BRILACIDIN AS A BROADSPECTRUM INHIBITOR OF ENVELOPED, ACUTELY INFECTIOUS VIRUSES

by

Carol Anderson
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Committee:

_____	Dr.Mariaelena Pierobon, Thesis Chair
_____ _____	Dr. Aarthi Narayanan, Committee Member
_____	Dr. Ancha Baranova, Committee Member
_____	Dr. Iosif Vaisman, Director, School of Systems Biology
_____	Dr. Donna Fox, Associate Dean, Office of Student Affairs & Special Programs, College of Science
_____	Dr. Fernando Miralles-Wilhelm, Dean, College of Science
Date: _____	Fall Semester 2021 George Mason University Fairfax, VA

Brilacidin as a Broad-Spectrum Inhibitor of Enveloped, Acutely Infectious Viruses.

A Thesis Project submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

Carol Anderson
Bachelor of Arts
Virginia Polytechnic Institute and State University

Director: Mariaelena Pierobon, Research Associate Professor
George Mason University

Fall Semester 2021
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to my amazing husband, Jacob Baisden, who has supported me through everything, my sisters and parents, and my amazing in-laws.

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I would like to thank my thesis director, Dr. Aarthi Narayanan, who has guided and mentored me through this program. I am so grateful to have been able to work with her. Her continued support has truly allowed me to grow in every aspect of research. No words can truly express how much the opportunities she has given me have changed my life. I would also like to thank my committee chair, Dr. Mariaelena Pierobon, for taking me under her wing. I am also grateful to Dr. Ancha Baranova, my final committee member, who was always willing to help and guide me.

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I would like to thank my amazing husband, Jacob Baisden, for always supporting me. I am so lucky to have someone in my life who makes me laugh like he does. I am grateful to have him in my life every single day.

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LIST OF ABBREVIATIONS

Antimicrobial Peptides..... AMPs
Brilacidin .....BRL
Biosafety Level-2 .....BSL-2
Biosafety Level-3 .....BSL-3
Complementary RNA ..... cRNA
Dulbecco's Modified Eagle Medium ..... DMEM
Dimethyl sulfoxide ..... DMSO
Essential Modified Eagle Medium.....EMEM
Fetal Bovine Serum ..... FBS
Human small airway epithelial cells ..... HSAECs
Multiplicity of Infection.....MOI
Non-essential amino acids.....NEAA
Organ on a Chip .....OoC
Penicillin and streptomycin.....P/S
Phosphoate Buffered Saline ..... PBS
Plaque forming unit ..... pFu
Rift Valley Fever Virus.....RVFV
Sindbis virus..... SINV

ABSTRACT

BRILACIDIN AS A BROAD-SPECTRUM INHIBITOR OF ENVELOPED, ACUTELY INFECTIOUS VIRUSES.

Carol Anderson, M.S.

George Mason University, 2021

Thesis Project Director: Dr. Mariaelena Pierobon

Rift Valley Fever virus (RVFV) is a Bunyavirus known to cause severe disease in livestock. Its zoonotic capability makes it a serious threat as it has become endemic in many parts of the world. While primarily spread through *Aedes* and *Culex* mosquitos, it can also be transmitted via aerosol and direct contact with infected bodily fluids. In humans it is known to cause hemorrhagic fever and liver failure. There are currently no FDA approved treatments for RVFV, making it an area of serious concern for the development and implementation of novel therapeutics. In this investigation Brilacidin (BRL), a defensin mimetic, was tested against RVFV among other viruses such as Sindbis (SINV) and an Echovirus. BRL was previously designed for antibiotic-resistant bacteria and its amphipathic nature enables it to disrupt membranes. It has been shown to be effective on both gram-positive and gram-negative bacteria, including MRSA. This work on bacteria has since been expanded to enveloped viruses such as SARS-COV-2, the causative agent of COVID-19. RVFV was tested in Human small airway epithelial cells (HSAECs) via plaque assay, and then this was repeated in other cell lines including Huh7s and HepG2s. Cytotoxicity analyses were conducted to determine cell viability when BRL was applied to the cells. Through direct viral treatment, a significant decrease was seen in viral load, that was not seen with pre-treatment and post-treatment of the cells. Cell viability was also tested for, demonstrating that the presence of BRL aids cell survival. This work was then

expanded out to Sindbis virus (SINV), and an Echovirus. The Echovirus served as a negative control due to its unenveloped nature. In both viruses, BRL was once again found to be effective at reducing viral titer.

INTRODUCTION

Rift Valley Fever Virus

Rift Valley fever virus (RVFV) is part of the *Bunyaviridae* family (1). It is a zoonotic, negative stranded RNA that is transmitted through *Aedes* and *Culex* mosquitos. Its genome consists of three segments: Small (S), Medium, (M), and Large (L). The S segment codes for N and NSs proteins. The M segment codes for NSm, Gn, and Gc proteins. Finally, the L segment codes for an RNA-dependent RNA polymerase (1,2). The fully formed virion is roughly 90-100 nm in diameter. It is surrounded by a lipid membrane formed from a host cell in conjunction with the Gn and Gc proteins. Within the virion are a copy of the N protein and the L protein so that upon new infection cycle it is able to begin transcription from negative sense RNA into positive sense RNA. It will also generate complementary RNA (cRNA) to start the formation of new viral RNA (2).

RVFV employs a variety of mechanisms to evade host defense systems (2). Primarily, it prevents host transcription of mRNA so that cellular proteins cannot be made. It will also down regulate production of RNA-dependent protein kinase which plays a role in promotion of apoptosis upon a cell signaling that is virally infected. It will also downregulate the production of IFN-β to prevent the SAP30 complex from being activated (2).

Rift Valley Fever

RVFV is the causative agent of Rift Valley Fever, which was first observed in Kenya in 1931 but is believed to have an ancestor it diverged from between 1880-1890. (2). The introduction of livestock to Africa likely resulted in the disease becoming endemic and spreading throughout the continent and into the Middle East. The first major outbreak occurred in South Africa from 1950-1950, where it is believed that it

resulted in the deaths of 100,000 sheep, making it particularly deadly to livestock. There were 9 serious outbreaks from 1997-2010, all following periods of heavy rainfall and heavy mosquito breeding (2).

This virus causes very severe disease in humans, including such symptoms as hemorrhagic fever, blindness, and neurological disorders (4). In livestock, it is even more severe and causes febrile illness, spontaneous abortion, or fetal malformation during pregnancy (4). It is currently endemic in many parts of Africa and appears to be spreading to the point where it now classified as an NIAHID Category A Priority Pathogen. In this study, a 48-hour variant of MP-12 variant will be used, which is a BSL-2 level pathogen (1). This strain is currently being tested as a potential vaccine candidate with great success so far in livestock (3). It has been suggested that variants of this strain could be developed that exhibit even higher safety standards than MP-12 alone (3). Since all current serotypes of RVFV emerged from a recent common ancestor, it is likely that a vaccine targeting the glycoproteins will provide protection against all currently endemic variants (2).

Antibiotic Resistance and the Need for Antimicrobial Peptides:

Antibiotic resistance has been on the rise since the inception of antibiotics themselves. Resistance is primarily due to overuse, including inappropriate prescription, agricultural use, and a more recent limited ability to develop new antibiotics (4). With the advent of resistance, it is also no longer seen as a potentially profitable area to develop new antibiotics. A strategy proposed to circumvent this issue involves the introduction of novel drugs based on innate human peptides, specifically defensins, that can attack the membranes of bacteria directly.

Defensins are a family of antimicrobial peptides (AMPs) that are usually less than 100 amino acids in length, consisting of α-, β-, and θ-subtypes (5). Leukocytes show high concentrations of defensins in their granules, and upon phagocytizing microorganisms into vacuoles, granules bind and release defensins. Defensins have a distinct β-sheet rich fold and six disulfide-linked cystines. It remains unclear how the cyclization of these disulfide-linked cysteines occurs, and these linkages can make defensins expensive to manufacture. This elucidates the need to develop defensin mimetics that model the properties of naturally occuring defensins without the cysteine linkages. That said, their structure allows defensins to

disrupt cell membranes and replication cycles of bacteria (5). Defensins can accomplish this due to their amphipathic characteristics, referring to their positively and negatively charged ends. The cationic side of these molecules can target and disrupt bacterial membranes, which are overwhelmingly negatively charged (6).

It is also believed that antimicrobial peptides can regulate immune function, including potentially enhancing leukocyte and monocyte action (7). AMPs were not truly recognized until 1996, but more than 3,000 have been identified since their discovery and they are taking the world by storm. While the need for more testing remains, it appears that their multiple mechanisms of action and the ability to target membranes directly will give them an edge that likely mitigates antibiotic resistance; bacteria are unlikely to completely alter their membranes in defense of a synthetic peptide while also mutating to evade other forms of attack (7).

Brilacidin: A Defensin Mimetic

In addition to bacteria, the application of peptides extends to other pathogens including viruses, parasites, cancer, and fungi (7). The synthetic drug Brilacidin (PMX-30063) is defensin mimetic, meaning it is modeled after a naturally occuring defensin. It is among 44 antimicrobial peptides currently in clinical trials, but one of only 16 that appear to have broad-spectrum activity against both gram-positive and gram-negative bacteria (7). Brilacidin (BRL) is modeled after a naturally occuring host defensin and has been shown to be effective on MRSA and vancomycin resistance Enterococcus (8). More recent studies have elucidated that it is able to disrupt viral integrity and disabling viral entry. This is believed to be due to its amphipathic nature, make it ideal for membrane disruption while being easier to manufacture than traditional defensins (8).

Preliminary studies have already been done on SARS-CoV-2, the agent causing COVID-19, where it was able to inhibit in ACE2 positive human lung cell lines (9). More recently, it showed antiviral acitivity in both Calu-3 and Vero cell lines, suggesting an ability to prevent viral entry and potentially break down the viral membrane, especially when combined with remedsivir (6).

This study aims to further explore BRL's ability to combat viral infections. While primarily focused on Rift Valley Fever virus, a *Bunyavirus*, other viruses will be investigated as well to analyze potential broad-spectrum effects against enveloped viruses. It was believed that BRL would be effective against enveloped viruses due to its ability to cause lipid membrane disruption. This same mechanism we believed would render it ineffective against non-enveloped viruses. The most obvious difference between virions and human cells is size. The RVFV virion is estimated to be between 90-100 nm in diameter (12,13). The average lung cell is anywhere from 7.54-8.77 μm in diameter (14). Liver cells are even bigger, from 25-30 μm in diameter (15). These cells are roughly 100-300x times that of the virion. While BRL will disrupt the membrane of the viruses, it also has the potential to do this for cells in a given system. As a result, we must account for cell viability and test concentrations that are shown to be safe for the cell but may still impact the virion.

Sindbis Virus

Sindbis virus (SINV) is an *Alphavirus* transmitted through *Culex* mosquitos (16). While *Bunyaviruses* are negative stranded, *Alphaviruses* consist of a positive RNA genome. Both of these viruses are enveloped. Their genome codes for five proteins, making it one of the smaller viral genomes known. These five proteins are: the capsid protein, E1, E2, E3, and 6K. E1 and E2 are glycoproteins that will make up the icosahedra shell of the virus. It is able to leave the host cell through a budding process (16). Importantly, this virus primarily uses receptor-mediated endocytosis for early viral entry, but appears to be able to use alternative entry techniques when this method is no longer available to it (17). Targeting viral receptors for viral entry may be a good antiviral therapeutic strategy to develop (17). That said, BRL should be able to attach the viral envelope and potentially cause degradation leading to lower infectious viral titer.

This virus has spread across the globe where it is now endemic in parts of Africa, Asia, Europe, and Australia (18). Migratory birds appear to be natural hosts and can amplify the disease. There are a wide range of symptoms of SINV infection, but they typically manifest as a flu-like symptoms including arthritis, a rash, and nausea. Joint pain can sometimes be long-lasting and have detrimental effects on

quality of life of those infected. Infection with SINV has been linked to many existing diseases across the globe, including Ockelbo and Pogosta disease (18).

Echovirus

Echoviruses belong to the family *Picornavirus* and are single-stranded positive sense RNA viruses (20). This is a non-enveloped virus, so we hypothesized that BRL would be ineffective against it. This virus codes for four primary proteins, VP1-VP4. When translated in a host cell, it forms a polyprotein that must then be cleaved into smaller proteins for assembly of new virions (20). Symptoms range widely for these viruses, causing everything from viral meningitis, a life-threatening condition, to asymptomatic infection. It causes rashes, central nervous system disease and failure, and respiratory illnesses (21). It's ability to affect the nervous system has been known to cause paralysis. In fact, it is estimated that 80% of viral meningitis cases now come from this family of viruses. It primarily affects young children, where it could potentially cause severe disease (21). Due to its non-enveloped nature, they were selected to be a negative control for this study (12).

MATERIALS AND METHODS

Viral Stocks:

The RVFV stock used was the recombinant (r)MP-12 strain. The SINV utilized was originally isolated from *Culex pipiens* and *Culex univattutus* mosquitos in 1952. Both the SINV and EV stock were amplified in Vero and tested in` E6 cells (African Green Monkey Kidney Cells).

Cell Culture:

Human small airway epithelial cells (HSAECs) were grown with Ham's F-12 modified media, 5% fetal bovine solution (FBS), 1% penicillin and streptomycin (P/S), 1% Non-Essential Amino Acids (NEAA), 1% sodium pyruvate, and 0.1% β-mercaptoethanol. Veros were grown with Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS, 1% P/S, and 1% L-glutamine. Huh-7 cells (human hepatocyte carcinoma cells) were cultured with 10% FBS, 1% L-glutamine, 1% P/S, 1% NEAA, and 1% sodium pyruvate. HepG-2 cells (human hepatocyte carcinoma cells) were cultured with Eagle's Minimuim Essential Medium (EMEM), 10% FBS, and 1% P/S. All cell lines were incubated at 37°C and 5% CO_2.

Cytotoxicity Assay:

96-well plates were seeded with HSEACs, HepG-2s, or Huh-7s and left to become confluent overnight. Blank wells were left empty. Once confluent, media was removed and replaced with new media mixed with the various concentrations of BRL or dimethyl sulfoxide (DMSO), with all concentrations run in triplicate.

After 24 hours, the Brilacidin or DMSO media was removed and replaced with 50 µL of CellTiter-Glo® Reagent and 50 µL of media. Luminescence was then analyzed.

Viral Infections:

For testing BRL's effectiveness, four viral treatments were conducted in RVFV. Pre-treatment consisted of diluting BRL down to 20 µM in media and placing 200 µL of it onto cells for 1 hour. Direct

viral treatment involved diluting the virus down in media and suspending 20 μM of BRL in it and allowing it to incubate for 1 hour. Post-treatment followed the same methodology as pre-treatment, but instead of leaving for 1 hour, it remained on the cells until collection time. This was 16 hours for RVFV accounting for two replication cycles of the virus. For SINV this was 18 hours, accounting for three replication cycles of the virus. This was 20 hours for the EV, allowing for two replication cycles. The following combinations of treatments were conducted: Pre-treatment and post-treatment, direct viral treatment alone, post-treatment alone, and a combination of pre-treatment, post-treatment, and direct viral treatment. All infections took plates in a 96-well plate and were infected at an MOI of 0.1.

Plaque Assays:

12-well plates were seeded with Veros and left overnight. Confluency of cells will be checked prior to infecting. Frozen viral supernatants were defrosted and 450μL of DMEM+++ was added to a 96 deep well dilution plate. 7, ten-fold serial dilutions were done on each virus sample, adding 50 μL of sample into the 450μL well. Overlay was be removed from plates with 2 mL aspirating pipet. Starting with the lowest dilution and working the way up, 200μL was placed into the 12-well plates. Plates were incubated for 1 hour, rocking them every 15 minutes. After the 1 hour, cells were overlayed with 1:1 mixture of EMEM+++++ and 1.0% Agarose in diH_2O. Plates are then incubated for 48-hours for RVFV and SINV. For the EV, 6-well plates were used instead of 12-well plates and plates were left for 72 hours. This was due to the virus resulting in small plaque so visualization had to be optimized.

After incubation, plates are removed and fixed with 10% formaldehyde for 1 hour. Plugs were removed and then plates were dyed with crystal violet, allowing to stain for 10 minutes. Plaques were then counted.

Cell Viability Assay

Viral infections were conducted according to protocol previously outlined. Mock infected wells were added as a baseline comparison as BRL is known to kill some cells. These mock infected cells

received BRL and DMSO treatment but no viral infection. Cell viability was measured using CellTiter-Glo® as previously described. Luminescence was analyzed and BRL and DMSO were compared to see statistical significance.

Statistical Analysis:

All experiments described here were performed in triplicate. Results underwent a T-tests and ANOVA one-way tests as necessary, to describe their significance. P values less than 0.05 were considered of statistical significance and standard deviations were also reported. Graphs were generated in Prism.

RESULTS

Establishing BRL as effective against RVFV infection at varying concentrations:

We began by testing BRL's cytotoxic effect on HSAECs (Fig 1A). BRL was left on HSAECs at varying concentrations for 24 hours and then treated with Cell Titer-Glo® in order to establish safe concentrations for testing. The CC_{50} value was found to be 61.32 μM. From there, three different concentrations (10 μM, 20 μM, and 30 μM) of BRL were selected for initial testing. This phase of testing consisted of only directly incubating the virus with BRL for 1 hour then infecting the HSAECs with RVFV for 1 hour. After this, inoculum was replaced with fresh media. At 10 μM there was no statistical difference between BRL and the DMSO control (Fig 1B). At 20 μM there was approximately a $1\log_{10}$ reduction in viral titer resulting in an 89.2% decrease in viral titer (Fig 1C). There was roughly a $1\log_{10}$ reduction at 30 μM as well, resulting in a 89.6% decrease in viral titer (Fig 1D).

BRL decreases viral titer at 16 hours but less effective at 24 hours:

Since the effectiveness of BRL remained roughly the same for 20 μM and 30 μM, the remainder of the experiments in HSAECs occurred at 20 μM to maximize viral inhibition while also preserving cell viability. Three more treatments were conducted at 20 μM and samples were collected at 16 hours and 24 hours post-infection. Pre-treatment and post-treatment consisted of placing media with 20 μM of BRL on the cells for one hour, removing the treatment, and infecting with RVFV for 1 hour. Inoculum was then removed and replaced with BRL at 20 μM until collection. A combination of these three treatments was also tested. Post-treatment only consisted of placing BRL on the cells after the infection had already taken place. Pre-treatment and post-treatment (Fig 2A) saw no statistical decrease in viral titer between BRL treatment and DMSO. Direct viral treatment (Fig 2B) showed roughly a $1\log_{10}$ decrease in viral titer. The largest difference in viral titer was seen when pre-treatment, post-treatment, and direct viral treatment were combined (Fig 2C). This resulted in roughly a $2\log_{10}$ decrease in viral titer, or a 99.72% decrease in viral titer. Post-treatment was similarly found to be not significant resembling the pre-treatment and post-treatment combination (Fig 2D). Then, we wanted to investigate how long this viral titer decrease would last. Samples were collected 24 hours post-infection allowing for one more replication cycle of RVFV to

occur. Pre-treatment and post-treatment, as well as post-treatment alone remained statistically not significant (Fig 3A, Fig 3D). Comparing direct viral treatment from 16 hours to 24 hours, there was now no significance between BRL and DMSO, meaning the virus had caught back up to the control group (Fig 3B). However, the combination of pre-treatment, post-treatment, and direct viral treatment maintained the $2\log_{10}$ reduction that was seen at 16 hours (Fig 3C). There was a 99.23% decrease in viral titer.

BRL is effective against the hot strain of RVFV:

All previous experiments had been conducted with an attenuated strain of RVFV under BSL-2 conditions. We decided to also test BRL on a BSL-3 strain of RVFV. The BSL-2 strain is 23 mutations different from that of the hot strain, accounting for its non-infectious nature (2). Similar results were seen to the attenuated strain where there was no decrease in infectious viral titer for pre-treatment and post-treatment as well as post-treatment alone. Direct viral treatment saw about a $0.5\log_{10}$ reduction in titer, which is equivalent to a 69.8% decrease in infectious viral particles. A combination of treatments saw this even further decrease to slightly less than a $1\log_{10}$ drop in infectious viral titer. Viral titer decreased by 85.41%. While this is not quite on par with what was seen in the BSL-2 strain, it still indicates that BRL is effective at reducing viral titer. Due to the similarity between pre-treatment and post-treatment and post-treatment alone and the ineffectiveness of both, moving forward only pre-treatment and post-treatment were run for all viruses and cell lines.

BRL increases cell viability in HSAECs:

Another important area of interest is whether BRL can increase cell survival during infection. Analyzing cell viability with Cell-Titer Glo® we were able to measure this for our three treatment strategies. Mock infections were also conducted to serve as a baseline for cell viability under BRL and DMSO conditions as BRL has been shown to kill some HSAECs at 20 μM concentration. The pre-treatment and post-treatment saw 78.5% of cell surviving infection with BRL treatment, whereas the DMSO saw only 68.0%. BRL increased cell viability by 10.5%. For direct viral treatment 85.5% of cells survived infection with BRL, but with DMSO only 77.25% survived, meaning cell viability increased by 8.25%. For a combination of the treatments, 71.25% of cells survived when BRL was applied and for

DMSO 70.5% survived. This is likely due to repeated treatments stressing the cells. This was not found to be statistically significant.

BRL is robust against RVFV in other cell lines:

After working with RVFV in HSAECs, we decided to expand this work out to two other cell lines: Huh7s and HepG2s. Both Huh7s and HepG2s are human liver carcinoma cells. RVFV is known to cause liver failure, so we wanted to expand out this study to other cell lines of interest. Similarly, to HSAECs, we began with conducting a cytotoxicity assay on both. The CC_{50} was 12.03 μM for Huh7s (Fig 6A). For HepG2s the CC_{50} was found to be 153.3 μM (Figure 7A). Due to the low nature of the Huh7 CC_{50} value, we decided to proceed with BRL treatment at 5 μM instead of 20 μM and see if it would still be effective at reducing viral titer. Similarly, to RVFV infection in HSAECs, pre-treatment and post-treatment saw no statistical difference in viral titer compared with DMSO (Fig 6B). Direct viral treatment saw just under $1\log_{10}$ in viral titer, reducing viral titer by 72.8% (Fig 6C). A combination of the three treatments saw roughly a $2\log_{10}$, or 84.9%, decrease in viral titer that was very statistically robust (Fig 6D). For Pre-treatment and post-treatment at 5 μM in Huh7s, roughly 52.45% of cells survived infection with BRL, 1 13.1% increase compared with DMSO (Fig 6E). For direct viral treatment there was a 9.10% increase in cell viability (Fig 6F). For Pre-treatment, post-treatment, and direct viral treatment there was the greatest increase in cell viability at 16.0% increase in cell viability (Fig 6G).

Finally, in HepG2s, treatment was conducted at 20 μM. Pre-treatment and post-treatment surprisingly saw a $0.5\log_{10}$, or 81.0%, reduction in viral titer (Fig 7B) and a 25.73% increase in cell survival (Fig 7E). For direct viral treatment, there was a $2\log_{10}$ decrease (96.6%) in viral titer (Fig 7C). BRL cell survival increased by 11.25% (Fig 7F). For pre-treatment, post-treatment, and direct viral treatment there was a similar BRL survival was $2\log_{10}$, or 98.7%, decrease in viral titer (Fig 7D). Cell survival was also high at 20.33% increase in cell viability (Fig 7G). Overall, in this cell line cell survival was roughly around 98% for all treatment strategies, indicating that BRL is helping cell survival significantly.

Brilacidin is effective against enveloped and non-enveloped viruses:

After establishing that BRL is effective in RVFV, we moved onto look at it in the Alphavirus Sindbis. All treatment strategies remained the same as before, including BRL at a concentration of 20 μM, infecting at an MOI of 0.1, and using U87MGs cells.

Pre-treatment and post-treatment saw a surprising $2\log_{10}$ reduction in viral titer, or 97.9% decrease (Fig 8A). This also revealed an increase in cell viability of 6.5% (Fig 8D). For direct viral treatment there was a lower reduction in viral titer, of $1\log_{10}$, or 90.9% decrease (Fig 8B). This also showed a 6.5% increase in cell viability (Fig 8E). A combination of the three treatment strategies saw an effect similar to that of the pre-treatment and post-treatment, resulting in a $2\log_{10}$ reduction in viral titer, or a 99.4% decrease (Fig 8C). Cell viability for this treatment strategy was comparable to that of the DMSO control group (Fig 8F).

The original intent of this study was to use the EV as a negative control due to its unenveloped nature. We predicted that BRL would not be effective at reducing EV titer as there is no membrane to disrupt. Pre-treatment and post-treatment results closely aligned with that of all previous viruses (Fig 9A). However, cell viability increased by 25.25% (Fig 9D). Surprisingly, there was a slight reduction in titer with direct viral treatment of roughly $0.5\log_{10}$ equaling 50.6% lower viral titer (Fig 9B). Cell viability also increased by 16.50% (Fig 9E). The greatest reduction in viral titer was seen for the three treatment combinations. This was roughly $1.5\log_{10}$, or 95.2%, reduction in viral titer (Fig 9C). That said, cell viability decreased from 90% in the control group to 82.0% (Fig 9F). This is likely due to repeated treatments agitating the cells. There remains a mechanism of action to be discerned for non-enveloped viruses.

FIGURES

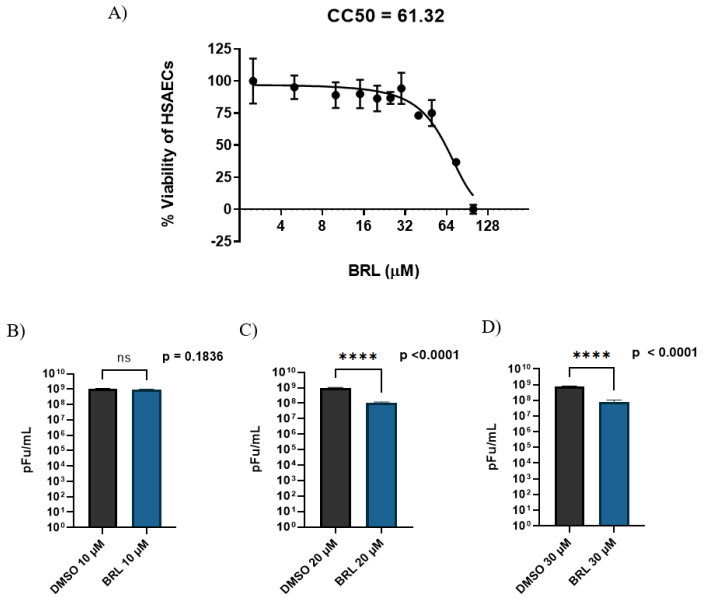

Figure 1: Establishing Brilacidin as effective on RVFV at varying concentrations. (A) HSAECs were treated with BRL for 24 hours and cell viability was measured using Cell Titer Glo® and compared with DMSO. The CC50 value was found to be 61.32 μM. **(B-D)** RVFV was directly treated with BRL for 1 hour at three concentrations (10 μM, 20 μM, 30 μM) and then left to infect cells for 16-hours. Viral titer was measured via plaque assay.

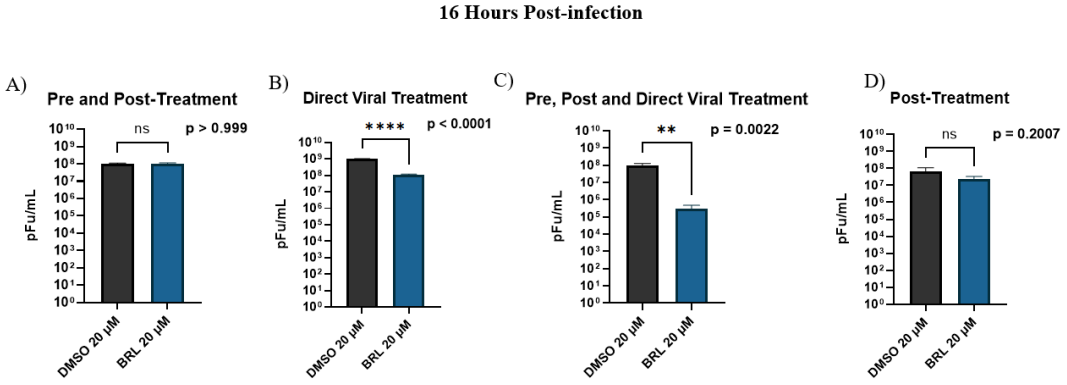

Figure 2: Testing four treatment strategies with Brilacidin for RVFV infection in HSAECs at 16 hours post-infection. (A) HSAECs were pre-treated with 20 μM of BRL for 1 hour, then infected with RVFV for 1 hour (MOI 0.1). Post-treatment was left on for 15-hours. **(B)** RVFV was incubated with 20 μM of BRL for 1 hour and then placed on HSAECs for 1 hour. Inoculum was replaced with fresh media and collected 15 hours later. **(C)** Treatment plan combined what was outlined in **(A)** and **(B)**. **(D)** BRL was only administered after RVFV infection and left for 15 hours**.**

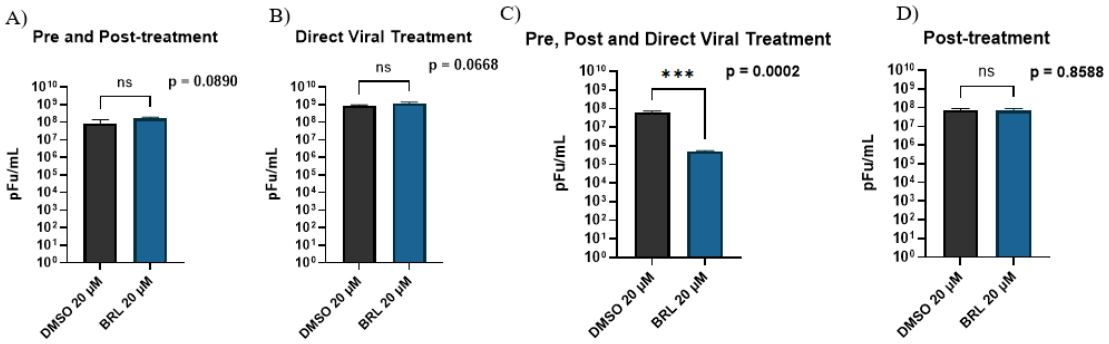

Figure 3 : Testing four treatment strategies with Brilacidin for RVFV infection in HSAECs at 24 hours post-infection. (A) HSAECs were pre-treated with 20 µM of BRL for 1 hour, then infected with RVFV for 1 hour (MOI 0.1). Post-treatment was left on for 23 hours. **(B)** RVFV was incubated with 20 µM of BRL for 1 hour and then placed on HSAECs for 1 hour. Inoculum was replaced with fresh media and collected 23 hours later. **(C)** Treatment plan combined what was outlined in **(A)** and **(B)** which was the only statistically significant portion of this data set. **(D)** BRL was only administered after RVFV infection and left for 23 hours.

Figure 3

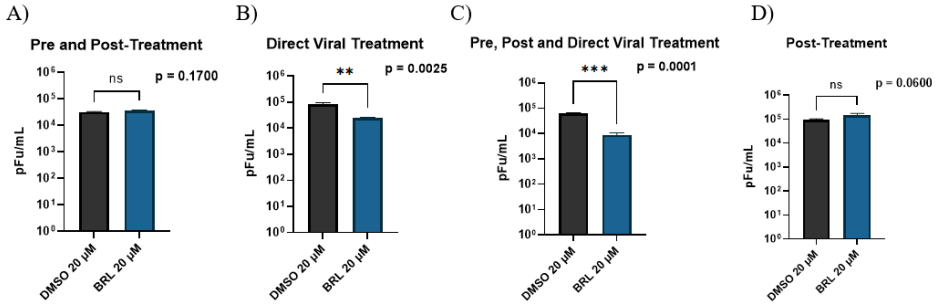

Figure 4: BRL is effective in the hot strain of RVFV. (A) HSAECs were pre-treated and post-treated with BRL at 20 µM and infected with RVFV (MOI 0.1) **(B)** Hot strain of RVFV was directly treated with 20 µM of BRL for 1 hour before infecting HSAECs. **(C)** Pre-treatment, post-treatment, and direct viral treatment were used at µM. **(D)** Post-treatment was conducted alone at 20 µM.

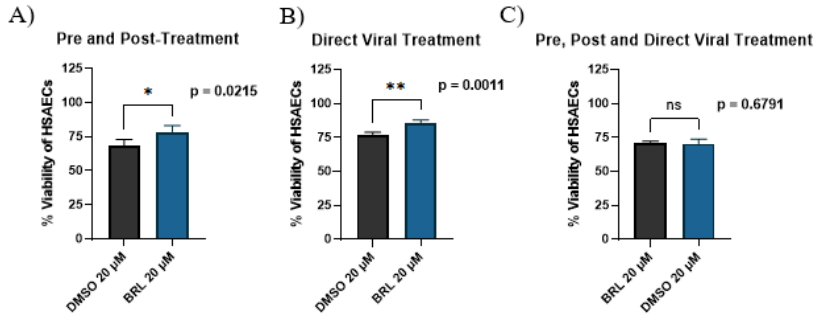

Figure 5: BRL increases cell viability of HSAECs over the course of infection. (A) HSAECs were pre-treated and post-treated with BRL at 20 µM and infected with RVFV (MOI 0.1). Cell Titer Glo was used to measure cell viability. **(B)** RVFV (MOI 0.1) was directly treated with BRL at 20 µM and infected HSAECs for 1 hour before inoculum was replaced with fresh media. (**C)** A combination of **(A)** and **(B)** were used to measure cell viability.

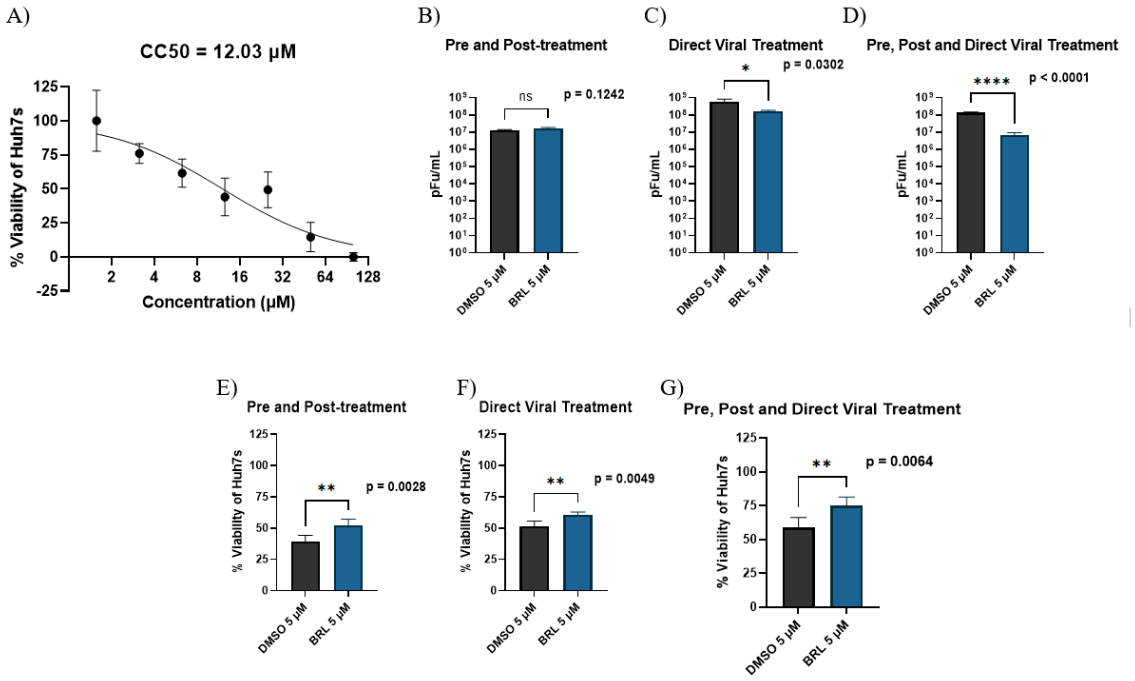

Figure 6: BRL is effective at preventing RVFV infection in Huh7 cells. (A) Huh7 were treated with BRL for 24 hours and cell viability was measured with Cell Titer Glo® and compared with DMSO control. The CC_{50} value was found to be 12.03 µM. **(B & E)** Huh7s were pre-treated with 5 µM of BRL and then infected with RVFV (MOI 0.1). Inoculum was removed and cells were post-treated with BRL. Viral titer was measured via viral plaque assay. Cell viability was measured using Cell Titer Glo® and compared with mock infection. **(C & F)** RVFV was treated directly with 5 µM of BRL and incubated for 1 hour. Then virus was left to infect cells for 1 hour before inoculum was removed and replaced with media. **(D & G)** A combination of the treatments outlined in **(B)** and **(C**) were applied to the cells and virus.

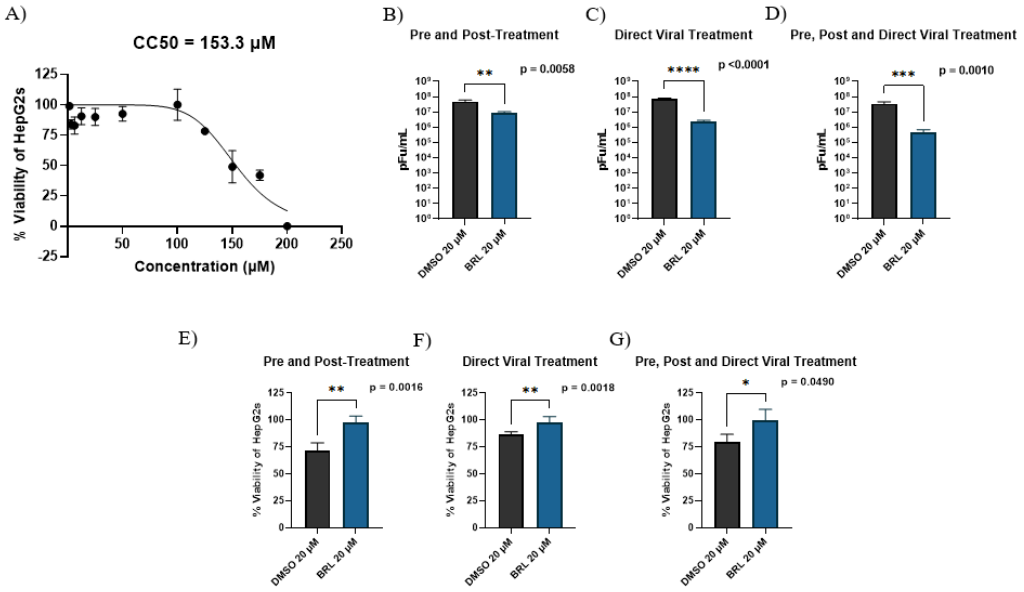

Figure 7: BRL is effective at preventing RVFV infection in HepG2 cells. (A) HepG2 were treated with BRL for 24 hours and cell viability was measured with Cell Titer Glo and compared with DMSO control. The CC50 value was found to be 153.3 µM. **(B & E**) HepG2s were pre-treated with 20 µM of BRL and then infected with RVFV. Inoculum was removed and cells were post-treated with BRL. Viral titer was measured via viral plaque assay. Cell viability was measured using Cell Titer Glo and compared with mock infection. **(C & F)** RVFV was treated directly with 20 µM of BRL and incubated for 1 hour. Then virus was left to infect cells for 1 hour before inoculum was removed and replaced with media. **(D & G)** A combination of the treatments outlined in **(B)** and **(C)** were applied to the cells and virus.

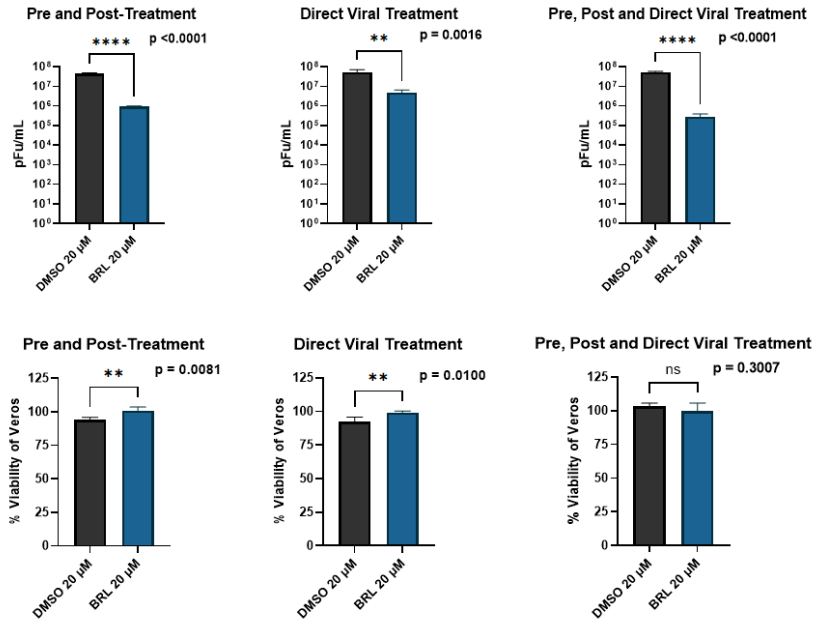

Figure 8: BRL is effective at preventing SINV infection in Veros. (A & D) Veros were pre-treated with 20 µM of BRL and then infected with SINV (MOI 0.1). Inoculum was removed and cells were post-treated with BRL. Viral titer was measured via viral plaque assay. Cell viability was measured using Cell Titer Glo and compared with mock infection. **(B & E)** SINV was treated directly with 20 µM of BRL and incubated for 1 hour. Then virus was left to infect cells for 1 hour before inoculum was removed and replaced with media. **(C & F)** A combination of the treatments outlined in **(A)** and **(C)** were applied to the cells and virus

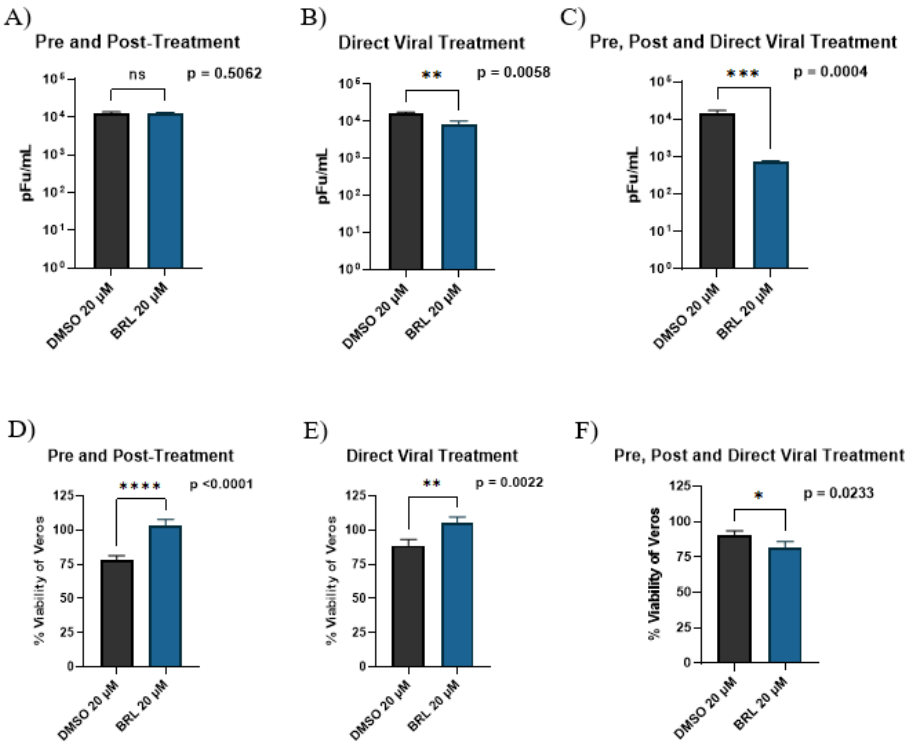

Figure 9: BRL is effective at preventing Echovirus infection in Veros. (A & D) Veros were pre-treated with 20 µM of BRL and then infected with an EV (MOI 0.1). Inoculum was removed and cells were post-treated with BRL. Viral titer was measured via viral plaque assay. Cell viability was measured using Cell Titer Glo and compared with mock infection. **(B & E)** The EV was treated directly with 20 µM of BRL and incubated for 1 hour. Then virus was left to infect cells for 1 hour before inoculum was removed and replaced with media. **(C & F)** A combination of the treatments outlined in **(A)** and **(C)** were applied to the cells and virus.

DISCUSSION

RVFV is a serious, zoonotic disease that spills over into humans primarily from *Aedes* and *Culex* mosquitos but can also be spread through livestock via contact with infected bodily fluids (1, 2). Its ability to cause hemorrhagic fever in humans make it a particular risk for bioweapon development. In livestock, it is known to cause spontaneous abortions and have a high mortality rate, making it detrimental to agricultural economies in the areas in which it is endemic and causes outbreaks (1, 2). All of these factors result in a pressing need to find antiviral therapy for those who are potentially exposed.

In this study we aimed to tests Brilacidin, a defensin mimetic, against RVFV infection in HSAECs (Fig 1, 2, 3). BRL is a small molecule that is known to interact with both gram-positive and gram-negative bacterial membranes, forming pores to cause cell death (7,8,11). This same mechanism of action can then be applied to enveloped viral membranes (8,11). We were able to demonstrate that when BRL was directly applied to the virus there was a $1\log_{10}$ reduction in viral titer compared with DMSO (Fig 1C). This reduction was dose dependent, where lower concentrations were no longer effective and greater concentrations were limited due to cell death (Fig 1B, 1D). From there, three more treatment strategies were tested to elucidate BRL's mechanism of action analyze its role in infection. Post-treatment alone and pre-treatment combined with post-treatment appeared to have no significant effect on viral titer. However, when combining pre-treatment, post-treatment, and direct viral treatment a decrease of $2\log_{10}$ was seen. This even held up 24-hours post-infection, where direct viral treatment on its own did not. This indicated that BRL is directly disrupting the viral membrane, preventing and slowing early entry (8,11).

When this was compared with the hot strain of RVFV, similar results were seen. Under BSL-3 conditions, pre-treatment and post-treatment as well as post-treatment alone saw no statistical decrease in viral titer (Fig 4A, 4D). However, the direct viral treatment saw a 69.8% decrease in infectious viral titer (Fig 4B). The combination of the three treatments saw roughly an 85.4% decrease in infectious viral titer (Fig 4C). This indicated that while not as effective as in the BSL-2 strain, it was still preventing early infection.

BRL was also shown to be effective at increasing cell viability for pre-treatment and post-treatment as well as direct viral treatment alone in the HSAEC cell line. Interestingly, there was a decrease in cell viability when treatment strategies are combined. This is likely due to constant exposure to BRL irritating cells and causing them to die. This further emphasizes the need to balance dosage to reduce viral titer with cell survival. Then we investigated Huh7 and HepG2 cell lines where BRL was once again found to be effective. Huh7s were highly sensitive to BRL and a lower concentration of it was applied, leading to less but still significant viral reduction (Fig 6) (20,21). HepG2s were more robust against BRL and had the greatest reduction in viral titer of all the cell lines, including a reduction seen with the pre-treatment and post-treatment strategies (Fig 7B). Higher concentrations remain to be tested in this cell line. Whether or not BRL is able to directly inhibit the virus, we have shown that still administering the drug to cells can improve cell viability which could be profoundly important for increasing immune response during infection. This could potentially increase survival during serious course of infection and decrease recovery time. Drug-induced liver injury is a very serious issue when developing drugs and can potentially lead to liver failure (25). BRL increased cell survival here but needs to be studied further. Cell stress still needs to be taken into account as BRL potentially could rupture cell membranes.

Then, Sindbis was tested, following up on previous Alphavirus studies already in progress. We found results paralleling that of RVFV, but most notably pre-treatment post-treatment did result in a $2\log_{10}$ decrease in viral titer (Fig 8A). Direct viral treatment on the other hand saw a $1\log_{10}$ reduction in viral titer, comparable to that of RVFV (Fig 8B). Finally, a combination of treatment strategies resulted in a $2\log_{10}$ reduction in viral titer once again(Fig 8C). Cell viability only increased for pre-treatment and post-treatment and direct viral treatment alone (Fig 8D-F). This is likely cell line specific as these cells are known to proliferate extensively, making up for any cell death. It is not currently understood why there was such a significant decrease in this cell line with pre-treatment and post-treatment with BRL.

Surprisingly, BRL was found to be effective against the Echovirus we had selected. That said, it was not nearly as effective, $0.5\log_{10}$ compared with $1\log_{10}$ as seen in the other viruses and cell lines (Fig 9B). This indicates the BRL's primary mechanism of action remains viral envelope disruption, but it may still be able to signal internal cellular mechanisms to prevent infection. Further testing in more non-

enveloped viruses is required to confirm this finding and further a distinct mechanism of action for BRL in these viruses. Interestingly, the cell viability for the three treatment combinations decreased compared with DMSO controls. This is likely due to the stress that repeat exposure to the drug caused on these cells and would have to be explored further.

Further impacts need to be studied to further this data. Most importantly is the need to elucidate a mechanism of action against non-enveloped viruses. This thesis marks the first time an enveloped, negative sense RNA virus was tested with BRL. Expanding this to both negative and positive sense RNA viruses could reveal BRL's continued broad-spectrum antiviral effects. Flaviviruses would be natural next steps in continuing to understand BRLs effect on hemorrhagic fever viruses. Organ on a chip (OoC) experiments could also further this research with the ability to more accurately model tissue interactions and infection signaling pathways in primary cell lines (26). An estimated 80% of new drugs fail clinical trials, 30% of which are due to toxicity. OoC is a step towards understanding BRL's toxicity in a dynamic model (26). There are also other FDA approved drugs, such as Bortezomib, that BRL could potentially be used in conjunction with to increase effectiveness (27). With the goal being to decrease viral titer enough to prevent infection, continuing to test cytotoxicity remains an important goal of this research.

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BIOGRAPHY

Carol Anderson graduated from Fairfax High School, Fairfax, Virginia, in 2016. She received her Bachelor of Arts in Physics from Virginia Polytechnic Institute and State University in Spring 2020. She then went on to start her Master of Science in Biology from George Mason in Fall of 2020.