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David John Holthausen Date

An effector of amphibian innate immunity protects against human influenza A viruses

By

David John Holthausen
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____

Joshy Jacob, Ph.D.
Advisor

_____ _____

Lawrence Boise, Ph.D.
Committee Member

Max Cooper, M.D.
Committee Member

_____ _____

Brian Evavold, Ph.D.
Committee Member

Suryaprakash Sambhara, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

An effector of amphibian innate immunity protects against human influenza A viruses

By

David John Holthausen
B.S., Boston College, 2010

Advisor: Joshy Jacob, Ph.D.

An abstract of
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Abstract

An effector of amphibian innate immunity protects against human influenza A viruses

By David John Holthausen

While the current program of influenza vaccines is effective at conferring protection against influenza A viruses, varying from 19-60%, antiviral treatments are a necessary second line of defense when vaccine mismatch and pandemic outbreaks occur. With the increased reliance on the current antiviral drugs, there is also an increased susceptibility to the emergence of drug resistant viral strains. As such, it is crucial to develop new antiviral therapies. We studied a host defense peptide secreted from the skin of the South Indian frog *Hydrophylax bahuvistara.* This peptide, which we named "urumin", is virucidal for H1 hemagglutinin-bearing influenza viruses. Urumin specifically targets the conserved stalk domain of H1 hemagglutinin and demonstrates effectiveness against drug-resistant H1 influenza viruses. Electron microscopy studies demonstrated that urumin physically destroys influenza virions. Urumin also protected against lethal influenza infection *in vivo.*

To determine the mechanism of urumin's antiviral activity, we conducted bioinformatic analysis and 3-dimensional modeling of hemagglutinin. We identified an extremely conserved amino acid loci, 417L, in the stalk of HA that is crucial for urumin activity. Using an A/Memphis/8/2003 H1N2 influenza virus, which contains an L417F mutation, we found that urumin's antiviral activity is ablated. When this L417F mutation is inserted into a urumin-susceptible virus, A/Puerto Rico/8/1934, antiviral activity is absent.

Western blot analyses demonstrated that urumin acts by separating the HA trimer and disrupting the disulfide linkage between HA1 and HA2. This activity is dependent upon urumin having free cysteine residues. Given the mechanism of action, and the nature of the HA cleavage site, we speculate that peptide modification strategies can improve urumin activity against influenza subtypes other than H1.

Urumin represents a unique class of anti-influenza virucide, acting like a thiol reductase, specifically targeting the HA disulfide bridge, bonding to HA1, and releasing HA2. These findings indicate the potential for urumin as a clinical anti-influenza therapeutic, especially given that peptide modification strategies should enhance effectiveness against other influenza strains. Overall, these studies establish a novel mechanism of anti-influenza activity, and demonstrate the importance of research into non-human host defense peptides.

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Chapter 1: Introduction

Influenza is the most common reoccurring human respiratory viral infection and represents a primary burden on world health. These enveloped, single-stranded, segmented RNA viruses, belonging to the orthomyxoviridae family, are classified and subtyped based upon the nature of strain specific antigenic differences. Influenza viruses are characterized into three major types - A, B, C, based upon the nature of their nucleoprotein (NP) and matrix protein (M1). Type A influenza viruses are further classified into subtypes based upon the nature of the two major surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA). There are currently 18 known HA (H1-18) and 11 NA (N1-11) subtypes. The most recent of these subtypes were discovered in bats in 2012 and 2013 (1, 2). Due to the divided nature of the influenza RNA genome into 8 distinct segments, reassortment of these segments produces new viral variants and an antigenic evolution of predominant influenza strains. (3-6).

Global and Financial Impact of Influenza

Worldwide, there are 3 to 5 million severe cases of influenza infection annually; it infects 5-10% of the adult population, 20-30% of children, and transmits across the globe (Figure 1) (7). Annually 250 to 500 thousand deaths are a result of influenza infection (8, 9). Seasonal influenza epidemics occur annually, and over the past century there have been five major pandemic outbreaks. These pandemics are the result of antigenically new viruses arising from the assortment of human influenza with avian or swine influenza strains, causing dramatic increases in infection rate and mortality. These massive outbreaks include:

the 1918 H1N1 Spanish influenza, which caused the death of at least 700 thousand Americans and 40 million people worldwide (10, 11); the 1957 H2N2 Asian influenza containing reassorted segments (HA, NA, and viral RNA polymerase: Polymerase Basic 1 (PB1)) from an avian virus; 1968 H3N2 Hong Kong influenza containing avian HA and PB1 segments; and, a 1977 H1N1 Russian influenza (12, 13). More recently than these other pandemic outbreaks: the 2009 H1N1 swine influenza pandemic spread to 214 countries and caused an estimated 9 to 18 thousand deaths, 200 to 400 thousand hospitalizations, and 45 to 90 million infections in the United States alone (14, 15).

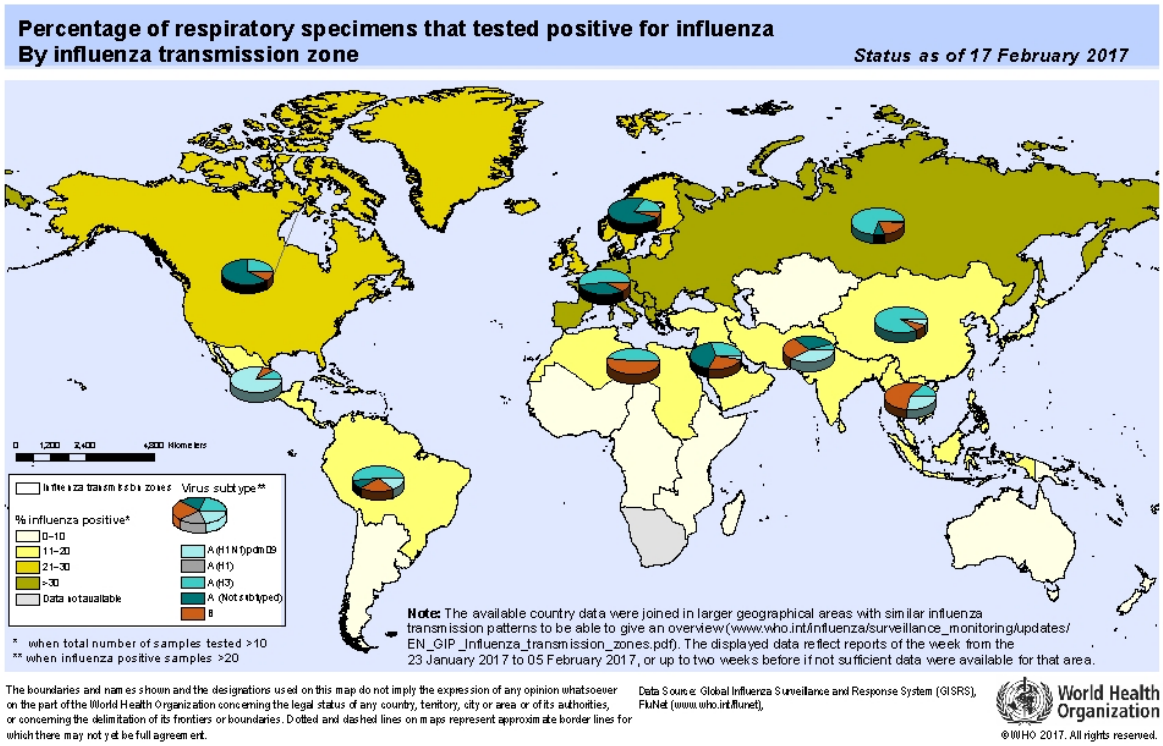

Figure 1: Map of Worldwide Influenza Transmission

In addition to the pandemic outbreaks that occur, seasonal influenza infection epidemics present a substantial burden on the population and economy as a major cause of

morbidity and mortality. Recently, in the United States, deaths attributed to influenza infection has reached highs of approximately 50 thousand per year due to seasonal epidemics (16). Beyond the mortality rate, the economic losses in the United States each year due to lost work hours and hospitalizations from seasonal influenza reaches almost $100 billion annually (17). These figures do not account for pandemic influenza outbreaks. It is estimated that for the next influenza pandemic, the cost burden for the American economy will be between $100 billion and $235 billion (18).

Influenza A Viral Structure and Function

Influenza viruses are enveloped, single-stranded, RNA viruses belonging to the orthomyxoviridae family (Figure 2). These viruses are split into three major types, which are believed to have diverged evolutionarily. Influenza A, B, and C, differ in their structures, nucleoprotein and matrix specifically, host range, as well as pathogenicity. The single stranded RNA genome of these viruses is negative-sense. In comparison to influenza A and B viruses, which both have 8 segments that encode at least 11 proteins, the far less common influenza C viruses have 7 segments, which encodes only 9 proteins. While influenza A and B viruses are similar, influenza A viruses present the most significant risk to human health. This is due to the unique nature of influenza A viruses having an animal reservoir in birds, providing the possibility for zoonotic infections, host switching, and the generation of novel pandemic strains (19). In addition to influenza A, B, and C, a fourth type of influenza virus, tentatively characterized as influenza D was recently discovered in cattle (20).

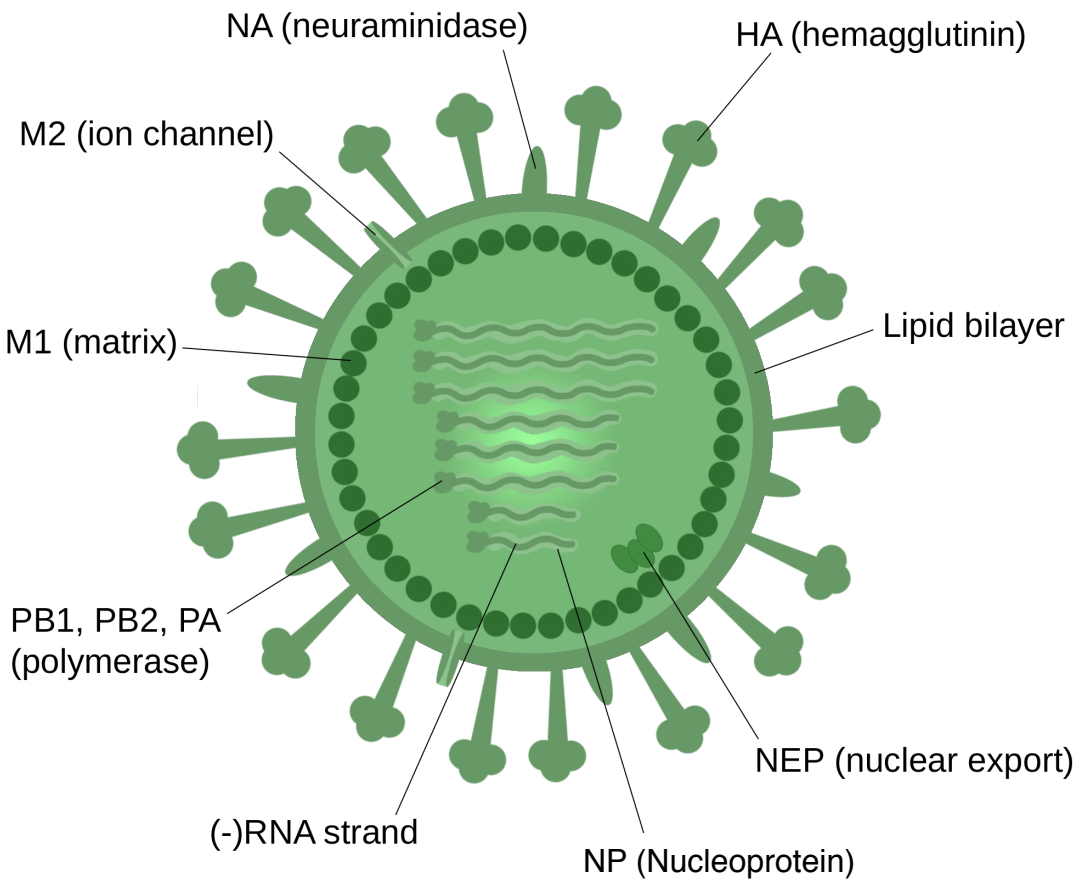

Figure 2: Influenza A Virus Structure

Envelope and Envelope Proteins

Influenza A viruses are enveloped in a lipid membrane. This lipid membrane is derived from the host cells from which they bud. Despite budding from host cells, the lipid composition of influenza A viruses does not mirror that of the host cells. This is due to a selective incorporation of lipids and budding from specific domains on the host membrane (21, 22). These specific domains are lipid raft zones, which are specialized clustering zones with higher concentrations of cholesterol and sphingolipids (23). In addition to having differential lipid composition to host cells by targeting lipid rafts, differences are also found

on a virus by virus level. The lipid distributions amongst influenza strains also demonstrate variance and are thought to be associated with differences in viral fusion, budding, and pathogenicity (24, 25). The targeting of influenza viruses to bud from lipid rafts is associated with the major surface proteins of the virus. Specifically, hemagglutinin (HA). Hemagglutinin is one of three proteins that covers the surface of influenza viruses. The other two surface proteins are neuraminidase (NA) and matrix 2 (M2), with HA and NA being predominant. With regards to the lipid structure of the viral envelope, hemagglutinin intrinsically associates with lipid rafts in the host membrane, though neuraminidase also plays a role, as the deletion of the cytoplasmic tail of either HA or NA reduces the association of viral assembly and budding with lipid rafts (26, 27).

The roles of matrix proteins, neuraminidase, and hemagglutinin as envelope proteins are far greater than just targeting fusion and budding to preferential membrane locations. The influenza matrix genome segment gives rise to two proteins. Matrix 1 (M1) is found underneath the viral envelope and forms a layer that interacts with the cytoplasmic domains of HA, NA, and M2, as well as viral RNA and ribonucleoprotein (RNP) complexes. M1 acts as a bifunctional protein that binds the viral envelope and mediates RNP encapsidation into the envelope (19, 28). The other matrix protein, M2, is an integral membrane protein and pH-gated proton channel that is required for viral replication, as acidification of the inside of the virus is necessary for viral genome unpacking from nucleoprotein (29). Neuraminidase, one of the two spike proteins on the surface of influenza, is an enzyme required for releasing newly formed virions that are budding off from the host cell. NA is a

Type II integral membrane glycoprotein that cleaves sialic acid found on the surface glycoproteins of the host cells, which influenza utilizes for binding.

Hemagglutinin

The last of the influenza surface proteins, like neuraminidase, is a surface spike protein and interacts with sialic acid on the host cell surface. Hemagglutinin is a glycosylated Type I integral membrane protein that acts in influenza attachment, binding to sialic acid, as well as is responsible for fusion of the viral envelope with the host membrane. HA exists on the surface of influenza virions as a homo-trimer, consisting of a variable globular head region and a conserved stalk region (30, 31). These HA trimers recognize different variants of sialic acid depending upon their linkage to galactose on the host sugars. Avian-adapted HAs specifically bind to sialic acid with an α2-3 linkage to galactose, while human-adapted HAs bind to sialic acid with α2-6 linkage to galactose (32). After the binding of HA to sialic acid on the cell surface, the influenza virus is internalized. During this internalization process, host proteases cleave the hemagglutinin HA0 precursor into disulfide-bonded HA1 and HA2 polypeptides prior to their activation. After cleaving into HA1 and the HA2 fusion protein, the acidification of the pH in the endosome causes conformational changes, which are required for activation and membrane fusion, releasing viral contents into host cells for replication (19, 33-36).

Viral polymerase, nucleoprotein, and non-structural proteins

In addition to the viral proteins on or associated with the envelope required for viral entry, fusion, and budding, influenza A genomic RNA also encodes crucial polymerase subunits, nucleoprotein, and non-structural proteins. Genomic segments PB1, PB2, and PA encode for the three polymerase subunit proteins Polymerase Basic protein 1 (PB1), Polymerase Basic protein 2 (PB2), and the Polymerase Acidic protein (PA). PA, PB1, and PB2 form a hetero-trimer and are responsible for transcription and replication of the influenza genome inside the nuclei of infected cells. While PB1 acts as the RNA-dependent RNA polymerase, PB2 binds host mRNA 5' caps during mRNA synthesis, and PA has endonuclease activity, protease activity, and is utilized by the virus for "cap-snatching" from cellular mRNA transcripts (19, 37-40). In addition to encoding the RNA-dependent RNA polymerase PB1 viral polymerase subunit, the PB1 genomic segment also encodes a second protein, PB1-F2, which is a non-structural protein with apoptotic activity (41).

The viral nucleoprotein (NP) encoded by the NP segment of the genome encapsidates each of the 8 RNA segments of the influenza A virus genome. Each single-stranded viral RNA segment is wrapped around monomers of the nucleoprotein and packaged with the viral RNA polymerase hetero-trimer of PB1, PB2, and PA into viral RNP complexes within each influenza virion. In addition to its role in packaging of viral RNP complexes, nucleoprotein also is required for the nuclear transport of the RNPs into and out of the host nucleus, interaction with host proteins such as actin, RNA transcription, and replication (19, 40, 42).

The last type of protein encoded in the influenza A genome are non-structural proteins. As has been mentioned, one of these non-structural proteins is PB1-F2, which is encoded in the PB1 polymerase subunit segment and has apoptotic activity (41). The NS gene segment encodes two non-structural proteins; Non-Structural protein 1 (NS1) and Non-Structural protein 2 (NS2). The NS1 protein is multifunctional, with certain activities being attributed to specific domains. The N-terminal portion, residues 1-73, exists as a homo-dimer, binds to double-stranded RNA and signals for nuclear localization. This N-terminal domain of NS1 functions to inhibit type I interferon (IFN) signaling by sequestering double-stranded RNA away from the 2'-5' oligo (A) synthase/RNase L pathway (43). Additionally, NS1 is crucial in the inhibition of the Retinoic Acid-Inducible Gene I (RIG-I) pathway of sensing influenza A viruses' RNA, triggering an immune response (44). Influenza inhibits the RIG-I pathway by binding to RIG-I, preventing downstream activation of Interferon Regulatory Factor 3 (IRF3) (45, 46) The central domain of NS1, residues 73-207, referred to as the effector domain, signals for nuclear export and binds to several cellular proteins, including the p85β subunit of Phosphotidyl-Inositol 3 Kinase (PI3K) and Protein Kinase R (PKR), and a subunit of Cleavage and Polyadenylation Specific Factor (CPSF), which leads to PI3K signal activation, PKR inhibition, and the inhibition of 3'-end processing of cellular pre-mRNAs including IFN-β pre-mRNAs. Overall, these activities by the NS1 effector domain promote viral replication and translation, and inhibit host mRNA processing and interferon response (47-49). The C-terminal portion of NS1, residues 207-230 contain a PDZ domain ligand, which interact with PDZ protein-protein recognition sites in human cells, interrupting cellular processes (50, 51). PDZ domains (Post synaptic density protein 95,

Drosophila disc large tumor suppressor, and Zonula occludens-1 protein) consist of approximately 80-100 amino acids and contain 6β-sheets and a short and long α-helix (52). These modules associate with short amino acid motifs at the c-terminus of the proteins they are targeting. As such, they function in modulating processes, especially signal transduction pathways, including transport and ion signaling (53). The second non-structural protein NS2, also known as the Nuclear Export Protein (NEP) acts to facilitate the export of viral RNPs from the virion (19, 40).

Influenza Infection, Pathogenesis, and Virulence

Infection with influenza A viruses presents as an acute respiratory infection in humans. With the onset of influenza infection, symptoms include a high fever, inflammation in the mucus membrane, malaise, cough, headaches, and severe fatigue. Viral replication peaks approximately 48 hours after inoculation with virus and symptoms persisting for seven to ten days (54). Many different factors, both from the host being infected, as well as from the virus itself, impact the severity of disease and the ability of the virus to replicate. Influenza viruses, like all viruses have evolved key mechanisms that allow for them to endure, transmit in the population, and evade host immune responses.

Antigenic Drift and Antigenic Shift

The high rate of mutagenesis in influenza A viruses, as well as the segmented nature of the RNA genome has provided two key mechanisms by which these viruses evolve and adapt to evade pre-existing host immunity and thrive. Antigenic drift is characterized by the

high rate of mutagenesis in influenza A viruses, which occurs at a rate of approximately 1 x 10^{-3} to 8 x 10^{-3} mutations per site, per year (55). As hemagglutinin is responsible for binding and entry into host cells, antibodies produced that target HA, inhibit binding of influenza to host cells and can neutralize infection and be effective at preventing reinfection (56). Mutations that cause substitutions, specifically in the two surface spike proteins HA and NA, allow for minor variations and can produce selectively advantageous viral variants that are able to evade the pre-existing host immune response, and are not susceptible to neutralizing antibodies generated from viruses from previous years. This yearly level of mutation and evolution in influenza A viruses is what accounts for and helps to produce new yearly, seasonal epidemics. Subversion of the host immune response also is attributed to reassortment of the eight RNA genome segments. When co-infection of host cells by two or more different influenza A viruses occur, the progeny that are released from those cells can contain gene segments from either of the inoculating, parental viruses. Antigenic shift is characterized by the HA or NA from one virus reassorting with gene segments from another influenza A virus, producing a completely new influenza virus. Like with antigenic drift, these reassortment events account for a key evolutionary mechanism in influenza A viruses and have been associated with the emergence of pandemic influenza viruses (6, 12, 19, 57-59).

Viral Factors of Pathogenesis and Virulence

In addition to evolutionary mechanisms by which influenza A viruses can subvert the host immune response and thrive, there are other essential viral factors that are associated with increased pathogenesis as well as virulence. As previously discussed, with the ability of

hemagglutinin mutations to be critically important for evading pre-existing host immune responses, other modifications in HA have also been associated with the severity of influenza A infections. The nature of the HA cleavage site, where HA0 separates into HA1 and HA2 has been found to be a critical determinant of pathogenesis. In most low-pathogenic influenza A viruses, the HA cleavage site contains a single arginine amino acid, which is only recognized by trypsin-like proteases in the mucosa of the airway and in the intestine. In contrast, the more virulent influenza A strains, such as H5N1 viruses, contain a multi-basic cleavage site, which is recognized by proteases in many other organs, making systemic infections much more likely and increases virulence (60). However, the multi-basic cleavage site is not a full indicator of high pathogenesis as the H1N1 1918 pandemic strain has a mono-basic cleavage site. Rather, in the case of the 1918 pandemic H1N1, the nature of the RNA polymerase complex and nucleoprotein, as well as HA and NA, attributed to the high level of viral replication and the severe yet un-protective immune response. This impact on the immune response led to high severity and lethality because of a lack of antibodies to control secondary bacterial infections (61-63).

PB1-F2, which has apoptotic activity (41), is another viral virulence factor. PB1-F2 acts to disrupt the mitochondrial membrane of infected cells to induce apoptosis. A single serine to asparagine mutation at position 66 of PB1-F2 (66N is predominant amongst other influenza A strains) drastically reduces the pathogenicity of the 1918 pandemic influenza strain (64). PB1-F2 has also been associated with the anti-interferon activities of the NS1 protein (65). The last major viral virulence and pathogenesis factor is the NS1 protein, which

has already been extensively discussed in the viral structure and function section on page 8 (6).

Host Factors of Pathogenesis and Virulence

There are also key host factors that influence the susceptibility and course of influenza A infections. Age is a factor of susceptibility; infants under the age 2, or senior citizens over the age of 65 are more susceptible to more severe influenza disease. Also, pregnant women, and individuals with pre-existing health conditions such as asthma, cardiovascular disease, and not surprisingly, immune compromised individuals are more susceptible to severe disease. More recently, with the 2009 H1N1 pandemic, obesity and diabetes were associated with increased susceptibility to severe influenza A-mediated disease (66-68). There are also certain host factors that directly or indirectly interact with viral proteins. These factors are involved with most viral processes and are necessary for pathogenesis and virulence. Viral fusion, transport of the viral RNP complexes into the nucleus of infected cells, transcription, translation, and replication of the viral genome, export of RNP complexes from the nucleus, and lastly packaging, assembly, and budding of new virions are all impacted by host factors. ATPV0D1, an element of the endocytic pathway is required for influenza viral entry (69). CAMK2B, a calcium sensor, CDC-Like Kinase 1 (CLK1), which acts in regulating alternative splicing in mammalian cells, and CDKN1B (also known as p27), a cell cycle regulator, are all required for optimal replication levels of influenza A viruses (70, 71). While some host factors are required for influenza virus infection, others are crucial for regulating influenza. The WNT pathway and IFN-Induced

Transmembrane protein 3 (IFITM3) are associated with increased type I interferon production and reduced viral replication (6, 72-74).

Influenza Vaccines, Antivirals, and Resistance

The principle methods for combatting influenza infection in the human population is duel fold, including vaccine and antiviral strategies. While the current program of influenza vaccines is effective at controlling virus, they are also limited. Due to the phenomenon of antigenic drift already discussed, new influenza vaccines need to be reformulated each year. When there is mismatch of the circulating influenza strain to the formulated vaccines, efficacy is reduced (75). Furthermore, vaccines are ineffective at protecting against pandemic influenza strains (76, 77). The second line of defense are antiviral therapeutics. Currently, there are five licensed antiviral drugs on the market, that are split into two classes. The adamantanes inhibit viral replication by interfering with the M2 proton channel. The second class of influenza antivirals are neuraminidase inhibitors (NAIs), which includes oseltamivir, also known as Tamiflu, which was approved by the United States Food & Drug Administration (FDA) in 1999. Despite having two classes of antiviral drugs, resistance has become a major concern. Currently, no adamantane drugs are recommended due to high numbers of resistant strains (78, 79). Furthermore, natural isolates of influenza A viruses containing single point mutations in NA have been found to be resistant to NAI drugs (80).

Influenza Vaccines

Originating in the 1940's, vaccination strategies have been a fundamental manner by which the impact of influenza on human health has been diminished. Early flu vaccines consisted of purifying influenza viruses grown in chicken embryos and then inactivating the viruses using formalin and phenyl mercuric nitrate (81, 82). However, the effects of antigenic drift were found rapidly, and within a few years, influenza vaccines had to be reformulated to be effective against the evolving viruses (83, 84). In addition, adverse reactogenicity events in children led to the development of subunit and split vaccines (85). More recently, live attenuated influenza vaccines have been produced, as well as inactivated variant vaccines. The live attenuated vaccines, which were licensed in the United States in 2003, and are delivered as a nasal spray, are temperature-sensitive and cold-adapted so that they will replicate in the upper, but not the lower respiratory tracts (86-88).

The current influenza vaccination strategy accounts for the four distinct variants of influenza viruses circulating the human population. These variants are the influenza A 2009 pandemic H1N1 strains and influenza A H3N2 strains, as well as two influenza B strains, which differ greatly. The trivalent vaccine produced to combat the ever changing variants of these strains, consist of formulations against at least two influenza A viruses and one influenza B virus (89). Despite improvements in the formulation of the yearly influenza vaccines, protection is only sustained for a short time and efficacy in children receiving the live attenuated vaccine is 83%, while adult vaccination is only approximately 75% effective, diminishing as adults age (90-94). Additionally, more recently, for the past two flue seasons, the cold-adapted influenza vaccines (CAIV) has not been recommended due to low efficacy.

Though they elicit decent levels of protection, at 75% effectiveness, and even lower levels of efficacy considering mismatch, the current vaccine strategies are ultimately suboptimal (75, 76).

Influenza Antivirals

While vaccination strategies present a manner to prevent severe influenza infections by conferring a memory immune response, their lack of optimal effectiveness have made a second level of protection crucial. Anti-influenza antiviral drug therapies are this second line of defense against influenza infections. As was previously described, there are two major classes of influenza antivirals, neuraminidase inhibitors and adamantanes, which interfere with the M2 proton channel. The three licensed neuraminidase inhibitors are oseltamivir (Tamiflu), which is taken orally, zanamivir (Relenza), which is inhaled, and peramivir (Rapivab), which is administered intravenously. These three NAIs act as analogues of sialic acid, blocking the active site of neuraminidase, preventing the release of budding virions from host cells (95). The other class of influenza antivirals are the adamantanes. These drugs, amantadine and rimantidine act as antiviral agents by inhibiting the M2 proton channel, which prevents the unpacking of nucleoprotein from viral RNPs and the viral replication process (29, 79).

Influenza Resistance

Despite having these antiviral therapies against influenza to supplement the vaccination strategies, resistance to these drugs poses a major problem to their effectiveness.

The adamantane class of influenza antiviral drugs are no longer recommended as a treatment by the CDC due to over 99% resistance by current circulating H3N2 and pandemic 2009 H1N1 influenza A viruses. In addition to this incredibly high level of resistance, adamantanes are ineffective against influenza B viruses. Thankfully, the level of resistance in circulating strains against neuraminidase inhibitors is low, at less than 1%. However, natural influenza strains that are resistant to these antivirals have been isolated. Moreover, it has been found in these resistant viruses that a single mutation, one of which is an H275Y mutation in neuraminidase, can confer resistance against NAIs (78, 79). Given the danger that the expansion of drug-resistant influenza A viruses pose, it is crucial to develop new and alternative therapies for fighting influenza infection.

Host Defense Peptides

Host defense peptides, also often known as anti-microbial peptides, are a critical and ancient defense mechanism of the innate immune system. Shared amongst all living organisms, from insects and plants to higher mammals, host defense peptides are an evolutionarily conserved defense mechanism. Despite being a conserved effector of innate immunity for millennia, host defense peptides continue to be an effective defensive mechanism against bacteria, viruses, and fungi that normally evolve and develop resistance against inhibitory substances. These small 15-50 amino acid residue in length peptides are often cationic and are separated into families based upon the nature of their amino acid composition, structure, and charge. Since the first discovery of host defense peptides in the 1970's, over 2,700 different peptides from bacteria, archaea, protists, fungi, plants, and

animals have been discovered (96). The activities of these peptides range from antibacterial and antiviral activity, to wound healing, inflammation, and signal transduction. While most host defense peptides with antibacterial and antiviral activity act by destabilizing membranes, specificity for surface molecules and intracellular activities have also been described (97-99).

History of Peptide Discovery

The nuances of innate immunity and the nature of host defense peptides are relatively new discoveries. The first reporting of what would be known as antimicrobial peptides occurred in the 1972 when Hans Boman and colleagues discovered that *Drosophila melanogaster* vaccinated with live bacteria were able to neutralize the infection with some sort of broad spectrum substance (100). Boman would than go on from these studies in *Drosophila* and move onto studies into the silk worm *Hyalophora cecropia*. In 1981, Boman and colleagues determined that the silk worms survive bacterial infection because of a substance in the worms' hemolymph. This substance was the first isolated host defense peptide, which they called cecropin (101). Because of this discovery and further research into what were specifically termed antimicrobial peptides (AMPs), it was found that many other similar peptides had already been discovered and had yet to have been identified as being an AMP. Some of the most notable examples of previously discovered host defense peptides are human lysozyme discovered by Alexander Fleming in the 1920's, gramicidin isolated from *Bacillus brevis,* the peptide melittin isolated from bee venom, as well as a family of peptides released from the granules of neutrophils that have broad spectrum antimicrobial and antiviral capabilities called defensins (102-105). In 1987, the field of host defense peptides

broadened with the elegant discovery by Michael Zasloff and Dudley Williams that the frog *Xenopus laevis* secretes large quantities of unique peptides from its skin to protect itself from microbial infections (106, 107).

Peptide Structural Diversity

The structure of host defense peptides is as varied as the diversity of the peptides themselves. These peptides derive from large precursor segments which include a peptide signal sequence and can be post-translationally modified in numerous ways from glycosylation and proteolytic processing, to carboxy-terminal amidation, amino acid isomerization, and halogenation (97, 108). The extant of the peptide diversity is so massive that two identical peptide sequences are rarely, if ever, isolated from different species. However, conservation can be found in peptide precursors, suggesting that there is greater constraint on the mechanism of peptide production and trafficking (97, 109).

All host defense peptides rely on a fundamental underlying ability to adopt configurations that produce a clustering of hydrophobic and cationic amino acid residues into an amphipathic domain. The manner and environment in which these amphipathic regions organize and interact is shape specific. Linear peptides organize amphipathic regions upon entering a membrane, at which point the secondary structure arranges into an α-helical confirmation (109, 110). Peptides with more rigid secondary structures, those containing disulfide bonds and forming anti-parallel β-sheet secondary structures have specific regions of cationic and hydrophobic residue clustering (111, 112). A third large family of peptide structure is characterized by a preponderance of one or two different amino acids, which

contain specifically hydrophobic and hydrophilic side chains that arrange around a peptide scaffold when associating with a membrane (113, 114). The array of peptides that most organisms express, include a diverse variety of peptides from these structural classes. Overall, for such small biologically active compounds, the extent of the diversity demonstrates the adaptive nature of these molecules to the evolutionary pressures on each given species, as single mutations can have a drastic effect on their activity (97).

Host Defense Peptide Mechanisms

The most common function of host defense peptides is to act as a defensive mechanism against bacteria, viruses, and fungi. To conduct this activity, most host defense peptides utilize a fundamental difference between the membranes of host plant and animal cells and the membranes of bacteria and enveloped viruses. In contrast to host cells, which have relatively neutral outer leaflets of their membranes, and negatively charged lipids segregated to the inner leaflet by flippases, bacterial and viral envelope membranes are highly negatively charged on the outer leaflet (115, 116). The proposed mechanism for how the cationic nature of host defense peptides act upon this difference is described in the Shai-Matsuzaki-Huang model. This model proposes that host defense peptides that function by charge carpet the outer leaflet of the bacteria or virus, and then integrates into the membrane. This integration process causes a thinning and expansion of the outer leaflet of the bacterial or viral membrane in comparison to the inner leaflet, and the formation of holes and pores in the membrane as lipids and peptides move into the inner leaflet. At this

stage, peptides that can also function intracellularly are able to access their targets as the membrane of the bacteria or virus collapses and is destroyed (Figure 3) (97, 116-118).

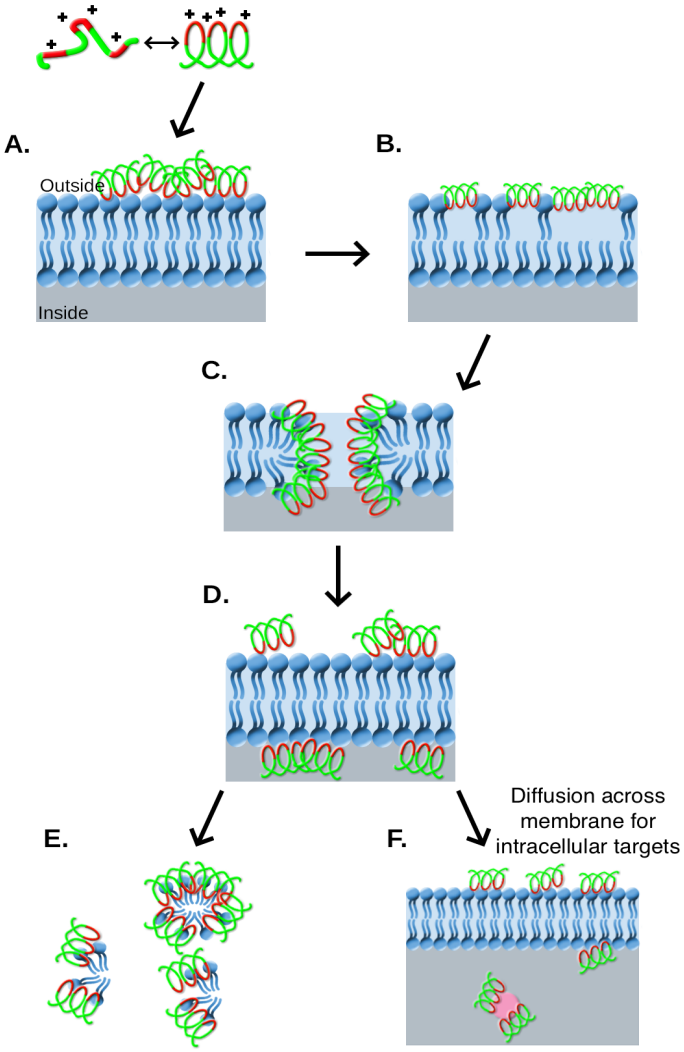

Figure 3: Shai-Matsuzaki-Huang Model of Host Defense Peptide Mechanism

The Shai-Matsuzaki-Huang model and charge-based destruction of membranes is not the only mechanism by which host defense peptides act. Peptides have also been discovered that directly target surface molecules. One of the best examples is the peptide Nisin produced by Lactococci bacteria. Nisin is 14 amino acids in length and is amphipathic. This peptide binds

with high affinity to the fatty acyl proteoglycan anchor in the bacterial membrane, Lipid II. Upon binding to Lipid II, Nisin diffuses into the membrane (119). This mechanism, has also been described in plant defensins (120). While many studies and hypotheses have been made on how antimicrobial peptides kill bacteria in the processes just described, less is known on the mechanism of antiviral activity for antiviral peptides (116, 117, 121-123).

Antiviral Mechanisms

α-defensins, β-defensins, θ-defensins, and cathelicidins are host defense peptide families that have shown activity against human viruses, in addition to having antimicrobial activity. These amphipathic and cationic peptides, which range in structure from the triple-stranded β-sheets of α and β defensins, to circular θ-defensin octadecapeptides, and the α-helix containing cathelicidins, have shown antiviral activities against Herpes Simplex Virus (HSV), Human Immunodeficiency Virus (HIV), influenza A viruses, Human Papillomavirus (HPV), adenovirus (AdV), Respiratory Syncytial Virus (RSV), SARS coronavirus, and vaccinia virus (124-127).

α-defensins antiviral mechanisms include binding to viral envelope proteins on HSV, which blocks viral entry, blocking AdV viral entry and uncoating, binding to CD4 and the envelope glycoprotein on HIV to down-regulate CD4 and CXCR4, which inhibit fusion, protein kinase C, and viral replication, blocking HPV viral escape from endocytic vesicles, and inducing influenza A viral aggregation and inhibiting protein kinase C and viral trafficking (128-138). β-defensins antiviral mechanisms include binding the viral envelope glycoprotein of HSV, down-regulating CXCR4 in HIV, modulating cytokines to effect

influenza viral entry, and blocking RSV viral entry, as well as destabilizing the RSV envelope (129, 139-145). θ-defensin antiviral mechanisms include binding of HSV viral envelope glycoproteins to prevent viral entry, binding to gp120 and CD4 to block HIV fusion, cytokine modulation in SARS infection, and inducing influenza A viral aggregation and inhibiting the virus from fusing with the endosome membrane (146-155). Cathelicidin antiviral mechanisms include damaging the vaccinia viral envelope which removes the outer membrane, binding and inhibiting HIV reverse transcriptase, and binding and disrupting the membrane of influenza A viruses (156-161). These peptide families demonstrate nuanced mechanisms for targeting the destruction or blockade of many different viruses (124).

Resistance to Host Defense Peptides

In contrast to more conventional antibiotic and antiviral drugs where bacteria and viruses often develop methods for evading therapeutic effects, the acquisition of resistance to host defense peptides occurs much less frequently. For a defense mechanism that has existed for millennia, that microbes and viruses have yet to evolve around their effect, is striking. While the susceptibilities of different bacteria, viruses, and fungi to host defense peptides can differ greatly, the nature of different responses to peptides remains an unknown (162). Despite these differences, overall, the theory for why resistance mechanisms haven't evolved is because these host defense peptides exploit crucial and basic aspects of their target, such as the membrane of a bacterium or virus. To evade the peptides, escape mutants would have to reorganize and redesign their lipid membranes, which is likely too great of an evolutionary burden to overcome. Furthermore, since host defense peptides lack any

specifically defining characteristics, being short amino acid chains with no unique epitopes, and are produced as an array of peptides, development of a resistance mechanism that destroys peptides is likely also exceedingly difficult (97). This challenge of circumventing the effects of host defense peptides is a clear advantage for their development as therapeutics.

Amphibian Host Defense Peptides

While all organisms produce their own unique sets of host defense peptides, in the past 30 years of research into the field, anuran amphibians, frogs and toads have proven to be model species for peptide discovery and research. Though host defense peptides, like dermicidin, which is expressed and secreted in human sweat have been isolated, in comparison, anuran amphibians secrete tremendous quantities of peptides from their skin (163). These quantities dwarf analogous peptides produced by mammalian tissues. In addition, species diversity and population isolation has yielded far greater breadth and variety of frog skin peptides. Within even individual frogs, often several unique members of the same peptide family are produced. Given the extant of the quantity and variety of these peptides, with no two identical peptide sequences being observed even in closely related species, the skin of frogs is an ideal source for discovering, isolating, and characterizing new peptides (164-166). Furthermore, as the host defense peptides produced by anuran amphibians have evolved alongside the host to defend against pathogens they would encounter, they may recognize similar motifs on pathogens that infect other species.

Host Defense Peptides from Asian Frogs

Asia is a region of high diversity and large populations of frog species. In the tropical regions in Asia, the rain forest ecosystems present a large resource for obtaining unique host defense peptides. Though, more recently, the temperate regions of this area, which are often thought of as areas of lower biodiversity, have been a zone for isolating new amphibian host defense peptides that have shown promise as potential therapeutics (166). Host defense peptides from 15 different genera of Asian frogs have been reported. Three of the most studied genera of Asian frogs are genera Amolops, Hylarana, and Rana from the family Ranidae. These frogs, which are endemic from regions of China to India, each produce host defense peptides that are novel, as well as peptides from known families with anti-bacterial, anti-fungi, and anti-cancer activities. These known host defense families include the brevinin-1, brevinin-2, esculentin-1, esculentin-2, and temporin peptide families, amongst others (167-190). Many of these peptides are highly effective against bacteria, viruses, fungi, and cancer cells. However, a key concern, like with all potential therapeutics is their toxicity to host cells. Some of the most potent host defense peptides secreted on the skin of Asian frogs prove to be highly toxic when internalized. Peptides that are highly effective, while eliciting minimal toxicity to normal host cells, provide the greatest potential for future therapeutics. For example, the temporin peptide isolated from *Rana chensinensis* is highly effective against breast cancer cells, and at the same time, demonstrates little toxicity towards normal host cells (191).

Anti-influenza peptides

With both the increasing global threat and the increasing resistance to current anti-influenza strategies, the development of antiviral peptides has become a major target of anti-influenza research. Some of the more recent research into anti-influenza peptides can be broken down into three distinctive mechanistic groups. These peptides either disrupt the viral envelope of the influenza virus, demonstrating a virucidal mechanism, interact or bind with hemagglutinin to block viral entry of influenza into host cells, or interact with the viral polymerase complex to inhibit viral replication (192). One of the major issues with the current peptides that are candidates for therapeutics is that they often degrade *in vivo* and are cleared quickly. Though, given the nature of influenza, infecting the lungs, the pulmonary route of drug delivery presents an easy method for delivering peptide and circumventing proteolytic enzymes in the gastrointestinal tract (193). Despite this drawback, many peptides demonstrate specificity and selectivity for influenza, while also eliciting few side effects.

Virucidal peptides that disrupt the envelope

As has been previously discussed, Influenza A viruses are enveloped in a lipid membrane. This lipid membrane is derived from the host cells from which they bud. Despite budding from host cells, the lipid composition of influenza A viruses does not mirror that of host cells. This is due to a selective incorporation of lipids and budding from specific domains on the host membrane (21, 22). These specific domains are lipid raft zones, which are specialized clustering zones with higher concentrations of cholesterol and sphingolipids

(23). Because of the specialized nature of viral membranes, some anti-influenza peptides target the influenza viral membrane. The human cathelicidin LL-37 acts to disrupt the envelope of influenza by the Shai-Matsuzaki-Huang carpet model, where the peptide creates a layer around the lipid bilayer of the viral envelope and destabilizes the membrane (159). By doing so LL-37 may be acting as a component of the initial innate defense to influenza viruses during human infection (160). A second set of anti-influenza peptides that act in a similar manner are a trio of peptide sequences derived from the protein lactoferrin, which demonstrate activity against H1N1 and H3N2 viruses (194). Melittin is a 26-amino acid peptide derived from honeybee venom, which disrupts the viral envelope by developing pores in the lipid bilayer and ultimately leads to viral lysis (195-197). A last peptide currently being studied as an anti-influenza disruptor of the membrane is an optimized variant of the peptide mucroporin, mucroporin-M1. This peptide variant takes the sequence of mucroporin, isolated from the scorpion *Lychas mucronatus,* and replaces glycine and proline amino acid residues with lysine and arginine residues (198). This optimized variant of mucroporin, which has an enhanced net positive charge in comparison to the isolated peptide, demonstrates activity against H5N1 influenza, via what is believed to be a direct interaction with the viral envelope (199).

Peptides that block viral entry and fusion with cell membrane

A second major mechanism of peptide-induced anti-influenza activity is by inhibiting the binding of influenza to host cells and the prevention of viral envelope fusion with the cell membrane. This class of anti-influenza peptides can be broken down into two types:

peptides that inhibit hemagglutinin binding by competing with the sialic acid binding site on host cells and peptides that prevent the necessary hemagglutinin conformational changes for viral fusion with the late endosome. The first class of peptides, which inhibit hemagglutinin binding, present very promising candidates for antiviral therapy. A peptide, which was derived from the signal sequence of fibroblast growth factor 4 demonstrates broad activity against human, avian, and swine influenza A viruses (H1N1, H2N2, H3N2, H5N1, H5N9, H7N3), as well as influenza B viruses (200). This peptide demonstrated effectiveness, both when mice were pre-treated with peptide, as well as when viral infection was treated therapeutically, though the effect was diminished in therapeutic treatment. The activity of this peptide, as a blocker of hemagglutinin was confirmed by the inhibition of chicken red blood cell agglutination, when treated with influenza virus. The antiviral activity was retained when the optimal and minimal peptide sequence was assessed (200, 201). In addition to this peptide derived from the signal sequence of fibroblast growth factor 4, FluPep, which is a mixture of mostly hydrophobic α-helical peptides can interact with HA, blocking viral fusion (202). Flufirvitide, in addition to inhibiting viral entry, also acts to modulate the immune response by activating the production of anti-inflammatory cytokines and chemokines, enhancing macrophage phagocytosis, and increasing neutrophil activity (203). Anti-lipopolysaccharide peptides or SALPs, which were originally discovered as peptides that elicit antimicrobial activity against both Gram-positive and Gram-negative bacteria, also demonstrate anti-influenza activity (204, 205). These SALPs act to inhibit H1, H3, and H7 influenza viruses by preventing viral attachment by binding the N-acetylneuraminic acid component of the influenza viral receptor.

Peptides that inhibit viral replication

The last set of anti-influenza peptides are peptides that inhibit viral replication. As has been previously described, genomic segments PB1, PB2, and PA encode for the three polymerase subunit proteins Polymerase Basic protein 1 (PB1), Polymerase Basic protein 2 (PB2), and the Polymerase Acidic protein (PA). PA, PB1, and PB2 form a hetero-trimer and are responsible for transcription and replication of the influenza genome inside the nuclei of infected cells. While PB1 acts as the RNA-dependent RNA polymerase, PB2 binds host mRNA 5' caps during mRNA synthesis, and PA has endonuclease activity, protease activity, and is utilized by the virus for "cap-snatching" from cellular mRNA transcripts (19, 37-40). One method for creating antiviral peptides that impede viral replication is to produce PB1-derived peptides which prevent polymerase function. PB1-derived peptides $PB1_{1-25}$ and $PB1_{1-37}$ demonstrate binding to the PA subunit, which inhibits viral polymerase activity and influenza replication (206, 207). Additionally, $PB1_{715-740}$ binds to a conserved region of the PA subunit. $PB1_{731-757}$ and a peptide derived from the PA-binding domain of PB1 also inhibit influenza replication by blocking polymerase activity (206, 208, 209). Another peptide that inhibits influenza viral replication is "Killer Peptide". This peptide, which was isolated from yeast and elicits both anti-HIV-1 and antimicrobial activity, also significantly inhibits influenza A viral replication. In addition to inhibiting influenza replication, Killer Peptide also reduced the synthesis of viral proteins and the production of viral particles (210).

Peptides which act to either disrupt the viral envelope, block viral entry and fusion with the cell membrane, or inhibit viral replication account for the major classes of known anti-influenza peptides. However, these are not the only known potential mechanisms. The

defensin peptide class has demonstrated the ability to impact infections from enveloped, as well as non-enveloped viruses. Unlike the other mechanisms, these peptides act by stimulating the innate immune response, increasing the activity of the mucosal epithelium (138, 211). Though, α-defensin-1 also demonstrates the ability to effectively inhibit influenza viral replication and protein synthesis. Ultimately, the anti-influenza properties of defensin peptides appears to be linked to the ability to modulate protein kinase C activity in infected cells (138). The potential involvement of the protein kinase C pathway has led to the suggestion that zinc finger peptides may prove to be an effective class of anti-influenza peptides (212, 213).

Summary

Overall, influenza presents a global health burden as the most common human respiratory virus. With a worldwide burden of 3 to 5 million cases of severe influenza annually, the dangers of major pandemic outbreaks, vaccines that can be ineffective, and drug-resistant influenza strains, efforts to produce new and innovative anti-viral therapies have become paramount. Antiviral peptides present a new way of combatting influenza viruses. Pioneering research in the 1970's and 1980's introduced host defense peptides, an evolutionarily conserved set of innate immune effectors produced by all organisms. These small peptides of roughly 15-50 amino acid residues in length belong to many different families with unique sequences across species. Of the organisms studied, anuran amphibians have proven to be a prime resource for host defense peptide discovery, with immense species and peptide diversity. These peptides have demonstrated the ability to be effective at

neutralizing various bacteria, viruses, fungi, as well as having other important cellular roles. As such, host defense peptides present a potential revolutionary step for developing new anti-influenza therapies.

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Chapter 2: An Amphibian Host Defense Peptide Is Virucidal for Human H1 Hemagglutinin-Bearing Influenza Viruses

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Authors

David J. Holthausen[1], Song Hee Lee[1], Vineeth Kumar T.V[2], Nicole M. Bouvier[3], Florian Krammer[3], Ali H. Ellebedy[1], Jens Wrammert[1], Anice C. Lowen[1], Sanil George[2], Madhavan Radhakrishna Pillai[2] and Joshy Jacob[1,4,*]

Affiliations

[1]Emory Vaccine Center, Yerkes National Primate Center, Emory University, 954 Gatewood Road Atlanta, GA 30329, USA

[2]Rajiv Gandhi Center for Biotechnology, Poojapura, Thiruvananthapuram, Kerala, India

[3]Department of Microbiology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1124, New York, NY 10029 USA

[4]Lead Contact

*Correspondence: joshy.jacob@emory.edu (J.J)

Summary

Although vaccines confer protection against influenza A viruses, antiviral treatment becomes the first line of defense during pandemics because there is insufficient time to produce vaccines. Current antiviral drugs are susceptible to drug resistance, and developing new antivirals is essential. We studied host defense peptides from the skin of the South Indian frog and demonstrated that one of these, which we named ''urumin,'' is virucidal for H1 hemagglutinin-bearing human influenza A viruses. This peptide specifically targeted the conserved stalk region of H1 hemagglutinin and was effective against drug- resistant H1 influenza viruses. Using electron microscopy, we showed that this peptide physically destroyed influenza virions. It also protected naive mice from lethal influenza infection. Urumin represents a unique class of anti-influenza virucide that specifically targets the hemagglutinin stalk region, similar to targeting of antibodies induced by universal influenza vaccines. Urumin therefore has the potential to contribute to first-line antiviral treatments during influenza outbreaks.

Introduction

Influenza is the most common recurring human respiratory virus (3-5). Worldwide there are 3-5 million cases of severe influenza infection annually, which affects 5-10% of adults and 20-30% of children and results in 250-500 thousand deaths (8, 9). Current vaccination strategies do offer protection; however, pandemic outbreaks occur unexpectedly, limiting our ability to develop vaccines in a timely manner. Additionally, for seasonal Influenza A viruses (IAV), mismatches frequently occur between the vaccine and circulating

strains (77). The emergence of drug-resistant IAV is also a major concern; currently, only neuraminidase (NA) inhibitors are recommended, as adamantane-resistant influenza strains have become widespread (78, 79). Thus, there is a pressing need to develop new antivirals against IAV.

One strategy for creating new influenza antivirals was to take advantage of pre-existing host innate defense mechanisms of other species. Host defense peptides (HDPs) are a critical innate defense mechanism across all species that are far less prone to resistance than conventional drug therapies (78, 97, 125). In 1981, Hans Boman showed that the silk moth *Hyalophora cecropia* survived bacterial infections because of HDPs in their hemolymph (101). In 1987, Michael Zasloff (214) and Dudley Williams (107), studying *Xenopus laevis,* elegantly demonstrated that amphibians secrete large quantities of unique peptides to defend against microbial infection (97, 164, 215, 216). These peptides have evolved to protect the frogs against pathogens that they might encounter, but other studies have shown that amphibian HDPs also are active against human viruses like HSV-1, HSV-2, and HIV-1 *in vitro* (217-222). HDPs have also been shown to have anti-cancer (165, 222) and anti-diabetic (165) activity. Therefore, HDPs may represent an untapped resource for novel drug discovery.

As amphibian HDPs have shown activity against other human viruses, we sought to determine whether they also contained HDPs against human IAV. We identified HDPs from skin secretions of frogs and identified a peptide that was virucidal against H1 hemagglutinin(HA)-bearing human IAV. This peptide targeted the conserved stalk region of H1 HA, physically destroyed influenza virions, and protected naïve mice from lethal influenza infection. Urumin specifically targets the stalk region of hemagglutinin, like the

way that antibodies induced by universal influenza vaccines target the virus, and may prove a useful antiviral therapy, especially given that it is effective against drug-resistant H1 influenza strains.

Results

A peptide from *hydrophylax bahuvistara* exhibits anti-A/PR/8/1934 influenza virus activity *in vitro*

Cohorts of the Indian frog, *Hydrophylax bahuvistara*, a species currently being studied for antimicrobial peptides (223), were captured and skin secretions were collected after mild electrical stimulation. Animals were subsequently returned to their natural habitat, unharmed. We identified 32 HDPs from the secretions by molecular cloning of HDP cDNAs and synthesized the peptides for screening. We screened these peptides for potential activity against the human H1N1 influenza A virus A/Puerto Rico/8/1934 (PR8) using a plaque assay. We incubated individual peptides or control ovalbumin (OVA) peptide with the virus for 2 hours, plated them with Madin-Darby Canine Kidney epithelium (MDCK) cells, and counted virus plaques 3 days later. We identified four peptides that showed greater than 50% reduction of viral titers as compared to the control OVA peptide (Figure 1A, Figure S1). As HDPs are often toxic to mammalian cells, especially at higher concentrations, we measured the toxicity of these peptides by incubating them with human red blood cells (RBCs) for 1 hour and assessed hemolysis. Of the 4 peptides, #25, showed no toxicity up to 320μM in PBS (Figure 1B). This peptide reached a 20% level of toxicity only at 1430μM (Figure 1C). We named this peptide urumin from the word “urumi”, a deadly whip sword

that originated from the same geographical region as *H. bahuvistara*. Urumin is a 27-amino acid peptide with a net 2+ positive charge and no known homology to any other HDP (Figure 1D). Of the four peptides with antiviral activity, urumin was chosen for further characterization due to its low toxicity.

Next, we assessed the dose response and the kinetics of urumin's antiviral activity. The IC_{50} of urumin was 3.8μM (Figure 1E) and increasing the peptide concentration led to significantly increased inhibition of viral growth ($p=0.0004$, Figure S2). The therapeutic index (TI) of a host defense agent compares the therapeutic effect to the toxicity and is often used as a measure of the safety of a therapeutic agent. The higher the TI is over 1, the more favorable the safety of the therapeutic. For urumin, the toxic dose (TD_{50}) was 2450μM (Figure 1C) and IC_{50} was 3.8μM (Figure 1E). TD_{50}/IC_{50} produces a TI of 644.7, indicative of a favorable therapeutic profile. Additionally, the antiviral activity occurred within 15 minutes of incubation with the peptide ($p<0.0001$, Figure 1F). The antiviral effect of urumin was specific to IAV as urumin had no antiviral effect on any of the other viruses tested, which included HIV, SIV, HSV-II, Hepatitis C, Ebola, Zika and Dengue viruses (not shown).

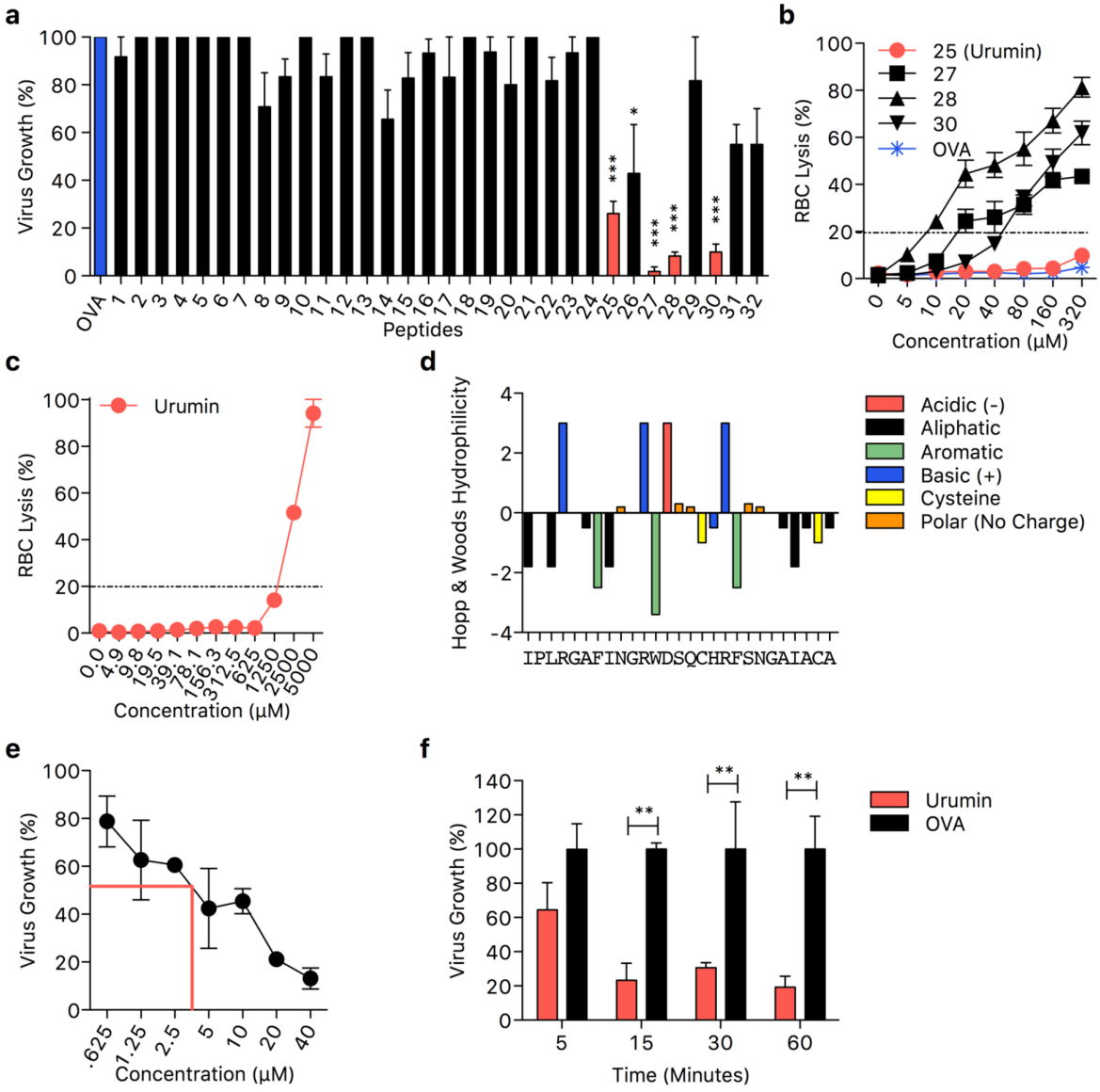

Figure 1. A Peptide from Hydrophylax bahuvistara Exhibits Anti-A/PR/8/1934 Influenza Virus Activity In Vitro

(A) 32 peptides isolated from the skin of *H. bahuvistara* were incubated with H1N1 PR8 influenza for 2 hr and virus growth was assessed by plaque assay. OVA control-treated virus was set as 100% virus growth. Peptides were used at 10 mM, except peptides 11, 26, 28, 29,

32, which were tested at lower concentrations due to hemolytic activity at 10 mM. Peptides highlighted in red significantly reduced viral titers in comparison to OVA control.

(B and C) Serially diluted frog peptides or OVA in PBS were incubated with human RBCs for 1 hr. Peptide toxicity was compared to lysis induced by 0.1% Triton X-100, which was set as 100% cell lysis, and PBS as 0% lysis.

(D) Sequence of urumin on the Hopp & Woods amino acid hydrophilicity scale (224).

(E) Graded concentrations of urumin (0.6–320 mM) were incubated with PR8 for 2 hr and IC50 was determined by peptide-induced virus growth inhibition as assessed by plaque assay.

(F) 10 mM urumin or OVA was incubated with PR8 for 5–60 min and kinetics of urumin activity was determined by virus growth inhibition as assessed by plaque assay.

Pooled results from three independent experiments shown in (A)–(C), (E), and (F). Statistical significance *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ was determined by one-way ANOVA ($p < 0.0001$) with Bonferonni post-tests against OVA control (A) and two-way ANOVA (peptide $p < 0.0001$) with Bonferonni post-tests (F). Error bars denote means ± SEM. Please also see Figures S1 and S2.

Urumin is specific for H1 hemagglutinin and targets the conserved stalk region of H1 HA

As urumin showed specificity for PR8 influenza virus, we sought to determine whether this antiviral effect was strain or subtype specific. We tested urumin against eight different H1N1 and four different H3N2 IAVs. These viruses were circulating strains from 1934 to 2013 and included the 2009 pandemic strain (A/California/04/2009) and a 2013

drift variant of this virus (A/Tennessee/F5001/2013). Of the 12 viruses, urumin inhibited all 8 H1N1 viruses by at least 60%, with titers of some strains decreasing by over 90%. In contrast, all 4 of the H3N2 viruses were reduced by less than 50%, with 3 of 4 being reduced by less than 30% (Figure 2A). Overall, urumin had a significantly greater effect against H1N1 strains than H3N2 influenza strains ($P<0.0001$, Figure 2B). These results suggested that urumin could be targeting H1 HA, N1 NA, or both. To more precisely determine the specificity of urumin, we used reassortant PR8 viruses that bear the same six internal gene segments but differ in their HA and NA expression. We tested four PR8 reassortant viruses - H1N1, H1N2, H3N1, and H3N2 against urumin or the OVA control. Of these viruses, urumin significantly inhibited H1N1 and H1N2 viruses, while the H3N1 and H3N2 were unaffected, demonstrating that urumin inhibited IAV that bear H1 HA but not H3 HA, or N1/N2 NA ($p<0.0001$, Figure 2C).

The HA protein is a trimer that consists of a conserved stalk region and a variable globular head region (30). Targeting the conserved stalk region of HA, as in broadly neutralizing antibodies (225, 226) or computationally designed stalk binding proteins (227), present a promising approach to neutralizing human IAVs. As urumin targeted H1 HA, and inhibited 8 H1N1 viruses that circulated over a span of 75 years, we reasoned that it probably targeted the conserved stalk and not the variable globular head region. To test this, we utilized the H9N3 virus, A/guinea fowl/Hong Kong/WF10/1999 and a chimeric form of this virus, wherein the HA consists of the H9 head region, and the PR8 H1 stalk (228). As expected, urumin inhibited the chimeric virus with the H1 stalk and H9 head and produced a similar level of reduction observed with PR8. In contrast, the non-chimeric wild-type

H9N3 virus (H9 stalk and H9 head) was not inhibited (Figure 2D), suggesting that urumin specifically targeted the stalk region of H1 HA. To confirm this, we tested whether urumin would block the binding of monoclonal antibodies (mAbs) directed to the stalk but not head region of HA. We conducted a competitive ELISA wherein recombinant purified HA was incubated with serially diluted urumin or OVA and assessed the ability to block the binding of mAbs directed at the head or stalk regions of HA (229). Urumin inhibited the binding of the anti-stalk but not anti-head mAbs (Figure 2E, 2F, 2G). These data demonstrated that urumin binds to the conserved stalk region of H1 HA to inhibit IAV.

Urumin is effective against drug-resistant influenza viruses

Current anti-influenza drugs target NA and M2 proteins (78, 79). As urumin targets HA and not NA or M2, we sought to determine whether urumin is effective at neutralizing drug-resistant H1N1 viruses. We tested the against seven drug-resistant influenza strains. These viruses included isolates resistant to oseltamivir carboxylate (A/Louisiana/08/2013 H275Y, A/Texas/23/2012 H275Y), zanamivir and peramivir (A/District of Columbia/02/2014 Cl1-7), or oseltamivir carboxylate and zanamivir (A/Chile/1579/2009 I223K). Viruses generated by reverse genetics were also tested, including a control drug-sensitive pandemic H1N1 rgCal/04/09 wild-type, oseltamivir-resistant mutants rgCal/04/09 H275Y and rgCal/04/09 H275Y S247N, an oseltamivir-sensitive A/New York/08-1253/2008 (rg1253 (S)) and oseltamivir-resistant A/New York/08-1326/2008 (rg1326(R)) (230, 231). Our data showed that urumin significantly reduced the viral titers of all drug-

resistant and drug-sensitive H1N1 virus strains assessed (Figure 2H) and if drug-resistant H1 HA influenza strains become prevalent, urumin could be used against these isolates.

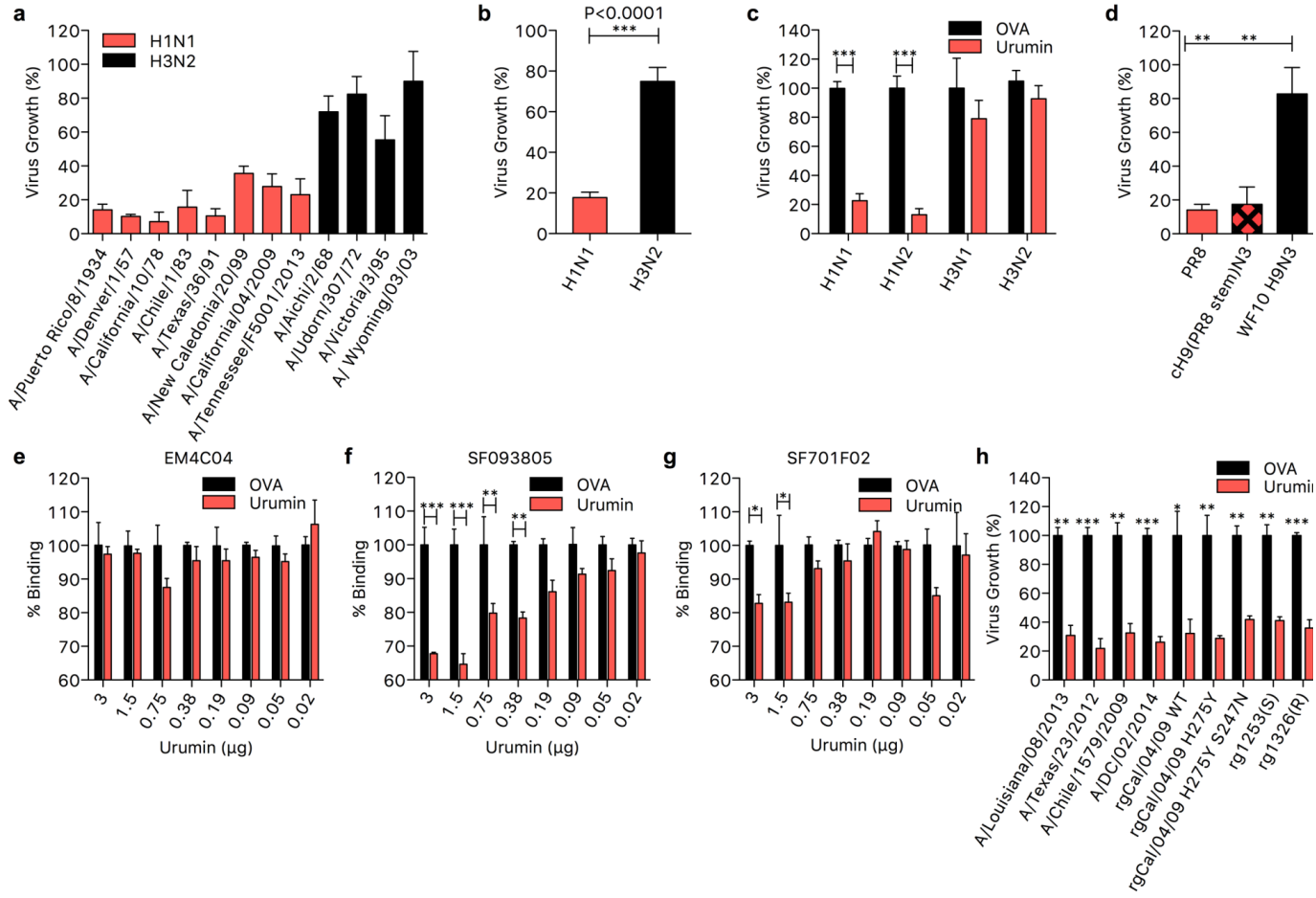

Figure 2. Urumin Is Specific for H1 Hemagglutinin, Targets the Conserved Stalk Region of H1 HA, and Is Effective against Drug-Resistant Influenza Viruses

(A and B) 100 mM urumin or OVA peptide was incubated with eight H1N1 influenza viruses from 1934 to 2013 and four H3N2 influenza viruses from 1968 to 2003 for 2 hr and peptide-induced virus inhibition was assessed by plaque assay. Pooled data from (A) are shown in

(B).

(C) 100 mM urumin or OVA peptide was incubated with four reassortant PR8 viruses that shared all six internal gene segments and differed only in HA and NA segments for 2 hr and peptide-induced virus growth inhibition was assessed by plaque assay.

(D) 100 mM urumin or OVA was incubated with PR8, H9N3 A/guinea fowl/Hong Kong/WF10/99, and a chimeric H9N3 including the WF10 head region and the PR8 conserved stalk for 2 hr and peptide-induced virus growth inhibition was assessed by plaque assay.

(E–G) We incubated plate-bound purified HA with serially diluted urumin or OVA peptide (3 to 0.02 mg) followed by anti-HA head-specific mAbs EM4C04 (E), or stalk-specific mAbs, SF093805 (F), and SF701F02 (G). Binding of mAbs was determined by ELISA, OVA control was set as 100% binding. This competitive ELISA shows that urumin peptide inhibits binding of anti-stalk but not anti-stem mAbs. (H) 100 mM urumin or OVA was incubated with drug-resistant viruses A/Louisiana/08/2013, A/Texas/23/2012, A/Chile/1579/2009, and A/District of Columbia/02/ 2014, reverse genetic drug-sensitive controls (rgCal/04/09/WT and rg1253(S)), and resistant strains (rgCal/04/09 H275Y, rgCal/04/09 H275Y S247N, and rg1326(R)) for 2 hr and peptide-induced virus growth inhibition was assessed by plaque assay. Urumin was effective against drug-resistant viruses.

Shown are pooled results from three independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ as determined by Student's t test (B, C, F), one-way ANOVA ($p = 0.003$) with Bonferonni post-tests (D) and two-way ANOVA (antibody $p = 0.0008$, concentration $p < 0.0001$) with Bonferonni post-tests (E–G). Error bars denote means ± SEM.

Urumin disrupts influenza virus integrity

Since we showed that urumin effectively inhibited growth of H1 HA-bearing IAV, we next determined how the peptide functioned. It was possible that urumin was actively destroying influenza virions or that it was blocking viral growth. To test this, we incubated PR8 virus with urumin or OVA for 2 hours and then imaged virus by electron microscopy. Our data showed that OVA-treated virions were intact (Figure 3A), while urumin-treated influenza virions were destroyed (Figure 3B). These data indicate that urumin is actively destroying influenza virions.

Urumin requires sequence fidelity and chirality for activity

To determine which amino acid residues in urumin were most critical for anti-influenza activity, we generated 23 alanine scan mutant urumin peptides (Figure S3A) (232). First, we assessed whether these mutations increased toxicity to human RBCs. None of the alanine mutants showed toxicity to RBCs, except for a mutation at the 3rd residue, where we observed 20% lysis at the high concentration of 160μM (Figure S3B). To test the mutants for anti-influenza activity, we utilized a focus-forming assay (FFA) against PR8, which allowed us to move from 6- to 12-well plates to a 96-well format. We infected MDCK-SIAT1 cells, which over express α2,6-linked sialic acid (233) and increase experimental sensitivity. Of the 23 mutants, 13 (57%) showed less antiviral activity than urumin, 10 (43%) had equivalent activity to urumin, and no mutations significantly improved peptide-induced viral reduction (Figure 3C).

We also tested whether peptide chirality had an impact on the effectiveness of urumin anti-influenza activity. To conduct this experiment, a stereoisomer variant of urumin was produced using D rather than L amino acids. This D amino acid urumin variant, like the L variant, elicited no toxicity to human RBCs (Figure S3C). We then assessed anti-PR8 activity of D-urumin in comparison to L-urumin as well as OVA control. In contrast to L-urumin, D-urumin showed no reduction of PR8 virus, demonstrating that Urumin recognizes a target with a chiral center (Figure 3D). Thus, the specificity of urumin is exquisite, requiring sequence fidelity and chirality.

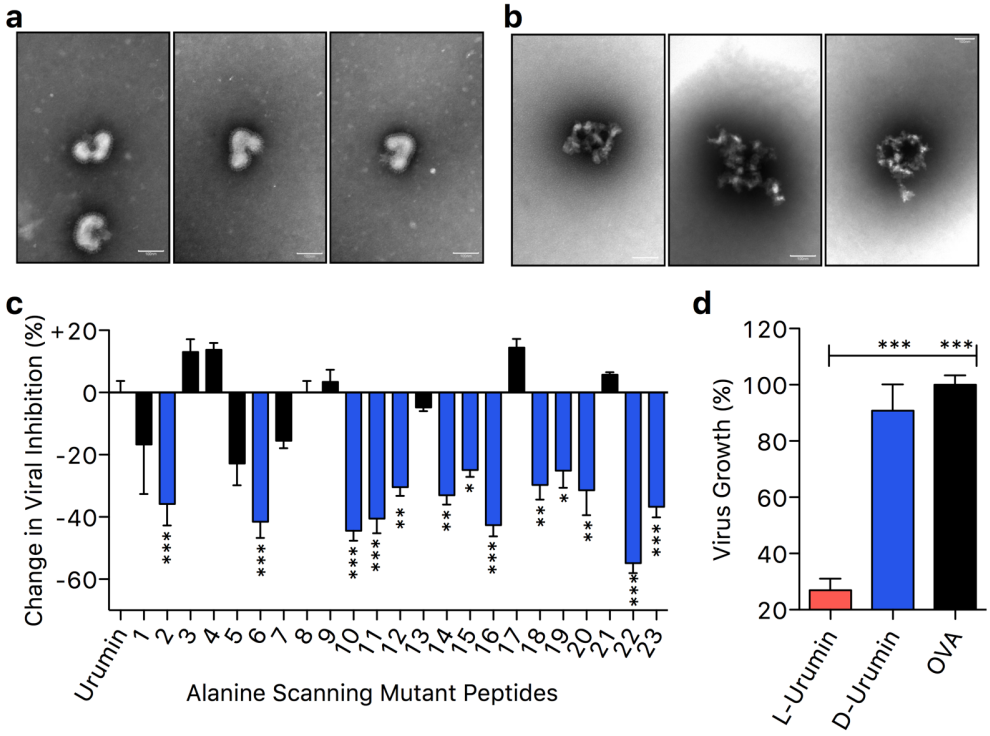

Figure 3. Urumin Disrupts Influenza Virus Integrity and Requires Sequence Fidelity and Chirality for Activity

(A and B) Electron microscopy of PR8 virus incubated with 40 mM OVA (A) or urumin (B)

for 1 hr.

(C) 40 mM alanine scanning mutants of urumin were incubated with PR8 for 2 hr and peptide-treated virus growth was assessed by FFA. The reduction of viral titers by WT urumin was set as 0% and the change in antiviral activity of each mutant peptide was compared to this baseline.

(D) 100 mM of L- or D-enantiomer urumin or OVA peptide were incubated with PR8 virus for 2 hr and peptide-induced virus growth inhibition was assessed by FFA.

Pooled results from three independent experiments shown in (C) and (D). Statistical significance *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ was determined by one-way ANOVA ($p < 0.0001$, C and D) with Bonferonni post-tests. Error bars denote means ± SEM. See also Figure S3.

Intranasal administration of Urumin reduces influenza-induced morbidity, mortality, and lung viral titers *in vivo*

Next, we determined whether urumin could exert its antiviral activity *in vivo* by testing the extent to which peptide treatment could protect naïve mice infected with live mouse-adapted PR8 virus. Cohorts of BALB/c mice were treated with 20μg urumin or an OVA control, administered intranasally (i.n.). Five minutes later, they were infected with 2 x LD_{50} of live mouse-adapted PR8 influenza i.n. The mice were then treated daily for the next 3 days with 20μg of urumin or OVA i.n. (Ideally, the peptide should be delivered systemically, but because that strategy still needs to be resolved, we chose the suboptimal i.n. route for this proof of principle confirmation that urumin is functional *in vivo*.) We assessed

morbidity (percent mass reduction) and mortality (survival) over 14 days following infection. Urumin-treated mice had significantly less morbidity than control mice ($p<0.0001$, Figure 4A and 4B). Furthermore, 70% of urumin-treated mice survived 2 x LD_{50} influenza infection while only 20% of OVA-treated control mice survived (Figure 4C).

We also determined the extent to which i.n. administration of urumin reduced lung virus titers in infected mice. Cohorts of BALB/c mice were administered with 6.6μg, 20μg, or 60μg of urumin or OVA and infected with 2 x LD_{50} of live mouse-adapted PR8 in the same manner as the morbidity and mortality analysis described above. The animals were euthanized at days 3 and 6, and lung viral titers were assessed by FFA. We found that there is a significant decrease in viral focus-forming units per gram of lung tissue in the urumin-treated cohort as compared to control mice at day 6 (80% with 6.6μg $P<0.01$, 82% with 20μg $P<0.01$, and 92% with 60μg $P<0.05$, Figure 4D). Taken together, the data demonstrates that urumin is effective at reducing influenza-induced morbidity, mortality, and lung viral titers *in vivo*.

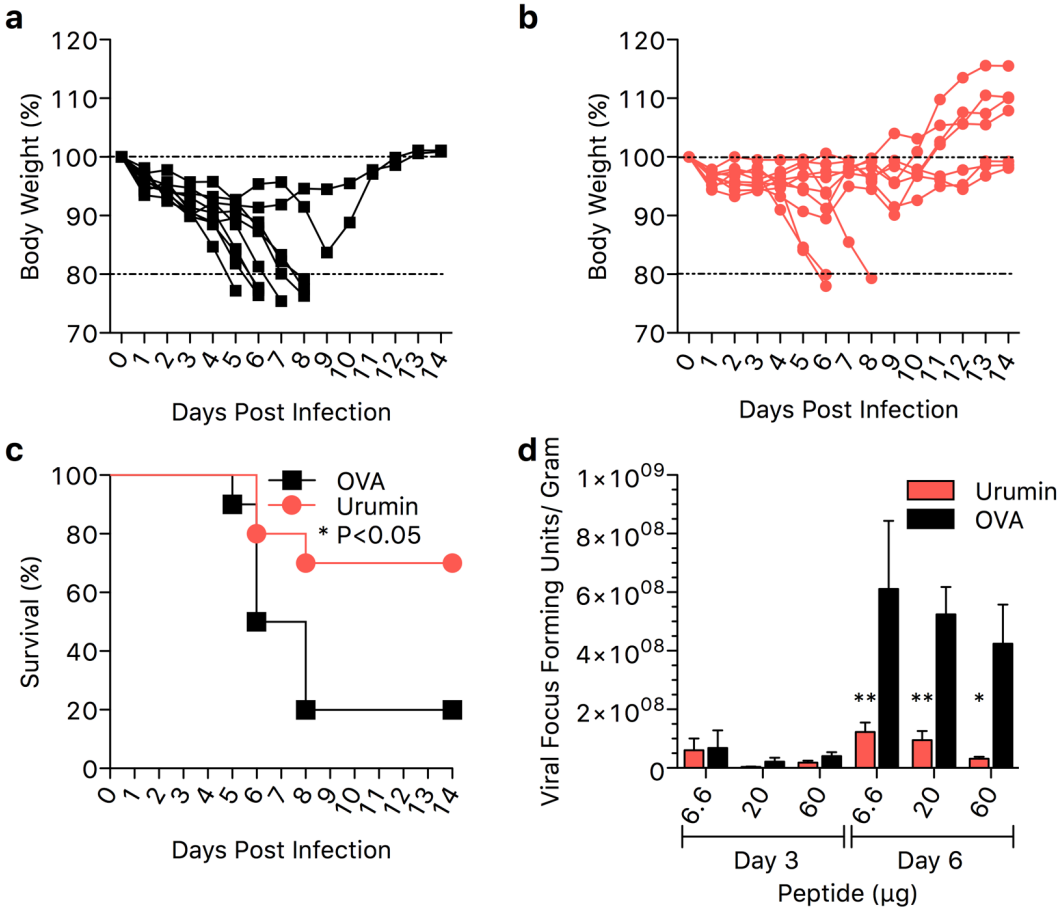

Figure 4. Intranasal Administration of urumin Reduces Influenza-Induced Morbidity, Mortality, and Lung Viral Titers In Vivo

(A–C) Cohorts of BALB/c were infected with 2 x LD_{50} live mouse-adapted PR8 influenza virus and treated with 20 mg of urumin or OVA peptide on days 0–3. Morbidity was assessed by percent of initial body weight loss over 14 days for control OVA- (A) or urumin peptide (B) -treated mice. If the body mass fell below 80% of original mass, mice were euthanized. Mortality was assessed by survival over 14 days (C).

(D) Cohorts of BALB/c were treated with graded doses of OVA or urumin (6.6–60 mg on

days 0–3) and infected with 2 x LD_{50} in the same manner as in (A)–(C). At day 3 or 6 after infection, animals were euthanized, lung tissues harvested, and viral titers assessed by FFA. Results from one of three independent experiments with ten mice per group shown in (A) and (B); pooled results from the ten mice per group shown in (C); data represent pooled results from experiment with five mice per group in (D). Statistical significance *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ was determined by two-way ANOVA (A versus B $p < 0.0001$), *$p = 0.0290$ log-rank (Mantel-Cox) test (C), and two-way ANOVA ($p < 0.0001$ for peptide and time/concentration) with Bonferonni post- tests (D). Error bars denote means ± SEM.

Discussion

We demonstrated that an amphibian innate defense peptide can be harnessed for combating human IAV. The peptide, urumin, is specific for H1 HA and targeted the conserved stalk region much like broadly neutralizing stalk-specific antibodies (234, 235). The specificity of this peptide is exquisite in that, in contrast to the L-form of urumin, the D-enantiomer peptide, which has the same amino acid sequence but mirror image conformation with respect to the L-peptide, failed to inhibit virus. While most HDPs act by destabilizing membranes, peptides with specificity for cell surface molecules, though rare, have also been described. Nisin is a 14-amino acid amphipathic peptide produced by Lactococci bacteria. Nisin binds with high affinity to Lipid II, the fatty acyl proteoglycan anchor in the bacterial membrane. After binding, Nisin diffuses into the surrounding membrane and causes killing (119). Similarly, urumin specifically targeted a cell surface protein, but the mechanism by which it lyses virus still needs to be elucidated.

There is a pressing need to develop new antiviral agents against IAV, particularly those that can be used during pandemic outbreaks where there is insufficient time to produce vaccines, or when the predominant seasonal strains do not match those selected for the annual vaccines (236). Current antivirals target the M2 or NA proteins, and a single mutation is often sufficient to render the viruses drug-resistant (78, 79). Because of these concerns, antiviral peptides against influenza provide a strategy for combating these viruses (192). Antiviral peptides against influenza are often separated into three target categories, peptides that inhibit viral replication by interfering with the viral polymerase like an inhibitory peptide designed to bind and block protein-protein interaction (206), peptides that use electrostatic interactions to disrupt the membrane like the human cathelicidin LL-37 (160), and peptides that interact with HA to block viral fusion and entry like a peptide derived from the signal sequence of fibroblast growth factor 4 (200). The mechanism of urumin does not fit into one of these categories. By specifically binding to HA and causing viral disruption, we believe that urumin represents a unique class of antiviral agent. By binding the conserved stalk region of H1 HA, we speculate that a combination of conformational changes in HA and electrostatic forces upon the membrane could presumably lead to viral destruction. Future studies will determine the specific binding site and mechanism of urumin's virucidal activity. Additionally, strategies to systemically deliver urumin *in vivo* will be undertaken.

Experimental Procedures

Skin secretion harvesting

Performed as previously described (237). Adult *H. bahuvistara* frogs (both sexes, n=15) were collected from Kerala India, under license from the Kerala Forest Department. Skin secretions were collected by mild transdermal electrical stimulation and fixed in liquid nitrogen. Frogs were released back into habitat in a healthy state.

Molecular Cloning of cDNAs encoding HDPs

Performed as previously described (237). RNA was isolated from secretions using Dyna beads (Dynal Biotech) and a cDNA library was constructed using SMARTerTMcDNA Amplification Kit (Clontech). Advantage DNA Polymerase (Clontech) was used for PCR and gel purified PCR products were cloned into pGEM-T easy vector system (Promega) and sequenced.

Peptides

32 peptide sequences were identified from *H. bahuvistara*. Peptides were synthesized at Genscript, Genemed Biotechnologies Inc., and Dr. Brian Evavold's laboratory at Emory University. All peptides were synthesized at purity greater than 80% without specific disulfide bridge modifications. For a control, $OVA_{257-264}$ (InvivoGen) was used.

Influenza viruses

Influenza strains used in study: A/Puerto Rico/8/1934, A/Denver/1/57, A/California/10/78, A/Chile/1/83, A/Texas/36/91, A/New Caledonia/20/99,

A/California/04/2009, A/Tennessee/F5001/2013, A/Aichi/2/68, A/Udorn/307/72, A/Victoria/3/95, A/Wyoming/03/03, A/guinea fowl/Hong Kong/WF10/1999, chimeric A/guinea fowl/Hong Kong/WF10/1999 with A/Puerto Rico/8/1934 H1 stalk region, and recombinant A/Puerto Rico/8/1934 viruses with HA and/or NA from A/Netherlands/602/2009 (H1N1) or A/Panama/2007/1999 (H3N2), A/Texas/23/2012 H275Y, A/Louisiana/08/2013 H275Y, A/District of Columbia/02/2014 Cl1-7, A/Chile/1579/2009 I223K, rgCal/04/09 wild-type, rgCal/04/09 H275Y, rgCal/04/09 H275Y S247N, A/New York/08-1253/2008 (rg1253(S)), and A/New York/08-1326/2008 (rg1326(R)).

Plaque assay

Assay was performed as previously described (238). IAV (50-100 pfu) were treated with peptides for 2 hours at 37°C and the extent of viral inhibition was measured by plaque assay.

Hemolysis peptide toxicity assay

Serially diluted peptides in PBS were incubated with single donor human RBCs (Innovative Research) for 1 hour at 37°C. PBS was used as a 0% lysis control and 0.1% Triton X-100 as 100% lysis. Plates were centrifuged at 300 x *g*, 4°C for 5 minutes to pellet non-lysed RBCs. Supernatants were removed and hemoglobin release was detected by absorbance at 450nm.

Influenza focus-forming assay

Peptides were incubated with IAV (80-100 FFU/well) in Opti-MEM at 37°C for 2 hours. Peptide-treated virus was used to infect MDCK-SIAT1 cells and incubated at 37°C for 1.5 hours, overlaid with methylcellulose and incubated overnight at 37°C. Cells were fixed with ice-cold methanol/acetone (1:1) for 30 minutes and spots developed using anti-Influenza NP-antibody (Millipore MAB8257B).

Anti-HA mAb competitive ELISA

100pg Purified recombinant HA (FR-180 recombinant H1 HA with histidine tag, from influenza A/California/04/2009 (H1N1) pdm09 (International Reagent Resource)) was coated overnight at 4°C and incubated with serially diluted (3-0.02μg) urumin or OVA for 1 hour at 37°C. Anti-HA mAbs were then added at a concentration determined to be 75% of binding and incubated for 1 hour at 37°C. The mAbs were EM4C04 directed at the globular head of HA, and SF093805 and SF701F02, which bind to the stalk region. Bound human IgG was detected using human IgG-HRP (Jackson ImmunoResearch Laboratories 109-036-098) and TMB substrate and read at 450nm.

Electron microscopy

40μM urumin or OVA was incubated with 100pfu PR8 for 2 hours in serum-free DMEM with 1% antibiotics and then processed for Transmission electron microscopy at the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University.

Mice

BALB/c mice, purchased from The Jackson Laboratory were maintained specific pathogen-free in accordance with the institutional guidelines of Emory University's Animal Care and Use Committee. All control and experimental mice were age- and sex-matched.

Peptide administration and live virus challenge

Cohorts of BALB/c mice were anesthetized and administered 20μg urumin or OVA in 20μ,l i.n., and then five minutes later infected with 2 x LD_{50} of mouse adapted PR8 in PBS (30μl volume, i.n.). On each of the following three days, mice were anesthetized and given 20μg of urumin or OVA in 20μl, i.n. For lung viral titer assessment, cohorts of mice were administered with 60μg, 20μg, or 6.6μg doses of urumin or OVA (20μl volume, i.n.) and infected with 2 x LD_{50} mouse adapted PR8 influenza five minutes later. On each of the following 3 days, mice were given doses of urumin or OVA i.n.

Morbidity and mortality assessment

Cohorts of BALB/c mice (n=10 per group) that were administered peptide and infected with live influenza, as described above, were weighed daily starting on day 0 through 14. Morbidity was assessed by percent weight loss, in comparison to each individual mouse's mass prior to infection. If the mass of a mouse dropped below 80% of initial body weight, it was euthanized as per IACUC guidelines and counted towards virus-induced mortality.

Lung viral titer assessment

On day 3 or 6 post-infection, after being administered peptide and virus, BALB/c mice (n=5 per group) were euthanized and their lung tissue removed and weighed. The lung tissue of each mouse was homogenized, cleared by centrifugation for 10 minutes at 600 x *g* and 4°C. The supernatant was taken and the viral titers were assessed using the influenza virus FFA described above.

Statistical analysis

Statistical significance was verified by the Student's t test, ANOVA, and Kaplan-Meier survival analysis (Log-rank (Mantel-Cox) test).

Supplemental Information

Peptide #	Amino Acid Sequence	Molecular Weight (Daltons)
(Urumin) 25	**IPLRGAFINGRWDSQCHRFSNGAIACA**	2961.4
27	**SIFSLFKMGAKALGKTLLKQAGKAGAEYAACKATNQC**	3820.6
28	**SFVTKLKDVAIGVAKGAGLGILKTLTCKLDNSCA**	3436.1
30	**SFITKLKDVAIGVAKGAGLGILKTLTCKLDNSCA**	3450.2

*Peptide purity greater than 80%

Figure S1. Peptides that demonstrate anti-A/PR/8/1934 activity in vitro.

Related to figure 1.

Amino acid sequences of peptides #25, 27, 28, and 30 from *Hydrophylax bahuvistara* and the identified molecular weights. Peptide #25 (Urumin) shows no homology to known peptide

families, peptide #27 shows homology to Esculentin 2 peptide family, peptides #28 and 30 show homology to Brevinin 2 peptide family.

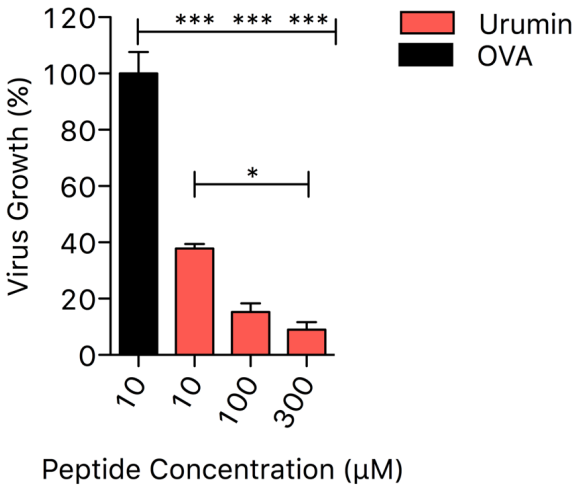

Figure S2. Increasing urumin peptide concentration leads to increased virus growth inhibition.

Related to figure 1.

10μM, 100μM, and 300μM urumin and OVA were incubated with PR8 for 2 hours and peptide-treated virus growth was assessed by plaque assay. The data presented are pooled results from three independent experiments. Statistical significance **p<0.005, ***p<0.0005 as determined by one-way ANOVA (P<0.0001) with Bonferonni post-tests. Error bars denote means ± SEM.

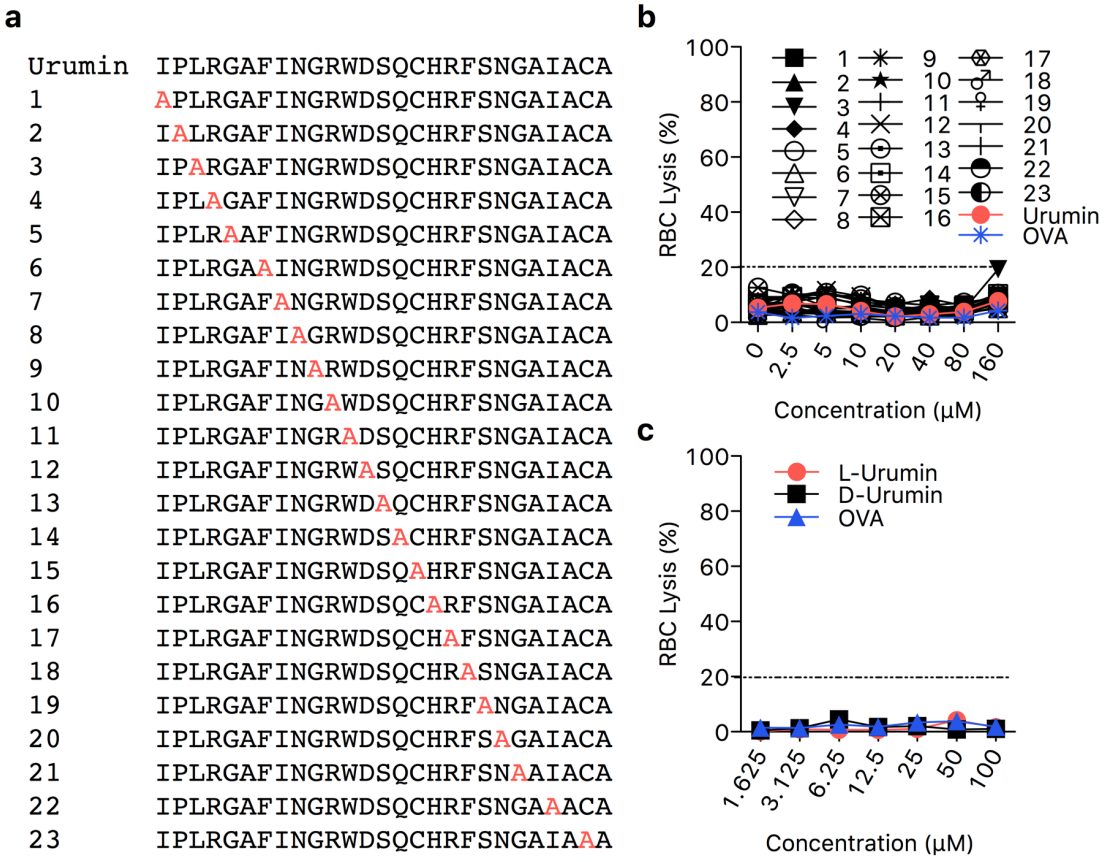

Figure S3. Alanine scan mutants and D enantiomer of urumin peptide are not toxic to human red blood cells.

Related to figure 3.

(A) Alanine scanning mutants of urumin peptide. The inserted alanine residue in each mutant is shown in red.

(B and C) Toxicity analysis of alanine mutant peptides (B) and D-enantiomer peptide (C). Tested by human RBC hemolysis and compared to lysis induced by 0.1% Triton X-100 which was set as 100% cell lysis, and PBS as 0% lysis.

(B and C) pooled results from three independent experiments. Error bars denote means ± SEM.

Author Contributions

D.J.H. and J.J. designed experiments and wrote the manuscript. D.J.H. and S.H.L performed the experiments. S.G., V.K.T.V, and M.R.P. provided frog peptide sequences. A.H.E. and J.W. generated the anti-HA mAbs. A.C.L., F.K. and N.M.B. generated reassortant IAV, H9 chimeric virus, and drug-resistant IAV, respectively.

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Chapter 3: The virucidal peptide urumin acts like a thiol reductase, binds to and destabilizes hemagglutinin on human influenza viruses

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Authors

David J. Holthausen[1], Song Hee Lee[1], Gordon A. Dale[1], Jessica R. Shartouny[1], Anice C. Lowen[1], Sanil George[2], and Joshy Jacob[1,3,*]

Affiliations

[1]Emory Vaccine Center, Yerkes National Primate Center, Emory University, 954 Gatewood Road Atlanta, GA 30329, USA

[2]Rajiv Gandhi Center for Biotechnology, Poojapura, Thiruvananthapuram, Kerala, India

[3]Lead Contact

*Correspondence: joshy.jacob@emory.edu (J.J)

Summary

Urumin is an anti-influenza peptide, which binds to the conserved stalk of H1 hemagglutinin-bearing influenza A viruses and acts in a virucidal manner to physically destroy influenza virions. Here we studied the mechanism of urumin's antiviral activity. By bioinformatics analysis and 3-dimensional modeling of hemagglutinin, we identified an extremely conserved location, 417L, in the stalk of hemagglutinin, critical to urumin's effectivity. Using an A/Memphis/8/2003 H1N2 virus, which has a mutation at that locus, we demonstrate that urumin is ineffective against this virus. When the L417F mutation is inserted into the susceptible H1N1 A/Puerto Rico/8/1934 virus, anti-viral activity is lost. Western blot analysis revealed that urumin activity leads to the separation of the HA trimer, breaks the disulfide bond between HA1 and HA2, and is dependent on open-cysteine residues to be effective. As such, urumin acts like a thiol reductase, specifically targeting the HA disulfide bridge, bonding to HA1, and releasing HA2.

Introduction

Influenza virus infection is a major burden on world health with 3-5 million severe cases and 250-500 deaths caused by influenza each year (8). As the most common recurring human respiratory viral infection, influenza affects 5-10% of the adult population and 20-30% of children annually (3-5, 9). Despite the protection offered by current vaccination and antiviral strategies, occurrences of mismatching of the vaccine to the circulating influenza strains, the unexpected nature of pandemic outbreaks, and the emergence of drug-resistant influenza strains impede the effectiveness of these strategies (77-79, 236). With these

impediments to current regiments of vaccines and anti-viral drugs, antiviral peptides that target influenza viruses have become a focus for new therapies (192).

Originally discovered in 1981, when Hans Boman isolated antimicrobial peptides from the hemolymph of the giant silk moth, *Hyalophora cecropia*, (101), host defense peptides play an essential role in the innate immune system of all organisms (97, 125). As a target for new therapeutics, in comparison to conventional drug therapies, host defense peptides are less susceptible to resistance (97), which is critical due to the increase in drug-resistant influenza strains (78). Currently, anti-influenza antiviral peptides are split into three types based upon their mechanisms. Peptides which utilize charge and electrostatic interactions to disrupt the membrane of influenza virions, an example of which is LL-37 a human cathelicidin (160). Peptides like one derived from the signal sequence of fibroblast growth factor 4, which interacts with surface hemagglutinin to inhibit viral fusion and entry into host cells (200). Or, peptides that block viral replication by inhibiting viral polymerase activity, like an inhibitory peptide designed to bind to and interfere with protein-protein interaction (206).

Recently, a peptide isolated from the skin of the frog *Hydrophylax bahuvistara,* named urumin, was shown to have antiviral activity against human influenza viruses bearing H1-hemagglutinin (239). Since their discovery in 1987, when Michael Zasloff (106) and Dudley Williams (107) studied *Xenopus laevis,* amphibians have been an exquisite source of host defense peptides, secreting large quantities of diverse peptides as a mechanism of defense (97, 164, 215, 216). In destroying human influenza viruses, urumin both specifically binds to the conserved stalk region of HA, as well as acts in a virucidal manner, destroying influenza

virions. As urumin activity is distinct with regards to known categories of influenza antivirals, determining how urumin disturbs H1 influenza viruses is of critical importance. Identification of this mechanism defines a new class of host defense peptides and provides insight on a key weak point in HA stability that in principle can be translated for the targeting of other influenza HA subtypes.

Results

Position 417L on the stalk region of H1 hemagglutinin is an extremely conserved and accessible locus at the center of HA

We conducted bioinformatic sequence analysis of 9,896 unique HA sequences to map out potentially crucial loci for the activity of the anti-H1 peptide urumin to the HA stalk. These HA sequences come from human H1 isolates from across the world. Briefly, these sequences were aligned and analyzed for the level of mutagenesis at each of the 590 amino acid residues of the HA protein as compared to the H1N1 A/Puerto Rico/8/1934 (PR8) virus (Protein Data Bank 1RU7). To quantify the extent of mutation at each of these amino acid positions, the percentage of sequences with variability away from the consensus sequence was determined (Figure 1A). Of the 590 residues, we find that 132 (22%) have variability indexes below 0.04%, or, 4 or less mutations amongst the 9,896 sequences.

While many of the amino acid positions are quite variable between the human isolates, we hypothesized that urumin targets a highly conserved amino acid loci, given that the urumin peptide elicited antiviral activity against highly variable H1 viruses isolated from 1934-2013 (239). Specifically, we targeted a portion of the HA stalk, at the center of the HA

trimer, accessible through an opening in the globular head region of HA (Figure 1B). This section of the stalk region, accessible from the opening created by the HA head trimer, corresponds to amino acid positions 415-421 of the HA stalk (Figure 1C). Analysis of the percent variability away from the consensus sequence in this region, reveals that position 417, a leucine residue, is the most highly conserved amino acid in the centrally accessible stalk region (Figure 1D). Of the 9,896 sequences analyzed, only 3 H1 influenza isolates contain a mutation at 417L. Taken together, these analyzes suggest that the 417L position is critically important to the structure of the H1 HA trimer.

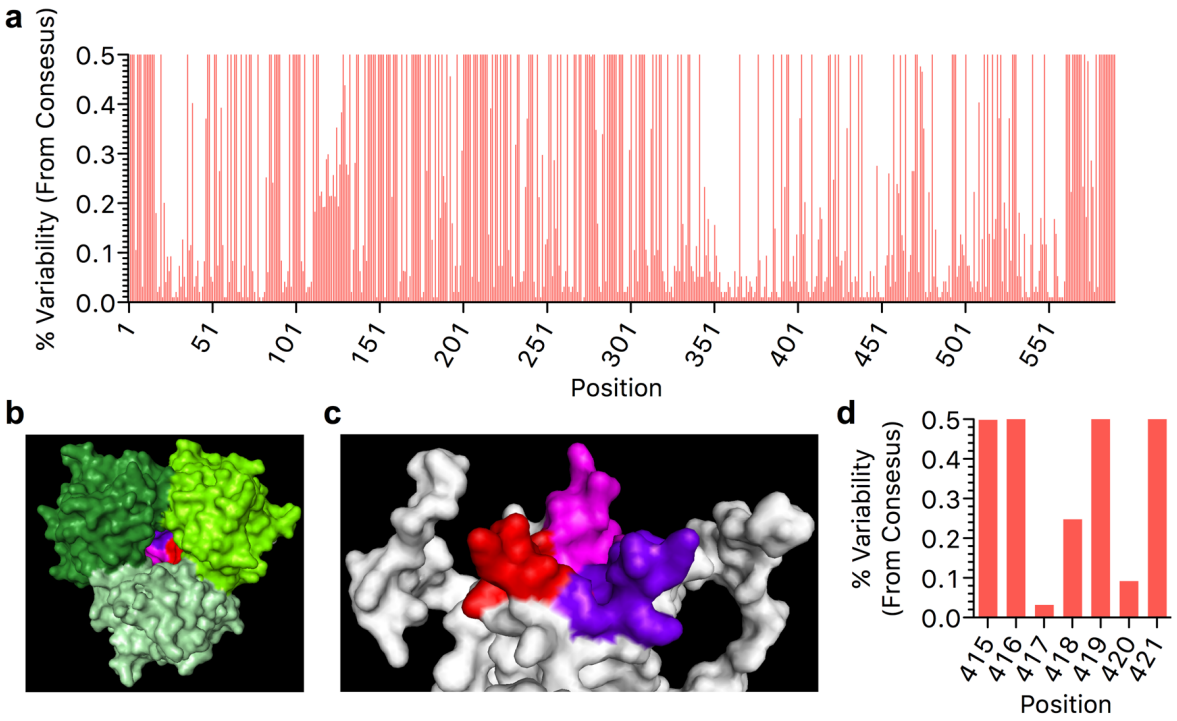

Figure 1. Position 417L on the stalk region of H1 hemagglutinin is an extremely conserved and accessible locus at the center of HA

(A) Percent variability from the consensus sequence of each amino acid position in hemagglutinin from 9,896 H1 influenza human isolates.

(B and C) 3D-modeling of A/Puerto/Rico/8/2934 hemagglutinin trimer. HA globular head monomers shown in green (B) and internal residues of conserved HA stalk monomers accessible through opening in HA head trimer shown in Red, Pink, and Purple (B and C).

(D) Percent variability from the consensus sequence of positions 415 to 421 in the hemagglutinin stalk, accessible through opening in HA head trimer from 9,896 human H1 influenza isolates.

Urumin does not inhibit the H1N2 A/Memphis/8/2003 virus containing an L417F mutation

One of the H1 viruses that contains a mutation at position 417 is the H1N2 virus A/Memphis/8/2003 (Me8). This H1N2 virus contains a leucine to phenylalanine L417F mutation at this position, replacing the aliphatic leucine with an aromatic amino acid. This mutation at position 417 is one of many differences between Me8 and PR8. However, when we compare the sequence of Me8 HA to the HA of 8 H1N1 viruses that have been shown to be susceptible to urumin antiviral activity (239), we find that there are 3 unique mutations in the stalk region of Me8 in comparison to these 8 other viruses (Figure S1). These mutations are D52N, K419R, and L417F. While the D52N mutation is located on the exterior of the HA stalk, both L417F and K419R reside in a region of the HA trimer where the fusion peptide of each HA monomer meet (Figure 2A). Additionally, in comparison to the 99.97% conservation of the 417L locus amongst H1 isolates examined, of the 9,896 H1

HA sequences analyzed, the 52D locus is conserved in 91% of sequences and 419K is conserved in 83% of sequences (Figure 2B). The Me8 virus is the only virus to contain mutations at both position 417 and 419. We obtained the Me8 H1N2 virus and tested whether urumin was effective at inhibiting the virus *in vitro* by focus-forming assay. We incubated urumin or an OVA control peptide with Me8 and PR8 viruses for 2 hours, plated them with MDCK-SIAT1 cells which overexpress sialic acid, overlaid with methylcellulose overnight, and enumerated focus-forming units the following day. In contrast to the antiviral activity of urumin against the PR8 virus, we find that the effect is significantly diminished against the Me8 virus (Figure 2C). Based upon this finding, one of these three loci must be the crucial for urumin's antiviral activity.

Urumin is effective against PR8 mutant viruses that retain a leucine at position 417 on the HA stalk

Having narrowed down potential loci on the stalk of H1 HA that can impact antiviral activity with the resistance to the Me8 virus, we next sought to determine which of these residues, or a combination of these residues, is essential for urumin resistance. To assess this, we generated mutant PR8 viruses containing the D52N, L417F, and K419R replacements. We also generated mutants that combine these mutations to determine if multiple replacements are necessary for urumin binding. While combinatorial mutants containing the D52N and L417F replacements and triple mutant were created, PR8 viruses containing only the D52N and K419R or L419F and K419R double replacements were not able to be recovered for further analysis. Despite not having the full complement of combinatorial

mutant viruses, we assessed urumin activity *in vitro* by focus-forming assay. We incubated urumin or an OVA control peptide with PR8, the PR8 mutant viruses and the Me8 virus for 2 hours, plated them with MDCK-SIAT1 cells, overlaid with methylcellulose overnight, and enumerated focus forming units the following day. While the D52N and K419R mutant PR8 viruses elicit similar levels of urumin susceptibility to that of wild-type PR8 virus, we find that viruses containing the L417F replacement mutation, whether as a single mutation, or part of a double or triple mutant virus are resistant to urumin akin to the wild-type Me8 virus (Figure 2D). Based upon these results, we have demonstrated that the highly conserved 417L locus is crucial for urumin's antiviral activity against H1-bearing influenza viruses (Figure 2E).

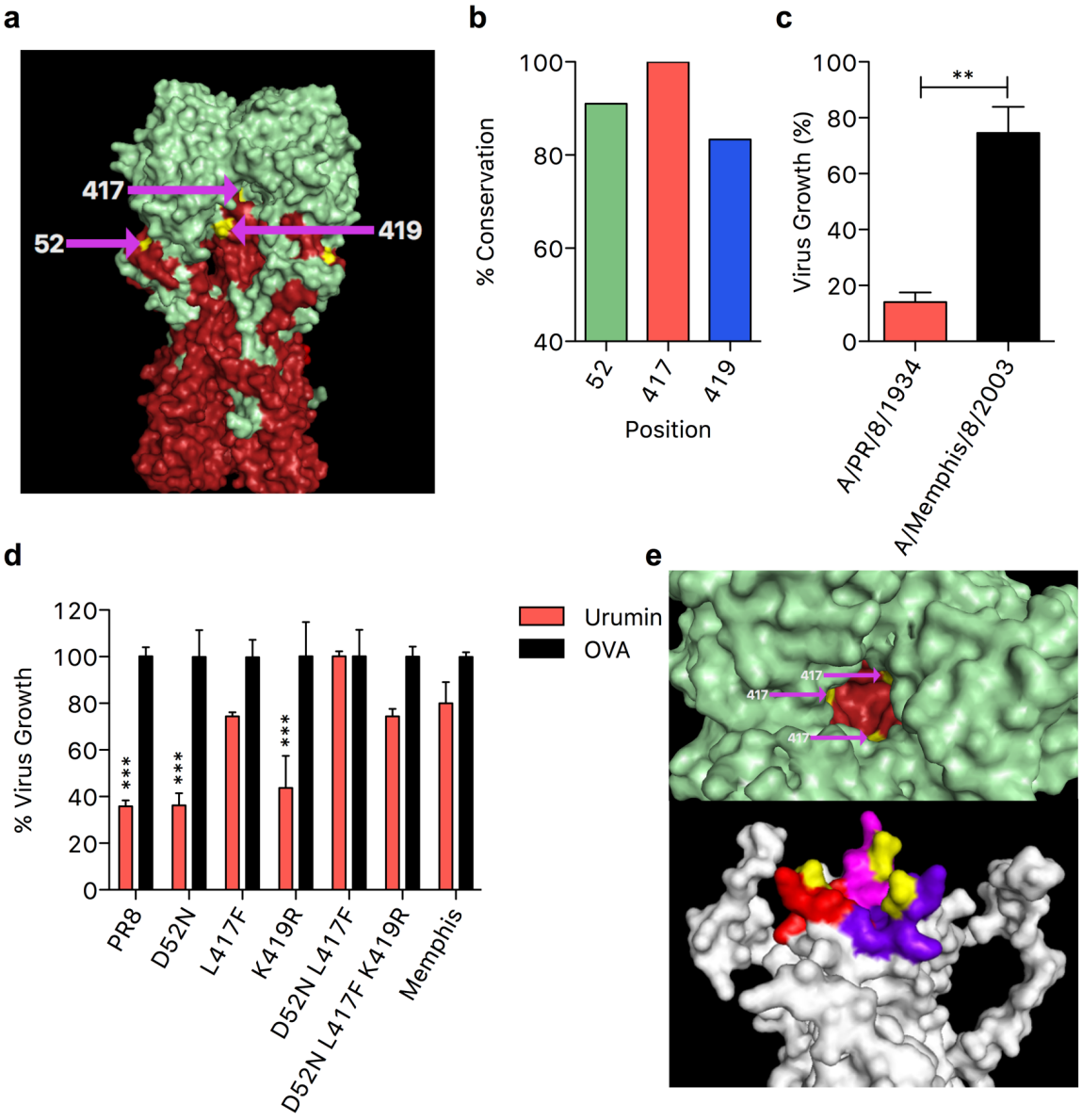

Figure 2. Urumin does not inhibit the H1N2 A/Memphis/8/2003 virus containing an L417F mutation and is effective against PR8 mutant viruses that retain a leucine at position 417 on the HA stalk

(A) 3D-modeling of A/Puerto Rico/8/1934 hemagglutinin trimer. Globular head HA monomers in green, stalk region in red, and positions 52, 417, and 419 are highlighted in yellow.

(B) Sequence conservation of positions 52, 417, and 419 of H1 HA. Percent conservation based upon the percent variability from the consensus sequence of 9,896 H1 HA sequences from human isolates.

(C) 100μM urumin and control OVA peptide were incubated with A/Puerto Rico/8/1934 and A/Memphis/8/2003 H1 influenza viruses for 2 hours and peptide-induced virus growth inhibition was assessed by focus-forming assay.

(D) 50μM urumin and control OVA peptide was incubated with A/Puerto Rico/8/1934, A/Memphis/8/2003, and 5 mutant PR8 viruses containing replacements from Me8 HA for 2 hours and peptide-induced virus growth was assessed by focus-forming assay.

(E) 3D-modeling of A/Puerto Rico/8/1934 hemagglutinin trimer with position 417 of the conserved stalk region highlighted in yellow. Images include globular HA head (above) or illustrates only HA stalk region (below).

(C and D) represent pooled results for three independent experiments. Statistical significance *P<0.05, ** P<0.005, *** P<0.0005 was determined by student's t-test (C) and two-way ANOVA with Bonferroni post-tests (D). Error bars denote means ± SEM.

Urumin acts as a reducing agent to physically disrupt hemagglutinin

Next, having determined that a specific mutation can ablate urumin's activity, we wanted to assess whether urumin directly disrupts HA. Specifically, whether the binding of

urumin to HA disrupts the structural stability of the protein itself. To discover the impact urumin has on the stability of HA, we incubated urumin or an OVA control peptide with recombinant HA derived from a susceptible A/California/07/09 H1N1 influenza virus, and analyzed the pattern of the protein by western blot. If urumin is acting to destabilize surface trimeric HA, we would anticipate that urumin-treated recombinant HA would have diminished amounts of HA trimer in comparison to the OVA control. Briefly, we incubated urumin or the OVA control peptide with the recombinant A/California07/09 HA for 2 hours, conducted SDS-PAGE in a native non-reducing gel and western blot analysis utilizing an α-HA stalk antibody. In the native gel as well as the α-HA stalk western blot we found that urumin treatment yields a reduction in the HA trimer and the appearance of a much smaller protein band of approximately 20 to 30 kDA in size (Figure 3A). Utilizing the same experimental setup as has just been described, we conducted SDS-PAGE under native non-reducing and western blot analysis with α-HA serum antibodies. In addition to reducing the amount of HA trimer, bands with sizes corresponding to HA2 (28 kDa), HA2a (18kDa), and HA dimers (140 kDa) and trimers (210 kDa) are present (Figure 3B). HA2 is a product of the cleavage of the HA0 precursor into HA1 and HA2 during this internalization process of influenza. Serine proteases in the endosome cause the cleavage of HA0 into the disulfide-bonded HA1 and HA2 polypeptides. After cleavage, pH induced conformational changes activate and facilitate membrane fusion (33-36). Interestingly, no band corresponding to HA1 was detected. We hypothesize that the band seen at approximately 100 kDa is a dimer of HA1 linked together by disulfide bonding to each of urumin's cysteine residues. Additionally, we assessed the effect of temperature on urumin activity releasing the HA2

subunit and decreasing the amount of HA trimer. Urumin activity is visible, though diminished, at room temperature and 4°C, in comparison to 37°C (Figure S2). This demonstrates that urumin's activity does not rely on, but is improved by increased thermal energy. The production of HA2 due to urumin treatment suggests that urumin not only disrupts the HA trimer, but also induces the breaking of the HA disulfide linkage.

To compare urumin's activity to conventional disulfide linkage reduction techniques, western blot analysis was conducted on OVA-treated HA using a beta-mercaptoethanol reducing gel. This treatment under reducing conditions produced banding patterns in the OVA treated HA that mirror those of the urumin-treated HA under non-reducing conditions (Figure 3C). The urumin-induced reduction of the recombinant HA trimer demonstrates that the peptide physically disrupts the integrity of the protein. Additionally, the appearance of the HA2 bands and identical banding pattern of urumin treatment in the non-reducing conditions to the HA banding pattern of OVA under reducing conditions demonstrate that urumin's mechanism of action against influenza HA is as a cysteine: cysteine reducing agent, specifically targeting the HA disulfide bond.

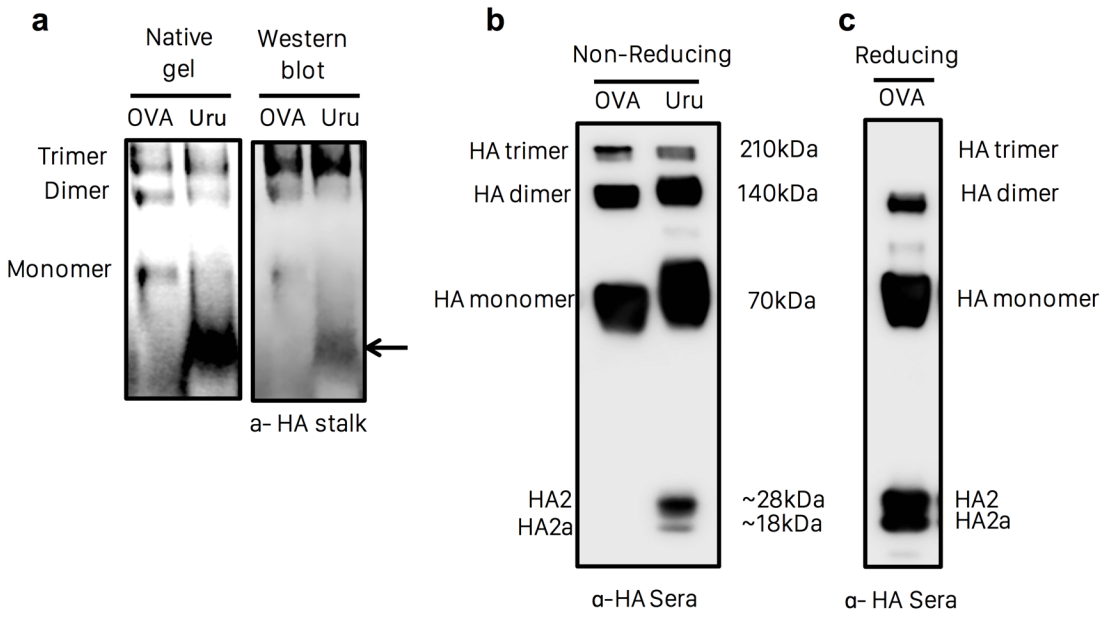

Figure 3. Urumin acts as a reducing agent to physically disrupt hemagglutinin

(A, B, and C) 100μM urumin or a control OVA peptide was incubated with 10μg of recombinant HA (A/California/07/2009) for 2 hours, protein samples were separated by non-reducing SDS-PAGE (A and B) or reducing SDS-PAGE (C) and analyzed by western blot using an α-HA stalk antibody (A) or α-HA serum antibodies (B and C).

Urumin acts like a thiol reductase, breaks the HA disulfide bond, and remains bound to the HA1 subunit

As data suggested that urumin may act as a reducing agent, we next sought to confirm this mechanism of action, as well as determine if urumin is covalently bonding to either the HA1 or HA2 subunits. To confirm that urumin is acting like a thiol reductase, utilizing its free cysteines to break the HA disulfide bond and bond itself to HA, we generated a variant of urumin that has an intramolecular disulfide bond between its two cysteine residues. We

conducted the same peptide treatment, SDS-PAGE, and western blot analysis as has been described. Briefly, OVA control peptide, free cysteine urumin, and the cysteine bonded urumin were incubated with the recombinant A/California07/09 HA for 2 hours, and non-reducing SDS-PAGE was conducted, followed by western blot analysis using α-HA serum antibodies. Unlike free cysteine urumin, which breaks the HA disulfide bond, releasing HA2, the cysteine-bonded urumin variant does not, demonstrating the same band pattern as the OVA control peptide (Figure 4A). Cysteine-bonded urumin's inability to break the disulfide bond between HA1 and HA2 demonstrates the crucial activity of urumin's cysteine residues.

Lastly, knowing that urumin breaks the HA disulfide bond, we sought to determine which subunit of the HA protein urumin is binding to. Given that subunit HA2 monomer bands are observed, but not HA1 monomer units, we hypothesize that urumin is releasing HA2 and linking dimers of HA1. To assess this, we obtained biotinylated urumin peptides tagged at either the N-or C-terminus. Full length recombinant A/California/07/09 HA or recombinant A/California/07/09 HA1 were incubated with an OVA control peptide, urumin, N-biotinylated urumin, or C-biotinylated urumin. SDS-PAGE under non-reducing conditions, Ponceau S staining for rapid detection of protein bands, and western blot analysis was conducted. Ponceau S staining observed protein bands that correlate with the HA monomer (70 kDa) and urumin (2.9 kDa) from the full-length HA incubation, and bands that correlate with HA1 (48 kDa) and urumin (2.9 kDa) (Figure 4B). Western blot analysis using α-HA sera demonstrated that both N-biotinylated and C-biotinylated urumin are effective at reducing the HA precursor and releasing HA2 (Figure 4C). Western blot

analysis using Streptavidin-HRP, which binds to biotin, confirmed the hypothesis that urumin binds to the HA1 subunit (Figure 4D). Overall, urumin requires the use of its free cysteine residues to induce the breaking of the HA disulfide bond, releasing HA2, binding to the HA1 subunit (Figure 4E).

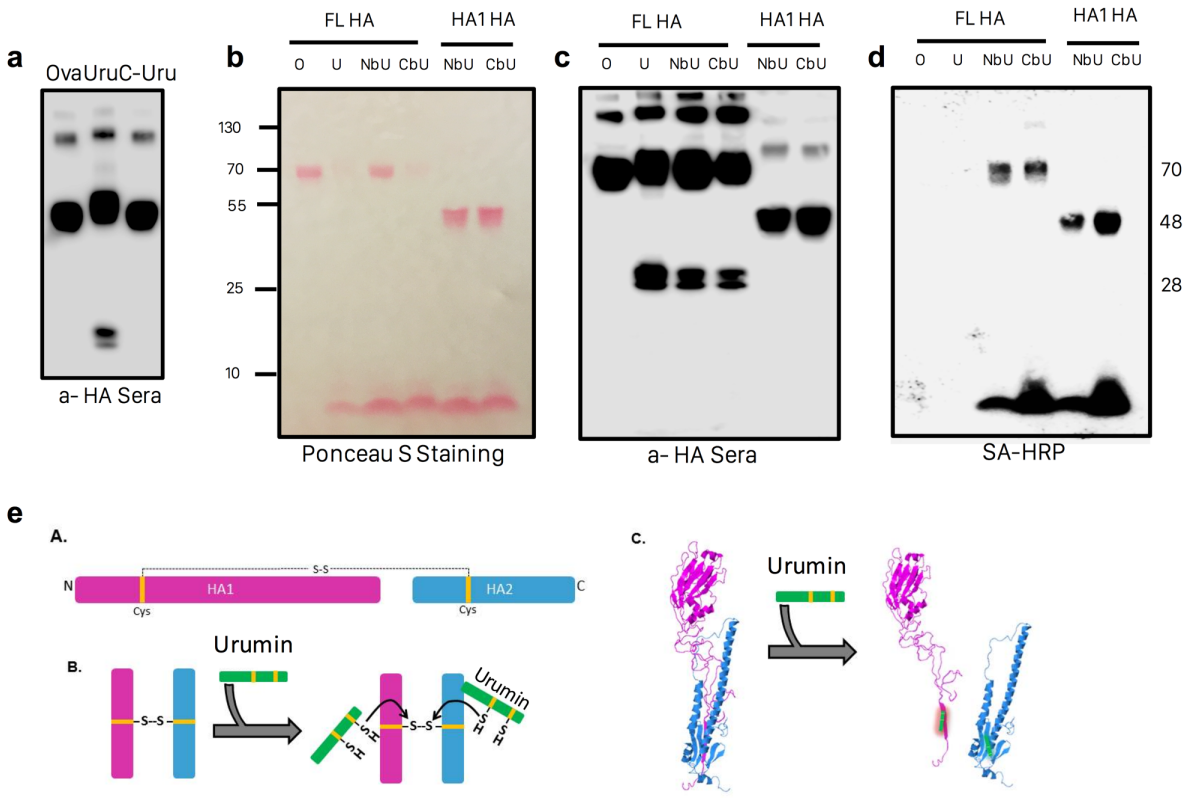

Figure 4. Urumin acts like a thiol reductase, cleaves the HA disulfide bond, and remains bound to the HA1 subunit

(A) 100μM urumin, control OVA peptide, or cysteine-bonded urumin (C-Uru) was incubated with 10μg of recombinant HA (A/California/07/2009) for 2 hours, protein samples were separated by non-reducing SDS-PAGE and analyzed by western blot using α-HA serum antibodies.

(B, C, and D) 100μM urumin, control OVA peptide, N-biotinylated urumin (NbU), or C-biotinylated urumin (CbU) was incubated with 10μg of recombinant full length (FL) HA or HA1 HA (A/California/07/2009) for 2 hours, protein samples were separated by non-reducing SDS-PAGE and analyzed by Ponceau S staining (B), or western blot using α-HA serum antibodies (C) or streptavidin-HRP (D).

(E) Mechanism of urumin is thiol reductase like activity. A, HA1 and HA2 are bound by a disulfide bond between two cysteine residues. B, Urumin's free cysteine residues attack the HA disulfide bond, forming a covalent bridge. C, HA2 is released from HA1 and urumin remains bound to HA1.

Discussion

In conclusion, we demonstrate a unique mechanism of peptide-mediated destruction of influenza viruses. The frog host defense peptide urumin, which we have previously reported to be effective against H1-bearing human influenza viruses (239) acts in a manner like a thiol reductase, reducing the disulfide bridge that bonds the 2 HA subunits, HA1 and HA2. In this process, HA trimers are disrupted, diminishing into dimers and monomers, and urumin binds to HA1, releasing HA2. Thiol reductases are a family of enzymes which carry out the reduction, oxidation, or isomerization of protein disulfide bonds (240). These enzymes often have similar three dimensional structures and all contain a conserved active site consisting of -CXXC-, often -CGPC- (241, 242). While urumin conducts activity like thiol reductases, it is unlike any thiol reductase. The cysteine residues in urumin are 10 residues apart, rather than the two amino acids between the cysteines in other thiol

reductases. Additionally, while urumin is a 2.9 kDa peptide, most thiol reductases like thioredoxin, which is 12 kDa in size and gamma interferon-inducible thiol reductase (GILT), which is a 35 kDa precursor protein with a 30 kDa active form, are much larger proteins (243, 244). Urumin presents what is likely the smallest known molecule with thiol reductase like activity, as well as the one with the most unique sequence that conducts such activity.

While urumin acts upon the HA disulfide bridge, there are also critical loci that are not near the HA disulfide bond or cleavage site. The extremely conserved 417L residue central to the HA stalk, is accessible through the opening in the head region of the HA trimer, and has been shown to be crucial for urumin's activity. Given that this is not the binding site of urumin, we speculate that the replacement of the leucine residue at this position with the much larger phenylalanine ringed structure stabilizes the HA trimer, and blocks the ability of urumin to separate the protein monomers. Single mutations in hemagglutinin can drastically impact the intermolecular interactions between HA monomers, effecting the HA trimer stability and overall viral fitness (245). As such, the mechanism of resistance demonstrated by the H1N2 Me8 virus is not by inhibiting binding of the peptide, but rather is by reducing the rate of reaction to the extent that viral activity is not significantly reduced. Therefore, future studies will assess whether modifications to urumin can overcome this effect and can be susceptible against the Me8 virus, other subtypes of influenza like H5N1 viruses, as well as H3N2 viruses, where urumin demonstrated a lack of antiviral activity (239).

Experimental Procedures

Bioinformatic Analysis

9,896 H1 hemagglutinin sequences from human isolates were downloaded from the Influenza Research Database (www.fludb.org). Sequences covered all geographical regions except Asia. Alignments were performed via MUSCLE (Multiple Sequence Comparison by Log-Expectation) sequence analysis online at the Influenza Research Database. Aligned sequences were imported into Bioedit version 7.1.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A positional amino acid frequency table was generated from the alignment to identify the frequency of each amino acid per residue of H1. Using this table, we calculated the percent of sequences that differ from the consensus residue at any given site (D) using the formula below, where N is the number of sequences (9,896), and C is the maximum number of sequences that share a common amino acid residue at each site.

3D-Modeling of Hemagglutinin

Modeling of H1 Hemagglutinin was done using Pymol (https://www.pymol.org/). Coordinate data used was from the Protein Data Bank entry 1RU7.

Frog Peptides

Urumin peptide was isolated from *H. bahuvistara*. Peptide was synthesized at Genscript (Piscataway, NJ), Genemed Biotechnologies Inc. (San Francisco, CA). and Dr. Brian Evavold's laboratory at Emory University. Urumin was synthesized at purity greater than

80% with and without specific disulfide bridge modifications. N-terminal and C-terminal biotinylated urumin was also produced. As control, $OVA_{257-264}$ peptide (InvivoGen) was used. Skin secretion harvesting of peptide and molecular cloning of cDNAs encoding antimicrobial peptides have previously been described (239).

Influenza Viruses and Recombinant Hemagglutinin

Influenza viruses used in this study include: A/Puerto Rico/8/1934 (H1N1), A/Memphis/8/2003 (H1N2), A/Puerto Rico/8/1934 mutant viruses containing D52N, L417F, K419R, D52N and L417F, or D52N, L417F, and K419R replacements. Recombinant H1 HA with histidine tag, from influenza A/California/07/2009 (H1N1) pdm09 (FR-559 International Reagent Resource) and recombinant H1 HA1 with histidine tag, from influenza A/California/07/2009 (H1N1) pdm09 (FR-695 International Reagent Resource).

Influenza Focus-forming Assay

Focus-forming assays were performed as previously described (239). Peptides were incubated with IAV (80-100 FFU/well) in Opti-MEM at 37°C for 2 hours. Peptide-treated virus was used to infect MDCK-SIAT1 cells and incubated at 37°C for 1.5 hours, overlaid with methylcellulose and incubated overnight at 37°C. Cells were fixed with ice-cold methanol/acetone (1:1) for 30 minutes and spots developed using anti-Influenza NP-antibody (Millipore MAB8257B).

SDS-PAGE and Western Blot Analysis

Peptides were incubated with 10μg of protein for 2 hours at 37°C (unless different temperature is specified). Reaction samples were prepared by mixing with 2x Laemli buffer and then boiled at 70°C for 10min for non-reducing condition and sample butter containing 2-mercaptoethanol for reducing condition. Total protein was separated by SDS-PAGE (NuPAGE, 4-12% Bis-Tris gel) and transferred to Nitrocellulose membranes (Hybond). Membranes were blocked with 5% non-fat dry milk in 0.5% TBST (100 mM Tris, pH 8.0, 1.5M NaCl, 0.5% Tween 20) and incubated with primary antibody diluted in blocking buffer. Antibodies used were: hamster anti-HA sera (Dr. John Steel), anti-HA stalk (SF093805, Dr. Ali Ellebedy), anti-Streptavidin-HRP (Southern Biotech 7100-05). Membranes were washed with TBST, followed by incubation with HRP conjugated secondary antibody diluted in blocking buffer. Following incubation, membranes were washed again in TBST and imaged using an Odyssey Fc Imager and Image Studio, version 2.1.10, software (Li-Cor).

Statistical analysis

Statistical significance was verified by the Student's t test and ANOVA.

Supplemental Information

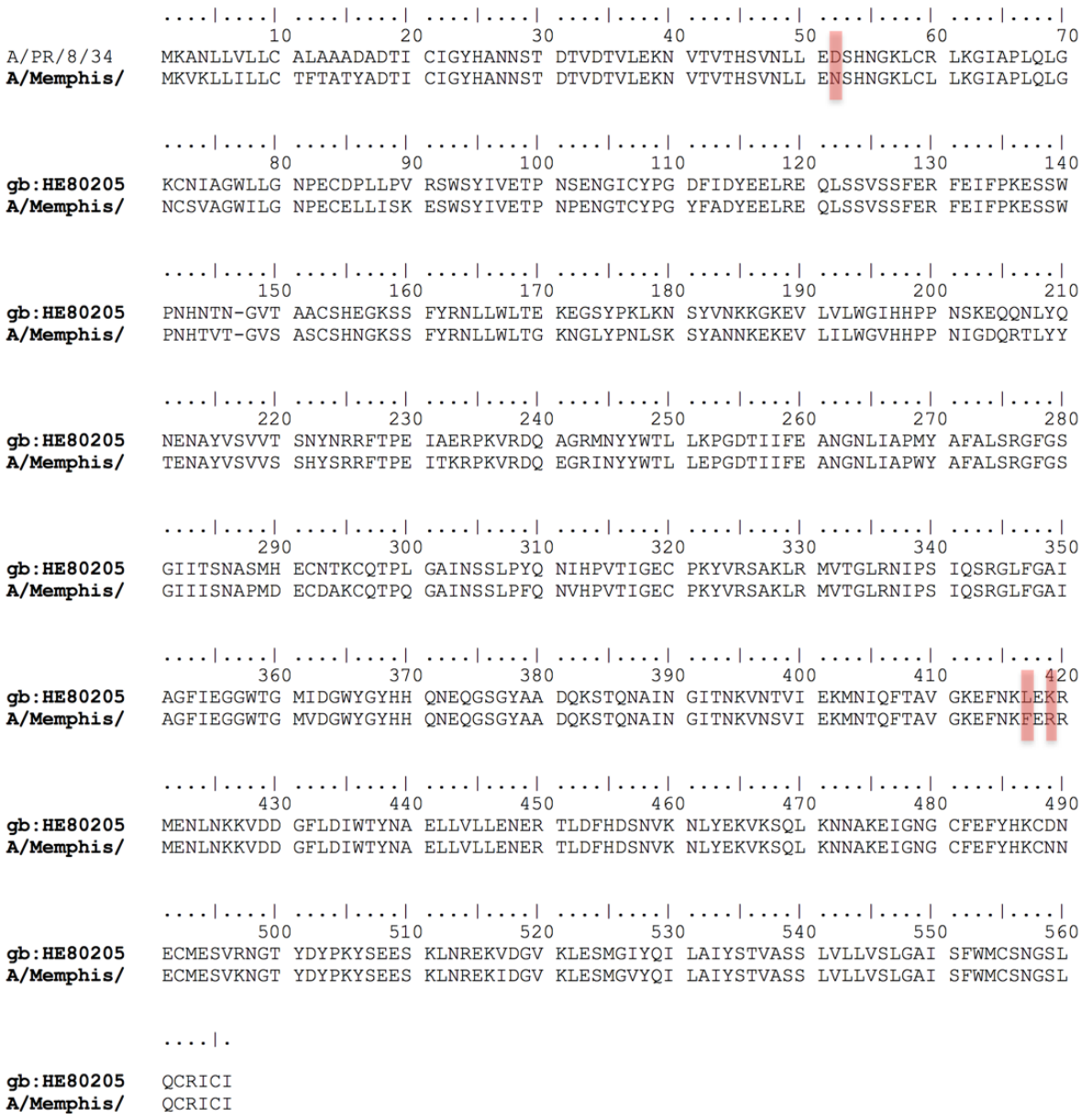

Figure S1. Sequence analysis of A/Memphis/8/2003 H1N2 HA against 8 HA from H1N1 influenza viruses. Related to Figure 2.

Sequence analysis of the Ha stalk sequence of A/Memphis/8/2003 H1N2 influenza against the HA stalk sequences of A/Puerto Rico/8/1934, A/Denver/1/57, A/California/10/78,

A/Chile/1/83, A/Texas/36/91, A/New Caledonia/20/99, A/California/04/2009, and A/Tennessee/F5001/2013 H1N1 influenza viruses.

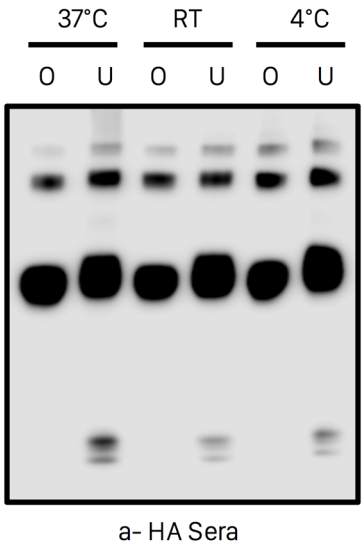

Figure S2. Urumin is effective but has reduced activity at low temperatures. Related to Figure 3.

100μM urumin or control OVA peptide was incubated with 10μg of recombinant HA (A/California/07/2009) for 2 hours at 37°C, room temperature (23°C), or 4°C, protein samples were separated by non-reducing SDS-PAGE and analyzed by western blot using α-HA serum antibodies.

Author Contributions

D.J.H., J.J., S.H.L, and G.A.D. designed experiments and wrote the manuscript. D.J.H, S.H.L., and G.A.D. designed the experimental procedures. D.J.H., S.H.L., and J.R.S. performed the experiments. G.A.D. conducted sequence and bioinformatic analysis. S.G. captured frogs and isolated urumin sequence. A.C.L. generated mutant PR8 viruses.

Acknowledgements

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Chapter 4: Discussion

Urumin as an Influenza Antiviral Therapy

In the second chapter, we demonstrated that the peptide urumin is an effective antiviral agent against human H1 hemagglutinin-bearing influenza viruses. This amphibian host defense peptide, which is naturally secreted by *Hydrophylax bahuvistara*, acts to destroy influenza virions and protects against lethal influenza infection *in vivo (239)*. In and of itself, this ability to protect against influenza infection is not unique to urumin. The annualized influenza vaccines protect millions of people from being infected with the seasonal circulating strains of influenza viruses. Additionally, available antiviral drugs, like Tamiflu (Oseltamivir), a neuraminidase inhibitor, which inhibits viral release from infected cells, shortening the length of severity of symptoms, are available (246). Despite having these vaccines and antiviral drugs, influenza remains the most prevalent human respiratory infection (7-9). Vaccine mismatch and pandemic outbreaks reduce the effectiveness of vaccination approaches and resistant influenza strains to current antiviral drugs renders them ineffective (75-80). Given that landscape, finding new and unique influenza therapies has been of great importance. Our research assessing amphibian host defense peptides for anti-influenza activity is just one of many studies that are discovering and adapting naturally occurring peptides with anti-influenza properties for potential clinical use. These studies have isolated anti-influenza peptides which primarily act by disrupting the influenza envelope, blocking viral entry and fusion with the cell membrane, or inhibiting viral replication (192). Even though anti-influenza peptides are primarily grouped into these classes, urumin does not fit into one of these categories.

What makes urumin unique as an anti-influenza antiviral peptide is that it disrupts the integrity of the virus similarly to peptides that interact with lipid membranes, but does so with the specificity of targeting and binding to hemagglutinin. The preponderance of peptide drugs or conventional therapeutics that target hemagglutinin or neuraminidase on the surface of influenza act as antivirals by either inhibiting viral entry and fusion with the cell, or by inhibiting the ability of newly produced virions to bud from infected cells. Unlike these antivirals, urumin specifically targets and binds to hemagglutinin, and in doing so, actively destroys the virus. An implication of this specificity towards targeting influenza hemagglutinin is that urumin can be used at effective concentrations where there is high influenza viral destruction and low toxicity to the host. Moreover, while the major influenza antivirals on the market today are neuraminidase inhibitors, urumin's specificity for targeting hemagglutinin makes it a potentially important alternative in the event of widespread resistance against that class of drugs. While the current level of neuraminidase inhibitor resistant influenza strains is very low, they are a cause for concern. A single point mutation in neuraminidase can render neuraminidase inhibitors ineffective (78, 79). The danger and consequence would be of the highest severity if one of these neuraminidase inhibitor resistant strains became prevalent and spread amongst the population. To protect against this potential, it is imperative to develop alternative antiviral therapeutics that are functional against resistant influenza strains.

Urumin Clinical Development

As a potential influenza antiviral with activity against neuraminidase inhibitor resistant strains, developing urumin as a clinically viable drug is crucial. The anti-influenza effect is only as impactful as its ability to be translational. In our initial studies, we demonstrated that lyophilized urumin, reconstituted in dimethyl sulfoxide (DMSO), diluted to concentration in PBS and administered intranasally was effective at significantly improving influenza-induced morbidity, mortality, and lung viral titers (239). This was encouraging as a proof of concept that urumin can be developed as a therapeutic. However, requiring patients to inhale peptide, to target the site of infection, as a protective measure is neither practical, nor the best way to protect prophylactically or therapeutically.

Proteases, enzymes which degrade proteins, are expressed by all organisms. In animals, proteases are found throughout the body with high concentrations of metabolic proteases in the blood stream and the gastrointestinal tract. Fortunately for the proof of concept studies, influenza is a respiratory virus, with a mucosal site of infection. By administering the peptide intranasally, urumin was in direct contact with the virus during infection, isolating it from host produced proteases. Amino acids can be produced as enantiomers, also known as two stereoisomers, which are mirror images of each other. Of these two configurations, the L-enantiomer, in contrast to the D-enantiomer, is almost exclusively produced naturally. Because of this, host proteases, for the most part, only demonstrate enzymatic activity against the L-enantiomer of peptides and proteins. D-enantiomer peptides and proteins are not susceptible to this natural degradation. As only the L-enantiomer of urumin demonstrated functionality, and D-urumin was unable to

effectively inhibit viral growth, we are unable to easily circumvent these natural metabolic processes. In a pilot experiment that was not published, we administered HA-tagged urumin intravenously into mice, and assessed their serum peptide levels by ELISA using an anti-HA antibody. L-urumin was found to be metabolized and fully cleared within 6 hours of administration. As such, one of the major focuses for developing urumin as a clinical antiviral is to produce a drug that can be sustained in the host system for an extended period, and is not easily metabolized.

There are different approaches that can be taken to extend the half-life of peptide drugs. As already mentioned, one approach is to use D-amino acids to produce a peptide variant that cannot be degraded by naturally occurring proteases. As urumin requires high specificity and is chirality dependent, the same amino acid sequence, but composed of D-amino acids was ineffective. An alternative method for utilizing D-amino acids is to produce a D-retro-enantiomer or "retro-inverso" peptide. The D-retro-inverso peptide consists of D-amino acids, but in the reverse order as the L-peptide. This reverse arrangement yields a peptide with the same arrangement of amino acid side chains as the naturally occurring L-peptide, but the carboxy and amino ends are in the opposite position (247). A D-retro-inverso urumin may be able to retain the anti-influenza functionality of D-urumin, while also preventing protease degradation.

Another potential approach for stabilizing and extending the lifespan of the peptide *in vivo* is by crafting fusion peptides that bind the peptide to antibodies. The advantages of an antibody-peptide fusion protein are not only extended lifespan, but also, in the case of an IgG-peptide fusion protein, stable, systemic circulation of the peptide in the bloodstream.

With a half-life of approximately 21 days, the IgG-peptide fusion would drastically increase the half-life However, as the site of infection is the mucosa of the lungs and urumin's antiviral activity requires direct interaction with the virus, a fusion peptide system that directs the peptide to that site would be optimal. IgA antibodies are Ig dimers, which while they can be found in the blood, are principally known for being secreted and present in the mucosa. By binding urumin to an IgA antibody with required J chain for dimerization, the administered urumin-IgA fusion would both traffic and be secreted at the site of influenza infection, as well as have approximately a 6-day lifespan. 6 and 21 day lifespans for protection between requiring another dose are not monumental, especially in comparison to protection provided by vaccination. Additionally, whether these Ig fusion peptides are effective in a therapeutic setting, in admitted hospital patients, day 3 or later post infection, will need to be determined. However, in the event of a pandemic outbreak of a neuraminidase inhibitor resistant influenza strain, prophylaxis regiments over a week or over several weeks would likely be a life-saving measure for millions of individuals.

Prophylactic and Therapeutic Strategies

Beyond manipulations to increase stability, target delivery, and extend the lifespan of the peptide, another major concern for urumin's clinical application is strategizing prophylaxis and therapeutic measures. As urumin is a directly virucidal peptide it lends itself more naturally for prophylactic uses. The IgA fusion peptide or nebulized administration of the D-retro-inverso peptide would protect at the site of infection. The prophylactic protection of urumin could also be potentially used for surface disinfection. In chapter 3 we

demonstrated that urumin is not only effective at physiological temperature, but also is effective at room temperature and almost freezing (4°C). This was not a surprising discovery as urumin, being naturally secreted by *hydrophylax bahuvistara,* would have evolved for functionality at ambient temperatures that the frog would experience. Being able to function at a range of temperatures should translate favorable to using a urumin formulation, or potentially the D-retro-inverso peptide in a disinfecting solution. Such a urumin solution would be highly useful for surfaces in medical facilities and for medical personnel. The most challenging development of urumin will be as a therapeutic. As urumin acts upon the virus directly, systemic coverage of the peptide would likely be necessary to reduce viral titers and symptoms of infected individuals. The IgG-urumin fusion peptide may be that solution. Alternatives may require a system that targets urumin to traffic into infected cells.

Modeling a Novel Mechanism of Anti-Influenza Activity

In the third chapter, we focused on the mechanism of urumin's anti-influenza activity. Previously, we concluded that urumin bound to the conserved stalk of hemagglutinin and that its specificity was against H1 hemagglutinin. During our studies in chapter 3 we demonstrated that urumin acts to disrupt hemagglutinin. No other host defense peptide or influenza antiviral has demonstrated activity that acts in such a manner. As such, urumin functions through what we believe to be a novel mechanism of anti-influenza activity. Urumin was ineffective against the H1N2 A/Memphis/8/2003 virus, as well as mutant viruses containing the L417F mutation derived from that virus. However, despite contributing to resistance, L417F is not the binding site for urumin.

Impact of Hemagglutinin Stability

The stability of hemagglutinin on the surface of influenza viruses has demonstrated an important role in contributing to the susceptibility to proteolytic cleavage, the pH threshold for activating conformational changes, and the inter-monomer associating forces (245, 248-251). Single substitution mutations in HA can disrupt the intermolecular interactions between monomers, destabilizing the HA trimer, impeding viral fitness (245). We hypothesize that the phenylalanine at position 417 is a stabilizing residue, which inhibits the effective separation of the hemagglutinin trimer. By preventing this effective separation, position 417 is conferring resistance against urumin. Preliminary experiments demonstrate that urumin can successfully bind to and break the HA disulfide bond in viruses containing the L417F substitution, however, this activity is significantly reduced. We hypothesize that this reduction, but not complete ablation in viral disruption, is indicative of the nature of the reaction kinetics of urumin's activity. While urumin can effectively disrupt 417L containing influenza viruses, the stability of the 3 phenylalanine rings in the hemagglutinin trimer of the L417F viruses shifts the activation energy of the reaction. This shift in activation energy skews the kinetics of the reaction to the extent that urumin's ability to disrupt influenza virions is no longer biologically significant.

Functionality beyond H1 Hemagglutinin

Due to the inability of urumin to be effective against viruses bearing H3 hemagglutinin in chapter 2, we concluded that urumin specifically targets the conserved stalk region of H1 hemagglutinin only. However, while the reduction of H3 virus growth was

significantly less than that of H1 viruses when treated with urumin, the reduction was not 0. The overall reduction of H3-hemagglutinin bearing influenza virus growth was 20%. Given what was learned of the mechanism of urumin activity and the site of binding during the studies detailed in chapter 3, the resistance elicited by H3 influenza viruses likely is not the result of urumin being unable to bind to the hemagglutinin. H3-bearing influenza viruses, like all influenza subtypes contain highly-conserved HA cleavage and disulfide linkage sites. Instead, we hypothesize that similarly to influenza viruses containing the L417F substitution, H3 hemagglutinin has greater stability than H1 hemagglutinin, and urumin activity requires greater activation energy to occur to the extent that the impact is biologically significant. As such, urumin's activity is likely broader than just H1-bearing influenza A viruses. Either by increasing the energy of the reaction to improve the rate of urumin's activity, or by manipulating urumin in a way that reduces the activation energy, increasing the rate of reaction, urumin should be able to significantly reduce H3 influenza viruses.

Based upon the mechanism, where urumin binds to the hemagglutinin disulfide linkage site conserved across influenza A viruses, once the activation energy has been sufficiently reduced, urumin should be effective across all influenza subtypes. Therefore, modifications to improve the effectiveness of urumin will likely lead to the characterization of urumin as an omnibus anti-influenza effector, not only as an agent that specifically targets H1 hemagglutinin. One method that we anticipate will decrease the activation energy of urumin's activity and increase the rate of reaction across all influenza A viruses is the binding of urumin to antibodies as fusion proteins. In the first section of this chapter, the production of IgA-urumin and IgG-urumin fusion proteins was discussed. In addition to increasing the

stability, half-life, and trafficking of urumin within a host, these fusion proteins will also substantially increase the mass of the molecule. The increased mass should increase the ability of urumin to separate the hemagglutinin trimers; which we will study experimentally. Another method to increase the reaction rate and assess urumin's antiviral activity against other influenza subtypes, is to test even higher concentrations of urumin. As the urumin toxic dose (TD_{50}) occurs at a concentration of 2450μM, much higher concentrations than 100μM (as tested in the *in vitro* studies in chapter 2 and 3) can be assessed and considered a viable option for *in vitro* and *in vivo* use. Significant increases in temperature to increase the reaction rate will not be viable as protein denaturation will be a concern with substantive increases in temperature beyond 37°C. Increases in temperature would also not be feasible in an *in vivo* setting.

Implications for future anti-influenza therapeutics

The initial discovery of an amphibian host defense peptide with virucidal activity against H1-bearing human influenza viruses demonstrated that powerful and specifically targeted molecules can transcend beyond the utility of the host species. As we have explored beyond the specificity of urumin's activity, into how it is functioning, we have uncovered a mechanism that is the beginning of a new class of anti-influenza therapeutics. We have importantly found a new weakness in influenza for exploitation. Previous focuses on hemagglutinin for antiviral properties have involved inhibiting the ability of HA to bind sialic acid on cell surfaces, as well as blocking the ability of it to cleave intracellularly as part of its replicative process. We have demonstrated that the separation of HA1 and HA2 has

disastrous effects for the virus with dissociation of the hemagglutinin trimer and the disruption of viral integrity. The major implication, beyond the scope of urumin as an antiviral, is that the hemagglutinin disulfide linkage is a crucial factor in viral stability. Targeting the HA disulfide bond and inducing hemagglutinin separation may be a key aspect for not only antiviral development, but also efforts to produce an influenza vaccine with broader activity than the current configurations.

The Untapped Resource of Host Defense Peptides

Overall, amphibian host defense peptides, and host defense peptides in general, present an important area for the future of drug discovery. For millennia, host defense peptides have evolved with their hosts to best protect them from the pathogens that have evolved with them. As host defense peptides often have activity against the most conserved and crucial elements of their targets, be they bacteria, fungi, or viruses, they are less prone to be subverted by resistance. Additionally, these targeted motifs are often not limited to pathogens that infect the given species. Because of this feature, if a pathogen contains the motif or element that the peptide acts upon, the host defense peptide can cross-neutralize that bacteria or virus, regardless of the species that encounters it. Since the pioneering discoveries in the 1970's, research into amphibian host defense peptides has expanded our understanding of these molecules and provided key insights in the development of novel therapeutics. The breadth of host defense peptide research should not specifically focus on the peptides of certain organisms. Amphibians present an ideal source because of the quantity that they secrete and their species diversity. However, sources from all types of

organisms; plants, invertebrates, and vertebrates have uniquely evolved sets of peptides that ought to be researched to augment our understanding of pathogens and their weaknesses that could be exploited through host defense peptides.

Natural selection has honed these host defense peptides. In the example of urumin, *hydrophylax bahuvistara* does not require a peptide with virucidal properties against human influenza viruses. Urumin likely evolved to protect these frogs from viruses, whether influenza or not, that contain a motif like found in influenza viruses. As every living organism produces host defense peptides, each species provides the opportunity to find a unique peptide with unique functionalities. One or many of these unique species may produce peptides containing key sequences that can act against human pathogens for which we do not have an effective vaccine, antibacterial, or antiviral therapy. The story of urumin should inspire creative therapeutics for breakthroughs in research on HIV, Zika virus, Dengue virus, SARS coronavirus, multidrug resistant bacteria, and Ebola virus.

Future Directions

While chapter 2 and 3 detail our findings to date in our studies of urumin, there is substantial work remaining. Some of the studies that are ongoing include isolating the bands from the SDS-PAGE gels of urumin-treated influenza viruses to be analyzed by mass spectroscopy so that we can confirm the contents of the bands as well as confirm where urumin is specifically binding molecularly to hemagglutinin. Another ongoing study is to assess urumin's functionality against L417F containing influenza viruses, as well as H3N2 influenza strains. Based upon a preliminary experiment and our working model, urumin can

disrupt the hemagglutinin trimer and break the disulfide bond between HA1 and HA2 of viruses containing the L417F mutation, however to a lesser extent than the 417L viruses. We are working to conduct SDS-PAGE and western blot analyses to determine if H1 L417F, H3, and H5 hemagglutinin is susceptible to urumin-induced trimer disruption and HA disulfide disruption. We hypothesize that they will be, but to a reduced extant in comparison to H1N1 viruses, as indicated by the lack of biological significance as measured by viral plaque and focus-forming assays.

In addition to these upcoming experiments, the long-term directions for this project are to better understand urumin's mechanism of action and to develop systems to improve urumin as a clinical therapeutic. Understanding the mechanism of urumin action will be a major effort in the future of this project. Truncated variants of urumin will be tested to specifically determine the crucial core aspects of the peptide for trimer separation and HA disulfide bond disruption. We hypothesize that at the very least, the segment containing the 2 cysteine residues will be necessary for activity. Additionally, as the specifics of the binding and cleavage mechanism are determined, these details will inform the ability to improve and generate a new class of virucidal hemagglutinin targeting anti-influenza peptides. To develop urumin as a clinical therapeutic, we will focus on modifications that improve stability and the half-life of the peptide, specifically traffic the peptide, and increase the rate of reaction of urumin's activity as outlined earlier in this chapter. We will also assess the host immune response to these various modifications and if antibodies against the peptide variants are produced. Beyond initial *in vitro* experiments and *in vivo* studies in BALB/c mice, ferret

studies will be conducted as ferrets are the premiere model for influenza transmission (252). These experiments will ultimately lead to an optimized system for human clinical trials.

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