowing whatever virus had not been inactivated to establish an infection. Another hypothesis is that the virus in the intranasal group had by this time migrated further away from the higher areas of A13r33 concentration (such as the nasal turbinates, upper trachea, and possibly the upper areas of the lung) and was able to establish an infection in this area as well. Bioavailability and absorption studies would help address which of these proposed scenarios was the cause. Further, it is warranted to evaluate the administration of mini-binder alone (no virus infection) in the ferret model to evaluate the contribution of the peptide to pathology and immune stimulation.

5.1 PROPOSED MODEL

We propose the following model for mechanism of action for A13r33 in the ferret model:

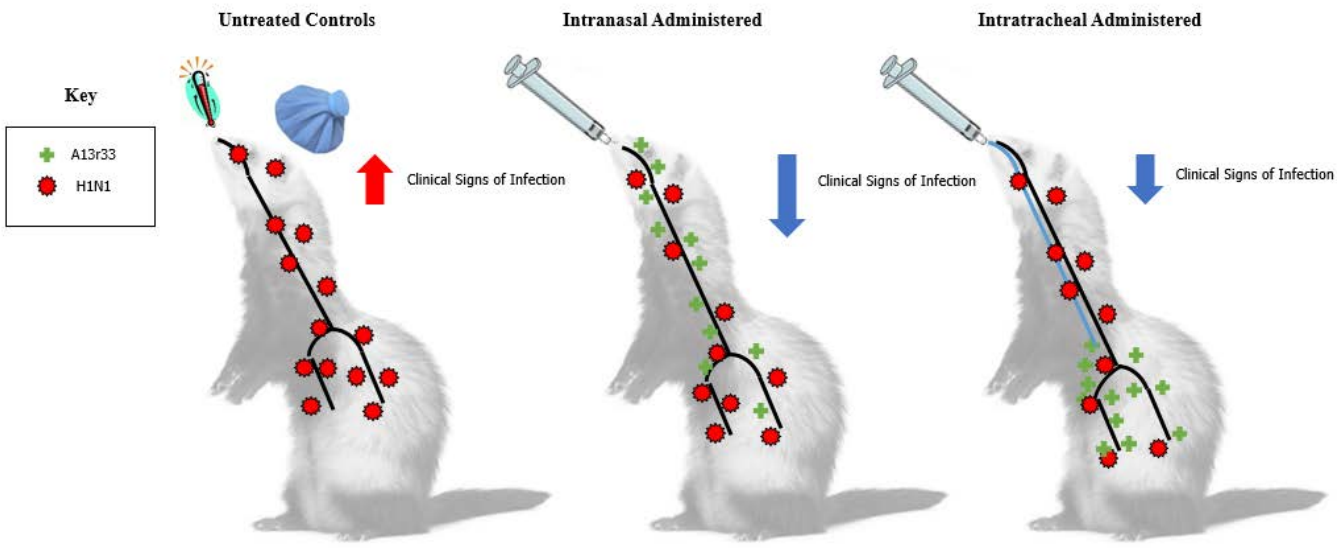


Figure 23 Proposed model for prophylactic treatment with A13r33 prior to aerosol challenge with A/California/07/2009 pH1N1.

As was mentioned prior we hypothesize that intranasally administered minibinder was able to coat the upper respiratory tract, including the nasal turbinates and nasal cavity, the pharynx, larynx, and trachea. Intratracheal administration delivered A13r33 to the carina of the lungs, likely causing a large volume of the minibinder to bypass the aforementioned physiological structures. Aerosolized virus was able then to bind to host epithelial cells in the upper respiratory tract in the intratracheal model more readily than in the intranasal model. This would explain the differences in clinical scores as ferrets in the intranasal were better protected against viral challenge due to the proximity of the antiviral to the site of infection. This could also identify the lag to day 5 for the clinical scores in the intranasal cohort to spike (Figure 21A). As the virus moved through the respiratory system it would have come into contact with less antiviral minibinder, allowing it to more readily infect and replicate in host epithelial cells. Intratracheal ferrets had lower clinical scores than controls (Figure 21A, C), but the highest clinical score for both groups spiked on the same day. This is likely due to the fact that neither

group had enough or any minibinder at the site of infection or the adjoining structures of the upper respiratory tract. This would allow the virus to establish an infection more quickly, leading the rapid increase in symptoms exhibited by both groups. Further studies to evaluate dosing and timing for administration pre- and post-exposure prophylaxis, elicitation of immune responses and pathology, and transmissibility of virus in the presence of mini-binder are warranted to fully understand the long term efficacy of A13r33.

5.2 PUBLIC HEALTH SIGNIFICANCE

Influenza is responsible for widespread illness and death each year worldwide. In 2003 influenza was responsible for causing and around 22,000 to 25,000 deaths as well as estimated cost of over $87 billion in the United States alone (12, 13). With issues of reduced vaccine efficacy, emerging influenza strains that are antiviral resistant, as well as issues of logistics, side effects, and a public that is increasingly turning against vaccinations, there exists a pressing need for effective antivirals that can interact and interfere with a broad variety of influenza strains. Certain strains of influenza, such as H5N1, are too pathogenic to be grown in chicken eggs, making vaccine production more difficult for these strains (23). H5N1 has also been shown to become readily pathogenic and transmissible in the ferret model (67). It is quite possibly that H5N1 or other highly pathogenic influenza strains might naturally evolve to become more readily infectious in humans while retaining their lethality. In such an event broadly neutralizing antivirals will be necessary to combat the potential pandemic.

Small molecules, like HB36.6 and A13r33, may be best way to provide protection against influenza strains. Smaller molecules are less likely to lead to an immune response while

providing protection against infection. They are also more likely to interact more discretely than larger molecules (68). Since influenza is made of ten different proteins there are a variety of targets to choose in designing proteins. Three proteins, NA, HA, and M2 are all found on the surfaces of viral particles. All are required to function properly if the virus is to be infective (M2 and HA) or spread beyond the initially infected cell (NA). Targeting any of these three proteins and disrupting their function could lead to interference or a complete halt to the viral infection. Antivirals like the two discussed in this thesis are critical in controlling the spread of influenza and providing protection to the public at large. There is a critical need for novel approaches that are both safe and effective in preventing the spread of the influenza virus. We hope that work like research that went into this thesis will ultimately lead to new antivirals capable of reducing the morbidity and mortality caused by this virus.

5.3 FUTURE DIRECTIONS

The minibinder A13r33 was designed to interfere directly with the vial hemagglutinin molecule, disrupting infection. Viral load data suggests that the virus was still able to replicate with little to no observable difference in titer amounts regardless of the route of antiviral administration. Additionally treated and untreated animals displayed similar viral load titers in both homogenized tissues and nasal washes. However there were clear differences in the observed clinical symptoms between all three experimental cohorts. It is possible that the minibinder was interacting or interfering with the host's immune response and quite possibly relieving symptoms while not reducing viral load.

Cytokine RT-qPCR work was attempted however data was not forthcoming in the pursuit of this research project. Future work should therefore focus on comparing certain cytokines, including IFN-ɣ, TNF-α, and IL-6, 8 10. These cytokines all play a roles in either pro-inflammatory or anti-inflammatory responses, suppression of viral replication, or are critical to signaling the presence of an infection. Additional work using flow cytometry and pathology should be used to analyze host immune cell response to infection in the presence or absence of A13r33 or other similar antivirals.

Finally there were only three animals used per cohort and only two experiments were performed. Additionally the control animals that were infected but untreated animals did not receive any form of sham control protein (such as lysozyme). In the future all animals should be treated either with A13r33/ antiviral or a sham control. Larger sample sizes using more ferrets per cohort should also be used. Furthermore only one dose of A13r33 was tested, future tests should include different concentrations of minibinder to see how this will affect viral loads as well as immune response. Comparison tests of A13r33 against antivirals currently available (such as oseltamivir) should also be performed. Finally studies should be performed wherein animals treated with the minibinder are left uninfected. This will allow researchers to determine if there is an immune response against A13r33.

APPENDIX A: PROTOCOLS

MDCK Cell Line Maintenance

Materials and Reagents

- Madin-Darby Canine Kidney (MDCK) Cells (ATCC CCL-34)
    - Store in liquid nitrogen until ready to be used.
- Maintenance Media
    - 500mLDMEM (1X, 4.5 g/L glucose, 4mM L-glutamine)
    - 5mLPenicillin/Streptomycin (10,000 U/mL/ 10,000 µg/ml)
    - 5mLHEPES Buffer (1M stock)
    - 5mLL-gluamine, 200 mM (100X), if needed
    - 25mLHeat Inactivated FBS
    - 5mLSodium pyruvate, if needed (may already be in DMEM)
- Virus Dilution Media
    - 500mLDMEM (4.5 g/L glucose, 4mM L-glutamine)
    - 5mLPenicillin/Streptomycin (10,000 U/mL/ 10,000 µg/ml)
    - 5mLHEPES Buffer (1M stock)
    - 12.5mLBovine Serum Albumin fraction V, 7.5% solution in PBS
- Viral Growth Media
    - 100mL Virus Dilution Media
    - 100µL 1:1000 2mg/mL TPCK Trypsin (stored in 2µg/mL stock)
- Trypsin/EDTA, 0.25%
- 96 well plates (flat, u-, and v-bottomed)
- 1X Phosphate-buffered saline (PBS) (pH 7.2)
- Water bath
- T25, T75, and T150 flasks with vented cap
- 37°C, 5% CO2 incubator
- 15mL conical tube
- Trypan Blue solution, 0.4% (Sigma, cat. #T8154)
- Hemocytometer
- 1.5mLcryovials
- Freezing media (90% FBS/10% DMSO)
    - Fetal Bovine Serum (FBS) (Atlanta Biological, Cat. #S11150)
    - Dimethyl Sulfoxide (DMSO) (Sigma, Cat. #D2650)
    - Freezing Container, 1°C/hour (NALGENE™ Cryo, cat. #5100-0001)
- 70% Ethanol and 10% Bleach

- -80°C freezer
- Liquid nitrogen storage
- Microscope
- Biosafety Cabinet class II
- Serological pipettes

Cell Propagation

Note: Perform all of these procedures in BSC class II

1. Thaw cells/ initiate culture
    a. Remove cells from liquid nitrogen and thaw in a 37°C water bath
    b. Resuspend in 10mL Maintenance Media
    c. Centrifuge at 400g for 5 minutes
    d. Remove the supernatant with a pipette and resuspend in 8mL of maintenance Media
    e. Pipette cells in media into T25 flask with vented cap
    f. Incubate flask in a 37°C, 5% CO2 incubator until cells are confluent, about 24 to 48 hours.
    g. Siphon media off with a serological pipette and wash twice with 8-10mL of 1X PBS
    h. Add 1mL of trypsin, place flask in 37°C, 5% CO2 incubator until cells lift of the plate. May take 15 to 30 minutes
    i. Add 18mL of maintenance media, remove total volume of media and cells and transfer to T75 flask.
2. Regular maintaining and splitting MDCK cells
   Note: Split cells at a 1:10 ratio every 3 to 4 days
    a. Siphon off media in the flask with a serological pipette and wash twice with 10mL of 1X PBS
    b. Add 2mL of trypsin for T75, 4mL for T150
    c. Place flask in 37°C, 5% CO2 incubator until cells lift of the plate. May take 15 to 30 minutes
    d. Resuspend cells in a 5:1 ratio of maintenance media to trypsin (8mL for T75, 16mL for T150)
    e. Siphon 1/10 of the volume into a new flask and add 9/10 volume of fresh maintenance media
    f. Place flask in 37°C, 5% CO2 incubator
3. Freezing cells for Liquid Nitrogen Storage
    a. Once flask reaches 80-90% confluency collect cells using methods elucidated in 2.a-d
    b. Take 20µL of Trypan Blue and mix well with 20µL of cell suspension in a single well of a 96 well flat bottomed plate.
    c. Place hemocytometer on microscope tray, add 10µL of trypan-cell suspension mix
    d. Count cells in all four corners of hemocytometer (appear as white dots surrounded with a blue halo
    e. Use following formula to determine cell count

i. (# of cells/4)*2*10^4= cells/mL

f. Resuspend cells in a dilution of ~10^7 cells/mL in freezing media
g. Aliquot into 1.5mL cryovials
h. Place aliquots into freezing vessel indicated for 1°C/hour rate
i. Place vessel into -80°C freezer
j. After 48-72 hours transfer vials to LN2 for long term storage

Viral Propagation and Growth

Materials and Reagents

- Embryonated Chicken Eggs, 9-12 days old
- Candling Flashlight
- Needle 23 gauge, 1 inch, imbedded in a rubber stopper
- Needle 18 gauge, 1 inch
- Disposable syringe
- 50ml centrifuge tube with 70% alcohol
- Sterile forceps
- Influenza virus
- Egg Incubator
- Absorbent pads (diaper)

Protocol

1. Upon delivery of eggs immediately place in incubator set to 100°C and ~ 60% humidity, and turn rocker on
2. Candle eggs daily
    a. Place candling flashlight on the top of the egg (where the air sac is located)
    b. Look for movement inside the egg, if there are distinct veins that connect the embryo to the air sac the egg is considered good.
    c. If there is no vein that connects to the air sac or there are no eggs, the egg is either dead or was never fertilized
3. On Day 11 the eggs are ready to be inoculated
    a. Prepare the virus inoculum: 10^{-3} to 10^{-10}, 10 eggs per dilution.
    b. Wipe top of eggs with alcohol wipes, let dry, and punch a small hole in the eggshell over the air sac using an 18 gauge, 1 inch needle. Make sure to punch hole close to fluid, so the needle can reach the fluid, and away from any major veins.
    c. Aspirate virus inoculum into 1ml syringe with 23 gauge, 1 inch.
    d. Insert the needle into the hole of the egg, pierce the allantoic membrane (about 0.5cm deep from egg shell) and inoculate 0.2ml per egg into the allantoic cavity.
    e. Change the syringe and needle when finished inoculating eggs in each tray or if the needle touches eggshell.
    f. Discard the syringe into a sharps container and seal the punched holes of the eggs with tape. Incubate the inoculated eggs at the appropriate conditions for virus strain. H1N1: 37°C, >80%RH humidity for 48 hours
    g. After 2 days of incubation remove the eggs from the egg incubator and candle to ensure eggs are still good.

h. Chill the eggs by placing them in a refrigerator (4°C) overnight (at least 12 hours) or chill at -20°C for 2-3 hours to sacrifice the embryo and harden the blood vessels for good fluid harvest.
i. When the virus is ready to be harvested remove from the fridge. Place eggs on absorbent pads in a class II BSC.
j. Dip the forceps in 70% alcohol to sterilize and peel off the top of the eggshell (open up the air sac). Insert the 18 gauge, 1 inch needle downward into the allantoic sac. Draw out the clear allantoic fluid and avoid disturbing the blood vessel and yolk sac and place in proper container depending on dilution.
k. Perform Hemagglutination assays to determine infectivity

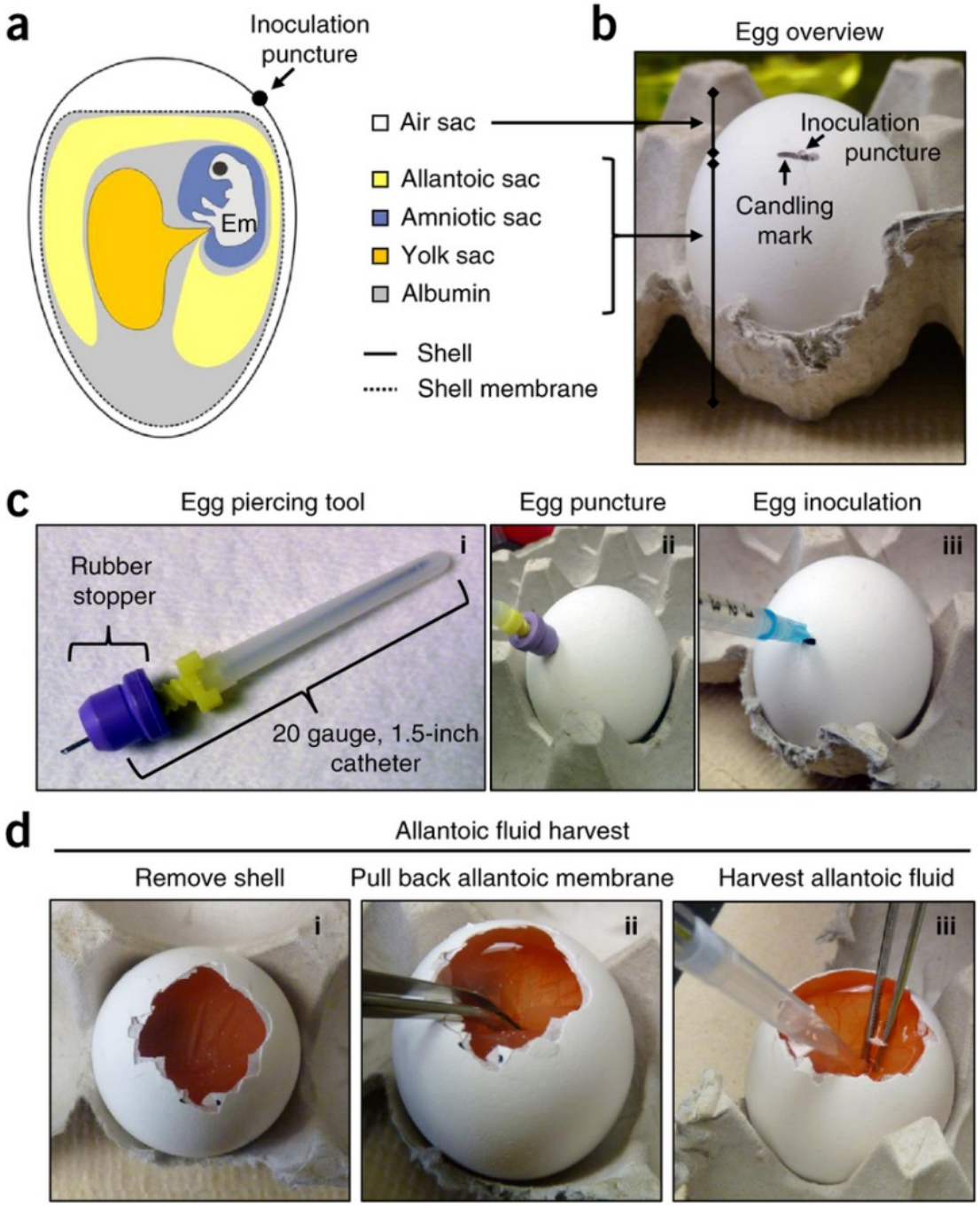


Figure 24. Egg Handling for Influenza Virus Production.

Hemagglutination (HA) and Hemagglutination Inhibition (HAI) Assays

Materials and Reagents

- Biosafety Cabinet class II
- V- and u-bottomed 96 well plates
- Microscope

- 15mL conical tubes
- Eppendorf 1.5mL snap cap tubes
- Serological pipettes
- Micropipettes (including multichannel) and aerosol barrier pipette tips
- 1X PBS
- Washed Pooled Turkey Red Blood Cells (RBC), 10% in Alsevers (Lampire Biological Laboratories, cat# 724908)
- Virus sample to test (Pandemic A/California/07/2009 H1N1)
- Serum samples (heat inactivated 56°C for 30 minutes)
- Reservoir Basins

Assays

Note: All Assays are to be performed in a class II BSC.

1. Hemagglutination Assay
    a. Make a 0.5% working solution of Turkey RBC
        i. Spin 1mL of RBC at 650 rpm for 5 minutes
        ii. Siphon off supernatant (should be clear or light pink, if red the cells are lysed the blood should be discarded)
        iii. Resuspend RBC in 10mL of 1X PBS
    b. Add 50µL PBS to each well of u-bottomed plate
    c. In the first column add 50µL of virus and mix well
    d. Transfer 50µL to the next well on the right and change pipette tips. Mix well and repeat until the end of the plate is reached, changing tips each time.
    e. Add 50uL of 0.5% RBC working solution to each well and mix very gently
    f. Leave plate at room for 30 to 60 minutes
    g. Negative results will appear as rounded dots of blood at the bottom of the well, positive results as a reddish uniform hazed across the total well.
    h. To find the viral Hemagglutinating Units (HAU) find the end point (the last positive dilution before the first negative well. Divide 1 by the dilution (i.e. if the dilution is 1:8, divide 1 by 1/8, 1/1/8, 8 is the HAU)

2. Hemagglutination Inhibition Assay
    a. Heat inactive all serum samples in a water bath at 56°C for 30 minutes
    b. Make a 0.5% working solution of Turkey RBC
        i. Spin 1mL of RBC at 650 rpm for 5 minutes
        ii. Siphon off supernatant (should be clear or light pink, if red the cells are lysed the blood should be discarded)
        iii. Resuspend RBC in 10mL of 1X PBS
    c. Make a 1.0% working solution of Turkey RBC
        i. Spin 2.8mL of RBC at 650 rpm for 5 minutes
        ii. Siphon off supernatant (should be clear or light pink, if red the cells are lysed the blood should be discarded)
        iii. Resuspend RBC in 14mL of 1X PBS
    d. Transfer 50µL of sera to Eppendorf tubes and add 200µL of 0.5% turkey RBC, mix well

e. Incubate room temperature for 30 minutes, vortexing every 10 minutes at medium speed
f. Centrifuge tubes at 14,000 rpm for 5 seconds
g. Add 50µL 1X PBS to wells B-H
h. Add 100µL sera supernatant from centrifuged tubes to row A (preferably in duplicates or triplicates)
i. Serially dilute down the plate 1:2 (take 50µL from A mix well into B and change tips, repeat), mix well
j. Remove 50µL from the final row (row H)
k. Dilute virus to 4 HAU
    i. For work done in Cole Lab with 5/2016 pH1N1 stock this is a 1:4 dilution
    ii. Dilute 3mLs of virus stock in 9mLs of 1X PBS. This is enough for two plates
l. Starting at row H and working up add 50µL of virus to each well, mixing well, and changing tips with each well.
m. Incubate plate at room temperature for 30 minutes.
n. Add 50µL of 1.0% turkey RBC to each well, changing tips each time. Mix well.
o. Incubate plates for ~45 minutes at room temperature
p. Rounded "buttons" of blood appearing at the bottom of the well are negative, meaning there are antibodies present specific to the virus. If no pellet forms but there is a hazy reddish color to the media that means there are no antibodies specific to the virus present.

Tissue Culture Infectious Dose 50 ($TCID_{50}$) Assay

Materials and Reagents

- Viral Growth Media
- 1X PBS
- flat-bottomed 96 well plates
- Serological pipettes and pipettor
- Wet Ice
- Sterile reagent reservoirs
- Multichannel pipette and tips

Procedure

1. Prepare MDCK cells from a confluent T-75cm_2 flask.
    a. Siphon off media in the flask with a serological pipette and wash twice with 10mL of 1X PBS
    b. Add 2mL of trypsin for T75, 4mL for T150
    c. Place flask in 37°C, 5% CO2 incubator until cells lift of the plate. May take 15 to 30 minutes
    d. Resuspend cells in a 5:1 ratio of maintenance media to trypsin (8mL for T75, 16mL for T150)
    e. Enumerate cell concentration using standard trypan blue staining visualized with a hemocytometer.
    f. Dilute cells to 3.0 (or 4.0) x 10^5 cells/ml in maintenance media

g. Dispense 100 µl (3.0, 4.0 x 10^4 cells) into each well of 96 well flat bottom plates
h. Incubate at 37°C, 5% CO_2 until confluent monolayer is obtained (~24-30 hours).
i. Remove media from MDCK cells using aspiration.
j. Wash each well with 200 µl of PBS and remove by aspiration.
k. Add 180 µl of viral growth media to rows B-H of 96-well sterile v-bottom plates using multichannel pipette.
l. Add 200 µl of virus sample to each of 5 wells for sample 1 (columns 1-5), sample 2 (columns 6-10) and 200 µl of viral growth media to column 11-12 as non-infection control.
m. Using multichannel pipette transfer 20 µl from row A to row B, discard tips.
n. Mix 10-20 times using swirling tip action and transfer again 20 µl from row B to row C, discard tips.
o. Repeat dilution down the plate.
p. Incubate plates at 37°C, 5% CO_2.
q. On days 3 and 4 post infection observe cytopathic effects (CPE) and score on a $TCID_{50}$ scoring sheet. Mark well with a "+" if CPE is observed, "-" if no CPE is observed. Use negative and positive controls to help determine if CPE is present.
r. Calculate the TCID50 using the method described by Reed and Meunch (ref)

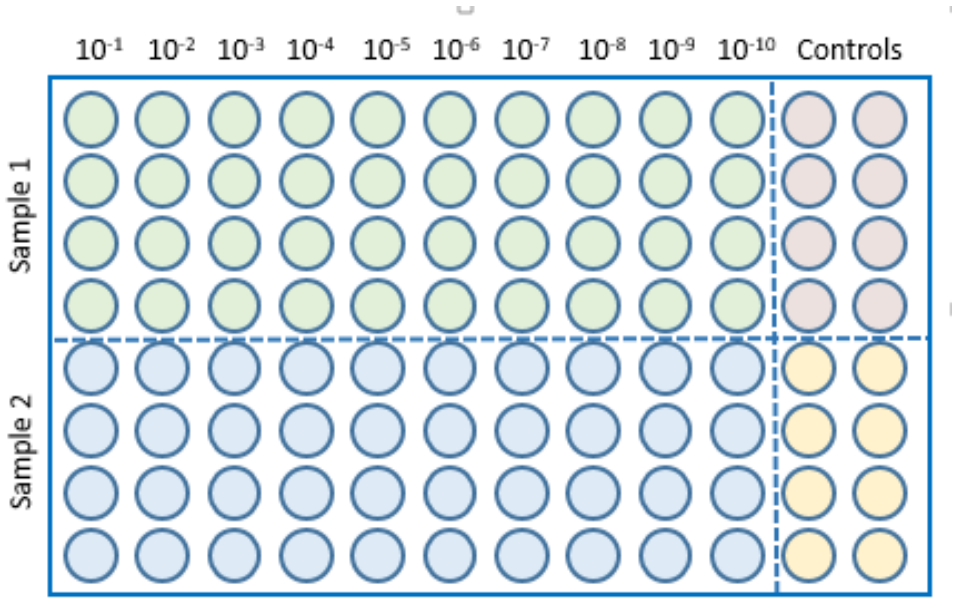


Figure 25. $TCID_{50}$ plate set up diagram.

Study Name: ______________ Notes: ______________

Investigator: ______________ ______________

Date: ______________ Agent: ______________

Lab Room Number: ______________ Observations by: ______________

Dilution		1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
	D												
	E												
	F												
	G												
	H												

Dilution		1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
	D												
	E												
	F												
	G												
	H												

Dilution		1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
	D												
	E												
	F												
	G												
	H												

Figure 26. Blank $TCID_{50}$.

Ferret Clinical Scoring Sheet

Parameter		Degree of Parameter	DATE:								
			Weight								
			Temp								
Appearance	Posture	Normal	0								
		Hunched or abnormal posture	5								
		Prostrate	10								
	Activity	Alert and playful	0								
		Alert but playful only when stimulated	2								
		Alert but not playful when stimulated	5								
		Neither alert nor playful when stimulated	10								
	Skin/Eyes	Normal	0								
		Ocular Discharge	2								
		Piloerection	5								
Movement	Activity	Normal	0								
		Lethargy	5								
		Moribund	10								
	Neurological	Normal	0								
		Tremors	10								
		Hind limb weakness /paralysis	10								
Pulmonary Function	Respiration	Normal	0								
		Sneezing	2								
		Nasal Discharge/Runny Nose	2								
		Shallow or Rapid	5								
		Open mouth breathing	5								
		Labored/coughing	10								
Consumption/ Elimination	Food/water	Normal	0								
		Decreased	2								
		Dehydrated	5								
		Inappetant	5								
		Anorexic	10								
	Feces/Urine	Normal	0								
		No stool	2								
		Mucoid/Soft stool	2								
		Diarrhea	5								
		Bloody feces	10								
	Vomiting	No	0								
		Yes	10								
Total Score											
Comments (clarify any abnormality not defined by parameter description):											

Figure 27. Blank Clinical Scoring Sheet.

Tissue Homogenization

Material and Reagents

- Cell Maintenance Media
- Pipettes (5, 10, 25, 50 mL)
- Pipettor
- Absorbent pads (“diapers”)
- Tissue samples in 15 mL or 50 mL conical tubes
- Scale
- Cryotubes, Nunc 375418
- Wescodyne (or other antiviral solution)
- 70% Ethanol
- 2x 1 L beaker
- OMNI tissue homogenizer
- Hard disposable homogenizer tips, autoclavable bags, OMNI 34750-AC-16
- Wet Ice
- Dry Ice

Procedure

1. Prior to Homogenization
    a. Pre-label at least three or four Nunc cryotubes for each sample.
    b. Prepare a spreadsheet to record sample IDs and organ weight to calculate the amount of media needed to prepare a 10% (or 5% or 20%) w/v suspension.
    c. Autoclave homogenizer tips in autoclavable bags.
    d. Samples can be weighed and organized into racks @ -80°C ahead of time if necessary.
2. Day of Homogenization
    a. Clean BSC and prepare beaker of antiviral solution (1% Wescodyne, 10% Bleach, etc.),
    b. Prepare a second beaker of 70% ethanol
    c. Lay down absorbent pads to cover the area around the homogenizer, it may be necessary to place absorbent pads against the walls of the BSC.
3. Organize and weigh tissues
    a. Organize the organs to be titered on wet ice starting with the samples anticipated to have the lowest virus titer and ending with the samples anticipated to have the highest titer of virus.
    b. Weigh tissues to be homogenized and record the weight on a spreadsheet.
        a. If there are multiple pieces of an organ (i.e. sections taken from a lung sample) weigh multiple pieces together.
4. Homogenization
    a. Add cell maintenance media to each tube; a minimum of 1.5 mL is needed
        i. A 10% w/v suspension is adequate for most organs
            1. EXAMPLE: tissue weighs 0.54 g, add 5.4 mL media for 10% w/v suspension
        ii. A 5% w/v suspension is appropriate for small organs like nasal turbinates.
            1. EXAMPLE: tissue weighs 0.12 g, add 2.4 mL media for 5% w/v suspension

ii. For large tissues such as a ferret brain, a 10% w/v results in a large volume
    1. EXAMPLE: brain weighs 6.2 g, 62 mL will not fit in 50 mL conical
        a. Add a set volume of media to grind (10 mL)
        b. Then add enough media to bring up to 20% (10 mL + 21 mL = 31 mL)
        c. Spin homogenized sample
        d. Put 1 mL of the 20% homogenate into cryotubes and add 1 mL of media to achieve a 10% w/v suspension and vortex well before use

b. Setup the homogenizer and prepare beakers containing antiviral solution to disinfect tips. Place absorbent pads in the work area.
c. Homogenize each organ using the tissue homogenizer.
d. The amount of time needed to homogenize organs varies with the size and consistency of the tissue and the speed of the motor. There are six speed levels on the OMNI homogenizer and we typically use the maximum (6) speed. Soft tissues such as brain and lungs take less time (~10-20 seconds) than harder tissues such as nasal turbinates (~30 sec - 1 minute). Homogenize until tissue is no longer recognizable. Nasal turbinates will be pulverized but not homogenized.
e. Before removing the used tip from the grinder, immerse it in a 50 mL conical tube containing antiviral solution to disinfect the tip.
f. Remove the grinder tip and place it in a beaker containing antiviral solution. Put a new grinder tip on the homogenizer and continue until tissues are homogenized.
g. Wipe down the homogenizer with bleach wipes, especially the on/off switch and the tip holder.
h. Spin the homogenized samples to pellet cellular debris 10 min. at 1500 RPM (~524 g) at 4°C.
i. Organize the cryotubes in racks.
j. Aliquot 1000 µL (or half the volume if < 2 mL total) of each tissue homogenate into duplicate (or more) cryotubes (300 µL minimum needed for titration).
    i. Place one cryotube on WET ICE if titering immediately.
    ii. Place duplicate cryotube(s) on DRY ICE to freeze

Add antiviral solution to 15 ml conical tubes to inactivate virus before discarding.

RNA Extractions

Materials

- Qiagen RNeasy Mini Kit (cat. # 74104 or 74106), used for homogenized tissues only.
- Qiagen Viral RNA Mini Kit (cat. # 52904 or 52906), used for Nasal Washes only.
- 10% Bleach, 70% Ethanol
- Micropipettes and aerosol barrier pipette tips
- Sterile, nuclease free water
- Wet ice
- Trizol/ TriReagent
- Chloroform

Assays

1. RNA Extraction for Homogenized Tissues
    a. Add 900µl TriReagent to sterile Eppendorf tubes and transfer 100µl of each homogenized tissue to a tube containing TriReagent
        i. For lung samples take 50 µl from the right and left homogenates and mix together, then proceed to the next step.
    b. Vortex all samples very well and let sit at room temperature for 5min with frequent vortexing until no visible clumps of cells are left
    c. Keep the samples at 4°C if you are not going to continue to the next step immediately or store them in -80°C freezer
    d. If samples are frozen, thaw to room temperature
    e. In BSC, add 200µl chloroform to each tube and invert vigorously for 20sec (DO NOT VORTEX)
    f. Let sit at room temperature for 3min
    g. Centrifuge at 12,000 x g for 15min at 4°C
    h. Transfer 500µl of aqueous phase (clear top layer) to a clean tube
    i. The remaining steps can be performed on a benchtop cleaned with RNase Away Reagent; remove tubes from BSC and clean hood with disinfectant
    j. Add 1 volume (500µl) 70% ethanol to each lysate and mix well by pipetting
    k. Transfer up to 700µl of sample at a time to RNeasy Mini Spin column inside a 2ml collection tube (supplied in kit)
    l. Centrifuge at >8000 x g for 15sec
    m. Discard flow through and repeat with remaining sample
    n. Add 350µl Buffer RW1 to spin column
    o. Centrifuge at max speed for 30sec and discard flow-through
    p. Add 10µl DNase 1 stock solution to 70µl Buffer RDD (Note: This calculation is per reaction. Multiply volumes for more samples.), mix by inverting tube, and briefly centrifuge
    q. Add 80µl DNase 1 – Buffer RDD mixture directly to spin column membrane and incubate at room temperature for 15mins
    r. Add 500µl Buffer RPE to spin column, centrifuge at max speed for 30sec and discard flow-through
    s. Repeat step r.
    t. Place spin column in a new collection tube (supplied in kit) and centrifuge at full speed for 1 min to dry the membrane
    u. Place spin column in a new 1.5ml collection tube with cap (supplied in kit), add 50µl nuclease-free water directly to the spin column membrane, and centrifuge at max speed for 1min to elute RNA
    v. Store samples in -80°C freezer

2. RNA Extraction for Liquid Samples (Nasal Washes)
    a. Add 900µl TriReagent to sterile Eppendorf tubes and transfer 100µl of each nasal wash to a tube containing TriReagent
    b. Vortex all samples very well and let sit at room temperature for 5min with frequent vortexing until no visible clumps of cells are left

c. Keep the samples at 4°C if you are not going to continue to the next step immediately or store them in -80°C freezer
d. If samples are frozen, thaw to room temperature
e. In BSC, add 200µl chloroform to each tube and invert vigorously for 20sec (DO NOT VORTEX)
f. Let sit at room temperature for 3min
g. Centrifuge at 12,000 x g for 15min at 4°C
h. Transfer 500µl of aqueous phase (clear top layer) to a clean tube
i. The remaining steps can be performed on a benchtop cleaned with RNase Away Reagent; remove tubes from BSC and clean hood with disinfectant
j. Resuspend the lyophilized 310µg of carrier RNA in 310µL Buffer AVE. Aliquot and store at -20°C. Avoid freeze thawing more than three times
k. Prepare a Buffer AVL/AVE- carrier RNA solution
    i. Calculate the volume of Buffer AVL with the following equation, n(0.56mL) =y mL
        1. n= number of samples
        2. y= volume of Buffer AVL
    ii. Calculate the volume of Buffer AVE-carrier RNA with the following equation, y mL * 10µL/ mL =z µL
        1. y= volume of Buffer AVL
        2. z= volume of Buffer AVE-carrier RNA
    iii. Gently mix the two reagents by gently inverting 10 times, DO NOT VORTEX
l. Into clean Eppendorf tubes pipette 560 µL of Buffer AVL/AVE- carrier RNA
m. Add 140 µL of the aqueous phase aliquoted in step h to the Buffer AVL/AVE-carrier RNA and mix by vortexing for 15 seconds
n. Incubate at room temperature for 10 minutes.
o. Centrifuge the tubes to collect droplets
p. Add 560 µL of 100% ethanol to the sample and mix by vortexing for 15 seconds
q. Centrifuge the tubes to collect droplets
r. Apply 630 µL of solution to the QIAamp Mini Column with attached 2mL collection tube. Centrifuge at 8,000 rpm for 1 minute. Place Mini column in a new 2mL collection tube and dispose old collection tube.
s. Add remainder of solution to the QIAamp Mini Column. Centrifuge at 8,000 rpm for 1 minute. Place Mini column in a new 2mL collection tube and dispose old collection tube.
t. Add 500 µL of Buffer AW1.Centrifuge at 8,000 rpm for 1 minute. Place Mini column in a new 2mL collection tube and dispose old collection tube.
u. Add 500 µL of Buffer AW2.Centrifuge at 8,000 rpm for 1 minute. Place Mini column in a new 2mL collection tube and dispose old collection tube.
v. Centrifuge QIAamp Mini Column at full speed for 1 minute. Place Mini Column in a 1.5mL Eppendorf tube
w. Add 60 µL of room temperature Buffer AVE to the column and incubate for 1 minute. Centrifuge at 8,000 rpm for 1 minute. Dispose of Mini column and store RNA at -80°C

Viral Titer Quantification by PCR

Material/Reagents:

- SuperscriptR III PlatinumR One-Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, CA, cat. #11745-100)
- MicroAmpR Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737)
- MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971)
- Quantstudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific)
- RNase/DNase-free microcentrifuge tubes
- Nuclease-free water
- Swine Influenza A (H1N1) real-time RT-PCR Assay (BEI Resources, NR-15577)
- Template RNA extracted with Qiagen Viral RNA Mini Kit (cat. # 52904 or 52906)

Set Up

1. Nucleic acids extracted from Swine Influenza A (H1N1) Positive Control (NR- 15590), and Novel H1N1 2009 (Swine) Influenza A (NR-15627) should be diluted 1:10 prior to use as template in the assay
2. Working stock aliquots of the primers and probes should be made at concentrations of 40µM (primers) and 10µM (probes) by adding 500µl nuclease free water to each

Table 4. PCR Primers and Probes for Influenza Viral Titers.

Primers and Probes	Cat #	Sequence
*Influenza A Probe	NR-15578	TGC AGT CCT CGC TCA CTG GGC ACG
Influenza A Forward	NR-15579	GAC CRA TCC TGT CAC CTC TGA C
Influenza A Reverse	NR-15580	AGG GCA TTY TGG ACA AAK CGT CTA
Swine H1 Probe	NR-15584	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A
Swine H1 Forward	NR-15585	GTG CTA TAA ACA CCA GCC TYC CA
Swine H1 Reverse	NR-15586	CGG GAT ATT CCT TAA TCC TGT RGC

*At the 5'end, TaqMan® probes are marked with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3'end with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) [41].

**Taqman® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'- end to prevent probe extension by Taq polymerase

Protocol:

1. Thaw RNA samples and PCR reagents on ice, keep on ice throughout procedure
2. Label one microcentrifuge tube for the master mix
3. All samples, standards and controls should be run in duplicate
4. Prepare master mix according to table below:

Table 5. Viral Titer PCR Master Mix.

Reagent	**Volume of Reagents** **(multiplied by number of reactions)**
Forward Primer	N* 0.5µL
Reverse Primer	N* 0.5µL
*Probe	N* 0.5µL
Superscript Taq Mix	N* 0.5µL
2x PCR Master Mix	N* 12.5µL
Nuclease Free Water	N* 5.5µL

* 6-carboxyfluorescein (6-FAM) probe must be protected from light

5. After adding water last, mix master mix by pipetting (DO NOT VORTEX)
6. Add 20µl master mix to all needed wells of a 96-well plate
7. Add 5µl of each unknown sample, standard or control to each well, mix well and then centrifuge the plate
    a. Controls:
        i. No Template Control (NTC) containing all reagents except RNA template sample, use nuclease free water instead
        ii. Positive Controls containing master mix and known positive virus samples:
            1. Swine Influenza A (H1N1) Positive Control (NR-15590)
            2. Novel H1N1 2009 (Swine) Influenza A Positive Control (NR-15627) should be included in each run for all primer and probe sets
8. Wearing gloves, smooth a clear adhesive cover over the 96-well PCR plate
9. Take plate to PCR machine on ice
10. Make sure the Quantstudio™ 6 qPCR machine is on by checking to see if there is a green light on the front. If there is not or there is a red light flip the switch on the back left side of the machine
11. Log onto the computer and click on the program “QuantStudio Real-Time PCR Software” located on the desktop
12. Click on “New Experiment” and select the following choices on the experimental properties tab
    - QuantStudio 6
    - 96 Well plate, 0.2mL
    - Standard Curve
    - TaqMan® Reagents
    - Standard

13. Click the “Define” tab on the left and make sure in the “Targets” section the Reporter is listed as FAM and the Quencher as NFQ-MGB
14. Under the “Samples” Section list samples as desired, but it is not necessary.
15. Check that the “Passive Reference” drop down menu is selected as ROX.
16. Click the “Assign” tab on the left and assign the Targets and Samples according to how the plate was set up.
17. Click on the “Run Method” tab on the left and chose the following:
    - Reverse Transcription: 50°C for 30 minutes
    - Taq Inhibitor Activation: 95°C for 2 minutes
    - PCR Amplification (40 cycles)
        - Denature: 95°C for 15 seconds
        - Anneal: 55°C for 30 seconds
18. Making the Standard Curve
    - Need the following
        - Virus stock with a known high titer
            - A/California/07/2009 pandemic H1N1, egg grown $2.3*10^8$ $TCID_{50}$/mL
        - Nuclease free water
        - Eppendorf tubes
        - Need all reagents/materials from RNA isolation from liquid samples and PCR protocols above
    - Protocol:
        - Add 100µl virus stock sample to 900µl TriReagent in six replicates (want a lot of sample so can make a lot of dilutions and aliquot)
        - Perform RNA extraction procedure for liquid samples and elute RNA in 50µl nuclease-free water
        - Combine all six replicates together (40µl x 6 ≈ 240µl)
        - Make 10-fold dilutions with nuclease-free water starting with 1:10 (10^{-1}) and going down to 10^{-8}
        - Run dilution PCR in duplicate against the remaining standard stock
            - Make sure to include a NTC
            - After PCR is complete, check the new standard against the old standard for accuracy
            - Calculate new standard quantities based off the original known virus titer (TCID50/ml)

Detection of Ferret Cytokines by Two Step RT-qPCR

Materials and Reagents

- RNA Template extracted with Qiagen RNeasy Mini Kit (cat. # 74104 or 74106)
- MicroAmpR Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737)
- MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971)
- Quantstudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific)
- RNase/DNase-free microcentrifuge tubes

- Nuclease-free water
- Fisherbrand™ 0.2mL flat cap PCR tubes (ThermoFisher Scientific)
- Maxima First Strand cDNA Synthesis Kit for RT-qPCR (cat# K1641)
- Maxima SYBR Green/ROX qPCR Master Mix (2X) (cat# K0221)

Protocol

1. cDNA Synthesis
    a. Label one microcentrifuge tube for the master mix
    b. All samples, standards and controls should be run in duplicate
    c. Prepare master mix according to table below:

Table 6. Reverse Transcription PCR Master Mix.

Reagent	**Volume of Reagents (multiplied by number of reactions)**
5X Reaction Mix	N* 4µL
Maxima Enzyme Mix	N* 2µL
Template RNA	1 pg to 5µg
Nuclease Free Water	up to 20µL
Total Volume	N* 20.0µL

    d. Add 15µl master mix to N+2 flat cap 0.2mL qPCR tubes.
    e. Add 5µl of each unknown sample, standard or control to each well, mix well and then centrifuge the plate
        i. Controls:
            1. Reverse Transcriptase Minus (RT-): includes all reagents except Maxima Enzyme Mix, replace this volume with nuclease free water
            2. No Template Control (NTC): includes all reagents except template RNA, replace this volume with nuclease free water
    f. Wearing gloves, smooth a clear adhesive cover over the 96-well PCR plate
    g. Take plate to PCR machine on ice to Thermocycler and set the following protocol
        i. Incubate for 10 minutes at 25°C followed by 15 minutes at 50°C, terminate the reaction by heating to 85°C for 5 minutes. Set an indefinite 4°C hold to follow.
    h. Remove tubes and either start qPCR or freeze samples at -80°C
2. qPCR
    a. Working stock aliquots of the primers should be made at concentrations of 5µM

Table 7. Cytokine PCR Primers

*Primers	Sequence
GAPDH Forward	AAC ATC ATC CCT GCT TCC ACT GGT
GAPDH Reverse	TGT TGA CGC AGG AGA CAA CCT
IFN γ Forward	CCA TCA AGG AAG ACA TGC TTG TCA GG
IFN γ Reverse	CTG GAC CTG CAG ATC ATT CAC AGG AA
TNF α Forward	TGG AGC TGA CAG ACA ACC AGC TAA
TNF α Reverse	TGA TGG TGT GGG TAA GGA GCA CAT

*All primers designed by Integrated DNA Technologies

b. Label one microcentrifuge tube for the master mix
c. All samples, standards and controls should be run in duplicate
d. Prepare master mix according to table below:

Table 8. Cytokine qPCR Master Mix.

Reagent	Volume of Reagent (multiplied by number of reactions)
Maxima SYBR Green/ROX qPCR Master Mix	N* 12.5µL
Forward Primer	0.4 µM
Reverse Primer	0.4 µM
Template DNA	≤ 500 ng
Nuclease Free Water	to 25µL
Total Volume	N* 20µL

e. After adding water last, mix master mix by pipetting (DO NOT VORTEX)
f. Add master mix to all needed wells of a 96-well plate
g. Add unknown sample, standard or control to each well, mix well and then centrifuge the plate
    i. Controls:
        1. No cDNA Control (NcDC): includes all reagents except template DNA, replace this volume with nuclease free water
h. Wearing gloves, smooth a clear adhesive cover over the 96-well PCR plate
i. Take plate to PCR machine on ice
j. Make sure the Quantstudio™ 6 qPCR machine is on by checking to see if there is a green light on the front. If there is not or there is a red light flip the switch on the back left side of the machine

k. Log onto the computer and click on the program “QuantStudio Real-Time PCR Software” located on the desktop
l. Click on “New Experiment” and select the following choices on the experimental properties tab
    i. QuantStudio 6
    ii. 96 Well plate, 0.2mL
    iii. Δ ΔCT
    iv. SYBR Green
    v. Standard
m. Click the “Define” tab on the left and make sure in the “Targets” section the Reporter is listed as SYBR and the Quencher as none
n. Under the “Samples” Section list samples as desired, but it is not necessary.
o. Check that the “Passive Reference” drop down menu is selected as ROX.
p. Click the “Assign” tab on the left and assign the Targets and Samples according to how the plate was set up.
q. Click on the “Run Method” tab on the left and chose the following:
    i. UDG pre-treatment: 50°C for 2 minutes
    ii. Initial denaturation: 95°C 10 minutes
    iii. PCR Amplification (40 cycles)
        1. Denaturation: 95°C for 15 seconds
        2. Anneal: 60°C for 30 seconds
        3. Extension: 72°C for 30 seconds

APPENDIX B: SUPPLEMENTAL RESULTS

B.1 PROPHYLACTIC STUDY

Table 9. Inhaled Dose of Influenza for Group 1.

Group Number (Date)	Ferret ID	Treatment A13r33	Inhaled Dose ($TCID_{50}$)
1 (4/18)	FT-001	5mg/ kg Intranasal	1.74E+06
1 (4/18)	FT-004	5mg/ kg Intratracheal	1.74E+06
1 (4/18)	FT-008	Control	1.39E+06
Run 1 Average			1.62E+06
2 (4/18)	FT-003	5mg/ kg Intranasal	5.41E+06
2 (4/18)	FT-005	5mg/ kg Intratracheal	5.13E+06
2 (4/18)	FT-009	Control	5.04E+06
Run 2 Average			5.19E+06
3 (4/18)	FT-002	5mg/ kg Intranasal	5.13E+06
3 (4/18)	FT-006	5mg/ kg Intratracheal	4.48E+06
3 (4/18)	FT-007	Control	5.04E+06
Run 3 Average			4.88E+06

Table 10. Inhaled Dose of Influenza for Group 2.

Group Number (Date)	Ferret ID	Treatment A13r33	Inhaled Dose ($TCID_{50}$)
1 (5/5)	FT-012	5mg/ kg Intranasal	5.04E+04
1 (5/5)	FT-015	5mg/ kg Intratracheal	5.32E+04
1 (5/5)	FT-016	Control	4.86E+04
Run 1 Average			5.07E+04
2 (5/5)	FT-010	5mg/ kg Intranasal	3.56E+05
2 (5/5)	FT-017	5mg/ kg Intratracheal	3.44E+05
2 (5/5)	FT-014	Control	3.37E+05
Run 2 Average			3.46E+05
3 (5/5)	FT-011	5mg/ kg Intranasal	8.20E+05
3 (5/5)	FT-013	5mg/ kg Intratracheal	7.54E+05
3 (5/5)	FT-018	Control	8.72E+05
Run 3 Average			8.15E+05

Table 11. Temperature (°C) of Ferrets in Group 1.

Ferret ID	Temperature(°C)							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-001	37.8	39.1	37.3	38.4	38.6	38.7	38.3	38.5
FT-002	38.4	38.7	39.2	38.4	38.3	37.7	38.2	38.8
FT-003	38.6	38.1	39.5	39.2	39.5	39.1	39	38.8
FT-004	35.4	36.1	36.3	36.5	36.4	36.2	36.2	36.3
FT-005	37.8	38.9	38.7	38.6	38.6	38.5	38.1	38.2
FT-006	37.6	35.1	39.4	38.1	38	38.1	38.6	38.5
FT-007	n/a	37	39.6	38.4	38.3	38.5	38	39
FT-008	37.9	39	39.3	38.5	38.1	38.2	39.1	39.1
FT-009	n/a	36.5	38.7	38.4	38.9	38.4	38.7	38.7

Table 12. Temperature (°C) of Ferrets in Group 2.

Ferret ID	Temperature(°C)							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-010	37.9	38.1	39.1	38.5	38.5	38.1	38.2	37.9
FT-011	37.4	38.6	38.3	38.7	38.6	36.8	37.4	37.4
FT-012	37.8	39.7	39.4	39.1	39.2	39	38.3	37.8
FT-013	40.3	40.5	41.1	41.4	41.1	40.8	40.7	39.9
FT-017	38.3	38.2	39.5	39	38.8	38.6	38.9	38.5
FT-015	38.2	38.9	38.9	39.4	38.8	38.7	38.4	38.7
FT-016	37.7	39	40	39.1	39.1	39.3	38.4	39.2
FT-018	37.8	38.8	39.7	38.9	38.3	38.2	38.2	39.9
FT-014	38.4	39.1	39.1	39.2	39.1	39.5	38.1	38.4

Table 13. Weights (kg) of Ferrets in Group 2.

Ferret ID	Weights (kg)							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-001	0.46	0.46	0.39	0.39	0.41	0.39	0.36	0.38
FT-002	0.42	0.4	0.33	0.34	0.35	0.32	0.3	0.35
FT-003	0.45	0.44	0.36	0.4	0.41	0.4	0.39	0.4
FT-004	0.46	0.44	0.41	0.41	0.41	0.42	0.41	0.43
FT-005	0.42	0.42	0.35	0.34	0.34	0.34	0.35	0.36
FT-006	0.35	0.37	0.33	0.34	0.35	0.34	0.31	0.31
FT-007	0.41	0.41	0.34	0.35	0.33	0.3	0.29	0.32
FT-008	0.34	0.36	0.3	0.32	0.31	0.31	0.31	0.32
FT-009	0.41	0.42	0.37	0.38	0.39	0.37	0.37	0.38

Table 14. Weights (kg) of Ferrets in Group 2.

Ferret ID	Weights (kg)							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-010	0.43	0.37	0.39	0.34	0.37	0.38	0.43	0.47
FT-011	0.47	0.43	0.4	0.38	0.36	0.34	0.36	0.37
FT-012	0.41	0.38	0.37	0.34	0.37	0.38	0.39	0.41
FT-013	0.42	0.42	0.41	0.38	0.4	0.4	0.39	0.4
FT-017	0.41	0.4	0.37	0.38	0.38	0.38	0.37	0.38
FT-015	0.44	0.45	0.41	0.41	0.43	0.43	0.43	0.44
FT-016	0.39	0.38	0.36	0.36	0.37	0.36	0.36	0.34
FT-018	0.51	0.5	0.48	0.48	0.49	0.48	0.53	0.48
FT-014	0.4	0.4	0.38	0.39	0.4	0.39	0.39	0.4

Table 15. Clinical Scores of Ferrets in Group 1.

Ferret ID	Clinical Scores							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-001	0	7	19	25	18	18	13	11
FT-002	0	5	22	24	26	26	13	11
FT-003	0	7	25	25	25	24	15	13
FT-004	0	9	11	12	15	11	11	9
FT-005	0	7	17	24	26	24	12	9
FT-006	0	7	15	22	19	19	18	18
FT-007	0	9	34	29	29	29	27	20
FT-008	0	12	29	27	27	24	24	20
FT-009	5	10	18	27	25	20	19	15

Table 16. Clinical Scores of Ferrets in Group 2.

Ferret ID	Clinical Scores							
	Pre-infection	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Necro)
FT-010	0	0	5	7	9	13	11	4
FT-011	0	9	11	11	11	20	18	2
FT-012	0	0	7	7	9	13	15	4
FT-013	0	2	4	19	18	14	11	9
FT-017	0	2	11	19	19	21	19	17
FT-015	0	2	7	19	19	19	16	14
FT-016	0	2	14	24	26	24	16	23
FT-018	0	2	7	17	19	16	21	16
FT-014	0	4	11	21	14	14	11	13

B.2 PILOT STUDY

Table 17. Inhaled Dose of Influenza.

Ferret ID	Infection Route	Titer Virus Received ($TCID_{50}$)
FT-003	Aerosol	$10^{4.45}$
FT-004	Aerosol	$10^{4.45}$
FT-002	Intranasal	$10^{4.2}$
FT-007	Intranasal	$10^{4.2}$
FT-005	Uninfected	Not Applicable
FT-006	Uninfected	Not Applicable

Table 18. Temperature (°C) of Ferrets.

Ferret ID				
	Pre-infection	**Day 1**	**Day 2**	**Day 3 (Necro)**
FT-003	38.8	38.8	38.7	38.8
FT-004	39	39	39.8	39.9
FT-002	38.3	38.3	39.2	39.
FT-007	38.6	38.6	39.2	39
FT-005	Not collected	Not collected	Not collected	Not collected
FT-006	Not collected	Not collected	Not collected	Not collected

Table 19. Weight (kg) of Ferrets.

Ferret ID	Weight (kg)			
	Pre-infection	**Day 1**	**Day 2**	**Day 3 (Necro)**
FT-003	0.93	0.93	0.93	0.9
FT-004	0.88	0.88	0.88	0.87
FT-002	1.04	1.04	1.04	1.06
FT-007	0.94	0.94	0.93	0.93
FT-005	1.05	1.06	1.06	1.07
FT-006	1.05	1.06	1.06	1.07

Table 20. Clinical Scores of Ferrets.

Ferret ID				
	Pre-infection	**Day 1**	**Day 2**	**Day 3 (Necro)**
FT-003	0	2	8	18
FT-004	0	2	8	21
FT-002	0	0	4	4
FT-007	0	2	4	4
FT-005	0	0	0	0
FT-006	0	0	0	0

Table 21. $TCID_{50}$ Raw Data for Nasal Washes, Right, and Left Lung.

CA0709 PA-nano Luc	Rt Lung	Aero	F003	D3	++	++++	++++	-+++	+---	----	----	----	----	----	----	----	----
			F004	D3	++	++++	++++	----	----	----	----	----	----	----	----	----	----
		IN	F007	D3	++	++++	++++	++++	---+	----	----	----	----	----	----	----	----
			F002	D3	++	++++	++++	++++	++++	----	----	----	----	----	----	----	----
	Lft Lung	Aero	F003	D3	++	++++	++++	-+++	+---	----	+---	----	----	----	----	----	----
			F004	D3	++	++++	++++	----	----	----	----	----	----	----	----	----	----
		IN	F002	D3	++	++++	++++	++++	--+-	----	----	----	----	----	----	----	----
			F007	D3	++	++++	++++	+++-	---+	----	----	----	----	----	----	----	----
	Nasal Wash	Aero	F003	D1		++++	+-++	+--+	----	----	----	----	----	----	----	----	----
				D2		++++	++++	++++	++++	----	----	----	----	----	----	----	----
				D3		+++-	++--	+---	----	----	----	----	----	----	----	----	----
			F004	D1		++++	++--	----	----	----	----	----	----	----	----	----	----
				D2		++++	++++	+++-	++--	----	----	----	----	----	----	----	----
				D3		++++	++++	----	----	----	----	----	----	----	----	----	----
		IN	F002	D1													
				D2		++++	++++	++++	++++	----	----	----	----	----	----	----	----
				D3		++++	++++	++++	-+--	----	----	----	----	----	----	----	----
			F007	D1													
				D2		++++	++++	---+	-+--	----	----	----	----	----	----	----	----
				D3		++++	++++	+++-	----	----	----	----	----	----	----	----	----

Table 22. $TCID_{50}$ Raw Data for Trachea, Soft Palate, and Nasal Turbinates.

CA0709 PA-nano Luc	Trachea	Aero	F003	D3	++	++++	+-+-	----	----	----	----	----	----	----	----	----	----
			F004	D3	++	++++	++--	----	+---	----	----	----	----	----	----	----	----
		IN	F002	D3	++	++++	++-+	----	----	----	----	----	----	----	----	----	----
			F007	D3	++	++++	++++	----	----	----	----	----	----	----	----	----	----
	Soft Palate	Aero	F003	D3	++	++++	++++	++++	++--	----	----	----	----	----	----	----	----
			F004	D3	++	++++	++++	++++	++--	----	----	----	----	----	----	----	----
		IN	F002	D3	++	++++	++++	++++	+--+	-+-+	----	----	----	----	----	----	----
			F007	D3	++	++++	++++	++++	++++	++-+	----	----	----	----	----	----	----
	Nasal Turbinate	Aero	F003	D3	++	++++	++++	++++	++++	-+++	----	----	----	----	----	----	----
			F004	D3	++	++++	++++	--++	----	----	----	----	----	----	----	----	----
		IN	F002	D3	++	++++	++++	++++	--+-	+---	----	----	----	----	----	----	----
			F007	D3	++	++++	++++	++++	----	----	----	----	----	----	----	----	----

APPENDIX C: FORMULAS

Table 23. Calculation to Determine $TCID_{50}$

	Number Infected Eggs/Wells (+ve)	Number Uninfected Eggs/Wells (-ve)	Accumulated numbers			Percentage infected
			Infected (A) Add from BOTTOM up	Uninfected (B) Add from TOP down	Total (A+B)	A/(A+B) × 100
10^{-6}	5	0	11	0	11	11/11 = 100%
$*10^{-7}$	4	1	6	1	7	6/7 = 86%
10^{-8}	1	4	2	5	7	2/7 = 29%
10^{-9}	1	4	1	9	10	1/10 = 10%
10^{-10}	0	5	0	14	14	0/14 = 0%

- Determine the dilution of inoculum producing 50 percent infection of eggs or cells
- If necessary, use the Reed Muench formula to calculate the index:

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected at dilution immediately above 50\%}) - (\%\text{ infected at dilution immediately below 50\%})} = \frac{86-50}{86-29} = \frac{36}{57} = 0.6$$

- Apply the index calculated using this formula to the dilution that produced the infection rate immediately above 50 percent = $10^{-7.6}$
- (Mark dilution with *)
- This dilution of the virus suspension contained one EID_{50} or $TCID_{50}$ unit of virus in 0.1 mL (or whatever volume virus was added to egg/first well 96- well plate (ex. 20ul/96 well plate)
- 1 mL of the virus suspension will contain ten times the reciprocal of the calculated dilution (if added 100ul) or 50x the reciprocal calculated dilution (if added 20ul).
- Therefore Infectivity Titer of virus suspension in EID_{50}/mL = $10 \times 10^{7.6} = 10^{8.6}$ EID_{50}/mL OR TCID50/mL = 50 x $10^{7.6}$ =

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