EFFICACY OF BRILACIDIN AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

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ABSTRACT

EFFICACY OF BRILACIDIN AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

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Venezuelan Equine Encephalitis Virus (VEEV) is a positive-sense, single-stranded, enveloped-RNA alphavirus of the Togaviridae family that is transmitted through the bite of an Aedes aegypti and Aedes albopictus mosquito species. There are no vaccines or therapeutic treatments available for VEEV, which exacerbates the need for better therapeutics. It is important to have therapeutics available that can inhibit viral replication and decrease the viral load early to stop the progression of neurological illness or damage and lessen the severity of other associated symptoms. Brilacidin is a potential antiviral drug that can be used to inhibit VEEV infection given its mechanistic ability to interfere with viral replication at the cell membrane. The efficacy of Brilacidin in VEEV will be evaluated through a series of experiments that will explore the dynamic of inhibition, safe testing concentrations, and its peak window of efficacy to gain a better understanding of the mechanistic impact on the host cell's phospholipid bilayer in cell culture.

VENEZUELEN EQUINE ENCEPHALITIS VIRUS

The Venezuelan Equine Encephalitis Virus (VEEV) is a positive-sense, single-strand, enveloped, RNA alphavirus of the Togaviridae family that is typically transmitted through the Aedes aegypti and Aedes albopictus mosquito species (15). There is no vaccine available for VEEV, which exacerbates the need for therapeutics until a safe and reliable vaccine is available. VEEV has the potential to re-emerge as an epidemic because it is easily transmitted through mosquitos with high rates of infection. VEEV is a category B select agent with the potential to be aerosolized and used as a biological weapon. If a large population of people were to be exposed to VEEV, the healthcare system would not be able to directly treat the viral infection because there are no vaccines or targeted therapeutics available. Infants and the elderly population with pre-existing comorbidities are the most vulnerable populations to VEEV infection (12). Maternal-fetal transmission is possible which can lead to high rates of infant mortality (12). The current standard treatment for VEEV relies heavily on non-steroidal anti-inflammatory drugs and analgesic agents used to treat fever, severe joint pain, and other associated symptoms of infection. There are no therapeutics available to directly treat and inhibit the viral infection. Over the last decade, there has been a re-emergence of alphavirus documented in the Americas, Asia, and Europe including o'nyong nyong virus (ONNV) and chikungunya virus (CHIKV) (4). Whether in the case of a re-emergence or biological

warfare event, it is important to have established safe and effective anti-viral therapeutics available to treat the infection. There is a pressing need for an anti-viral therapeutic that can reduce the viral load, lessen the severity of infection, and prevent the infection from progressing to encephalitis. The goal of my thesis is to evaluate the efficacy of Brilacidin as a potential antiviral drug to treat VEEV infection in cell culture. The efficacy of Brilacidin will be evaluated through a series of experiments that will explore appropriate dosing, the dynamic of inhibition, peak window of efficacy, and to gain a better understanding of Brilacidin's mechanistic impact on the host cell's phospholipid bilayer in cell culture. Brilacidin may prove to be an efficacious drug to treat VEEV infection given its mechanistic ability to interfere with viral entry and replication localized at the cell membrane.

Alphaviruses

Alphaviruses are zoonotic and encephalitic arboviruses of the Togaviridae family that are transmitted to humans through Aedes aegypti and Aedes albopictus mosquito species. The encephalitic alphaviruses are Western Equine Encephalitis Virus (WEEV), Eastern Equine Encephalitis Virus (EEEV), Ross River virus (RRV), Chikungunya virus (CHIKV), Highlands J virus (HJV), and VEEV lead to neurological symptoms in humans and equines (18). WEEV, EEEV, and VEEV can be further classified as the New World Alphaviruses known to cause a variety of arthritogenic and encephalitogenic diseases. The Old-World alphavirus are classified as Sinbus virus (SINV), RRV, and Semliki Forest virus (SFV), and CHIKV. The old world and new world classification of alphaviruses stems from differences in pathogenesis and the changes recognized in the

alphavirus genome over the years. WEEV, EEEV, and VEEV have a geographical distribution across North, South, and Central America. Today, VEEV transmission takes place mainly in North, South, and Central America. WEEV, EEEV, and VEEV were first recognized in the United States in diseased horses in California, Virginia, and New Jersey in the 1930s. Although, EEEV and WEEV have more widespread geographical distribution. These viruses are naturally transmitted by hematophagous arthropods (18).

WEEV was first recognized in the brains of horses in California in 1930 via a neutralization assay. "WEEV is maintained in an enzootic cycle between its natural vertebrate hoss, passerine birds, and its most common mosquito vector, *Culex tarsalis*, a species associated with irrigated agriculture and stream drainages in the western U.S. Transmission to horses and humans is mediated by so-called bridging mosquito vector specials including *Ochelerotatus melanimon* in California, *Aedes (Ae.) dorsalis* in Utah and New Mexican and Ae. Campestris in New Mexico (18)." Given the enzootic nature of the virus, transmission of the virus can occur relatively quickly with its many hosts. Also, it is possible for the virus to be reintroduced via migratory birds. According to the U.S. Centers for Disease Control and Prevention (CDC) there were 639 confirmed human cases of WEEV from 1964 to 2005 (18). The clinical presentation of WEEV is asymptomatic or mild non-specific illness following 2–7-day incubation period. Progression to encephalitis is seen in the minority of infected individuals. Overall, the fatality rate of WEEV is humans estimated between 3-7%. Today, there is an available vaccine for horses specific to WEEV. The fatality rate of WEEV in horses is 50% higher compared to the estimated fatality of humans.

The main vector of EEEV transmission in horses and humans are *Aedes*, *Coquillttidia*, and *Culux* species. Transmission mainly occurs around freshwater hardwood swamps in Florida, Georgia, Massachusetts, and New Jersey. According to the CDC, there were 220 confirmed human cases of EEEV in the U.S. from 1964 to 2004. The clinical presentation of EEEV is the most severe and virulent compared to WEEV and VEEV. The pathogenesis of EEEV is associated with progression of severe neurological disease couples with encephalitis, fever, headache vomiting and seizures that onset after 4–10-day incubation period. The fatality rate of EEEV is between 50-70% in humans. The mortality rate of EEEV is higher in horses compared to WEEV as well. The same vaccine used to inoculate horses from WEEV can be used for EEEV as a double vaccination to bolster similar efficacy seen with WEEV. EEEV is detected through the use of serological assays. Given its severe clinal presentation, there are further studies that utilize magnetic resonance imaging (MRI) and computed tomography (CT) to understand the acute physiological phenotype of encephalitis. The imaging studies revealed changes in the basal ganglia and thalami that suggest brain edema, ischemia, and hypoperfusion seen in the early stages of disease.

CHIKV is a reemerging mosquito-borne arbovirus characterized by febrile disease and severe polyarthritis known to be endemic in tropical and subtropical regions of sub-Saharan African and Southeast Asia. The disease was named chikungunya, from the Kimakonde language, which translates to “that which bends up”. CHIKV was isolated in the serum of an infected patient with debilitating arthritic disease characterized by stooped posture and rigid gait. The acute CHIKV infection present as fever,

incapacitating polyarthralgia, arthritis, rash, myalgia, and headache. The acute symptoms of CHIKV mostly resemble the dengue fever. There is reason to believe that CHIKV outbreaks have been taking place since 1779, and often misdiagnosed as dengue fever. The greatest difference between CHIKV and dengue fever is the recurrent musculoskeletal disease associated with CHIKV. “CHIKV disease is often self-limiting and has a low fatality rate (~0.1%) (16)”. Although, CHIKV has a low fatality rate, there are people who suffer from chronic arthritic musculoskeletal disease in the peripheral joints that can linger over the span of months to years leaving them disabled.

CHIKV was first recognized as a human pathogen in 1952 following an outbreak of severe polyarthritis in Tanzania. “Phylogenetic analyses of CHIKV sequences indicate that CHIKV originated in Africa over 500 years ago, and a common lineage diverged into two distinct branches, termed West African and East/Central/South Africa (16).” The West African strain is associated with enzootic transmission with smaller outbreaks of human disease. The East/Central/South African strain is known to have repeatedly spread to new regions and cause significant epidemics. This strain was recognized 70-150 years ago in Asia, and the virus continued to spread throughout Asia and develop its own strain with an Asian distinct genotype. At the end of 2004, this strain was recognized again in Kenya during one of the largest CHIKV epidemics. As result, the epidemic in Kenya led to new areas of expansion beyond its historical range. International air travel was the main facilitator of geographic expansion of CHIKV. For example, in 2005, there was a massive outbreak of CHIKV in several Indian Ocean islands that led to over 6 million cases of disease and created an economic burden within the region. Following the

original epidemic in Kenya from 2004, that same strain was recognized in Italy in 2007, France in 2010, and Brazil in 2014. In today's global and well-connected world, that only increases the chances of re-emergence and expansion of alphavirus.

VEEV was first isolated in 1938 through the brain of an infected animal found in Venezuela. The most recent outbreak of VEEV was seen in 1995 in Venezuela and Columbia with 75,000-1000,000 document cases in humans. In addition, there were more than 300 fatal cases secondary to the development of encephalitis. The clinical representation of acute VEEV infection is defined by fevers, headaches, fatigue, and other flu-like symptoms that can progress to severe neurological illness and/or encephalitis (2). If the disease does not progress to the point of neurological damage from encephalitis the fatality rate is < 1%. Cases of VEEV with known encephalitis have a fatality rate of 20% often with long-term neurological damage. VEEV has the potential to be developed as a biological weapon inherent to its ability to be easily aerosolized and mass produced. During World War II, VEEV was first developed as a biological weapon in the US and within the former Soviet Union. VEEV infection is highly infectious and more likely to develop the encephalitic phenotype of the infection when the host is exposed via aerosol. A murine model that mimics VEEV in equines and humans revealed at the later stages of infection the virus is undetectable in the peripheral organs and blood, but the levels and extremely high in the brain. Once the levels of virus are only detectable in the brain it is likely for the host to die within 5-7 days. VEEV infection establishes a high viral load that leads to inflammation in the brain and will override the blood-brain barrier. Neuronal cells are highly susceptible to infection via VEEV. Standard treatment

consists of non-steroidal anti-inflammatory drugs, analgesic agents to regulate fever and joint pains, and other treatments are symptomatic (4). From a treatment perspective, it is important to have therapeutics that can inhibit viral replication and decrease the viral load early to stop the progression of neurological illness or damage and lessen the severity of other associated symptoms. The need for anti-viral therapeutics against VEEV is immediate and should focus on preventing VEEV from spreading to the brain.

Structure

SINV is considered to be a structural prototype of alphaviruses. Through the use of electron cryomicroscopy, the three-dimensional structure of a SINV in the membrane has been established (14). The intact virion contains two icosahedral shells of viral encoded proteins separated by a membrane. The three-dimensional structure of the virus shows proteins in both shells with an icosahedral lattice. Electron cryomicroscopy revealed evidence of connection between the glycoprotein and nucleocapsid C protein from across the membrane. The interaction of the virion and the host cell at the membrane are critical to understanding the facilitation of infection and viral assembly. The protein-protein interaction that takes place at the host cell membrane is between the encoded proteins of the virus and the host cell membrane. These interactions may be an indicator of virion stability. The virion interacts with the host cell through two pathways for the majority of enveloped viruses. The first pathway the virus encoded membrane proteins have a specific conformational position into the membrane of the cell endoplasmic reticulum that will process and transport into the maturation of the virus membrane proteins while targeting the host cell membrane. The viral encoded proteins

E1 and E2, are positioned precisely as an icosahedral lattice stabilized by disulfide bridges within E1, and further stability is gained from the negatively charged host cellular membrane.

The alphavirus nucleocapsid is a positive sense single strand of RNA combined with a capsid protein C that has a three-dimensional icosahedral symmetry. The isochron symmetry is best explained as triangulation figure. The presence of the icosahedral lattice within the nucleocapsid implies communication and connection between the and the C protein. The C protein is 29 kDa. E1 and E2 maintain the icosahedral lattice structure. The disassembly of the virion membrane upon entering the host cell is likely to require a mechanism of disruption of the lattice via disassembly of disulfide reactions. Electron cyromicroscopy of VEEV TC-83 to a 4.4 resolution clearly defined the structure of E1 and E2. Surprisingly, this analysis also showed an E3 protein that is typically absent in a mature virion. The structural picture gained from the previous electron cryomicroscopy study provides insight on viral entry, viral attenuation, host recognition, and its innate immune response.

Genome

The genome of VEEV consists of 3 structural and 4 nonstructural proteins together is 11 to 12 kb in size (6). The RNA alphavirus has a 5’ cap and 3’ poly a tail. Upon infection the genome consists of one mRNA to encode the nonstructural proteins, and another smaller mRNA strand to produce the virion proteins. The four nonstructural proteins (nsPs) are recognized as nsP 1-4, and the structural proteins consist of a capsid protein and 2 envelope glycoproteins (E1 & E2). The alphavirus genome consists of an

early replication complex that leads to the encoding of the mature replication complex that consists of all four non-structural proteins. The early replication complex is made up of P1234 which breaks down to P123 and nsp4 and is responsible for negative sense RNA synthesis. The mature replication complex is made up of P123 which will break down into nsP1, nsP2, and nsP3 to encodes for the positive sense RNA synthesis.

nsP1 is responsible for the capping of mRNA to protect is from degradation by exonucleases. The alpha-helical amphipathic loop and palmitoylation sites anchors the replication complex directly to the plasma membrane secondary to its interaction with anionic phospholipids. nsP1 anchors the replication complex to the cell membrane, directs viral replication, is critical for RNA synthesis, and capping activities for viral RNA. nsP2 controls the innate antiviral defense by shutting down transcription and translation in the host to stop the innate immune response. nsP2 has the most conserved structural motifs of all the nsPs spanning across the OW and NW alphaviruses. The conserved structural motif is indicative of a Cys/His catalytic binding pocket, and cleavage site at the nsp2 protease. nsP2 is recognized as a virulence factor because of its direct role in inhibition of the interferon (IFN) mediated antiviral response (4). nsP3 is recognized as the N-terminal macrodomain that is defined by its phosphatase activity and nucleic binding ability. Within the macrodomain lies an alphavirus unique domain (AUD) with a zinc coordination site. The zinc coordination site works collaboratively with the C terminal domain to regulate viral transcription. In the absence of the C terminal there has been less viral pathogenicity. nsP3 contributes to viral RNA transcription and plays a supportive role in viral replication cycle as well. nsp4 contains

the RdRp polymerase that is responsible for viral transcription. The RdRp polymerase is highly conserved within the alphavirus genome. nsP4 is responsible for the addition of RNA synthetic properties to viral RNA. Overall, the nsPs contribute to the maintenance and execution of successful viral replication localized across the span of the four nsPs.

The transition between structural and non-structural is facilitated by the translation of 26S by P123. 26S is the sub genomic positive sense that generates a structural polyprotein that is cleaved into cleaved into five structural proteins. The first major structural protein is the capsid protein is critical in binding to the viral RNA to facilitate viral assembly. The remaining two major structural proteins are surface expressed enveloped glycoproteins known as E1 and E2. As the structural proteins are processed and encoded, there are two small cleavage products E3 and 6k left over. E1 and E2 is formed after the assembly of the capsid protein which buds at the cell membrane to acquire a lipid envelope contains the two glycoproteins.

Viral Replication Cycle

Alphavirus replication cycle is similar across the genus of Old World and New World viruses, but less is known specifically about VEEV. Viral replication takes place in the cytoplasm of the host cell. Upon infection, the virion will attach itself to a GAG receptor with the help of an unknown attachment factor. The attachment factor is believed to exhibit heparin binding properties, but its complete mechanism is unknown. "Glycosaminoglycans, which are expressed on many susceptible cell types, appear to serve as attachment factors to enhance infectivity (12)." Each virion contains a capsid protein with genomic RNA encapsulated inside of the virion and its lipid bilayer is

enveloped by E1/E2 heterodimers. The E1/E2 heterodimers play a critical role in viral attachment and entry. The E2 heterodimer aids in mediating viral attachment to the host cell's receptor before clathrin-mediated endocytosis takes over. Once the virion has made contact with the GAG receptor and its attachment factor, the virion can enter the cell via clathrin-mediated endocytosis. Endosomal acidification triggers a conformational change in the viral glycoproteins to aid in further exposure and insertion of the buried E1 fusion loop to directly insert itself into the host cell membrane. At this point, the virion will fuse into the endosomal membrane, and the genomic RNA is released inside the host cell. The nucleocapsid protein will dissemble itself to release the genomic viral RNA into the cytosol of the host cell to prepare for translation. Translation begins with the production of the nonstructural precursor protein P1234. P1234 is cleaved by the encoded nsP2 protease to produce P123 and nsP4. P123 and nsP4 together at this point in translation form the early processing stage of translation that is short-lived in order to continue into the mature processing stage of translation to synthesize a full-length negative sense strain of genomic viral RNA. Upon the completion of negative-strand synthesis, translation of the genomic RNA continues with the packaging of viral RNA into spherules localized on the cell membrane that will internalize to form a cytopathic vacuole (CPV-I) structure with markers for endosomal and lysosomal membranes. CPV-I serves as the site of genomic viral RNA replication at the cell membrane following infection. CPV-I will further break down to release the capsid protein and genomic RNA to form a nucleocapsid. The negative sense stand of the viral genomic RNA is used as a template for amplification of genomic positive-sense viral RNA that is used to encode the

structural proteins of the genome. The first major product released is E3-E2-6K-E1, followed by the minor product E3-E2-TF. 6K is also known as a transframe (TF). The 6K, TF, and E3-E2 complex serve as the viral accessory proteins that will be cleaved off in the final stages of processing. E1, E2, and the capsid proteins are the only major products once the structural proteins have been fully processed. After the completion of translation and processing of the viral genomic RNA, the nucleocapsids will form a CPV-II structure with the nucleocapsids on their cytoplasmic face with the ability to exit the cell or remain a part of the structure at any time. It may serve as an assembly intermediate, but it is unclear its specific role in the process. The complete function of the CPV-II structure in viral replication is not completely clear how it contributes to infection and pathogenesis. Viral replication is a highly controlled process. Clathrin-mediated endocytosis is one of the first steps in viral replication, that allows for the viral RNA and its genome to be released inside the host cell so that viral replication can proceed without any disruptions from activity outside in the cell. At this point in infection, Brilacidin is expected to interfere and exhibit the majority of its inhibition in the early stages of viral replication.

Innate Immune Response

The primary innate immune response to alphavirus infection is the type I interferon response (7). "Type I IFN signaling controls viral replication and pathogenesis during acute infection (16)." Type I IFN response leads to the activation of a downstream signaling cascade of interferon stimulated genes that try to stop viral replication. The CHIKV alphavirus acute infection is known to elicit a robust immune response defined

by the elevation of type I IFNs, proinflammatory chemokines, cytokines, and growth factors (12). Infection causes the host cell to shut down by suppression of the antiviral response and inhibition of antiviral response pathways. Type I IFNs are important to understand as the primary antiviral defense mechanism early on in viral replication. The immune system recognizes viral RNAs as pathogen associated molecular patterns (PAMPs) that are recognized by host receptors RIG-I-like (RLRs) and Toll-like receptors (TLRs). TLRs are pathogen recognition receptors that trigger the innate immune response. The body's natural immune response is not always sufficient to provide protection and survival from infection. RLRs take it a step further past recognition and serve as a cytoplasmic RNA helicase that signals the activation of transcription factors necessary to engage proinflammatory cytokines.

The brain tries to protect itself from VEEV through the production of B cells to prevent viral entry into the central nervous system. Once the virus has breached the blood brain barrier, the innate immune system has less ways to fight off infection and stop the progression on encephalitis. Understanding the viral replication cycle and innate immune response are crucial for the development of better therapeutics to treat the infection and reduce the viral load by inhibition of viral replication.

VEEV exhibits a known inflammatory response via inflammation-induced tissue damage, disruption of the blood-brain barrier integrity and aid in viral dissemination. The excessive inflammatory load elicited by VEEV can play a role in slowing disease pathogenesis and progression. Although the high levels of inflammation can have other

negative downstream affects that VEEV can use to its advantage in the progression of neurological disease development.

Inflammation is a natural response of the innate immune system that needs to be taken in consideration during the development of anti-viral therapeutics. It is equally important to control the viral load it is levels of inflammation throughout the body. Host Defense Peptides (HDPs) are naturally expressed by the immune system in response to foreign pathogens and viral infections. HDPs are good candidates for anti-viral therapeutics given their anti-viral activity an ability to modify and activate the immune response. LL-37 is a naturally occurring HDP in humans that has shown anti-viral activity against VEEV by inhibiting viral replication. "The antiviral activity of LL-37 has been attributed to its direct interaction with virions, which disrupts viral membranes, as well as its role in modifying and activating immune responses through the recruitment of immune cells such as monocytes and macrophages, downregulation of pro-inflammatory cytokines and upregulation of genes involved in anti-inflammatory responses (1)." Based on what is known about HDPs mode of action, this thesis was developed around exploring the efficacy of a synthetic host defense peptide against VEEV.

BRILACIDIN

Brilacidin is a computationally derived synthetic non-peptidic mimetic that mimics the structure and function of a host defense protein. It was developed and computationally derived from a series of small-molecule aryl amide mimics of antimicrobial peptides (AMPs) that feature a small arylamide backbone stabilized via hydrogen bonding, cationic and hydrophobic substitutions with a molecular mass of -1,000Da (12). The optimization of the aforementioned compounds against *staphylococcus aureus* resulted in a lead compound known as Brilacidin (PMX30063). Brilacidin exhibits anti-viral activity against SARS-CoV-2 by disrupting the viral integrity and impacting viral entry. Based on Brilacidin's mode of action against SARS-CoV-2, the efficacy studies for this thesis were designed based on the assumption Brilacidin will have a similar effect on viral integrity and entry. Brilacidin is a novel synthetic compound that has great potential as an anti-viral against VEEV TC-83 in cell culture.

History of Discovery

AMPs are evolutionary conserved molecules found in all living organisms. The lysozyme was the first antimicrobial peptide discovered in 1922 by Alexander Fleming. This discovery was overshadowed by Fleming's soon after discovery of penicillin in 1928 which was revolutionary in its time as an antibacterial treatment. The discovery of

AMPs began in plants until the 1960s brombinin was discovered in frogs and lactoferrin from milk. In the 1960s, multidrug resistance to antibiotics became a more common complication, and scientist began to refocus on assessing the therapeutic potential of AMPs. AMPs have the potential to evade multidrug-resistant that is seen with the use of standard antibiotics. AMPs are gene-encoded antibiotic proteins produced in nature. A study conducted by Hans Boman in 1981 reported the injection of bacteria into Cecropia silk moth induced a potent AMP called cecropins which was one of the first alpha-helical AMPs to be discovered. In 1994, AMPs were discovered in mammalian skin and in mice which led to the discovery of AMPS host defense mechanism. To date there are more than 2,500 AMPs collected in the Antimicrobial Peptide Database. AMPs have been discovered throughout the human body in places that are typically exposed to microbes' skin, lungs, intestinal mucosa, oral mucosa, and in the reproductive tract.

Antimicrobial Peptides

AMPs are their own class of peptides and proteins that are a part of the innate immune response in all multicellular organisms (19). This class of molecule has a wide range of inhibitory activity against bacterial, yeast, fungi, viral, and cancerous pathogens (19). AMPS are a diverse class of naturally occurring molecules that serve as the first line of defense against viral infection, inflammation, and/or injury. These proteins have a broad spectrum of activity against bacteria, yeast, fungi, viruses, and cancer cells. The majority of well-studied AMPS are in the context of bacteria, yeast, and fungi. There is a lot more to learn in the anti-viral context specifically.

Host Defense Peptides

Antimicrobial peptides are also referred to as host defense proteins secondary to their interaction the host's innate immune system seen in higher eukaryotic organisms. Host defense peptides have the ability to modulate host immunity. "In higher eukaryotic organisms, AMPs can also be referred to as host defense peptides', emphasizing their additional immunomodulatory activities (19)." The specific inhibitory activities are dependent on the type of AMP. AMPs vary in cytokines and growth factors that contribute to normal immune homeostasis. Defensins and cathelicidins make up the majority of AMPs seen in vertebras. Beta defensins are seen more within the gastrointestinal track. There is a class of beta-defensins produced by keratinocytes, which is the cell type that constitutes the majority of the epidermis. This specific class of AMPs is known to respond to wounds and infections. There are also amphipathic AMPs like the well-studied human cathelicidin peptide LL37 are derived from keratinocytes. It is named for its conserved amino-terminal domain called the cathelin because it was first recognized for its ability to inhibit cathepsin L. Cathelicidins were the first AMPs to be discovered in mammalian skin. Further research and studies have revealed several more classes of AMPs derived from all different cell types like eccrine sweat glands, neutrophils, dendritic cells, and bone-marrow derived cells. Intestinal AMPs are known to get rid of ingested pathogens and contribute to balancing the microbiome. Amphipathic AMPs adopt the amphipathic alpha-helical structure in a non-polar environment like the cell membrane. They do not exhibit much of a secondary structure

in aqueous solution. In addition, there is another class known as extended AMPs that lack a specific structural motif but are completely defined by their content of specific amino acid residues. For example, histatins AMPs seen in humans are rich in histidine residues. These types of extended AMPs can also be seen with high contents of arginine, glycine and tryptophan.

Structure

The structure of Brilacidin features a planar scaffold that is conformationally restrained by four positive guanadinyl and pyridinyl substitutions and two trifluoro methane hydrophobic substitutions (12). "Cationic AMPs typically consist of 10 to 50 amino acid residues with an overall positive charge (19)." AMPs tend to have an overall positive net charge paired with a strong presence of hydrophobic amino acids. The typical distribution of AMPs is made up of basic amino acids residues and hydrophobic residues. The electrostatic interactions between AMPs and the membrane of the virion are highly favorable. Most AMPs have an amphiphilic topology with a positively charged face intended to interact easily with the negatively charged cell membrane. "Many AMPs are derived from larger precursor proteins with a signal sequence at the amino terminus and the antimicrobial peptide domain at the carboxyl terminus (4)." The alignment of the residues is three dimensional facing opposing sides which leads to its water-soluble properties. AMPs can be further classified based on their secondary structure: alpha-helical, beta sheets, and extended AMPs. Alpha-helical AMPs like defensins are the most prevalent host defense peptide in human neutrophils. Beta-sheets are more likely bactenecins and defensins with two or more beta-sheets stabilized by disulfide bonds.

The primary mechanism of AMPS is in some way interfere with the membrane in order to exhibit immunomodulatory activities (19)." The specific inhibitory activities are dependent on the type of AMP. AMPs vary in cytokines and growth factors that contribute to normal immune homeostasis. Defensins and cathelicidins make up the majority of AMPs seen in vertebras. The structure of Brilacidin exhibits favorable interactions with the virion membrane of VEEV thus making it good potential anti-viral candidate.

Mechanism of Action

The primary mechanism of action for most AMPs is cell death secondary to dysregulation or loss of integrity of cell membranes. AMPs disrupt and alter cell membranes through dysregulation, pore formation, causing membrane leakage, depolarization, barrel stave mechanism, and/or carpet toroidal pore. Pore formation within the membrane leads to decreased membrane integrity and increases likelihood of membrane disassembly. This mechanism leads into direct inhibition and lysis of targeted microbes within the cell. AMPs are known to interact with bacterial cell membranes and initiate cell death by disrupting the properties of the phospholipid bilayer. Cell membrane and cell wall stress may also contribute to Brilacidin's mechanism of action. Some AMPS have been identified as having downstream cytoplasmic targets in addition to disruption of the cell membrane. The non-enzymatic disruption of the cell membrane secondary to the binding of an AMP. AMPs have the potential to disrupt the membrane through enzymatic digestion as well, but this is less common. For example, the lysozyme is known to hydrolyze its own bacterial membrane. There is a highly specific class of

AMPs that can cross the membrane without compromising its integrity but will directly kill targeted molecule/pathogen via the inhibition of an enzyme or biosynthesis of proteins and nucleic acids. The primary target of Brilacidin is the cell membrane and its associated properties. Brilacidin is known to cause dose-dependent membrane depolarization against Staphylococcus aureus. Brilacidin has been used as an ocular anti-infective to treat eye infections. In this context, its primary action is depolarization of the cell membrane that leads to membrane leakage and disassembly.

Brilacidin may have other downstream targets and secondary to membrane depolarization. A comparative study on Brilacidin and similar compounds utilized next generation sequencing technology to analyze the transcriptional profile and response. Transcriptional profiling asses how bacteria respond to physiological insult as result of drug treatments. Gaining a better understanding of the physiological response provides insight into the mechanism of action. "At the highest concentration of treatment, brilacidin caused the upregulation of 698 genes at a >3-fold change threshold (at any time point) ($P<0.05$) (12)." The majority of genes upregulated by the developed series of small molecule arylamide mimics of AMPs were genes associated with cell membrane and periplasmic misfolding stress. The transcriptional profile of Brilacidin showed a signification induction of the NsaSR, VraSR, WalKR, and GraSR two-component systems. GraSR is an antimicrobial peptide sensing. NsaSR is nisin sensitivity associated. WalKR regulon is involved in cell wall and membrane turnover and maintenance. VsaSR responds to cell wall stress and upregulated genes involved in lipoteichoic acid and peptidoglycan synthesis. NsaSR, GraSR, VraSR and WalKR are induced during cell wall

stress secondary to the accumulation of misfolded proteins. The induction of these systems is either a direct or indirect consequence of the AMP treatment.

Brilacidin and LL16 have an immediate effect on growth rate of treated cultures (12). "The kinetics of gene induction are well correlated with the effects of brilacidin treatment on culture growth (12)." Overall, this paper showed a global comparison of transcriptional responses of daptomycin and LL16 against brilacidin. Brilacidin had the most potent impact, but all showed similarities in their ability to induct two-components systems related to cell wall stress. In addition to the upregulated of the two component response systems these compounds showed an upregulation of pathways involved in alteration of cell surface charge. Also, Brilacidin showed a significant induction in the upregulation of cellular proteases and chaperones and the lysine biosynthesis pathway known as Dap operon. The upregulation of cellular proteases and chaperones are consistent with membrane depolarization and misfolded protein accumulation.

Antiviral and Immunomodulatory Properties

The antiviral properties of AMPs can vary based on the AMP classification and the specific viral species. This may suggest that a specific mechanism of action is dependent upon the structure of the viral particle and its replication cycle. AMPs have direct and indirect antiviral effects against viral particles and its viral replication cycle. Indirectly, AMPs can modulate the host immune system by initiating the innate immune response, pro-inflammatory cytokines, chemokines, and other AMPs. AMPs modulate immune response, exhibit chemotactic activity, attract leukocytes, stimulate angiogenesis, enhance differentiation of leukocytes/ monocytes, and lastly modulate the expression of

proinflammatory cytokines and chemokines. For example, LL37 exhibits chemotactic activity by attracting other neutrophils, monocytes, mast cells and T cells.

Direct inhibition is shown by directly targeting intracellular stages of viral replication and the steps preceding viral entry. Brilacidin has the ability to directly interact and alter viral particles by forming pores on the viral envelop secondary to their cationic and amphiphilic nature. Electron microscopy observational studies have been able to show disruption of the viral envelope following exposure to LL-37. EM also showed the AMP connected to the viral particle (20). This paper showed that pre-incubation with Host Beta-defensin incubated decreased virion infectivity, which is also indicative of its ability to alter individual viral particles. Another possible direct interaction with AMPs will cause viral particles to aggregate outside the cell membrane which will ultimately block viral entry and induce viral uptake by phagocytes. This paper showed this in an experiment with VEEV and LL-37 lead to clumping of the viral particles at the membrane and prevented the cell from being infection (2). Direct antiviral activity in AMPs can be seen as inhibition of viral attachment to its cell surface receptor. LL-37 is known to directly block HSV-1 infection and viral cell attachment in corneal epithelial cells. AMPs exhibit antiviral properties based on their ability to inhibit viral entry through interference of intracellular steps of viral replication. Even after viral entry, there are several studies that support Amps ability to inhibit replication of the viral genome within its host cell. AMPs intracellular antiviral activities are supported by the face that recombinant peptides added to cell culture medium can be internalized by the epithelial cells (8). The exact mechanism of post-fusion antiviral activity of AMPs and

Brilacidin is less understood although there is existing supporting evidence. Overall, there are still gaps in the understanding of direct anti-viral activities of anti-viral peptides and mimetics like Brilacidin.

AMPs have intrinsic pro-inflammatory properties through the recruitment of various inflammatory mediators. When AMPs bind to their cell surface receptor which will activate signaling pathways associated with up-regulation of cytokine and/or chemokine expression. "CC Chemokine Receptor 6 (CCR6), TLR4, and G Protein Couple Receptor (GPCR) are three receptors identified so far with which AMPs interact to induce the cellular inflammatory response. (8)" In VEEV infection, the IFN-1 signaling pathway is a part of the innate immune response, AMPs may take this action a step further to better protect the cell from viral entry and infection. AMPs have the potential to directly kill microbes and modulate host immunity. AMPs utilize their charged electrostatic interactions to bind directly to the membrane. Upon binding, the electrostatic interactions are strong enough to disrupt or rupture the cell membrane and inhibit intracellular functional activities like biosynthesis of proteins or inhibition of enzyme activity. Once the interaction with cell membrane has been secured, AMPS can modulate host immunity through the recruitment of immunocytes likes neutrophils and monocytes. The primary role of immunocytes is to respond to infection at the site of a host cell. AMPs are extremely complex and diverse in its natural host defense response to different pathogens.

Antimicrobial Peptides as Therapeutics

Brilacidin and other drugs in its class have the potential to serve as effective therapeutics due to their drug-resistant nature and inhibitory effects against multiple pathogens. AMPs have shown broad spectrum inhibitory activity towards rug-susceptible and multidrug-resistant gram negative and gram-positive bacteria as an anti-bacterial. Brilacidin is known to cause dose-dependent membrane depolarization in Staphylococcus aureus. Other key features of Brilacidin include broad-spectrum antibacterial activity that is unlikely to develop antibacterial resistance (10). Resistance to AMPs is unlikely given its primary target is the cell membrane. For example, the human cathelicidin peptide LL-37 has a general alpha-helical AMP structure whose interactions with the membrane via its electrostatic properties allow for the AMP to insert itself within the cell membrane leading membrane disruption. Most AMPs are induced in situations of infection, inflammation, or injury. Some AMPs are constitutively expressed, while the majority are induced in response to inflammation and/or infection. It is believed that his property of AMPs contributes to ability to serve as effective antibiotics without the fear of developing bacterial resistance. AMPs exhibit a wide range in terms of classification, mechanisms, target, and type of inhibition.

HYPOTHESIS & SPECIFIC AIMS

Brilacidin has the potential to effectively disrupt the viral replication cycle by decreasing membrane integrity of the virion, increasing the likelihood of membrane disassembly, which would significantly reduce the viral load, and lessen the severity of infection in the host cell. Brilacidin's complete mechanism is unknown but based on the current research we can conclude that it is targeting the early steps of viral replication and more specifically the membrane integrity of the virion which will impact viral entry. I hypothesize that Brilacidin will exhibit an anti-viral phenotype against VEEV TC-83 through interactions localized at the virion membrane in cell culture.

Aim 1: Does Brilacidin inhibit VEEV TC-83 infection in cell culture?

To evaluate the efficacy of Brilacidin *in vivo* I will carry out a series of efficacy studies to explore Brilacidin's impact on the viral load. If Brilacidin treatment is effective in vivo we expect to see a decrease in the number of infectious particles. This is indicative of an anti-viral phenotype against VEEV. Through its mechanism of action, we expect to see Brilacidin decrease the viral load in comparison to the control group. A cause of concern with AMPs is that the level of cytotoxicity can possibly be toxic in the cellular environment towards infected an uninfected cell. Exploring the cytotoxic concentration is pivotal in evaluating efficacy at a safe and effective dose.

Aim 2: How does Brilacidin inhibit VEEV TC-83 in cell culture?

This thesis will explore the efficacy of Brilacidin against VEEV by completing four different treatment schematics. First, the pre and post treatment schematic allow us to evaluate the anti-viral activity of Brilacidin on uninfected and infected cells. Second, in addition to the pre and post treatment, the virus will be directly treated for one hours prior to infection. Brilacidin is expected to have a direct impact on the integrity of the virion. This schematic allows us to evaluate Brilacidin's direct interaction with TC-83 in cell culture in addition to pre and post treatment. Third, TC-83 will be directly treated with Brilacidin prior to infection. This allows us to further evaluate Brilacidin's direct impact and mechanistic interaction with VEEV TC-83 in cell culture. Lastly, post treatment alone will give us an idea of how Brilacidin is efficacious in a post-infection setting. Overall, these experiments should provide insight on the efficacy and mechanistic impact of Brilacidin against VEEV TC-83.

MATERIALS AND METHODS

Cell Culture

Human astrocytoma (U87MG, HTB-14) and Vero cells (ATCC, CCL-81) cell lines were all acquired from American Type Culture Collection, Manassas, VA. U87MG cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 1% L-glutamine, 1% penicillin/streptomycin, and heat-inactivated Fetal Bovine Serum (FBS) at 10%. Vero cells were maintained in DMEM with 1% L-glutamine, 1% penicillin/streptomycin, and heat-inactivated Fetal Bovine Essence (FBE) at 5%. Both cell cultures were maintained at 37℃ with 5% CO2.

Toxicity Screens

Cell viability was measured using Cell Titer-Glo Luminescent Cell viability Assay (Promega, G7572) per the manufacturer's instructions. The Cell Titer Glo reagent is administered to cells at a 1:1 ratio following their initial 24-hour period of incubation with Brilacidin present. The plate was placed on a plate shaker for 2 minutes followed by a 10 min incubation at room temperature. Lastly, cell viability was measured as luminescence detected by the DTX 880 multimode detector (Beckman Coulter).

TC-83 Infections

VEEV TC-83 is the strain produced as result of 83 passages of its fully virulent counterpart IAK Trinidad donkey wild-type strain of the virus (2). The TC-83 strain will

be utilized for the experimental studies of this thesis. A VEEV TC-83 was obtained from BEI Resources. Experiments with TC-83 were performed under BSL-2 conditions. For all infections, cells were seeded and allowed 24 hours to reach confluency.

Plaque Assay

Vero cells were seeded in 6-well plates at a density of 5E5 cells/mL. The cells were grown overnight until confluent. Viral supernatants were serially diluted in Phosphate Buffered Saline (PBS) in a deep well plate and then will be used to infect the Veros. All plates were incubated for an hour with intermittent plate rocking every 15 minutes. An overlay made of 2X EMEM and agarose at 1:1 ratio was added to each well. Once the overlay has solidified, the plates were incubated for 48 hours. Following the incubation period, cells were fixed with 1mL/well of 10% formaldehyde solution to remove the overlay and stained with crystal violet solution to make the plaques visible for counting and determination of viral titers as plaque forming units (PFU/mL).

RESULTS AND DISCUSSION

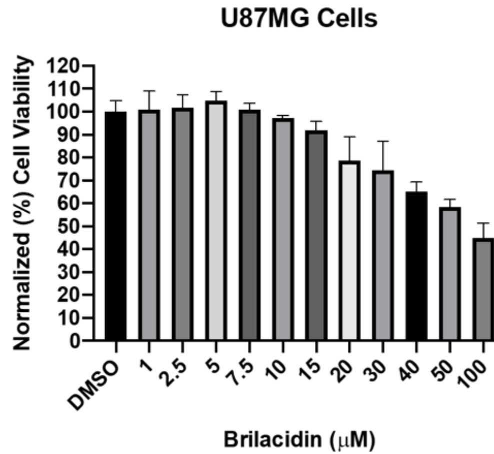

Figure 1 Cytotoxicity of Brilacidin

First, we must establish a safe and effective cytotoxic concentration (CC50) to test Brilacidin in the U-87 astrocyte cell line. Given the neurotoxic nature of the virus, the primary cell line for this study is human astrocytoma. It is important to establish the drug's cytotoxic potential to establish antiviral efficacy in a cell culture system (Gu, 2013). The cell viability experiment is completed by testing the drug at a range of different concentrations between 1-100 micromolar for 24 hours. CC50 testing will be used to establish the cytotoxic concentration in Veros as well that will be used in other experiments like the plaque assay. Based on the initial results, I will continue testing at 20 micromolar concentration for the remainder of the experiments. Determining the safe cytotoxic concentration range of this drug is important to evaluate before continuing with

efficacy studies. Based on this data, Brilacidin is largely non-toxic and safe to use in U87MGs up to 50 micromolar.

The viral plaque assay is used to evaluate the number of infectious virions by counting plaques. Plaques are infectious units and cellular dead zones in cell culture (3). The following viral plaque assays will be performed under these conditions: pre-treatment and post-treatment, pre-treatment, post-treatment and direct viral treatment, and post-treatment. For the pre/post-treatment, Brilacidin will be administered to cells for an hour prior to infection. Following infection, Brilacidin will be added back to the cells to treat for 18 hours post infection. In addition to the pre/post treatment, the virus TC-83 will be directly treated for one hour prior to infection for one hour, and post treatment will be applied as it was in the previous study. To directly treat the virus, following infection Brilacidin will be added to viral supernatant, incubate for 18 hours and then the viral supernatant will be collected. For post-treatment, following the infection, Brilacidin will be added to infection supernatant for 18 hours infection. All of the infections completed for the efficacy studies were done at MOI 0.1, and the viral supernatant was collected 18 hours post infection. In addition, all of the efficacy studies were completed with DMSO as the treatment to use these samples as the control group to compare to the Brilacidin sample. DMSO is toxic to the cells, hence it will be a good control to evaluate the efficacy of Brilacidin. Collectively, upon the completion of the aforementioned experiments I seek to gain a better understanding of how Brilacidin affects the phospholipid bilayer, viral load, viral replication, and the timing of when the drug is the most effective.

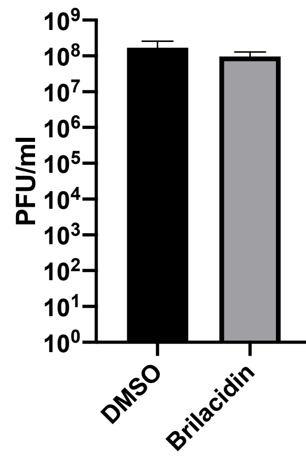

Figure 2 Pre and Post Treatment 10uM

The goal of the pre and post treatment efficacy study is to evaluate the anti-viral activity of Brilacidin on uninfected and infected U87MGs in cell culture. At 10 micromolar there is little to no inhibition seen in the Brilacidin samples compared to the control. Brilacidin does not appear to be efficacious against VEEV TC-83 at this dosage.

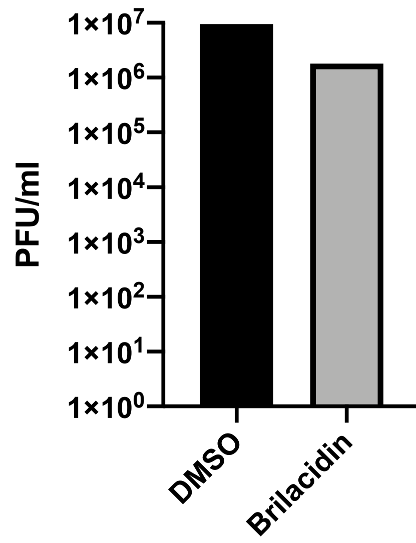

Figure 3 Pre and Post Treatment 20uM

Brilacidin is exhibiting anti-viral activity showing approximately one log in inhibition at 20 micromolar. One log of inhibition is indicative of a clear decrease in the number of infectious particles present 18 hours post infection. At this dosage, Brilacidin appears to safe and efficacious in cell culture against VEEV TC-83.

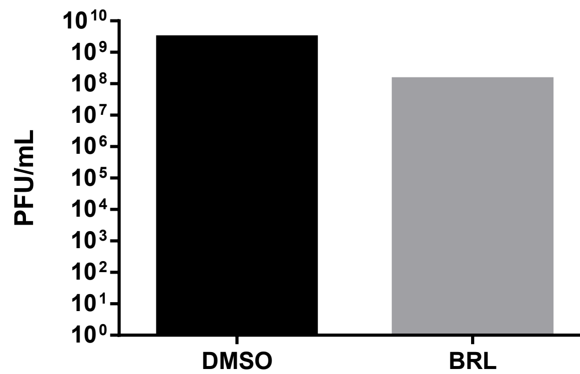

Figure 4 Pre and Post Treatment and Direct Viral Treatment 20uM

To evaluate Brilacidin's direct interaction with VEEV TC-83 in addition to the pre and post treatment allows us to further explore the role and efficacy of the pre and post treatment. This experiment provides information on the direct mechanistic impact of Brilacidin in cell culture. The pre and post treatment alone showed approximately one log of inhibition, but when the direct viral treatment is added, we can clearly see one log of inhibition if not more.

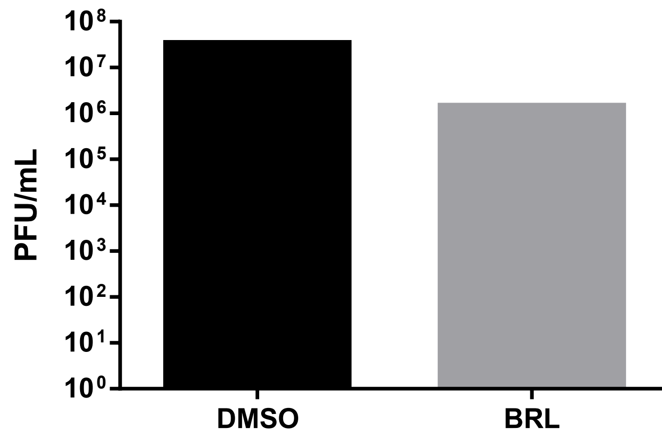

Figure 5 Direct Viral Treatment [1 hour] 20uM

Brilacidin exhibits anti-viral activity directly against the virion given there is a little more than one log of inhibition in the absence of pre and post treatment. At this treatment schematic, we see the most robust anti-viral response. This experiment gives us the most information about the mechanistic impact of Brilacidin in cell culture. It is most effective when treated directly against the virus.

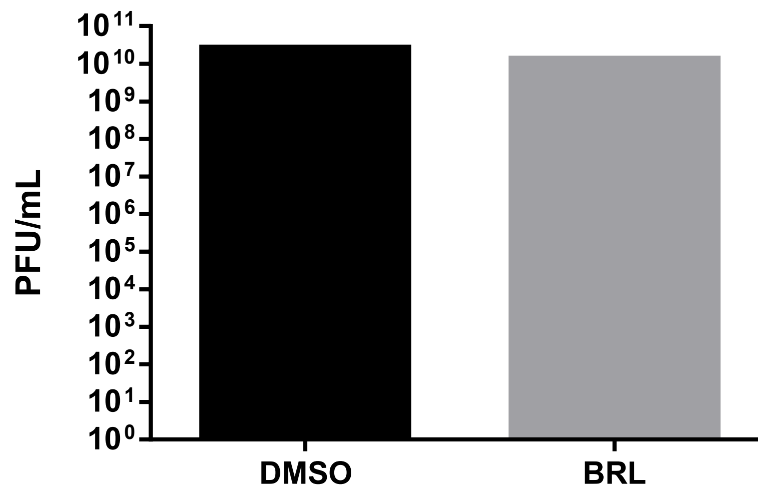

Figure 6 Post Treatment 20uM

Post treatment schematic was used to evaluate the efficacy of Brilacidin in cell culture against VEEV TC-83. Brilacidin does not appear to be exhibiting anti-viral activity post-infection.

Overall, the data from the efficacy studies reveal Brilacidin has an anti-viral phenotype against VEEV TC-83 in cell culture. Brilacidin appears to inhibit viral replication in VEE based on the decreased amount of infectious viral particles seen compared to the DMSO control throughout the efficacy studies. Brilacidin showed the greatest level of inhibition with direct viral treatment alone or in conjunction with the pre and post treatment. The data from the direct viral treatment indicates Brilacidin in most effective in the early steps of viral replication. Brilacidin is a strong anti-viral candidate to be further developed and explored as a therapeutic against VEEV.

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BIOGRAPHY

Miata Smith is a native of Prince George’s County, Maryland. She received her Bachelor of Science in Neuroscience from University of Delaware in 2019 and worked as a medical scribe for two years prior to pursuing her Master of Biology from George Mason in 2021. Miata will continue her career as a scientist in the pursuit of medicine.