THE EFFECT OF AUTOPHAGY INHIBITORS ON RVFV PRODUCTION

by

Niloufar Boghdeh
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. Kylene Kehn-Hall, Thesis Chair
_____	Dr. Aarthi Narayanan, Committee Member
_____	Dr. Yuliya Dobrydneva, 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: _____	Spring Semester 2021 George Mason University Fairfax, VA

The Effect of Autophagy Inhibitors on RVFV Production

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

by

Niloufar Boghdeh
Bachelor of Science
Virginia Commonwealth University, 2018

Director: Kylene Kehn-Hall, Professor
Affiliate Faculty Member, George Mason University
Professor, Virginia Polytechnic Institute and State University

Spring Semester 2021
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to my loving husband Eric Olson, and my amazing sisters Aisha and Rebecca.

ACKNOWLEDGEMENTS

I would like to thank my husband Eric Olson, my parents Marziyeh Ounagh and Azim Boghdeh, my sister Aisha Boghdeh, my in-laws Kathy and Michael Olson, and my sister in-law Rebecca Olson for supporting me throughout my pursuit of graduate studies. I would like to thank my extended family including Boghdeh's, Ounagh's, Olson's, Tucker's, and Brady's for providing support and encouragement during my graduate education.

I would like to especially thank my advisor and professor Dr. Kylene Kehn-Hall for supporting my pursuit of graduate studies throughout my time in George Mason Univeristy and giving me the opportunity to work in a leading virology lab. I am grateful for her guidance in my research and continuous support of my pursuit of higher education.

I thank my committee members Dr. Aarthi Narayanan, Dr. Ancha Baranova, and Dr. Yulia Dobrydneva for helping me through my graduate program and providing input and feedback regarding my thesis research.

I thank the Kehn-Hall lab members for providing traininsg and professional support in the lab. Dr. Nicole Bracci has been a tremendous support. Working directly with me throughout my research, and providing training on various lab techniques and experiments. I thank Nicole for her constant support, time, and encouragement of my thesis research. The Kehn-Hall lab members have been the best teachers any graduate student could ask for.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Biosafety Level 2……BSL-2
Biosafety Level 3……BSL-3
Centers for Disease Control……..……CDC
Complentary ribonucleuc acid……..…….……..cRNA
Dulbecco's Essential Modified Medium……...DMEM
Endoplasmic Reticulum……....ER
Hepatitis C virus……HCV
Human Bone osteosarcoma epithelial cells ……..…U2Os
Human Immunodefiency Virus……...……HIV
Human Small Airway Epithelial Cells……HSAECs
Messenger ribonucleuc acid……..…….mRNA
Mouse Hepatitis Virus……..MHV
Modified Vaccina Ankara……MVA
Mouse Embryonic Fibroblasts…….MEFs
National Institute of Allergy and Infectious Diseases……NIAID
Nonstructural protein……...……NSs
Protein Kinase RNA-activated……...PKR
Rift Valley Fever Virus...……....RVFV
Transcription Factor……..TF
Viral genomic ribonucleuc acid……...vRNA
United States Department of Agriculture……USDA

ABSTRACT

THE EFFECT OF AUTOPHAGY INHIBITORS ON RVFV PRODUCTION

Niloufar Boghdeh, MS

George Mason University, 2021

Thesis Director: Dr. Kylene Kehn-Hall

Rift Valley Fever Virus (RVFV) is an arbovirus that can infect ruminants and humans. It can cause many diseases including encephalitis, hemorrhagic fever, and ocular disease. A severe version of the disease is observed predominantly in pregnant and young livestock. While it is primarily transmitted by mosquitoes, most human cases are acquired through contact with blood or organs of an infected animal. Autophagy is an intracellular pathway that allows for the degradation of cytoplasmic organelles during cellular stress. The role of autophagy during viral infections is unclear. In some cases, it slows the progression of the infection, whereas, in many other cases the virus uses the autophagy system to enhance its replication. We hypothesized that inhibition of autophagy will cause RVFV titer reduction and provide evidence that the process of autophagy can be pro-viral to RVFV. To explore the impact of autophagy on RVFV replication, small molecule modulators of autophagy were utilized. CA-5F, DBBC661, and ML240, which are all

known autophagy inhibitors, were shown to be capable of reducing RVFV infectious titers. CA-5F was selected for further studies due to it being one of the most potent and least toxic inhibitors. CA-5F is a late-stage autophagy inhibitor that functions by inhibiting the autophagosome-lysosome fusion. Previous studies have shown CA-5F to have anti-tumor effects against lung cancer cells. HSEACs (Human Small Airway Epithelial Cells) were treated with non-toxic concentrations of CA-5F and a significant decrease in viral production was observed. Furthermore, the greatest decrease in RVFV titers was observed at 16 and 24 hours post-infection as compared to 8 hours post-infection. Additionally, intracellular RNA analysis showed that although CA-5F decreases RVFV infectious titers in a dose-dependent manner, it does not impact viral RNA production. This study provides evidence that the autophagy inhibitor, CA-5F, is capable of reducing RVFV production. Future studies will assess the importance of the viral protein NSs in CA-5F inhibition.

INTRODUCTION

Bunyaviruses: Phlebovirus

Bunyaviruses are a large and diverse order of negative-sense RNA viruses that are capable of infecting vertebrates, invertebrates, and plants (9,11,21,23). They are enveloped viruses with a tri-segmented [large (L), medium (M), and small (S)] negative-sense RNA genome, and are divided into 9 families based on biological and structural characteristics (11). The family Phenuiviridae that includes the genus phlebovirus contains 70 viruses that comprise 9 species (9). All *phleboviruses* have an ambisense coding region meaning their N protein is encoded in the negative sense and their nonstructural (NSs) protein is encoded in the positive-sense orientation on the S segment of their RNA (9).

Rift Valley Fever Virus

Rift Valley fever virus (RVFV) is the most well-known phlebovirus that can infect both humans and livestock (27). RVFV can be transmitted by mosquitoes or through contact with the blood or organs of infected animals (2,13). The virus can cause many diseases ranging from mild flu-like illnesses to a severe one that includes jaundice, encephalitis, hemorrhagic fever, and ocular disease (5,15). In livestock, the disease is more severe in pregnant females and young animals (27). RVFV is also difficult to control due to the ability of mosquito eggs to survive up to 10 years in soil, and due to climate change the outbreaks could increase in the near future, according to recent reports from the centers

for disease control (CDC) (6). The virus was first identified in the 1930s during an outbreak in sheep in Kenya. Then in the early 2000s, it was seen in the Middle East causing a large outbreak and infecting both livestock and humans (27,30). Although RVFV is more common in Sub-Saharan Africa, it has spread to the middle east, and with increasing international trade of livestock, it is an important virus to study. RVFV has the capability to be used as a potential bioweapon (24,31). New evidence also indicates that the aerosol route of infection is possible. (6) The recent MVA-vectored (Modified Vaccina Ankara) vaccine made for livestock shows promising results, however, the production is limited and there are currently no single-dose vaccines available for the treatment of RVFV in ruminants or humans (5,6).

Viral Replication and Nonstructural Proteins: NSs

Understanding the RVFV basic molecular structure is an important step in understanding its pathogenicity. The viral RNA genome of RVFV is composed of 3 segments L, M, and S (4). The L segment encodes the RNA-dependent RNA polymerase that is responsible for transcription and replication of the viral RNA, the M segment encodes for two glycoproteins important for cell attachment and viral-host membrane fusion, and the S segment codes for nucleoprotein (N protein) and nonstructural protein (NSs) (4,8,24). Once the virus enters the host cell through receptor mediated endocytosis, it releases its RNA into the host cell and from there it goes through replication and transcription (13,24). During the replication cycle, each genomic segment is transcribed into mRNA (messenger RNA) and is also used to make an anti-genome copy known as complementary RNA (cRNA). cRNA is then used as a template to make viral genomic

RNA (vRNA) which is packaged in the Golgi prior to the release of the virions from the cell through exocytosis (13).

In RVFV, the S cRNA segment is found in the virion which enables NSs to be expressed immediately after viral entry, indicating that it plays an important role during the early stages of viral infection (24). NSs is a major virulence factor, present in both the cytoplasm and nucleus of infected cells and inhibits cellular transcription by interacting with host transcription factor (TF) components, and promotes post-translational degradation of proteins including protein kinase RNA-activated (PKR) (21). PKR is an important antiviral protein kinase induced by interferons after viral infection (10). In many in vivo studies, PKR has been shown to restrict viral replication and results in apoptosis induction. The importance of PKR's antiviral action is illustrated by viral proteins of most animal viruses using many strategies to impair its action (10,26).

Attenuated RVFV strain

RVFV is classified as a Category A priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) and a select agent according to the CDC and the United States Department of Agriculture (USDA). Work with RVFV must occur in a high-containment biosafety level 3 (BSL-3) laboratory due to the lack of therapeutics and vaccines and has the potential to be transmitted via the respiratory route. However, the use of RVFV MP-12 vaccine strain allows studies to be performed in BSL-2 laboratories (6 ,30). This strain was made after a series of 12 passages in presence of a chemical mutagen. It is highly attenuated at the M and L segments while the S segment has the virulence phenotype, and this strain is capable of inducing high levels of antibodies (20). Recent

studies found that there are viral subpopulations of RVFV and a possible risk of reversion to virulence, causing many scientists to study MP-12 generated through a molecular clone (rMP-12), making it a model system to better understand the virus and to develop a long-lasting vaccine (4,20).

Autophagy

Autophagy is a major intracellular degradation system induced by both extracellular and intracellular stress (12). The three types of autophagy that can take place are macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is the major and most common form of autophagy. It occurs when large portions of cellular content are engulfed into a double membrane vacuole called the autophagosome, which later fuses with the lysosome for degradation (17,33). Microautophagy is the process where lysosomes directly engulf and digest small amounts of cytosolic substrates (33). Unlike these two, chaperone-mediated autophagy does not include vesicular trafficking. Rather, its target proteins are delivered straight to the lysosomal lumen for degradation (12,17). During these processes, if the carried materials are recognized and identified by specific receptors for degradation then it is called selective autophagy. However, when a group of proteins are transported together, all are degraded in a nonspecific manner then this is known as nonselective autophagy (17,33).

The process of macroautophagy takes place in 5 steps: induction, elongation, autophagosome formation, autophagolysome, and degradation (17). Extracellular or intracellular stress causes the cytoplasm to generate autophagosomes in several sites. In mammalian cells, studies suggest the endoplasmic reticulum (ER) associated structures

known as omegasomes may also be the initiation site for autophagy. After initiation, the membrane starts to expand forming a double membrane spherical autophagosome. In selective autophagy, the curvature of the phagophore membrane expands enough to fit the cargo being carried. The autophagosome delivers the material to lysosomes by fusing its membrane and forming autolysosome. The acidic lumen of the lysosome then causes the materials to degrade (12). Autophagosome formation is mediated by two ubiquitin-like conjugation systems and many autophagy related proteins such as Atg12 (17).

Small molecule modulators

Inhibition of protein-protein interactions and disease specific molecular mechanisms using small molecule modulators are emerging therapeutic strategies for targeting many viruses (22). Targeting the host cell and its proteins for antiviral effects carries advantages in understanding cellular processes during viral infection. When different protein-protein interactions within the host are targeted it is less prone to resistance unlike the proteins of a virus (14). There are many small molecule modulators of autophagy that have been previously studied, these include inhibitors such as CA-5F, DC661, and ML240 (7,29,34). Autophagy inhibitors can either be early suppressors of autophagy induction or late stage autophagy suppressors. Most autophagy inhibitors have high toxicities and are clinically limited in use. CA-5F is a curcumin analog and a late-stage autophagy inhibitor and blocks the fusion of autophagosomes with lysosomes (25,34). DC661 treatment of cancer cells showed inhibition of autophagic flux with greater lysosomal deacidification and induced apoptosis (29). ML240 inhibits autophagy by causing a defect in the autophagosome maturation and disrupts the protein homeostasis of

cells (7). Although most autophagy inhibitors are not specific, using these drugs will help us learn more about the importance of autophagy for RVFV and potentially find new ways of preventing RVFV infection.

Previous studies of autophagy in viral infections

The role of autophagy in virus infected cells and cancer cells have been previously studied in many different ways. There are many viral studies that consist of both activating and inhibiting autophagy in order to decrease viral infectivity showing both the proviral and antiviral role of autophagy (33, 34). Many viruses have evolved to induce autophagy in order to use the autophagic pathway to increase their replication and pathogenicity. In HIV (human immunodeficency virus) infection the host Atg genes enhance infection through their functions in autophagosome formation, and inhibition of autophagy in machrophages reduces HIV yields. (18) Another study showed that HIV Gag proteins promotes autophagosome formation in macrophages which in return promotes maximum extracellular viral yields (18). Many RNA viruses such as Influenza A, Poliovirus, and HCV (hepatitis C virus) have all shown to induce autophagy in vitro and use of autophagy inhibitors caused reduction in viral titers (1,18). HCV induces early stages of autophagy in hepatocyte cell lines through induction of unfolded protein response leading to increased viral replication (18). In poliovirus infected human cells, components of cellular apparatus of autophagosome formation stimulated poliovirus replication (1,18). Similarly, coronavirus mouse hepatitiv virus (MHV) infection also induced autophagy and components of autophagic pathway were used for forming viral replication complex. When cells without certain autophagic proteins were infected with MHV there was no induction

of autophagy, indicating proteins necessary for autophagy vacuole formation are needed for MHV to induce autophagy (28). All these studies show evidence of proviral role of autophagy and inhibition of autophagy reducing viral yields (18,33). With autophagy modulation showing effectiveness in reducing RVFV infection, we decided to further study the effect of inhibition of autophagy in RVFV infected mammalian cells. We hypothesized that inhibition of autophagy will cause RVFV titer reduction and provide evidence suggesting a pro-viral role of autophagy in RVFV infection.

MATERIALS AND METHODS

Cell Culture

Human small airway epithelial cells (HSAECs) were grown in Ham's F-12 medium according to the vendor's protocol. Ham's F-12 was supplemented with 50mL FBS, 5mL L-glutamine, 5mL penicillin/streptomycin, 5mL sodium pyruvate, 5mL nonessential amino acids, 500μL β-mercaptoethanol (Thermo Fisher, Lot 21985023). Vero cells (ATCC, CCL-81) were grown in DMEM medium and supplemented with 50mL FBS, 5mL L-glutamine, 5mL penicillin/streptomycin. Human hepatoma cells (Huh7, a kind gift from Charles M. Rice, Rockefeller University, New York, NY) were grown in DMEM medium and supplemented with 50mL FBS, 5mL L-glutamine, 5mL penicillin/streptomycin, 5 mL nonessential amino acids, 5 mL sodium pyruvate. All cell lines were maintained at 37 °C in incubators humidified 5% CO_2. All cells were plated at a density of 1.5 X 10^5 for 12-well plates and 2.0 X 10^4 for 96-well plates.

Viruses and Viral infection

RVFV recombinant (r)MP12 strain was tittered and rescued as previously described in (2). For experiments using rMP12, HSAECs were cultured in 96-well plate at 2 x 10^4 per well and cells were infected at MOI of 0.1 for 1 hour.

Drug treatment and Plaque Assay

HSAECs were seeded at 2.0 X 10^4 in 96-well plate pretreated for 1 hour with DMSO, CA-5F, ML-240, DC661. Cultured cells were infected at MOI of 0.1 for 1 hour with RVFV rMP12. The drugs were reintroduced to the cells and collected at different time points (8, 16, 24 post infection) and analyzed by plaque assays. For plaque assays, Vero cells were plated in 12-well plates at 10^5 cells per well. Samples were diluted in DMEM from 10^1 to 10^8 and infections were then carried for each dilution. After 1 hour of 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. The plates were allowed to solidify at room temperature and then incubated at 37 °C for 72 hours. At 72 hours, the cells were fixed using 10% formaldehyde for 1 hour at room temperature. At 1 hour, the agar plugs are discarded and fixed cells were stained with 1% crystal violet and 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 (CA-5F, ML240, DC661, DMSO) 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 using DTX 880 multimode detector (Beckman Coulter) and percent viability was calculated relative to the DMSO control.

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 15µL of master mix containing 1µL of enzyme mix, 4µL of 5X reaction mix, 0.4µL of 50mM magnesium sulfate, 0.4µL of ROX reference dye, 7.4µL of PCR water, 1µL of 10µM Taqman probe (6FAM-AAAGCTTTGATATCTCTCAGTGCCCCAA-TAMRA), 0.8µL of 10µM forward primer (AAAGGAACAATGGACTCTGGTCA), 10µM reverse primer (CACTTCTTACTACCATGTCCTCCAAT) added to 5µL of extracted RNA. The samples were heated at 50ºC for 30 minutes, 95ºC for 2 minutes, followed by 40 cycles of 95ºC (15 seconds), and 61ºC (60 seconds). RNA genomic copies were determined relative to a standard curve containing known amount of viral RNA.

Statistics

All quantifications are based on data obtained from triplicate samples unless indicated otherwise. Error bars in all figures indicate standard deviations. P values were calculated using an unpaired Student's t test.

RESULTS

Small molecule inhibitors of autophagy can decrease RVFV infection

We examined three different autophagy inhibitors for their impact on RVFV production in HSAECs. Autophagy is a cellular degradation process that helps keep the cell homeostasis in balance. The process of autophagy starts with stress or growth signals causing the induction of phagophore formation which then elongates while taking in the proteins and substrates that need to be degraded. This then becomes the autophagosome. The autophagosome then fuses with the lysosome creating an acidic environment for the degradation of proteins (Fig. 1). We examined three different autophagy inhibitors for their impact on RVFV production in HSAECs. CA-5F, ML240, and DC661 were tested on HSAECs using Cell Titer-Glo to determine non-toxic concentrations of each inhibitor. HSAECS were treated with various concentrations of each inhibitor for 24 hours and the percent viability was determined relative to the DMSO control. CA-5F was the least toxic out of the three inhibitors with its percent viability only dropping below 100% at 12.5μM (Fig. 2A and 2B). Cells treated with CA-5F at a 5μM concentration had a viability of 100%. Given that no concentration tested induced cell death rates of 50% or greater a CC_{50} for CA-5F was unable to be determined and was estimated as being greater that 12.5 μM. While ML240 and DC661 were highly toxic at concentrations higher than 2μM (Fig. 2A, 2C, and 2D). ML240 and DC661 CC_{50} values were determined to be 3.434 μM and 2.005

μM, respectively. Cells treated with ML240 and DC661 showed a 90-100 percent viability at 1.5μM. Thus, for initial antiviral testing CA-5F at 5μM, and ML240 and DC661 at 1.5μM concentrations were selected. Then, HSAECs infected with RVFV were treated with autophagy inhibitors (Fig. 3). After 24 hours of treatment, the supernatants were collected and viral titers determined using a standard plaque assay. The data showed that CA-5F and ML240 decreased RVFV infectious titers by more than $1\log_{10}$ in HSAECs. DC661 also showed some decrease with almost a $1\log_{10}$ decrease in RVFV infectious titer relative to the DMSO control (Fig. 3). We decided to test the effect of ML240 and CA-5F at different time points to further decide the best autophagy inhibitor.

CA-5F decreases RVFV infectious titers at 8, 16, and 24 hours

We tested CA-5F and ML240 on RVFV infected HSAECs at 5μM and 1.5μM concentration respectively to determine its impact at different time points after infection. The supernatants were collected at 8, 16, and 24 hours post infection. A standard plaque assay was performed to determine the effect of each inhibitor on RVFV infectious titers. In CA-5F treated cells, there was $1\log_{10}$ decrease at 16 hours post infection and half a log about $1\log_{10}$ decrease at 8 and 24 hours. While there was no significance difference at 8 and 16 hours post infection with ML240 compared to the DMSO control, at 24 hours post infection, ML240 decreased RVFV infectious titer by more than $1\log_{10}$. The data shows that CA-5F has a more significant impact on RVFV infectious titer at all time points post infection while ML240 only had a significant impact at 24 hours post infection (Fig. 4). Furthermore, ML240 inhibits valosin containing protein (VCP), which complicates the interpretation of our data as it relates to autophagy. Additionally, we have previously

shown that VCP is important for RVFV egress (3). Therefore, we turned our focus to CA-5F since it is an autophagy inhibitor that hasn't been studied in the context of RVFV, has more impact at 8 and 16 hours, as well as a more favorable cell viability profile.

CA-5F inhibits RVFV infectious titers in a dose-dependent manner, but does not impact viral RNA production.

In order to understand the impact of CA-5F on RVFV infection, HSAECs were treated with different doses of CA-5F at 16 and 24 hours post infection. As shown in Figure 2, HSAECs were viable when treated with 10μM of CA-5F, therefore a dose analysis was performed using 2.5, 5, and 10 10μM of CA-5F. HSAECs were treated with CA-5F for 1 hour, followed by infection with RVFV, and then post-treated with CA-5F. The supernatants and the cellular lysates of each sample were collected. A standard plaque assay was performed using the supernatants. Infected cells treated with 10μM of CA-5Fdecreased RVFV infectious titer by $2\log_{10}$ at 16 hours post infection and $1\log_{10}$ at 24 hours post infection (Fig. 5A). RNA extraction followed by RT-qPCR of was used to measure intracellular viral RNA levels. The intracellular RNA levels in all samples were relatively the same compared to the DMSO control (Fig. 5B). These results suggest that CA-5F does not impact viral RNA production, but rather inhibits a later stage of viral replication such as viral assembly or egress.

The effect of CA-5F on RVFV in different cell types.

CA-5F was tested in Vero and Huh7 cells to determine a viable concentration in each cell type. Vero cells are African Green Money kidney epithelial cells and are interferon deficient, while Huh7 cells are physiologically relevant as RVFV causes hepatic

damage in vivo. Huh7 cells have also previously have been used in studies with autophagy inhibitors (16). The cell viability assay showed that CA-5F is not toxic to the cell lines at up to 25μM (Fig. 6A-B). Based on these data, 25μM was used to test CA-5F's ability to affect viral production in both cell types at 16 and 24 hours post infection. With Vero cells, CA-5F was tested at concentrations of 5, 10, and 25uM at 16 and 24 hours post infection to compare with the HSAECs dose analysis. In Vero cells, CA-5F decreased viral titers by $3\log_{10}$ at 16 hours when treated with 25uM and about a log when treated with 10μM. There was no significant difference at 16 hours when Vero cells were treated with 5μM of drug (Fig. 7A). There also was more than a $2\log_{10}$ decrease at 24 hours with CA-5F 25uM and about a log with 10μM, while a minimal statistically significant change was observed when treated with 5μM. (Fig. 7A) RNA extraction followed by RT-qPCR of the was used to measure intracellular viral RNA levels. The intracellular RNA levels in samples at 5μM and 10μM were relatively the same compared to the DMSO control (Fig. 7B). There was a log decrease at both 16 and 24 hours in samples treated with 25μM of CA-5F, but there was no statistical significance at 16 hours and a minor significance at 24 hours (Fig. 7B). These results suggest that CA-5F has limited impact on viral RNA production in Vero cells, but rather inhibits a later stage of viral replication such as viral assembly or egress. Due to limited Huh7 cells available, we were only able to test CA-5F at 25μM. At 16 hours post infection there was more than a $2\log_{10}$ decrease of infectious RVFV titers and at 24 hours post infection there was almost $5\log_{10}$ reduction of infectious titer. This suggests that CA-5F reduces viral infectious titer in Huh7 cells (Fig. 7C). These data indicate that CA-5F inhibits RVFV infectious titers in multiple cells types.

FIGURES

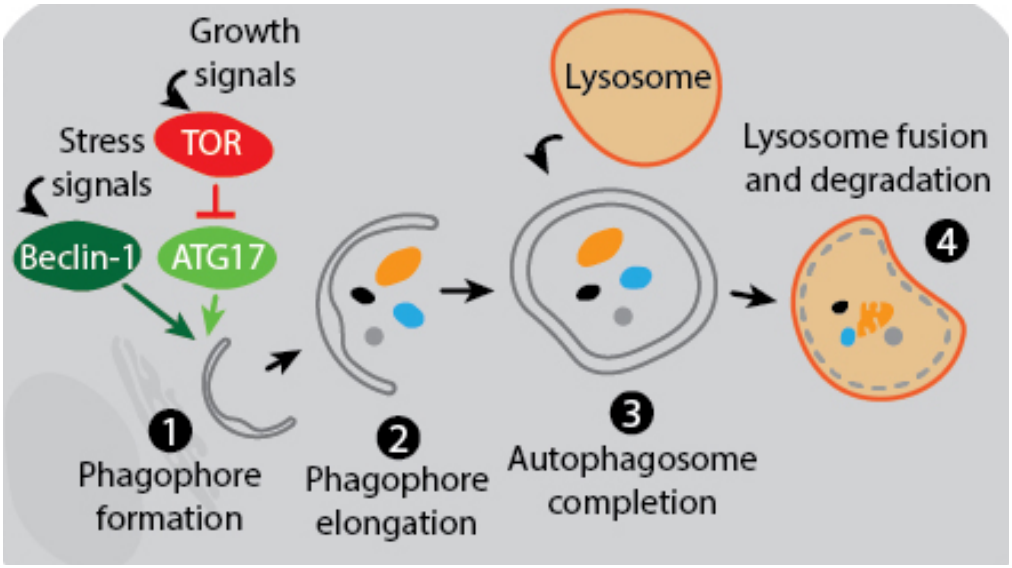

Figure 1 Model of Cellular Autophagy Pathway. Autophagy is a regulated multi-step process that leads to cargo degradation. Autophagy can eliminate cargo such as viruses and viral-derived antigens. It can be organized into 5 distinct steps beginning with (1) the initiation of phagophore formation which (2) nucleates around the intended cargo. The cargo can be selectively recruited by autophagy receptors such as p62, which can be regulated by TBK1. (3) The phagophore elongates and completes to form a structure termed the autophagosome which then fuses with nearby lysosomes carrying hydrolytic enzymes. This eventually leads to (4) the acidification and hence degradation of the contained cargo. (BioRender.com)

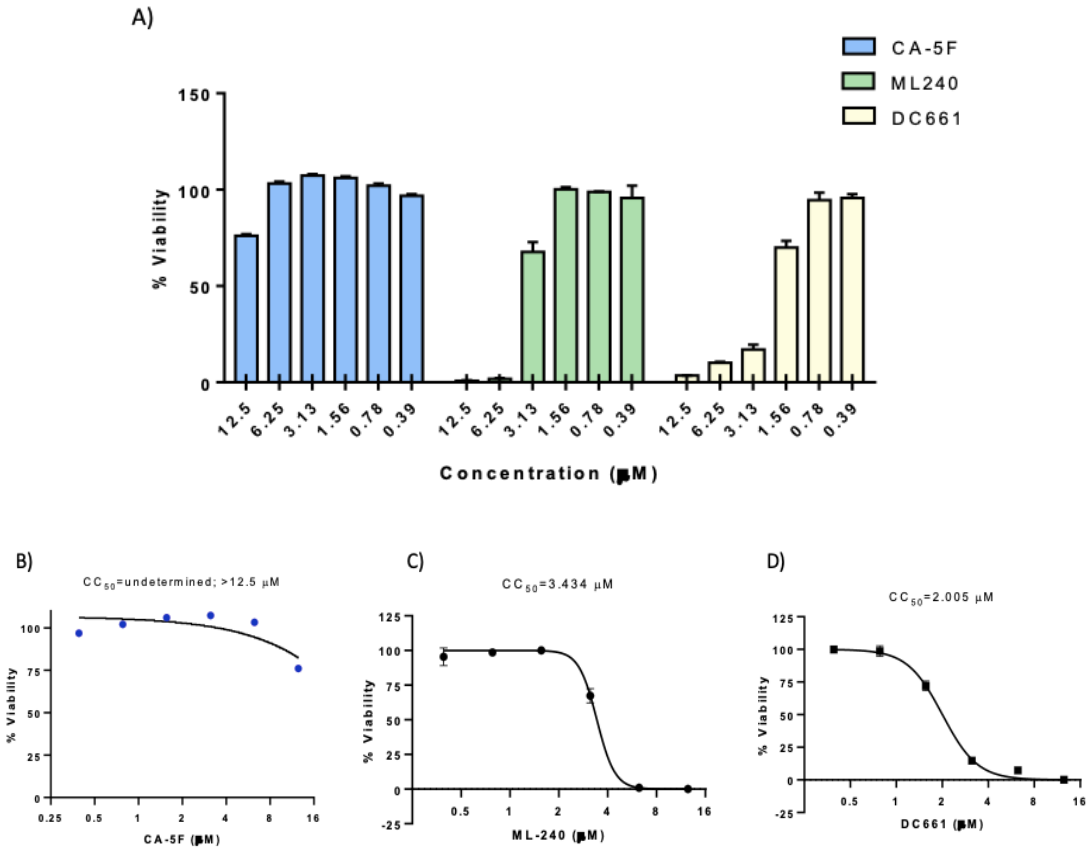

Figure 2 Determining Viable Concentrations of Autophagy Inhibitors in HSAECs. **(A)** HSAECs cells were treated with CA-5F, ML240, DC661, and cell viability was analyzed at 24 hours using Cell Titer-Glo. Percent viability of each was determined relative to the DMSO control. **(B-C)** CC_{50} of each inhibitor was determined. The means and standard deviation (N=3) are plotted.

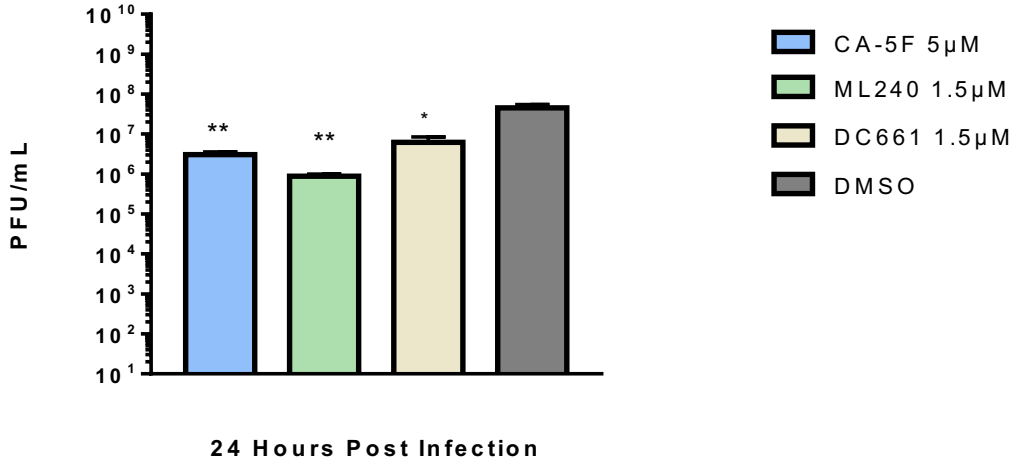

Figure 3 CA-5F and ML240 are more effective inhibitors of RVFV infectious titers than DC661. HSAECs cells were pre-treated for 1 hour with autophagy inhibitors (5µM CA-5F, 1.5µM ML240, 1.5µM DC661), then infected with rMP12 for 1 hour (MOI 0.1), followed by removal of viral inoculum and retreatment again with the saved autophagy inhibitors. At 24 hours post infection, the supernatants were collected for a standard plaque assay. The experiment was repeated twice for a more accurate representation of data. All sample data determined relative to the DMSO control in PFU/mL. The means and standard deviation from 2 experiments (N=3) are plotted.

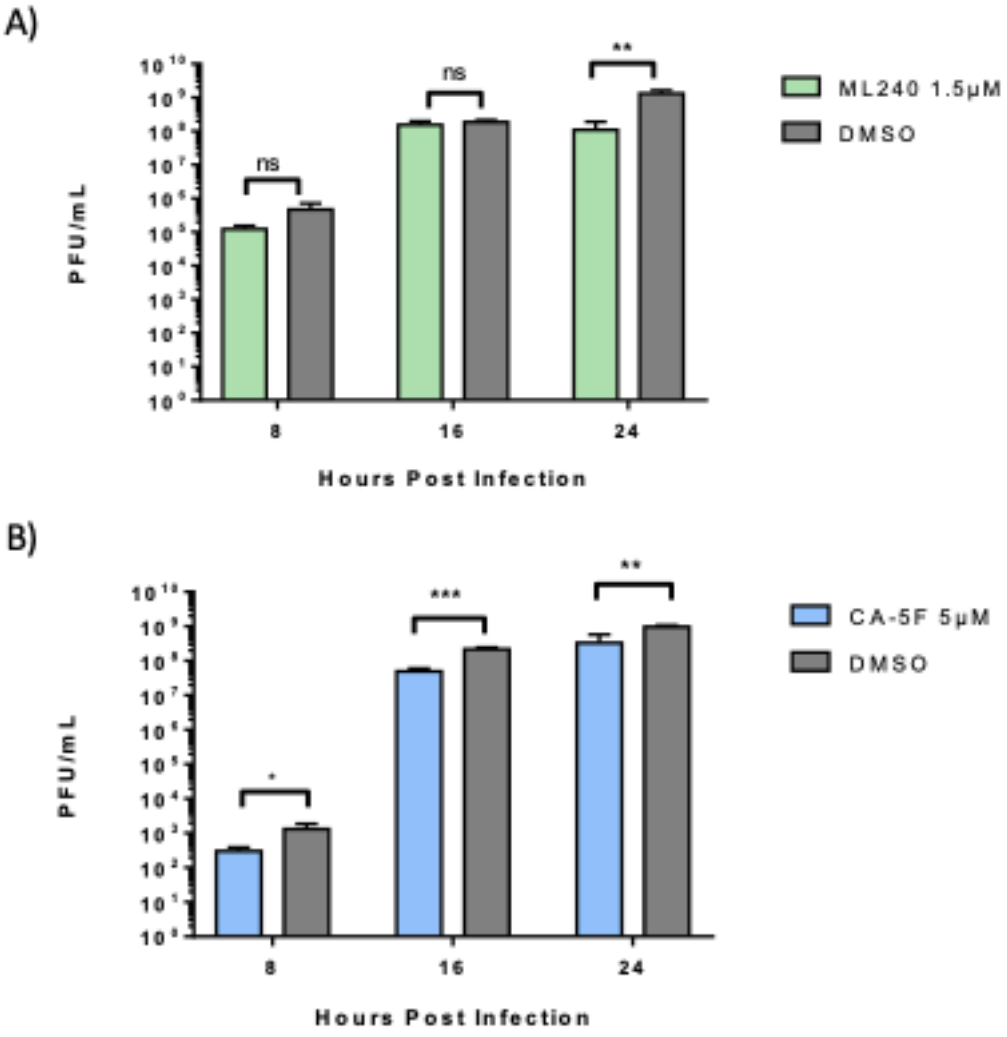

Figure 4 CA-5F and ML240 timepoint infection. (A-B) HSAECs cells were pre-treated for 1 hour with autophagy inhibitors (5µM CA-5F, 1.5µM ML240) then infected with rMP12 for 1 hour (MOI 0.1), followed by removal of viral inoculum and retreatment again with the saved autophagy inhibitors. The supernatants were collected for a standard plaque assay at 8, 16 and 24 hours post infection. All sample data determined relative to the DMSO control in PFU/mL The means and standard deviation from 2 experiments (N=3) are plotted.

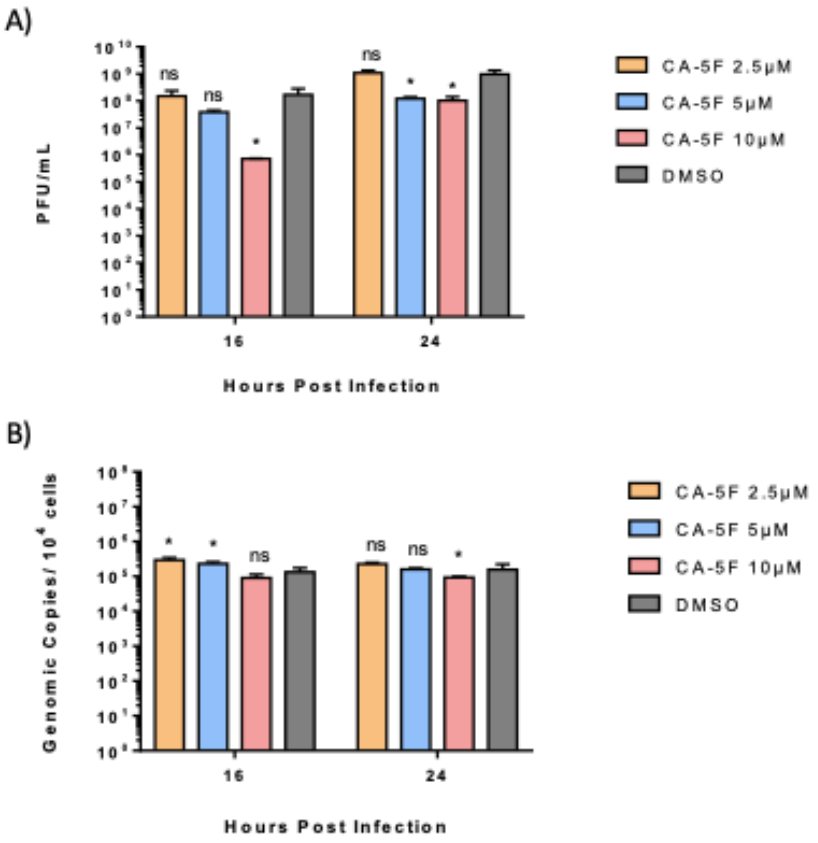

Figure 5 CA-5F inhibits RVFV infectious titers in a dose-dependent manner, but does not impact viral RNA production. (A) HSAECs cells were pre-treated for 1 hour with various viable concentrations of CA-5F (10 µM, 5 µM, 2.5 µM) then infected with MP12 for 1 hour (MOI 0.1), followed by removal of viral inoculum and retreatment again with the saved CA-5F doses. The supernatants were collected for a standard plaque assay at 16 and 24 hours post infection. The means and standard deviation (N=3) are plotted. **(B)** The Intracellular infectivity per infection was determined using RT-qPCR and genomic copies of each concentration were plotted.

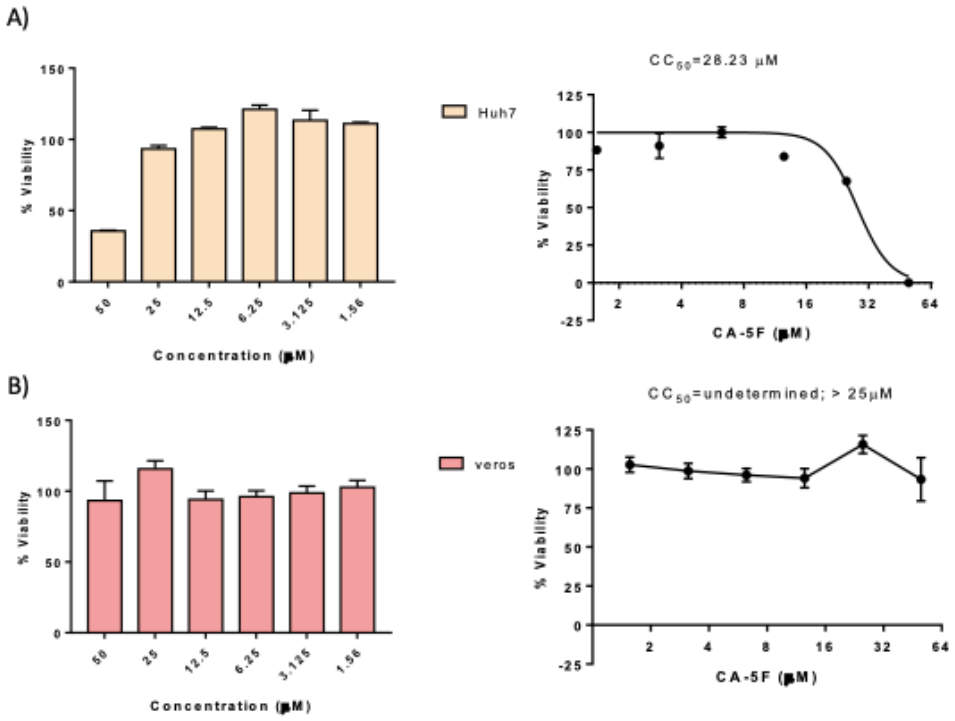

Figure 6 Determining Viable Concentrations of Autophagy Inhibitors in Huh7 and Veros. (A-B) Huh7 and Vero cells were treated with CA-5F at different concentrations and cell viability was analyzed at 24 hours using Cell Titer-Glo and the CC_{50} was determined. The means and standard deviation (N=3) are plotted.

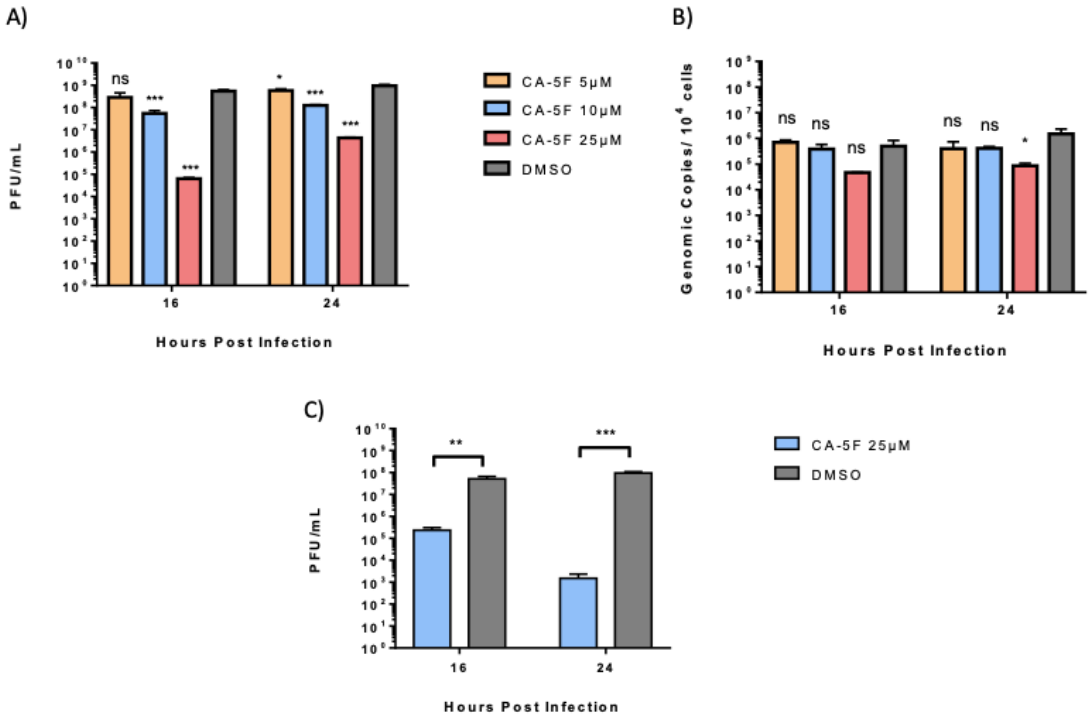

Figure 7 Determining the effect of CA-5F on RVFV in different cell types. (A) Vero cells were pre-treated for 1 hour with CA-5F (25, 10, 5 μM) then infected with MP12 for 1 hour (MOI 0.1), followed by removal of viral inoculum and retreatment again with the saved CA-5F. The supernatants were collected for a standard plaque assay at 16 and 24 hours post infection. **(B)** The Intracellular infectivity per infection was determined using RT-qPCR and genomic copies of each concentration were plotted. **(C)** Huh7 cells were pre-treated for 1 hour with CA-5F at 25 μM, then infected with MP12 for 1 hour (MOI 0.1) followed by removal of viral inoculum and retreatment again with the saved CA-5F. The supernatants were collected for a standard plaque assay at 16 and 24 hours post infection. All sample data determined relative to the DMSO control in PFU/mL. The means and standard deviation from 2 experiments (N=3) are plotted.

DISCUSSION

RVFV is a zoonotic arbovirus and an agricultural pathogen that can infect humans and livestock (30). RVFV can cause severe mortality rate in young animals and causes almost a 100% abortion rate in pregnant livestock (30). With the potential to be used as a bioweapon, finding potential therapeutics and understanding how host factors affect RVFV pathogenicity is an important research topic (3,25,27,30). In this study, we looked into treatment that target host cells rather than the virus directly. Autophagy is an essential cellular degradation process that keeps eukaryotic cellular homeostasis (12,34). Understanding the autophagic process in the innate immune response against pathogens is an emerging topic of research that has sent ripples through related scientific fields (24). The host response to viral pathogens has been an emerging target with the use of small molecular modulators (14). In this study, we examined 3 specific small molecule modulators of autophagy, CA-5F, DC661, and ML240 (7, 29,34).

We tested these autophagy inhibitors on RVFV infected HSAECs (Fig. 3). DC661 had the least effect on decreasing the RVFV infectious titer when compared to DMSO control, while ML240 and CA-5F had the best results with a 1 $\log_{10}$ or more decreased compared to the DMSO control. DC661 inhibits autophagy by deacidifying the lysosomes and is capable of inducing apoptosis. (29). CA-5F inhibits autophagic flux by blocking the fusion of the autophagosome with the lysosome instead of affecting the pH and the

hydrolytic function of lysosomes. Similarly, ML240 also impacts autophagy inhibition by disrupting autophagosome maturation preventing autophagosome from fusing with lysosome (7). The results could suggest increasing lysosomal pH could not have a significant effect on the role of autophagy in RVFV and steps prior to lysosome degradation in autophagy could impact RVFV infection. Since ML240 also inhibits VCP, which has previously been studied for its impact on RVFV replication, we selected CA-5F for further testing on RVFV infection (3). CA-5F is an analog of curcumin that was recently identified as a late-stage autophagy inhibitor (34). Curcumin has been previously shown to decrease viral load in RVFV infected mice, by binding and inhibiting IKK-Beta2 complex activity in infected cells (25). CA-5F is a relatively new compound and has previously only been studied on cancer cells (34). CA-5F showed promising results at 16 hours post-infection in cells treated with 5uM of CA-5F. Additional concentrations of CA-5F were tested for their impact of RVFV infectious titers at 16 and 24 hours post infection. Our data shows that CA-5F is an effective inhibitor of RVFV in HSAECs. CA-5F decreased RVFV infectious titer by 2 log and 1 log respectively when treated with 10μM of CA-5F at 16 and 24 hours post infection. Although our data showed there is a dose dependent decrease of viral titer, CA-5F does not affect viral RNA production (Fig. 5B). CA-5F targets proteins associated with cytoskeleton, gene expression, membrane trafficking, cellular redox state, and is a late-stage autophagy inhibitor that targets autophagosome-lysosome fusion (33). Our data combined with previous understanding of CA-5F shows that RVFV is affected by the inhibition of the autophagosome-lysosome formation. It also indicates that the

components of this process promote viral production possibly via enhancing the release of virions from the cell.

It has been shown the role of autophagy is different depending on which cell type and cellular environment it is in (16). To further investigate the role of CA-5F on host cells when infected with RVFV, we tested CA-5F in Huh7 and Vero cells. CA-5F was less toxic to Huh7 and Vero cells (Fig. 6 A-B) as compared to HSAECs (Fig. 2). CA-5F has been shown to be more toxic towards lung epithelial cells. A previous study showed that CA-5F was more toxic to most lung cells including A549 and other human bronchial epithelial cells compared to HUVECs and induced higher levels of apoptosis and necrosis when treated with higher doses of CA-5F (34). HSAECs are similar to A549 which can explain why CA-5F was more toxic to HSAECs. Another reason for the toxicity observed in HSAECs could be due to the fact that they are non-transformed cells. Cell-titer Glo assay of CA-5F treated Huh7 and Vero cells showed the cells were viable up to 25μM of CA-5F at 24 hours (Fig 6.A-B). Various concentrations (5, 10, 25μM) were tested on Vero cells and supernatants were collected at both 16 and 24 hours post infection. (Fig. 7A) CA-5F at 25μM decreased viral titers by $3\log_{10}$ at 16 hours and about a $\log_{10}$ when treated with 10uM. There was no significance at 16 hours when Vero cells were treated with 5μM (Fig. 7A). Additionally, there was more than a 2log10 decrease at 24 hours with CA-5F at 25uM of concentration and about a log with 10uM. Very little significant change was observed when treated with 5μM (Fig. 7A). In Huh7 cells, CA-5F 25μM was able to reduce viral infectious titers by almost 3log10 at 16 hours and by almost 5log10 at 24 hours post infection (Fig. 7C). The results indicate that CA-5F can reduce viral infectious titers in

multiple cell types. Furthermore, the results also show even in interferon deficient Vero cells, CA-5F is able to reduce RVFV infectious titer suggesting that there is a role autophagy plays in RVFV infection independent of interferon. Intracellular RNA analysis of infected Vero cells also showed little to no significant reduction in viral RNA production (Fig. 7B), which is in agreement with the data obtained in HSAECs.

CA-5F is a curcumin analog and has previously been only studied on cancer efficacy. Although CA-5F has previously not been studied with RVFV or any other virus, curcumin has been previously tested on RVFV and by interfering with protein-protein interaction it was found to reduce the RVFV extracellular infectious virus as well as partially inhibit RVFV replication. (25) Additionally, a previous study found that loss of key autophagy proteins such as ATG5 in *Drosophila* cells, mouse embryonic fibroblasts, and U2OS human bone osteosarcoma cells increased RVFV replication, suggesting that autophagy activation is antiviral (24). The study also showed in *Drosophila* that Toll-like receptor-7 limited RVFV replication by activating autophagy. They showed in mammalian cells that pattern recognition receptors are needed for activation of anti-viral autophagy and tested autophagy activating compounds (rapamycin and SMER28) on RVFV infected primary hepatocytes and neurons. The use of these compounds inhibited RVFV infection in mammalian cells (24). In contrast, our results suggest inhibition of autophagy reduces RVFV infectious titers, and reduction in CA-5F concentration increases RVFV infectivity. Furthermore, our experiments were all done in human cell lines while the antiviral autophagy study focused on mouse cells (MEFs) and a human bone cell line U2Os. Additionally, this study utilized rapamycin as an autophagy modulator which induces

autophagy by inhibiting mTOR, which is upstream of autophagy preventing induction step (24,33). In constract, CA-5F is a late-stage inhibitor preventing autophagosome and lysosome fusion. The difference in the experiments also can be due to the difference in measurement of viral infectivity. While similarly our studies look into RNA levels, the plaque assays are the main form of experimental method we used to measure the infectious viral titer while the paper used intracellular RNA and viral protein levels as a form of viral measurement (24). While our results suggest a different way autophagy might impact RVFV replication, it is clear that autophagy plays an important role in the infectivity of viruses in cells. Future studies on the impact of autophagy inhibitors and autophagic flux in viral replication is needed for better understanding the significant role of autophagy in viral infections. It is also important to investigate both host response to pathogenesis and virus utilization of host cellular processes for the same purpose.

REFERENCES

1. **Ail-Goughoulte, M, Kanda T, Meyer K, et al.** "Hepatitis C virus genotype 1a growth and induction of autophagy." *Journal of virology* vol. 82,5 (2008): 2241-9. doi:10.1128/JVI.02093-07
2. **Benedict A, Bansal N, Senina S, Hooper I, Lundberg L, de la Fuente C, Narayanan A, Gutting B, Kehn-Hall K**. "Repurposing FDA-approved drugs as therapeutics to treat Rift Valley fever virus infection." *Front Microbiol.* 2015;6:676.
3. **Brahms A, Mudhasani R, Pinkham C, et al.** Sorafenib Impedes Rift Valley Fever Virus Egress by inhibiting Valosin-Containing Protein Function in the Cellular Secretory Pathway. J Virol. 2017;91(21):e00968-17. Published 2017 Oct 13. doi:10.1128/JVI.00
4. **Brown JL, Dominik JW, Morrissey RL.** "Respiratory infectivity of a recently isolated Egyptian strain of Rift Valley fever virus." *Infect Immun.* 1981;33:848–853.
5. **Calvo-Pinilla, E., Marín-López, A., Moreno, S. et al.** "A protective bivalent vaccine against Rift Valley fever and bluetongue." *npj Vaccines* 5, 70 (2020). https://doi.org/10.1038/s41541-020-00218-y
6. **Centers for Disease Control and Prevention.** "The Story of the Rift Valley Fever Virus Vaccine." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 7 Nov. 2018, www.cdc.gov/onehealth/in-action/rvf-vaccine.html.
7. **Chou, Tsui-Fen et al.** "Structure-activity relationship study reveals ML240 and ML241 as potent and selective inhibitors of p97 ATPase." *ChemMedChem* vol. 8,2 (2013): 297-312. doi:10.1002/cmdc.201200520

8. **Ellenbecker, Mary et al.** "Inhibition of Rift Valley fever virus replication and perturbation of nucleocapsid-RNA interactions by suramin." *Antimicrobial agents and chemotherapy* vol. 58,12 (2014): 7405-15. doi:10.1128/AAC.03595-14
9. **Elliott, Richard M, and Benjamin Brennan.** "Emerging phleboviruses." *Current opinion in virology* vol. 5,100 (2014): 50-7. doi:10.1016/j.coviro.2014.01.011
10. **García, M A et al.** "Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action." *Microbiology and molecular biology reviews : MMBR* vol. 70,4 (2006): 1032-60. doi:10.1128/MMBR.00027-06
11. **Gaudreault, N.N., Indran, S.V., Balaraman, V. et al.** "Molecular aspects of Rift Valley fever virus and the emergence of reassortants.: *Virus Genes* vol. 55, 1–11 (2019). https://doi.org/10.1007/s11262-018-1611-y
12. **Glick, Danielle et al**. "Autophagy: cellular and molecular mechanisms." *The Journal of pathology* vol. 221,1 (2010): 3-12. doi:10.1002/path.2697
13. **Harmon, Brooke et al.** "Rift Valley fever virus strain MP-12 enters mammalian host cells via caveola-mediated endocytosis." *Journal of virology* vol. 86,23 (2012): 12954-70. doi:10.1128/JVI.02242-12
14. **Harrison, Charlotte.** "Drug Researchers Pursue New Lines of Attack against COVID-19." *Nature News*, Nature Publishing Group, 15 May 2020, www.nature.com/articles/d41587-020-00013-z.
15. **Ikegami, Tetsuro, and Shinji Makino**. "The pathogenesis of Rift Valley fever." *Viruses* vol. 3,5 (2011): 493-519. doi:10.3390/v3050493
16. **Jackson, William T.** "Viruses and the autophagy pathway." *Virology.* vol. 479-480 (2015): 450-6. doi:10.1016/j.virol.2015.03.042
17. **Khandia, Rekha et al.** "A Comprehensive Review of Autophagy and Its Various Roles in Infectious, Non-Infectious, and Lifestyle Diseases: Current Knowledge and Prospects for Disease Prevention, Novel Drug Design, and Therapy." *Cells* vol. 8,7 674. 3 Jul. 2019, doi:10.3390/cells8070674
18. **Kudchodkar, Sagar B, and Beth Levine.** "Viruses and autophagy." *Reviews in medical virology* vol. 19,6 (2009): 359-78. doi:10.1002/rmv.630

19. **Kuo, Szu-Yu, et al.** "Small-Molecule Enhancers of Autophagy Modulate Cellular Disease Phenotypes Suggested by Human Genetics." *PNAS*, National Academy of Sciences, 4 Aug. 2015, www.pnas.org/content/112/31/E4281.
20. **Lokugamage, Nandadeva, et al**. "Genetic Subpopulations of Rift Valley Fever Virus Strains ZH548 and MP-12 and Recombinant MP-12 Strains." *Journal of Virology*, American Society for Microbiology Journals, 15 Dec. 2012, jvi.asm.org/content/86/24/13566.
21. **Ly, Hoai J, and Tetsuro Ikegami.** "Rift Valley fever virus NSs protein functions and the similarity to other bunyavirus NSs proteins." *Virology journal* vol. 13 118. 2 Jul. 2016, doi:10.1186/s12985-016-0573-8
22. **Mabonga, Lloyd, and Abidemi Paul Kappo.** "Protein-protein interaction modulators: advances, successes and remaining challenges." *Biophysical reviews* vol. 11,4 (2019): 559-581. doi:10.1007/s12551-019-00570-x
23. **Maes, Piet et al**. "Taxonomy of the family Arenaviridae and the order Bunyavirales: update 2018." *Archives of virology* vol. 163,8 (2018): 2295-2310. doi:10.1007/s00705-018-3843-5
24. **Moy, Ryan H et al.** "Antiviral autophagy restricts Rift Valley fever virus infection and is conserved from flies to mammals." *Immunity* vol. 40,1 (2014): 51-65. doi:10.1016/j.immuni.2013.10.020
25. **Narayanan A, Kehn-Hall K, Senina S, et al.** Curcumin inhibits Rift Valley fever virus replication in human cell. *J Biol Chem*. 2012;287(40):33198-33214.
26. **Ogolla, Pauline Sebby et al.** "The protein kinase double-stranded RNA-dependent (PKR) enhances protection against disease cause by a non-viral pathogen." *PLoS pathogens* vol. 9,8 (2013): e1003557. doi:10.1371/journal.ppat.1003557
27. **Pepin, Michel et al.** "Rift Valley fever virus(Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention." *Veterinary research* vol. 41,6 (2010): 61. doi:10.1051/vetres/2010033

28. **Prentice, Eric et al.** "Coronavirus replication complex formation utilizes components of cellular autophagy." *The Journal of biological chemistry* vol. 279,11 (2004): 10136-41. doi:10.1074/jbc.M306124200
29. **Rebecca, Vito W et al.** "PPT1 Promotes Tumor Growth and Is the Molecular Target of Chloroquine Derivatives in Cancer." *Cancer discovery* vol. 9,2 (2019): 220-229. doi:10.1158/2159-8290.CD-18-0706
30. **Rolin, Alicia I et al.** "The risk of Rift Valley fever virus introduction and establishment in the United States and European Union." *Emerging microbes & infections* vol. 2,12 (2013): e81. doi:10.1038/emi.2013.81
31. **Sidwell RW, Smee DF**. "Viruses of the Bunya- and Togaviridae families: potential as bioterrorism agents and means of control." *Antiviral Res.* 2003 Jan;57(1-2):101-11. doi: 10.1016/s0166-3542(02)00203-6. PMID: 12615306.
32. **Smithburn, K. C., et al.** "Rift Valley Fever." *The Journal of Immunology*, American Association of Immunologists, 1 June 1949, www.jimmunol.org/content/62/2/213.short.
33. **Yang, Ya-ping et al.** "Application and interpretation of current autophagy inhibitors and activators." *Acta pharmacologica Sinica* vol. 34,5 (2013): 625-35. doi:10.1038/aps.2013.5
34. **Zhang, Lu et al.** "Identification of compound CA-5f as a novel late-stage autophagy inhibitor with potent anti-tumor effect against non-small cell lung cancer." *Autophagy* vol. 15,3 (2019): 391-406. doi:10.1080/15548627.2018.1511503

BIOGRAPHY

Niloufar Boghdeh graduated from Albemarle High School in Charlottesville, Virginia in 2014. She completed a Bachelor of Science in Biology at Virginia Commonwealth University in Richmond, Virginia in 2018. Niloufar continued graduate level education at George Mason Univeristy in the form of Master of Science in Microbiology and Infectious Diseases from 2019-2021.