INHIBITORS OF UBIQUITIN SIGNALING PATHWAYS EXHIBIT ANTIVIRAL ACTIVITIES AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

by

Brittany McGraw

A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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 R. Miralles-Wilhelm Dean, College of Science
Date: _____	Fall Semester 2022 George Mason University Fairfax, VA

Inhibitors of Ubiquitin Signaling Pathways Exhibit Antiviral Activities against Venezuelan Equine Encephalitis Virus

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

by

Brittany McGraw
Bachelor of Science
James Madison University, 2020

Director: Aarthi Narayanan, Associate Professor
School of Systems Biology

Fall Semester 2022
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to my supportive family John, Tonya, and Jason McGraw, and to my caring Fiancé Andrei Ionita.

ACKNOWLEDGEMENTS

I would like to thank my parents Tonya and John McGraw, my little brother Jason McGraw, my fiancé Andrei Ionita and my future in-laws Tudor Ionita and Alina Ionita for the constant support throughout my graduate school endeavors. I would like to say thank you to my grandparents Cherie and Terry McGraw for the positivity that they have sent through this journey.

I would like to give a huge thank you to my advisor Dr. Aarthi Narayanan for supporting my pursuit of graduate studies throughout my time in George Mason University and for giving me the opportunity to work in Virology, it has meant more to me than you will know. I am beyond grateful for the guidance, advice, and support you have given me throughout my time in your lab.

I want to thank my committee members Dr. Ancha Baranova and Dr. Mariaelena Pierobon for helping me through my pursuit in my master's program and providing unconditional support and input regarding my thesis.

I thank the Narayanan lab members for providing support and training in the lab. Niloufar Boghdeh has been a tremendous help. She has provided training on various lab techniques and experiments all while working directly with me through my entirety of my research. In addition, Carol Anderson and Michael Barrera have made a huge impact on my time at in the lab. From taking me under their wing and helping me find my feet to teaching me lab techniques. I thank Niloufar, Michael, and Carol for the constant support, time, and care of my thesis research. You have all been the best mentors and lab members that I could have ever asked for. Thank you.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

African Green Monkey Cells..... VERO
Biosafety Level 2…… BSL-2
Biosafety Level 3…… BSL-3
Bardoxolone……. BARM
Bardoxolone Methyl……... BAR
Centers for Disease Control……. CDC
Deubiquitinase……..... DUB
Dimethyl sulfoxide……DMSO
Dulbeccos's Essential Modified Medium…….DMEM
Food and Drug Administration (U.S) ……...FDA
Hepatitis C virus……... HCV
Human immunodeficiency virus…… ...HIV
Human microglia cells 3…… HMC3
Inhibitor of nuclear factor kappa-B.....IKK-B
Nuclear factor erythroid 2- related factor 2.....Nrf2
Nuclear Factor Kappa B.....NfkB
Nonstructural proteins.....Nsps
Omavexolone.....OMA
RNA-dependent RNA polymerasae.....RdRp
Reactive Oxygen species.....ROS
Human astroglia cells.....SVG-p12s
Transcription factor.....TF
Ubiquitin-Specific Peptidase 7.....USP-7
Ubiquitin Proteosome Pathway.....UPS
Virus replication complex.....VRC
Venezuelan Equine Encephalitis Virus.....VEEV
Zika Virus.....ZIKV

ABSTRACT

INHIBITORS OF UBIQUITIN SIGNALING PATHWAYS EXHIBIT ANTIVIRAL ACTIVITIES AGAINST VENEZUELAN EQUINE ENCEPHALITIS VIRUS

Brittany McGraw, MS

George Mason University, 2022

Thesis Director: Dr. Aarthi Narayanan

Acutely infectious viruses including alphaviruses, Venezuelan equine encephalitis virus (VEEV) is vector transmitted, resulting in increased disease burden in the civilian and military population. Several of these pathogens are highly infectious and transmissible as aerosols, thus posing an increased threat as a potential bioweapon. Studies from GMU-CIDR laboratory and from several others have demonstrated the critical role of the host ubiquitination pathway in the establishment of a productive infection for acutely infectious viral agents. For example, it has been demonstrated that proteasome inhibitor Bortezomib was a broad-spectrum inhibitor of alphaviruses and bunyaviruses. Published studies have demonstrated that many viral proteins including the capsid protein of VEEV, the NS1, 3, and 5 proteins of Dengue Virus, VP35 protein of Ebola virus are ubiquitinated in infected cells. As part of efforts to characterize the host: viral protein interactome of alphaviruses using VEEV, we have identified several proteins involved in the ubiquitin-proteasome pathway as interacting with all the viral nonstructural proteins. These observations and evidence in the scientific literature prompted us to hypothesize that ubiquitin-proteasome inhibitors will constitute an excellent broad-spectrum strategy to address emerging/re-emerging viral challenges. As several viral proteins are known to be ubiquitinated in multiple sites, inhibiting such a broadly critical post-translational modification

event is highly unlikely to result in resistant viruses.

We examined multiple inhibitors of host signaling pathways using Venezuelan Equine Encephalitis Virus as a prototype pathogen. Of eight inhibitors that were tested for antiviral activities against the TC-83 strain of VEEV in three different cell lines (Vero, HMC3, and SVG-p12), NSC697923, Bardoxolone methyl (BARM), Omavelexelone (OMA) demonstrated robust inhibition of TC-83 at 1μM concentration. In vitro dosing studies conducted thus far have quantified viral loads following combined pre/post-treatment of cells, pre-treatment only, and post-treatment only conditions. The results identify combined pre/post-treatment as the most effective strategy in robustly reducing viral load between 2-4 logs.

INTRODUCTION

Togaviridae: Alphaviruses

Alphavirus is one of two genera in the family Togaviridae and Rubivirus (rubella virus), the other togavirus genus. The alphaviruses are arthropod-borne (arboviruses), whereas the rubiviruses are transmitted via the respiratory tract (1). All arthropod-borne alphaviruses are antigenically related, but most can be distinguished in cross-reactivity tests with which they have been divided into 8 antigenic complexes: Eastern, Western, and Venezuelan equine encephalitis, Trocara (complex assigned based only on genetic divergence), Middelburg, Ndumu, Semliki Forest and Barmah Forest (1). All alphaviruses share antigenic sites on the capsid and at least one envelope glycoprotein, but viruses can be differentiated by several serological tests, particularly neutralization assays (2). The virions are spherical, around 60. To 70mm in diameter with a positive-sense, single-stranded RNA genome. The lipid-containing envelope has two (rarely three) surface glycoproteins that mediate attachment, fusion, and penetration while the icosahedral nucleocapsid contains capsid protein and RNA (2).

Venezuelan Equine Encephalitis Virus

Venezuelan equine encephalitis virus (VEEV) is a member of genus Alphavirus in the family Togaviridae. VEEV complex is a group of 14 antigenic varieties divided into 7 species (3). The VEEV species include four antigenic varieties namely IA/B, IC, ID, and IE, all of which cause human disease that is indistinguishable between the antigenic varieties (3). Subtypes IA/B and C are epizootic strains that cause fulminant disease and high mortality in equines while Subtypes ID and IE are enzootic strains that are typically avirulent in equines. VEEV is an enveloped viruses

which is continuous in naturae by a cycle between rodents and mosquitoes. However, epizootic strains can sporadically cause outbreaks in equines and human. The virus is widely distributed throughout the Americas. Outbreaks of VEE in humans and equids have been reported in at least 12 countries, including Venezuela, Colombia, Peru, Ecuador, Costa Rica, Nicaragua, Honduras, El Salvador, Guatemala, Panama, Mexico and the USA (4). Human infection begins with an asymptomatic period of 1-5 days that is followed by onset of febrile illness (3). Usually, the febrile illness is defined by fever, nausea, vomiting, headache, myalgia, diarrhea, lower back pain and ocular pain lasting for around 1-4 days. However, the short febrile illness can progress into what is called fulminant encephalitis. During this time, convulsions, hemiparesis, behavioral changes, and alterations of consciousness can occur (3). A more severe infection can occur which is associated with hemichorea, seizures, and stupor or coma (3). While mortality of VEEV is low (less than 1%), neurological disease can be up to 14% in infected humans. Due to the ease of aerosolization and an extremely low infectious dose, VEEV was developed as a bioweapon by the United States and the Soviet Union during the Cold War (5). Based off the low mortality rates but higher morbidity rates, VEEV was defined as Category B agent by the Centers for Disease Control and Prevention, and National Institutes of Health. To handle live virulent strains of VEEV, biosafety level 3 containment is required. There are currently no FDA approved vaccines or treatments for VEEV, however, the attenuated VEEV TC-83 strain is used to vaccinate military personnel and lab workers at risk of contracting the virus (5). VEEV is used in the laboratory as a model for alphavirus research, particularly in NW alphavirus research due to the ability to work with TC-83 at BSL-2 (5).

Viral Replication

In the first phase of infection, VEEV enters host cells by receptor-mediated binding of the virus to the host cell membrane (Figure 1). Virus containing endosomes fuse with lysosomes resulting in

formation of what is called an endolysosomes. From there, viral RNA is released in the cytoplasm following pH dependent conformational changes in the viral proteins, allowing fusion with the endolysosome membrane (3). VEEV is a single-stranded positive-sense RNA virus that replicates in the cytoplasm and does not have a nuclear phase of replication (3). In the second phase of infection, viral nonstructural proteins (Nsps) are translated as specific polyproteins from the viral genomic RNA. Nsp-2 cleaves the viral polyproteins into individual nsp-1, nsp-2, nsp-3, and nsp-4 proteins. Nsp-4 is a viral RNA-dependent RNA polymerase (RdRp), which with methyltransferase activity of nsp-2, drives synthesis of negative-sense viral RNA (3). Negative-sense viral RNA is transcribed into smaller positive-sense RNA and a full-length positive-sense viral RNA. The smaller positive-sense RNA is translated into viral structural proteins namely capsid, a polyprotein of E3 and E2 called PE2, 6k, and E1 (3). Viral nsp-2 plays a role in capping of viral genomic RNA while nsp-4 adds a poly-A tail to the viral genomic RNA. Full length positive- sense viral RNA is incorporated into a virus replication complex (VRC) (3). Assembly of VRC is aided by interaction of viral non-structural proteins with host proteins such as nsp-3 with IKK-β. In addition, viral Nsps interact with host factors that help promote VEEV replication. For example, capsid proteins can bind to components of the nuclear pore complex effectively blocking nuclear-cytoplasm-nuclear traffic and host protein translation (3). Furthermore, Nsp2 plays a role in loading of the viral RNA into nucleocapsid and maturation of virions. The last phase of infection, structural proteins E1 and E2 are embedded in the plasma membrane, and assembly and release of the mature virion particle occurs by encapsulating nucleocapsid and budding at plasma membrane (3).

Host Signaling Pathways and Ubiquitin-Proteosome Pathway (UPS)

As described in the previous section, VEEV utilizes host signaling pathways to establish infection. Such host signaling pathways include Nrf2, Nf-κB, Deubiquitinase (DUBS), Ubiquitin Specific Protease 7 (USP-7), and apoptosis. The ubiquitin–proteasome pathway (UPS) a major

lysosome-independent system and that is responsible for the removal of misfolded proteins. Recent studies show that the ubiquitin–proteasome machinery is a crucial system mediating Nrf2 activation in oxidative and electrophilic stressed cells (6). In normal cells, Nrf2 is maintained at a low protein level as it interacts with other proteins, which negatively regulates Nrf2 levels through promoting the degradation of Nrf2 via ubiquitin–proteasome pathway when the cell is in unstressed conditions (6). However, when the cell is under oxidative and electrophilic stress Nrf2 is released from a specific complex (KEAP1-Nrf2) and is translocated into nucleus to transcriptionally activate downstream antioxidant gene to help maintain homeostasis in the cell (6). Most viruses cause oxidative stress and increase the activity of radicals and reactive oxygen species (ROS), subsequently, the cellular protection system activates the Nrf2 and increases the expression of cytoprotective genes (7). However, some viruses inhibit the activation of Nrf2, in which means the virus also benefits this mechanism to maintain the homeostasis of the cell. Upon viral infection in host cells, cells initiate signaling events that activate (nuclear factor) NF-κB transcription factors that is highly involved in variety of physiological and pathological processes including inflammation, immune response, and cell survival (8). Many viruses are known to influence the NF-κB pathway where critical steps in the pathway are taken over to aid in viral replication. Several studies have implicated viruses and viral proteins as activators of the NF-κB cascade as well as serving as binding partners to proteins in the NF-κB pathway (8). Viruses such as Human Immunodeficiency virus (HIV), have been shown to not only activate the NF-κB signaling pathway, but also associate with the signaling components to enhance viral pathogenesis and viral life cycle (8). In addition to direct take over, viruses can indirectly interact with NF-κB by hijacking host proteins to stimulate activation and in so doing can change the elements of the NF-κB pathway to amplify viral replication. It has been shown that during VEEV infection, it can activate the NF-κB cascade by the phosphorylation of p65 (9). P65 regulates the expression of many target genes

that can control cellular stress response, apoptosis, proliferation, and a variety of other processes. Deubiquitinases or deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin from target proteins and are also involved in ubiquitin maturation, recycling, and editing (10) within the UPS. Some viral DUBs can down-regulate the production of antiviral molecules like cytokines allowing for viruses to evade host responses, but other viral DUBs can cause deubiquitinating of viral proteins that avoid targeting the proteosome. USP-7 deubiquitinates target proteins such as p53 (a tumor suppressor protein) and WASH (essential for endosomal protein recycling) and regulates their activities by counteracting the opposing ubiquitin ligase activity of proteins (11). Studies have shown VEEV requires a functional UPS for efficient replication. During the early stages of infection, VEEV capsid is ubiquitinated and most likely requires a functional proteasome system for degradation to allow viral RNA to be released into the cytoplasm for RNA translation and replication to occur (12). VEEV replicates in the cytoplasm of infected cells and eventually induces apoptosis (13) by delaying the cell cycle and disrupting host transcription, translation, and nucleocytoplasmic trafficking, which in turn results in atypical cell cycling (14).

Non-structural proteins

The genome of VEEV encodes 4 nonstructural proteins (nsP1-4) and five structural proteins: 6K, E1, E2, and E3 envelope glycoproteins, and capsid (5). nsP1 is involved in the synthesis and capping of viral RNA. The newly translated polyprotein is cleaved by the viral proteases nsP2 and nsP4 function as the RNA polymerase. The role of nsP3 has not yet been fully characterized; however, it has been implicated in RNA synthesis, has a role in pathogenicity in mice, and interacts with the host inhibitor κB kinase-β (IKKβ) component of the IKK complex (15).

Small molecule Host Signaling Pathway Inhibitors

In this study, 8 host signaling pathway inhibitors were selected based of their target pathways. The target pathways chosen were described in the host signaling pathway and ubiquitin-proteosome pathway section of this paper. Table 1 summarizes the chosen inhibitors for the study and describes their target pathway.

Previous studies of small molecule inhibitors in VEEV viral infection

The role of small molecule inhibitors in infected cells with VEEV has been an on studied previously in a plethora of ways. Most antiviral drugs are small-molecule inhibitors that target different stages of the viral life cycle by interacting with virus or host proteins critical for virus replication (16). For example, P5, a peptide extracted from the stem of Japanese encephalitis virus E protein, can inhibit ZIKV entry into host cells by changing the conformation of the E protein under low pH. The hydrophobicity of the last seven amino acid residues is also considered to be the key to the binding of the viral membrane (16). Other small molecule inhibitors have been shown to act like certain transcription factors to induce certain genes that affect expression. A small-molecule type I IFN receptor agonist called R08191 can directly transduces the IFN signal cascade and stimulates antiviral gene expression in acute hepatitis C virus (HCV) infections (17). Like type I IFN, the small-molecule compound induces IFN-stimulated gene (ISG) expression for antiviral activity in vitro and in vivo in mice and the ISG induction mechanism is attributed to a direct interaction between the compound and IFN-α receptor 2, a key molecule of IFN-signaling on the cell surface (12). In specific to VEEV, interactions of VEEV with host microRNA (miRNA) machinery has been studied using small molecule inhibitors against Ago2 noted as ACF (17). miRNAs are small non-coding RNAs that act as master regulators of gene expression by downregulating or degrading messenger RNA, thus suppressing production of the resultant proteins (17). ACF treatment promoted increased survival of neuronal cells over a non-treated, infected control and reduced viral titers of fully virulent VEEV as well as Eastern and Western Equine

Encephalitis Viruses and West Nile Virus, but not Vesicular Stomatitis Virus suggesting that inhibition of Ago2 results in decreased replication of encephalitic alphaviruses in vitro (17). In this study, we have hypothesized that small molecule inhibitors of ubiquitin mediated signaling events that impact the NFkB and Nrf2 signaling pathways will inhibit VEEV and decrease viral load in infected cells.

MATERIALS AND METHODS

Cell Culture

Vero African Green Monkey kidney epithelial cells (ATCC, CCL-81) were grown with Dulbecco's Modified Eagle's Medium (DMEM, Quality Biological, 112-013,101CS, Gaithersburg, MD, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin antibiotics (P/S) (Corning 30-003-CI, Corning, NY, USA), and 1% L-glutamine (Corning, 25-005-CI, Corning NY, USA). HMC3, human microglial cells (ATCC, CRL-3304), and SVG-p12, Human astroglia cells (ATCC, CRL-8621) were each cultured in Eagle's Minimum Essential Medium with 10% FBS and 1% P/S. All cell lines were maintained at 37°C incubators humidified with 5% CO_2. Cells were plated per well at a density of 1.5 X 10^5 for 12-well plates and 1.0 X 10^5 U87 MGs. For 96-well plates, at 100uL at 1.0 X 10^4 or 200uL at 2.0 X 10^4 per well was used for HMC3, and SVG-p12, and 100uL 5.0 X 10^4 for Vero Cells.

Viruses and Viral infection

VEEV TC-83 strain was studied as the BSL-2 model. TC-83 is a live attenuated vaccine derivative of the TrD strain of VEEV and was derived by 83 serial passages of the virus in guinea pig heart cells, and was obtained from BEI resources (Amaya, M. et al.). The TC-83 stain used during the study is registered under the Center for Infectious Disease Research (CIDR, formerly NCBID) and conducted at George Mason University's Biomedical Research Laboratory with registrations in accordance with Federal Select Agent regulations.

Drug treatment and Plaque Assay

Cells were seeded at 1.0 X 10^4 in a 96-well plate pretreated for 1 hour with selected inhibitors and DMSO control. Cultured cells were infected at MOI of 0.1 for 1 hour with VEEV-TC83. The drugs were reintroduced to the cells and collected at an 18 hour time point post-infection and analyzed by plaque assays. For plaque assays, Vero cells were plated in 12-well plates at $1.5x10^5$ cells per well. Samples were diluted in DMEM from 10^1 to 10^8 and infections were carried out for each dilution. 1 hour post-infection, 1 mL of a 1:1 solution of 1% agarose in distilled H_2O with 2x Eagle's minimal essential medium was added to each well. Plates were allowed to solidify at room temperature and then incubated at 37 °C for 48 hours. At 48 hours, plates were fixed using 10% formaldehyde for 2 hours at room temperature. At 2 hours, the agar plugs are discarded, and fixed cells were stained with 1% crystal violet in 20% methanol solution for 15 minutes. The plaques were counted for each plate and PFU/mL for each sample was determined. The mean and standard deviation was determined using the average of the 3 replicates for each sample.

Cell Viability Assay

Cell viability assays were performed on drug-treated cells using Cell Titer Glo Cell Luminescent Viability Assay according to vendor's instructions (Promega, G7570, Madison, WI, USA). The cell viability assay measures the relative ATP levels and is detected via luminescence detection and percent viability was calculated relative to the DMSO control. Similarly for viral infections cells were drug-treated and infected in 96-well white-wall plates. At specified collection time and percent cell survival was analyzed and compared with mock-treated wells.

RNA Extraction and reverse transcription, quantitative PCR

Cells were lysed with TriZol LS, and total RNA was isolated from cells with the Direct-zol RNA miniprep kit (Zymo Research) according to the manufacturer's protocol. The amount of intracellular RNA was determined by reverse transcription-quantitative PCR performed with the RNA UltraSenseTM One-step Quantitative RT-PCR System (Applied Biosystems). The

experiment was performed according to a standardized protocol using 20µL of master mix using Verso 1-step RT-qPCR Mix with ROX (Fischer Science) and 5µL of sample RNA. The samples were heated at 50ºC for 20 minutes, 95ºC for 15 minutes, followed by 40 cycles of 95ºC (15 seconds), and 60ºC (60 seconds). RNA genomic copies were determined relative to a standard curve containing a known amount of viral RNA. Intracellular RNA data was determined per 10,000 cells, while the extracellular RNA data was determined per volume.

Statistics

Plaque, RNA, and ELISA data calculations were determined using Microsoft Excel. Graphs and P-values were calculated using an unpaired two-tailed t-test in GraphPad Prism version 9.2.0 for Windows 10. Significance values are indicated using asterisks for * $p < 0.1$, ** $p < 0.05$, *** $p < 0.001$, **** $p < 0.005$, and ns for not significant.

RESULTS

Host Signaling Pathway Inhibitors decrease VEEV-TC83 infection in Vero Cells

We examined eight different host signaling pathway inhibitors for their impact on VEEV TC-83 infection. Many viruses have been shown to use or modulate the ubiquitin-proteasome system to enhance viral multiplication or create a persistent infection (21). In addition, The UPS regulates signaling pathways and hormones during viral invasion (22). For example, the sensing of pathogen-associated molecular patterns (PAMP) by pattern recognition receptor (PRR) initiates a series of signaling cascades which ultimately leads to the activation of major transcription factors driving inflammation, namely, the nuclear factor-κB (NF-κB) as well as the interferon regulatory factors (IRF) 3 and 7 (22). Following their translocation into the nucleus, NF-κB and IRF3/IRF7 induce the transcription of proinflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) and interferons (IFN), respectively, to counteract the invading pathogens (23). Here we examined eight different host signaling pathway inhibitors for their impact on VEEV productions in Veros. NSC, OMA, BARM, POO, YH1, BAR, JHS, and ML were screened using Cell Titer-Glo to determine the non-toxic concentrations of each inhibitor in Vero cells (Figure 2A). The cells were treated with various concentrations of each inhibitor for 24 hours. The percent viability of each inhibitor was then decided relative to the DMSO control in Vero cells. A table of tested inhibitors with their specific host targets and the cytotoxic concentration at 50% (CC50) was created in order of most (BARM) to least (ML) toxic and 2 lowest concentrations possible for

initial testing were determined (Figure 2A). For initial antiviral testing, Vero cells infected with TC-83 were treated with 1µM of OMA, BARM, and NSC, while the rest of the inhibitors were treated at 2µM. The supernatants were collected at 18 hours post-infection and viral titers were determined using a plaque assay. The data showed that while all the tested ubiquitin inhibitors decreased the level of TC83 infectious titer by at least a log compared to DMSO control, BARM, BAR, and OMA showed more than 2 log decrease. NSC, POO, ML, JHS, and YH1 showed more than a log decrease in TC83 infection compared to the control (Figure 2B).

The efficacy of Selected Host signaling pathway Inhibitors in Human Microglial cells (HMCs) and Human Astrocytes (SVG-p12)

OMA, BARM, NSC, YH1, and POO were selected for further testing in HMC3 and SVGp12 cells. YH1 and POO were selected to further explore TC-83 involvement in cell cycle and apoptotic pathway respectively instead of JHS and ML for further testing. This was due to POO being a selective inhibitor of ubiquitin-specific protease 7 (USP7) and YH1 being an apoptosis inducer. OMA, BARM, NSC, YH1, and POO were screened using Cell Titer- Glo to determine the non-toxic concentrations of each inhibitor in Vero cells. The cells were treated with various concentrations of each inhibitor for 24 hours. The percent viability of each inhibitor was then decided relative to the DMSO control in HMC3s, and SVG-p12s. Antiviral testing was like the initial antiviral testing, except HMCs and SVG-p12s cells were infected with TC-83 were treated with selected concentrations, DMSO and POO were treated at 1µM, OMA and BARM at 0.1µM, and YH1 and NSC at 0.5 µM (Figure 3a). These concentrations were selected using the preliminary results in Veros. The supernatants were collected at 18 hours post-infection and viral titers were determined using a plaque assay. The plaque data showed that there was at least a log decrease OMA, YH1, BARM, NSC in HMC3s and OMA and NSC in SVG-p12s (Figure 3b). To address whether the inhibitors may interfere with the extent of viral RNA replication, we

quantified the amount of extracellular and intracellular viral RNA during the early stages of the infectious process in the cytoplasm of drug-treated cells by q-RT-PCR using VEEV-specific primers. In HMC3s, the extracellular RNA levels in all samples were relatively the same compared to the DMSO control. Intracellular RNA levels were similar to the trend in the extracellular levels except OMA and BARM showed a log decrease from DMSO control. In SVG-p12s, OMA showed at least 1 log decrease in both extracellular and intracellular RNA whereas NSC showed at least 1 log decrease only in intracellular RNA (Figure 3c). These results suggest that NSC, BARM, and OMA have a significant impact at 18 hours post-infection in HMC3s. However, SVG-p12 data shows that there was not a significant impact in most inhibitors at 18 hours which could be how susceptible the cells are to VEEV infection. Therefore, we turned our focus to VEEV TC-83 infection in HMC3 cells.

Host signaling pathway inhibitors decrease VEEV TC-83 infection in HMC3 cells in a dose-dependent manner.

To understand the impact of NSC, OMA, and BARM on VEEV infection, HMC3s were treated with different doses of each inhibitor at 18-hours post-infection. A dose analysis was performed using 0.1, 0.5, and 1μM of NSC, BARM, OMA. HMC3s were treated with NSC, BARM, OMA in the following scenarios, Pre-only, Pre and Post, Direct, and Post only (Fig 4a). For Pre-only, HMC3s were pretreated with inhibitors for an hour and then inhibitor overlay was collected. For Pre-post, HMC3s were pretreated with inhibitors for an hour, collected, and VEEV was added for another hour, the virus was removed, and the collected pretreated was placed back on the cells. Direct viral the virus, inhibitors, and media were added together and used for treating the cells for an hour. Post-only is where inhibitors were added after viral infection. The supernatants and cellular lysates of each sample were collected. A standard plaque assay was performed using the supernatants. OMA, BARM, and NSC have a dose-dependent effect on

VEEV-TC83 infection in HMC3 cells when treated with both pre-and- post-infection. OMA and BARM had more than four log decreases at 1.0um and 0.5um concentration while at 0.1um there was 1 log difference. NSC was consistent across the concentrations with at least 1 log difference. However, with the inhibitors at a 1.0um concentration, showed the best results (Fig 4b). RNA extraction followed by RT-qPCR of was used to measure extracellular and intracellular viral RNA levels in Post-only and Pre-post infections. The intracellular RNA levels in all samples in Post-only were relatively the same compared to the DMSO control (Fig 5a). These results suggest that OMA, BARM, and NCS does not impact viral RNA production under post-only conditions, but rather impact viral extracellular RNA production in both post-only and pre-post infection (5b).

FIGURES

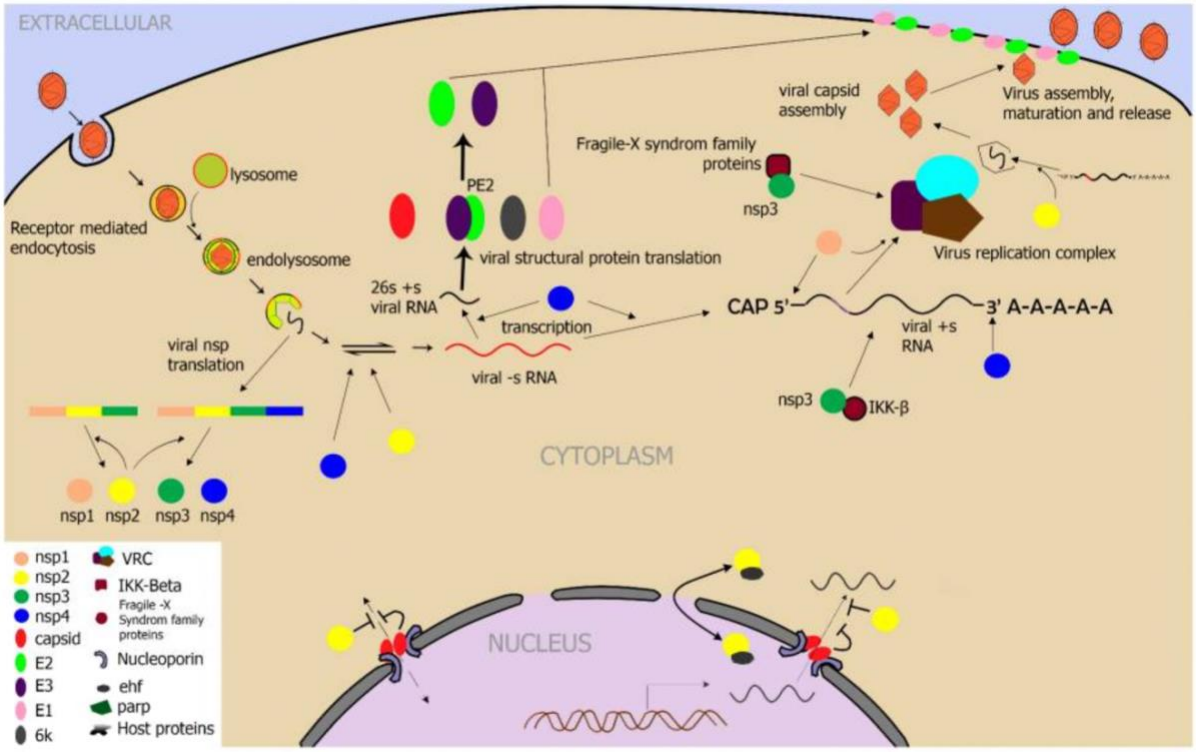

Figure 1. Model of Venezuelan Equine Encephalitis Virus (VEEV) viral replication in host cell. In the first phase of infection, VEEV enters in host cells by receptor mediated binding of the virus to the host cell membrane. Viral RNA is released in the cytoplasm following pH dependent conformational changes in the viral proteins, allowing fusion with the endolysosome membrane. In the second phase of infection, viral nonstructural proteins (Nsps) are translated as specific polyproteins from the viral genomic RNA. Negative-sense viral RNA is transcribed into smaller positive-sense RNA and a full-length positive-sense viral RNA. The smaller positive-sense RNA is translated into viral structural proteins namely capsid, a polyprotein of E3 and E2 called PE2, 6k, and E1. Full length positive- sense viral RNA is incorporated into a virus replication complex (VRC). The last phase of infection, structural proteins E1 and E2 are

embedded in the plasma membrane, and assembly and release of the mature virion particle occurs by encapsulating nucleocapsid and budding at plasma membrane.

Name	Abbreviation	Target
Bardoxolone methyl	BARM	IKK inhibitor, showing potent proapoptotic and anti-inflammatory activities and potent Nrf2 activator and nuclear factor-κB (NF-κB) inhibitor
Omaveloxolone	OMA	activates the transcription factor Nrf2 and inhibits NF-κB signaling
NSC697923	NSC	inhibitor of the Ub- conjugating enzyme (E2)
Bardoxolone	BAR	highly potent activator of Nrf2
YH239-EE	YH1	an apoptosis inducer
P005091	POO	selective and potent inhibitor of ubiquitin-specific protease 7 (USP7)
JSH-23	JSH	inhibitor of NF-κB transcriptional activity
ML-323	ML	UPS1-UAF1 inhibitor deubiquitinase (DUB)

Table 1. Selected Ubiquitin Host Signaling Pathway Inhibitors. Bardoxolone methyl (BARM) and Omaveloxolone (OMA) target Nrf2 and NF-κB signaling pathways. NSC697923 (NSC) targets specifically the E2 step in UPS. Bardoxolone (BAR) targets Nrf2 signaling pathway. YH239-EE (YH1) targets the apoptosis pathway. P005091 (P00) and ML-323 (ML) target specific deubiquitinase pathways. JSH-23 (JSH) targets NF-κB pathway.

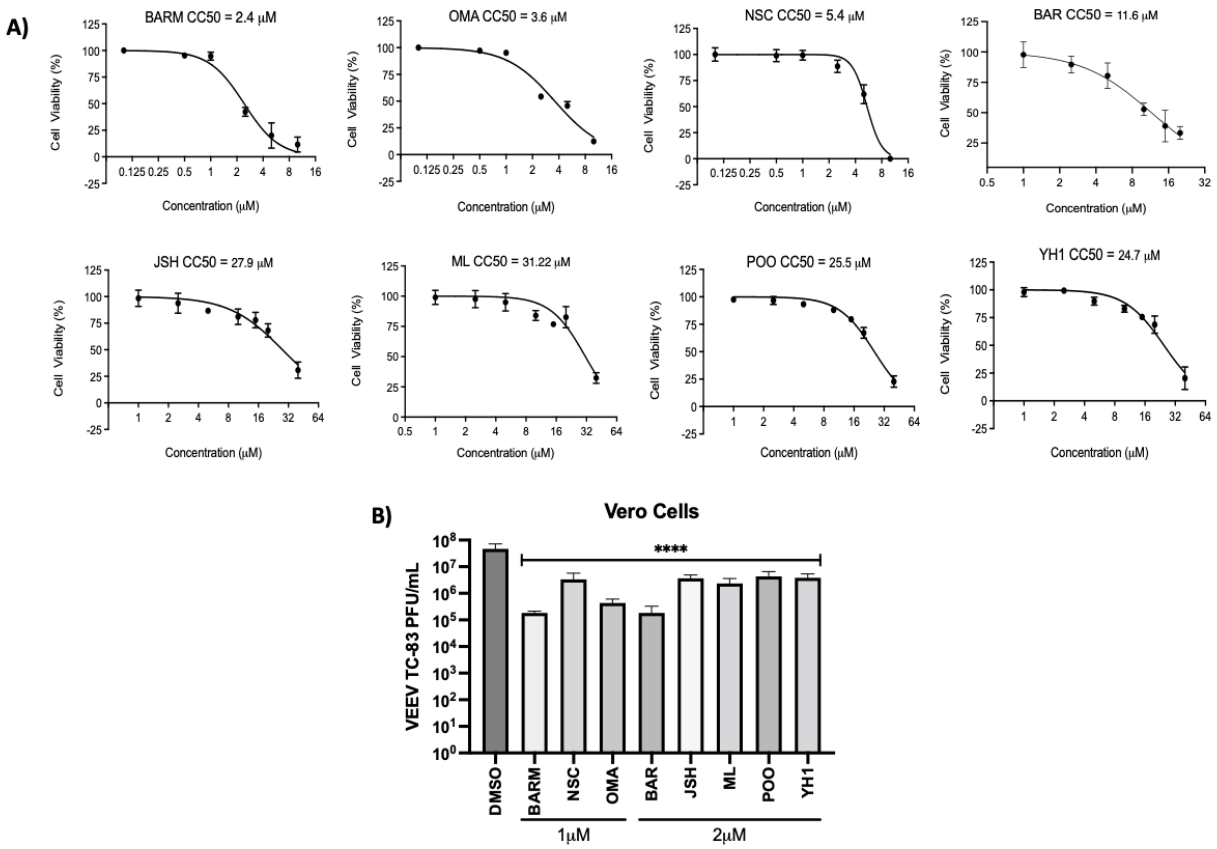

Figure 2. Ubiquitin cell viability and preliminary inhibitory effect on TC-83 in Vero cells. (A) Cytotoxicity of Ubiquitin Host Signaling Pathway Inhibitors in Vero cells. (B) TC-83 replication in Ubiquitin Host Signaling Pathway inhibitor-treated Vero cells.

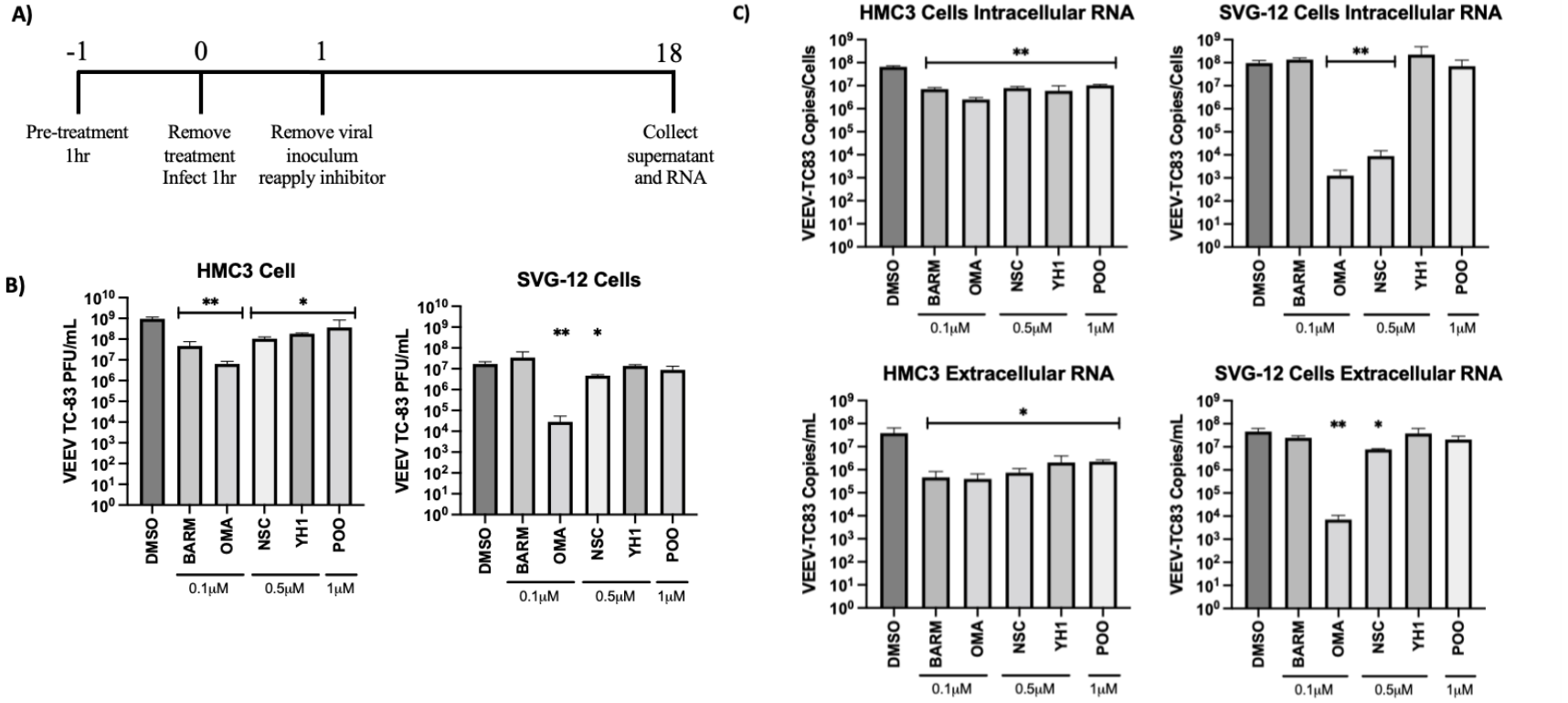

Figure 3. Selected inhibitors decrease VEEV TC-83 virus in 2 different cells lines. A) Schematic Representation of the experiment. B) Efficacy of Selected Inhibitors on VEEV-TC832 in HMC3 and SVG-p12 Cells. C) Intracellular and Extracellular RNA shows selected inhibitors decrease VEEV TC-83 virus in HMC3 and SVG-p12.

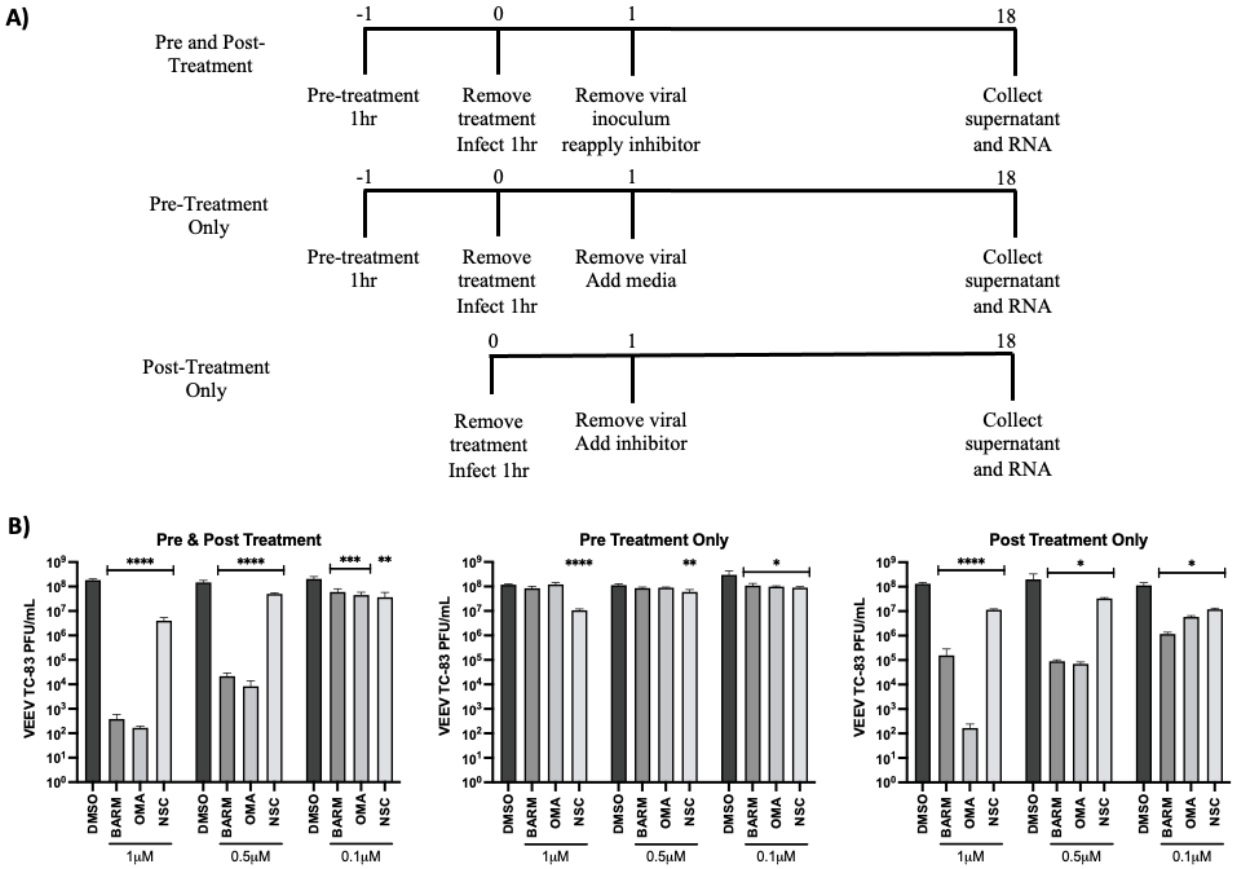

Figure 4. The Selected Inhibitors OMA, BARM, NSC Have a Dose Dependent Effect on VEEV-TC83 Infection in HMC3 When Treated both Pre & Post Infection. A) Schematic representation of the experiment. B) Efficacy of Selected Inhibitors on VEEV-TC832 in HMC3 in a dose-dependent manner.

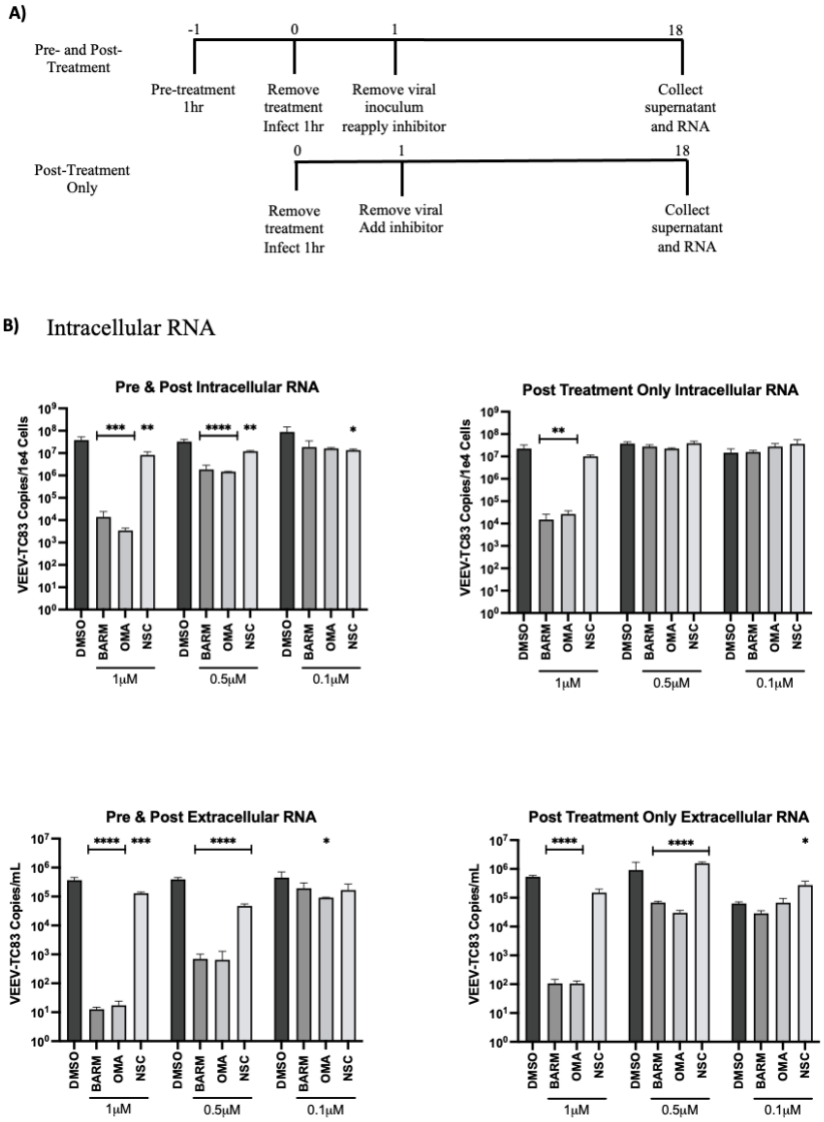

Figure 5. OMA, BARM, and NSC show a Dose-Dependent decrease of VEEV-TC83 RNA Production when Treated in both Pre & Post Infection. A) Schematic of infection. B) OMA, BARM, and NSC impact viral extracellular RNA production in bother post-only and pre-post infection.

DISCUSSION

VEEV is an emerging infectious virus that causes natural outbreaks in many parts of the world (18). VEEV was weaponized in the past and continues to be classified as a bio-threat agent owing to the retention of stability and infectivity in the aerosol form (18). However, since VEEV can exist as both a natural pathogen and a laboratory-developed biologic weapon, it raises concerns. In addition, global warming may facilitate the emergence of VEEV epidemics (19). Climate change can lead to longer transmission seasons, and changes in rainy seasons, which increase mosquito populations, and increase geographic distributions of tropical mosquito vectors (19). Due to these reasons, finding potential therapeutics and understanding how host factors affect VEEV pathogenicity is a detrimental research topic. In this study, we aimed to investigate the effects of ubiquitin host signaling pathways on VEEV replication.

Ubiquitination, a step in the nonlysosomal degradation of proteins, is a crucial post-translational modification in eukaryotic organisms (19). The different roles that the UPS plays in a cell make it an attractive target for pathogens (10,12,19). The UPS is involved in protein degradation, protein trafficking, transcription and cell signaling, hence viruses can easily manipulate this system to enhance replication (20). While the UPS plays an important role directly to the cell, many host signaling pathways such as Nrf2 and NF-κB are intertwined in the progression of viral infections (7,8,9). In this study, we examined 8 specific Ubiquitin host signaling pathway inhibitors, BARM, OMA, NSC, BAR, P00, YH1, JSH, and ML.

We tested these Ubiquitin host signaling pathway inhibitors on VEEV infected VEROs for preliminary testing (Fig. 2). All 8 inhibitors showed initially, at least 1 log difference compared to DMSO control. However, BARM, NSC, OMA, and BAR showed the most effective when compared to DMSO control with more than a 2 log difference. While that was the case, BARM and BAR are analogs of each other so it was decided to remove BAR from future studies as BARM and BAR should theoretically have similar results. In addition, P00 and YH1 were to be used in future studies since both inhibitors affect other areas of host signaling pathways compared to BARM, NSC, and OMA (Table 1). After down selecting to five ubiquitin host signaling pathways inhibitors: BARM, NSC, OMA, YH1, and P00, these were tested in new infected cell lines. The chosen cell lines were HMC3s and SVG-p12s. HMC3s which are microglia, are macrophages of the central nervous system and play an important role in the induction of inflammation following VEEV infection (20). Similarly, SVGp-12s or astrocytes is an integral component of the tissue microenvironment that is centrally involved in the development of VEEV-induced encephalitis (23). Therefore, we decided that using both cell lines we could look at the impact of the effects of various inhibitors on VEEV replication in cell lines that are directly involved in neurological development of VEEV infections. In efficacy testing of both cell lines, both YH1 at a 0.5uM concentration and P00 at 1uM a concentration had minimal impact on VEEV replication while BARM and OMA at 0.1uM concentration and NSC a 0.5uM concentration showed the most significance in VEEV infection with at least a 1 log difference. YH1 is an inhibitor of apoptosis indicating that the apoptosis pathway may have not been affected. VEEV has been known to inhibit both cellular transcription and translation in order to downregulate the innate immune system affecting the rate of apoptosis (1). P00 is an inhibitor of USP7 which is a type of DUBs. This could indicate that VEEV is utilizing the degradation of ubiquitinated host target proteins that may aid in further viral infection like the deubiquitnating

the capsid structural protein (12,13). In addition to our data showing that BARM, NSC, and OMA had decreased VEEV infectious titer by 1 log, 2 log, and 1 log difference at 0.1 uM, 0.5uM, and 0.1uM concentration, respectively in VEEV infected HMC3s, this trend continued in viral RNA. Both VEEV intracellular RNA and extracellular RNA production had decreased. In SVG-p12 infected cells, OMA and NSC showed a significant decrease in VEEV infectious titer with 3 log and 1 log difference at 0.1uM and 0.5uM, respectively. Similarly, to the effect of viral RNA in HMC3s, VEEV infected SVG-p12 showed a decreased in viral RNA in both extracellular and intracellular. Based off these results, BARM, NSC, and OMA show promising results thus, these three inhibitors were selected for further testing. Both BARM and NSC affect the NF-κB and Nrf2 pathway while OMA solely affects the NF-κB pathway. Both NF-κB and Nrf2 have been shown to effect proinflammatory response in host cells. NF-κB has long been considered a prototypical proinflammatory signaling pathway, largely based on the activation of NF-κB by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), and the role of NF-κB in the expression of other proinflammatory genes including cytokines, chemokines, and adhesion molecules (24). While Nrf2 contributes to the anti-inflammatory process by orchestrating the recruitment of inflammatory cells and regulating gene expression through the antioxidant response element (ARE) (25). Based on the properties of the best selected inhibitors, it was decided that only HMC3s were to be used. Past studies have shown that it is well accepted that microglia become activated during neurotropic viral infections, with activation leading to the expression of numerous downstream pro-inflammatory responses (25). Thus, selecting HMC3 as the better fitted cell line for further experiments. To determine at what point and what dose of inhibitor is most effect during viral infection, dose-dependent studies were performed for Pre-entry only, post-entry only, and both pre-post entry infections (Fig. 4). In Pre-post treatment, BARM, NSC and OMA decreased VEEV infectious titer by at least 1 log

compared to DMSO control at 1uM, 0.5uM, and 0.1uM concentrations. In Pre-treatment only, NSC decreased VEEV infectious titer by 2 log difference in 1uM concentration and 1 log difference in 0.5uM. There was not a significant decrease in VEEV infectious titer with NSC at 0.1uM concentration compared to DMSO control. In Post-only BARM, NSC, and OMA showed at least 1 log difference across all concentrations, indicating promising results of post-entry addition of inhibitor. While the 3 selected inhibitors: OMA, NSC, and BARM have a dose dependent effect on VEEV infection in HMC3 when treated with pre-treatment only, post-treatment only, and pre-post treatment infections, it was decided that RNA testing was to only be done on Pre-post treatment infections and post-only treatment infections. This was due to overall significant data in the selected inhibitors. In Pre-treatment only, NSC was the only inhibitor to show promising results. This could be due to the fact NSC only targets the NF-κB pathway. Several studies have implicated viruses and viral proteins can act as activators of the NF-κB cascade as well as serving as binding partners to proteins in the NF-κB pathway (8). Thus, by adding NSC pre-entry to viral it allows for the pathway to be inhibited prior to VEEV infection and in turn, decreasing VEEV hijacking. Again, while the date in pre-treatment only is to not go unnoticed, it was not used for further RNA experiments. In viral RNA experiments, OMA, BARM, and NSC showed a dose-dependent decrease of VEEV RNA production when treated with pre-post infection (Fig. 5). BARM and OMA showed best results when at 1uM concentration and 0.5uM concentration in both intracellular and extracellular RNA production with at least a 1 log difference in both cases. NSC had the least amount of impact at all 3 tested concentrations. In post-treatment only, BARM, NSC and OMA showed promising results with over a log difference in extracellular RNA at 1uM and 0.5uM. However, in intracellular RNA BARM and OMA at 1uM showed the best results with over a 2 log difference indicating that infecting HMC3s with inhibitor at pre-entry and post-entry has a greater impact on both VEEV

viral titer and RNA production. Furthermore, BARM and OMA were shown to have the most significant impact on ubiquitin host signaling pathway during VEEV infections. As said earlier in the paper, BARM and OMA are both inhibitors of NF- κB pathway and activator of Nrf2. The transcriptional factor nuclear factor E2-related factor 2 (Nrf2) regulates genes involved in the cellular response against various stressors, including oxidative stress[1,2]. The activity of Nrf2 is tightly controlled by a complex array of transcriptional regulators and post-translational modifications to ensure its proper activity, both under basal conditions and during adaptation to environmental changes. Under homeostatic conditions, a protein called Keap1 binds to Nrf2 and recruits a complex called Cul3/Rbx1complex, leading to its ubiquitination and subsequent proteasomal degradation. Under oxidative stress, however, the cysteine residues on Keap1 react with electrophilic compounds, resulting in a weaken interaction with Nrf2 and thus rescues Nrf2 from the degradation. Nrf2 moves into the nucleus, where it binds to antioxidant response element (ARE) sequences, thus activating genes encoding antioxidant, detoxification, anti-inflammation, proteasome, and autophagy proteins (26). During VEEV infections, oxidative stress will occur so by adding an inhibitor that will active nrf2 it will give aid to the host immune system by activating more nrf2 and in turn will help protect the host from reactive oxygen species helping bring the cell to homeostasis and suppressing the virus. In addition, when the host cells are under oxidative stress, certain proinflammatory responses are increased such as NF-kB. Too much of these proinflammatory responses can cause severe damage to the cells, Nrf2 has been shown to decrease the NF-kB signaling (26). Based on this, our hypothesis was proven that inhibition of ubiquitin signaling pathways protect host cells against VEEV infection. While our results show that Nrf2 and NF-kB signaling pathways have the most impact on VEEV viral replication, it is clear the ubiquitin host signaling pathways play an extraordinary role in the development in VEEV infections. Future studies on Nrf2 and NF-kB pathways in VEEV viral

replication is needed to better understand the mechanism of action these inhibitors place in the replication cycle.

References

1. Gould, E. A., Coutard, B., Malet, H., Morin, B., Jamal, S., Weaver, S., Gorbalenya, A., Moureau, G., Baronti, C., Delogu, I., Forrester, N., Khasnatinov, M., Gritsun, T., de Lamballerie, X., & Canard, B. (2010, August). *Understanding the alphaviruses: Recent research on important emerging pathogens and progress towards their control*. Antiviral research. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7114216/
2. *Medical Microbiology - NCBI Bookshelf*. (n.d.). Retrieved November 1, 2022, from https://www.ncbi.nlm.nih.gov/books/NBK7627/
3. Sharma, A., & Knollmann-Ritschel, B. (2019, February 18). *Current understanding of the molecular basis of Venezuelan equine encephalitis virus pathogenesis and vaccine development*. Viruses. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6410161/
4. Aguilar, P. V., Estrada-Franco, J. G., Navarro-Lopez, R., Ferro, C., Haddow, A. D., & Weaver, S. C. (2011). *Endemic Venezuelan equine encephalitis in the Americas: Hidden under the dengue umbrella*. Future virology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3134406/
5. Lundberg, L., Carey, B., & Kehn-Hall, K. (2017, September 29). *Venezuelan equine encephalitis virus capsid-the clever caper*. MDPI. Retrieved October 31, 2022, from https://www.mdpi.com/1999-4915/9/10/279
6. Li, N., Du, H., Mao, L., Xu, G., Zhang, M., Fan, Y., Dong, X., Zheng, L., Wang, B., Qin, X., Jiang, X., Chen, C., Zou, Z., & Zhang, J. (2022, June 11). *Reciprocal regulation of NRF2 by autophagy and Ubiquitin-proteasome modulates vascular endothelial injury induced by copper oxide nanoparticles*. Journal of nanobiotechnology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9188091/

7. E;, R. A. N. M. P. F. (n.d.). *The role of NRF2 transcription factor in viral infection*. Journal of cellular biochemistry. Retrieved October 31, 2022, from https://pubmed.ncbi.nlm.nih.gov/29737559/
8. Chen, J., & Chen, Z. J. (2013, February). *Regulation of NF-KB by Ubiquitination*. Current opinion in immunology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3594545/)
9. Amaya, M., Voss, K., Sampey, G., Senina, S., de la Fuente, C., Mueller, C., Calvert, V., Kehn-Hall, K., Carpenter, C., Kashanchi, F., Bailey, C., Mogelsvang, S., Petricoin, E., & Narayanan, A. (2014, February 19). *The role of ikkβ in Venezuelan equine encephalitis virus infection*. PloS one. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3929299/
10. Pfoh, R., Lacdao, I. K., & Saridakis, V. (2015, February). *Deubiquitinases and the new therapeutic opportunities offered to cancer*. Endocrine-related cancer. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4304536/
11. U.S. National Library of Medicine. (n.d.). *USP7 ubiquitin specific peptidase 7 [homo sapiens (human)] - gene - NCBI*. National Center for Biotechnology Information. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/gene/7874
12. Amaya, M., Keck, F., Lindquist, M., Voss, K., Scavone, L., Kehn-Hall, K., Roberts, B., Bailey, C., Schmaljohn, C., & Narayanan, A. (2015, April 30). *The Ubiquitin Proteasome System plays a role in Venezuelan equine encephalitis virus infection*. PloS one. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4415917/
13. Lundberg, L., Fontenot, J., Lin, S.-C., Pinkham, C., Carey, B. D., Campbell, C. E., & Kehn-Hall, K. (2018, December 18). *Venezuelan equine encephalitis virus capsid implicated in infection-induced cell cycle delay in vitro*. Frontiers in microbiology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6315117/
14. Bader, M., & Steller, H. (2009, December). *Regulation of cell death by the Ubiquitin-Proteasome System*. Current opinion in cell biology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2818673/
15. Wang, L., Liang, R., Gao, Y., Li, Y., Deng, X., Xiang, R., Zhang, Y., Ying, T., Jiang, S., & Yu, F. (1AD, January 1). *Development of small-molecule inhibitors against zika virus*

infection. Frontiers. Retrieved October 31, 2022, from https://www.frontiersin.org/articles/10.3389/fmicb.2019.02725/full

16. Konishi, H., Okamoto, K., Ohmori, Y., Yoshino, H., Ohmori, H., Ashihara, M., Hirata, Y., Ohta, A., Sakamoto, H., Hada, N., Katsume, A., Kohara, M., Morikawa, K., Tsukuda, T., Shimma, N., Foster, G. R., Alazawi, W., Aoki, Y., Arisawa, M., & Sudoh, M. (2012, February 10). *An orally available, small-molecule interferon inhibits viral replication*. Nature News. Retrieved October 31, 2022, from https://www.nature.com/articles/srep00259
17. Madsen, C., Hooper, I., Lundberg, L., Shafagati, N., Johnson, A., Senina, S., Fuente, C. de la, Hoover, L. I., Fredricksen, B. L., Dinman, J., Jacobs, J. L., & Kehn-Hall, K. (2014, October 18). *Small molecule inhibitors of ago2 decrease Venezuelan equine encephalitis virus replication*. Antiviral Research. Retrieved October 31, 2022, from https://www.sciencedirect.com/science/article/abs/pii/S0166354214002824
18. Lobaina, D. P., Tarazi, R., Castorino, T., & Vaslin, M. F. (2022). The Ubiquitin–Proteasome System (UPS) and viral infection in plants. *Plants, 11*(19), 2476. https://doi.org/10.3390/plants11192476
19. Çetin, G., Klafack, S., Studencka-Turski, M., Krüger, E., & Ebstein, F. (2021, January 5). *The Ubiquitin-proteasome system in immune cells*. Biomolecules. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7824874/
20. Risner, K., Ahmed, A., Bakovic, A., Kortchak, S., Bhalla, N., & Narayanan, A. (2019, December 12). *Efficacy of FDA-approved anti-inflammatory drugs against Venezuelan equine encephalitis virus infection*. Viruses. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6950191/
21. *Venezuelan equine encephalitis - statpearls - NCBI bookshelf.* (n.d.). Retrieved November 1, 2022, from https://www.ncbi.nlm.nih.gov/books/NBK559332
22. Li N;Chen XL;Li Q;Zhang ZR;Deng CL;Zhang B;Li XD;Ye HQ; (n.d.). *A new screening system for entry inhibitors based on cell-to-cell transmitted syncytia formation mediated by self-propagating hybrid VEEV-SARS-COV-2 replicon*. Emerging microbes & infections. Retrieved October 31, 2022, from https://pubmed.ncbi.nlm.nih.gov/35034586/
23. Keck, F., Kortchak, S., Bakovic, A., Roberts, B., Agrawal, N., & Narayanan, A. (2018). *Direct and indirect pro-inflammatory cytokine response resulting from TC-83 infection of*

glial cells. Virulence. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6141141/

24. Lawrence, T. (2009, December). *The nuclear factor NF-kappab pathway in inflammation*. Cold Spring Harbor perspectives in biology. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2882124/
25. Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J., & Tang, X. (2016, November 4). *Nrf2 signaling pathway: Pivotal roles in inflammation*. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. Retrieved October 31, 2022, from https://www.sciencedirect.com/science/article/pii/S0925443916302861
26. He, F., Ru, X., & Wen, T. (2020, July 6). *NRF2, a transcription factor for stress response and beyond*. International journal of molecular sciences. Retrieved October 31, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7369905/

BIOGRAPHY

Brittany A. McGraw graduated from North Stafford High School, Stafford, Virginia, in 2016. She received her Bachelor of Science from James Madison University in 2020. She was employed as an Associate Biologist at American Type Culture Collection (ATCC) for two years while continuing her Master of Science from George Mason University in 2022.