

**EFFICACY AND CYTOTOXICITY OF NOVEL ANTIVIRAL COMPOUNDS AGAINST
RIFT VALLEY FEVER VIRUS**

by

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EFFICACY AND CYTOTOXICITY OF NOVEL ANTIVIRAL COMPOUNDS

AGAINST RIFT VALLEY FEVER VIRUS

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ABSTRACT

Rift Valley Fever virus (RVFV) is a ss-RNA virus from the *Bunyaviridae* family found in Africa and the Arabian Peninsula. The virus is usually transmitted by mosquitoes and predominantly affects livestock; however, humans exposed to bodily fluids or tissue from infected animals can also be infected. In humans, Rift Valley Fever is usually characterized by mild febrile illness; however, in rare cases, the disease becomes more severe and can cause liver disease, encephalitis, vision loss, and hemorrhagic fever. Epizootic RVF can lead to abortion storms in which nearly 100% of pregnancies in infected ruminants result in abortion. RVFV is considered a bioterrorism threat and outbreaks have significant socio-economic impacts. Currently, there are no approved vaccines or therapeutics against RVFV for use in humans. Identification of a safe and effective therapeutic is crucial to public health's success in the prevention, treatment, and control of Rift Valley Fever. Antiviral properties of compound A3 were identified from a high-throughput screening assay with influenza-A virus. While the mechanism of action of M4 is unknown, A3 is thought to target the enzyme Dihydroorotate Dehydrogenase (DHODH) involved in the *de novo* pyrimidine biosynthesis pathway. Compound A3 has broad-spectrum antiviral activity and was shown to be effective against both DNA and RNA viruses including, VSV, HCV and HIV-1. To determine efficacy against RVFV, Vero E6 cells were incubated with compounds A3, M4, and Favipiravir (T-705), a known inhibitor of RVFV, at various concentrations with the MP-12 vaccine strain.

Viral titer reduction was measured using plaque assay. CC_{50} and inhibitory concentrations were estimated using non-linear regression and selectivity indexes were calculated. Compound A3 was highly effective with an IC_{50} of 48nM and low cytotoxicity with a CC_{50} of 453 μ M, for a SI of ~9,450 compared to a SI of ~1,550 for T-705. Due to high cytotoxicity, M4 had a relatively low selectivity index. However, comparison between CC_{50} and selectivity indexes of A3 with that of M4 and T-705 is limited due to differences in duration of compound exposure. Nevertheless, compound A3 was effective in inhibiting viral replication at very low concentrations and shows promise as a potential candidate for antiviral therapy against Rift Valley Fever.

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1.0 INTRODUCTION

1.1 RIFT VALLEY FEVER VIRUS

Rift Valley Fever Virus (RVFV) is a single-stranded RNA virus from the *Bunyaviridae* family and *Phlebovirus* genus ^[1]. The RVFV genome is composed of three segments: S, M, and L, which code for 7 proteins: L protein (viral RNA-dependent RNA polymerase), N nucleoprotein, the 78-kD protein, two nonstructural proteins NSs and NSm, and two envelope glycoproteins G_n and G_c ^[11]. The NSm protein, encoded by the M segment, is considered to be a virulence factor that suppresses apoptosis in infected host cells and enhances pathogenicity ^[6]. NSs protein, encoded by the S segment, is a major virulence factor that suppresses host mRNA transcription ^[6].

1.1.1 Epidemiology

Rift Valley Fever Virus was first identified in 1930 during an outbreak in the greater Rift Valley in Kenya ^[1]. The virus is currently found on the African continent, Madagascar, and the Arabian Peninsula ^[1]. The virus is usually transmitted by mosquitoes of the *Aedes*, *Culex*, and *Anopheles* genera; however, species of sand flies, midges, and ticks are also thought to play a role in the life cycle of the virus ^[2]. A wide range of animals, including sheep, cattle, goats, camels, and buffaloes, can be infected when bitten by an infected mosquito ^[3]. While humans can

also be infected via a mosquito bite, a majority of infections are thought to originate from contact with bodily fluids or tissue of infected animals ^[3]. Birthing, skinning, or slaughtering an animal, as well as handling aborted animal tissue have been significantly associated with increased risk for Rift Valley Fever infection ^[3].

Epizootic outbreaks of Rift Valley Fever are linked with periods of heavy rainfall. Long periods of heavy rainfall in the African grasslands leads to flooding of dambos, which are temporary ground pools, where mosquitoes of the *Aedes* genus lay eggs ^[4]. Transovarial transmission is believed to occur from female mosquitoes to progeny during periods of drought ^[1]. Flooding of the mosquito breeding sites stimulates hatching of the mosquito eggs and eventually a large increase in infected mosquito populations that proceed to infect animals, both wild and domestic ^[4].

Historically, outbreaks of Rift Valley Fever were limited to East Africa; however, a major outbreak occurred in Egypt in 1977 followed by outbreaks in Saudi Arabia and Yemen in 2000 indicating the spread of the virus to previously virgin territories ^[2]. Outbreaks have also been recorded in Somalia, Tanzania, Sudan, and most Sub-Saharan countries ^[2]. The outbreak in 2000 on the Arabian Peninsula had an estimated 2,100 human cases and nearly 300 deaths ^[2]. In the 2006-2007 outbreak in East Africa, more than 1,000 people were diagnosed with RVF and over 300 of those confirmed cases died from the disease ^[5].

1.1.2 Pathogenesis

In animals, the incubation period is between 12 hours and 6 days, depending on the species ^[2]. Rift Valley Fever epizootics are characterized by large-scale abortion events, referred to as “abortion storms”, in pregnant ruminants ^[1]. Nearly 100% of pregnancies in infected female

ruminants end in abortion ^[6]. Adult ruminants are moderately resistant to RVF, with a 20% mortality rate in experimentally infected adult sheep ^[6]. However, mortality rate among infected newborns and juvenile ruminants are high, nearly 100% in newborn lambs ^[7]. Susceptibility, severity of disease, and symptoms vary by species. For example, mice can exhibit acute hepatitis and lethal meningoencephalitis at late stages, while rhesus monkeys can develop hemorrhagic fever and adult sheep have been observed to develop ocular disease ^[6].

In humans, the incubation period of Rift Valley Fever is generally 4-6 days ^[6]. The majority of RVF cases are self-limiting, febrile illness with symptoms such as chills, malaise, dizziness, headache, and fever. However, in rare cases, the disease can lead to more severe disease such as encephalitis, meningitis, vision loss, and hemorrhagic fever ^[1,6]. The overall case fatality ratio is estimated to be between 0.5% and 2% ^[1]. The route of viral entry into the brain and the mechanism that causes hemorrhagic fever are aspects of RVF pathogenesis that require further research ^[6]. However, one study suggested an association between the variations in disease manifestation and severity with genetic polymorphisms in the innate immunity pathways ^[8].

1.1.3 Vaccines and Therapeutics

There are currently no licensed vaccines or therapeutic for use in humans. However, there are a limited number of vaccines have been shown to provide protection against RVFV in animal models and therapeutics that have been shown to be effective in inhibiting viral replication *in vitro* and *in vivo*.

The first RVF vaccine, the Smithburn vaccine, was developed in the 1940's by intracerebral passaging of the virus in mice ^[9]. The formalin inactivated Smithburn vaccine was

safe, but with low immunogenicity and short-term immunity. The live Smithburn vaccine, on the other hand, produced long-lasting immunity with a single dose; however, due to remaining virulence, caused abortions and fetal malformations when administered to pregnant animals ^[10].

The Clone 13 RVFV strain is a strain isolated from a RVF-infected patient from Central Africa ^[10]. The Clone 13 strain is missing 69% of the NSs open reading frame, leading to attenuation ^[6]. The RVF Clone 13 vaccine was shown to be highly immunogenic in sheep and goats and moderately immunogenic in cattle [10]. Unlike the Smithburn strain, the Clone 13 vaccine was safe to use in pregnant ewes without any teratogenicity or cases of abortion ^[10]. Vertical transmission of a vaccine strain would be advantageous in vaccinating large populations of animals and maintaining immunity; however, the Clone 13 virus does not develop detectable viremia in vaccinated animals and therefore, is unable to be transmitted from the mother to the offspring or by mosquito ^[9].

MP-12 is a RVFV strain that was derived from the wild type ZH548 strain that was isolated from a RVF patient during the 1977 Egypt outbreak ^[11]. The virulent ZH548 strain was serially passaged in the presence of a mutagen, 5-fluorouracil until the 12th passage ^[9]. The virus contains a total of 23 nucleotide mutations in the S, M, and L segments, but retains a functional NSs gene ^[12]. The specific mechanism of attenuation is unknown; however, the attenuating mutations were limited to the M and L segments, including a mutation that causes temperature sensitivity ^[11]. The MP-12 strain has been shown to provide protection from lethal virus challenge in lambs, cattle, and rhesus macaques ^[9]. Variants of the MP-12 strain with deletions in the NSs and NSm genes were shown to deliver protective immunity against a lethal virus challenge in rats ^[13]. The live attenuated MP-12 vaccine is one of the best-characterized RVFV vaccines and is the only RVF vaccine that has been conditionally approved for veterinary use in

the United States ^[11]. The vaccine has also been studied for human use in phase II clinical trials ^[11]. The MP-12 virus strain was used in this study because work with MP-12 can be done in BSL-2 conditions, whereas wild-type RVFV must be studied in a BSL-3 environment. Furthermore, the live attenuated MP-12 virus produces high viral titers *in vitro*.

While there are no licensed therapeutics approved for use against Rift Valley Fever Virus, compounds such as Suramin, an anti-parasitic drug, ^[14] and Curcumin, a compound extracted from turmeric, ^[15] were shown to inhibit viral replication *in vitro* by 1 log and 4 logs, respectively. Ribavirin was also shown to inhibit RVFV replication by 1 log at 12 μM ^[36], although it would only be considered an emergency option due to its associated toxicity ^[12]. Sorafenib, a drug primarily used to treat cancer, inhibited RVFV replication by 2-3 logs at 10 μM , depending on the cell line ^[16]. However, due to the toxicity of these drugs, development of novel compounds with improved safety and efficacy is critical for treatment and control of Rift Valley Fever.

1.2 PUBLIC HEALTH SIGNIFICANCE

1.2.1 Socio-economic Impact

Rift Valley Fever outbreaks have significant socio-economic impacts on the governments and pastoralist communities in Africa and the Arabian Peninsula. In pastoral societies, livestock represent the basis of human livelihood and culture ^[19]. In the East Africa, pastoralism has a vital role in national economies ^[19] and 90% of pastoralist income depends on keeping livestock ^[18]. Epizootic outbreaks of RVF not only result in enormous loss of livestock by death and abortion,

but also lead to the closure of markets and the ban of animal slaughter and the export of animals and animal products from affected countries ^[18]. In many communities in Africa including Arusha, Tanzania, livestock are used as sources of meat and milk as well as cultural roles, such as paying dowry, school fees, and healthcare costs ^[17]. In the 2007 RVFV outbreak in Tanzania and Kenya, over 40,000 livestock died from the disease and over 30,000 spontaneous abortions were recorded ^[2]. The economic impact on Kenya/Tanzania and Somalia during the 2007 RVFV outbreak was estimated to be \$66 million USD and \$471 million USD, respectively ^[19]. The calculated impact incorporates not only loss of livestock, but also costs for disposal of carcasses, healthcare costs for infected individuals and animals, reduction in trade and losses due to market closures ^[19].

1.2.2 Bioterrorism Threat

Prior to 1969, the US offensive biological weapons program developed Rift Valley Fever Virus as a biological weapon ^[23]. Rift Valley Fever Virus is categorized as a Category A high priority agent by the NIAID and as an overlap Select Agent between the USDA and the HHS ^[20]. The Working Group for Civilian Biodefense considers RVFV to be a likely biological weapon ^[21]. The National Veterinary Stockpile ranked RVFV the third highest threat to animals ^[23]. The virus can be infectious via aerosol; however, human-to-human transmission has not been recorded ^[22]. The risk of RVFV as a biological weapon against humans is limited; however, the impact on livestock and animals could destroy the livestock industry and cripple the economy ^[22]. Rift Valley Fever virus is known to be easily cultured *in vitro* and can be prepared in large

quantities ^[22]. Consultants from the WHO estimated that 50 kg of RVFV released from an aircraft over a population center of 500,000 people would leave 35,000 incapacitated and 400 dead ^[21].

1.2.3 Potential for Emergence in Europe and the United States

Recent spread of Rift Valley Fever Virus to the Arabian Peninsula heightens concerns that the virus could potentially spread to new areas, including Europe and the United States ^[23]. Trade of infected ruminants from endemic countries, movement of virus-carrying vectors, or intentional introduction of the virus are all possible pathways for emergence of RVFV in Europe and the United States ^[23]. Veterinary authorities are required to display certificates ensuring there was no evidence of RVF on the day of shipment or of vaccination 21 days prior to shipment in order to prevent importation of RVF-infected livestock ^[28]. Illegal importation of animals between North Africa and Southern Europe as well as the changing climate can all facilitate the spread of RVFV to Europe ^[26]. Several species of mosquitoes in both the United States ^[24] and Europe ^[25] have demonstrated potential for transmitting the virus. Surveillance, early detection, and containment of the virus are crucial to preventing the establishment of RVF in previously unexposed areas ^[27]. A 2004 USDA study estimated that 1 L of RVFV introduced into the United States would have a total economic impact over \$50 billion and would lead to an endemic status in the continental U.S. within 2 years ^[23].

1.3 COMPOUNDS

1.3.1 A3 and M4

Antiviral properties of compound A3 was identified from a high-throughput screening assay using A549 cells and influenza A virus ^[29]. A3 was able to inhibit influenza virus polymerase activity ^[30]. However, Uracil and orotic acid were found to reverse the inhibition of viral replication by A3, which suggested a mechanism of action involving *de novo* pyrimidine biosynthesis ^[30]. The enzyme Dihydroorotate dehydrogenase (DHODH) is thought to be the target protein; however, the hypothesis has not yet been confirmed ^[30]. A3 has been demonstrated to be effective against a variety of viruses, including Sendai virus, vesicular stomatitis virus, Sindbis virus, Hepatitis C virus, adenovirus 5, HIV-1, and Arenaviruses ^[30, 31]. For example, in the presence of A3, viral titer of adenovirus-5 was reduced by 6 logs, while vesicular stomatitis virus had a 5 log viral titer reduction ^[30]. A3 displayed limited antiviral activity in pig kidney (PK-15) cells and baby hamster kidney (BHK) cells. No antiviral activity with A3 was observed in chicken fibroblast (DF-1) cells, suggesting a species-specificity of the compound ^[30].

There is no published data on compound M4 and the mechanism of action is unknown.

1.3.2 Favipiravir (T-705)

The antiviral properties of Favipiravir, also known as T-705 or Avigan, were identified by Toyama Chemical Co., Ltd. through screening of a chemical library using a plaque reduction assay with the Influenza A virus ^[32]. Favipiravir showed no inhibitory activity during the viral

absorption stage or after 6-hours post infection, suggesting inhibition of the viral replication stage ^[32]. Favipiravir must be phosphoribosylated within cells to form the active form, Favipiravir ribofuranosyl-5'-triphosphate (Favipiravir-RTP) ^[32]. Favipiravir-RTP is either misincorporated into the nascent RNA or binds conserved domains in the viral RNA polymerase preventing further extension of the RNA strand; however, Favipiravir-RTP is 2,650 times more selective for the influenza virus RNA-dependent RNA polymerase than human DNA-dependent RNA polymerase ^[32]. Furthermore, Favipiravir-RTP did not display any inhibitory activity against human DNA polymerase ^[32]. T-705 was effective in inhibiting Rift Valley Fever virus, Arenaviruses, and other Bunyaviruses *in vitro* as well as *in vivo* in rat and hamster models ^[34,35]. Favipiravir was also shown to be effective against Zaire Ebola Virus (EBOV) *in vitro* and *in vivo*. In cell culture, T-705 inhibited EBOV replication by 4 log units at 110 μM ^[36]. Furthermore, treatment with Favipiravir 6 days post-infection with EBOV in mice lacking type-1 interferon displayed rapid virus clearance and protected 100% of mice from lethal disease ^[36].

2.0 STATEMENT OF THE PROJECT AND SPECIFIC AIMS

Identification of potential therapeutics is crucial to the prevention, treatment, and control of Rift Valley Fever. The goal of this project is to determine whether the compounds A3 and M4 are effective in inhibiting Rift Valley Fever Virus replication *in vitro*, are relatively safe with low cytotoxicity, and to compare selectivity indexes of these compounds with a known Rift Valley Fever Virus inhibitor. The ideal therapeutic would have low cytotoxicity and be effective in inhibiting viral replication even at low concentrations.

Aim 1: To determine cytotoxicity of the compounds in Vero E6 cells

Viability of cells treated with the compounds at various concentrations was determined. CellTiterGlo Luminescent cell viability assay measured relative luminescence of cells treated with compounds based on levels of ATP. Neutral Red assay measured absorbance of Neutral Red dye via active transport into metabolically active cells.

Aim 2: To determine efficacy of compounds in inhibiting viral replication by measuring reduction in viral titer and viral RNA and to calculate selectivity indexes

Inhibition of viral replication was determined by viral titer reduction and viral RNA reduction. Plaque assays measured viral titer or the number of plaque-forming units per volume in the supernatants of treated and untreated cells. Real-time polymerase chain reaction was used to quantify viral RNA in the supernatants. Results were compared between compounds and concentrations.

Selectivity indexes were calculated based on the cytotoxic concentration that kills 50% of cells and the inhibitory concentrations that reduced viral titer at specific intervals. Comparison of selectivity indexes of the compounds indicates which compounds are more suitable as therapeutics.

3.0 MATERIALS AND METHODS

3.1 BIOSAFETY

All experiments using live MP-12 Rift Valley Fever Virus were performed in Biosafety Level 2 in a Class II Biosafety Cabinet. Vesphene Ilse (diluted 1:128, Steris Corporation, SKU#646108) was used as the disinfectant.

3.2 CELL CULTURE

Vero E6 cells were cultured in D10 media (Dulbecco's Modified Eagle Medium with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin solution, and 1% L-Glutamine. Cells were split 1:12 every 72 hours using 0.05% Trypsin/EDTA. Cells were kept in an incubator at a 37°C and 5% CO₂.

3.3 EFFICACY DETERMINATION

Vero E6 cells were suspended with 0.05% Trypsin/EDTA and diluted in D10 media. Cells were counted using a hemocytometer and Trypan Blue solution. Cells were diluted to 25,000 cells per mL and 200 μ L of the diluted cell suspension was transferred to each well of two 96-well flat-bottom cell-culture plate and incubated for 24 hours at 37°C. Seven half-log dilutions of each compound were made in D2 media (Dulbecco's Modified Eagle Medium with 2% Fetal Bovine Serum, 1% Penicillin/Streptomycin solution, and 1% L-Glutamine) starting from 1000 μ M to 1 μ M from a 100mM stock. 100mM stock of compounds A3 and M4 was received from Dr. Megan L. Shaw, Mount Sinai Hospital. MP-12 virus stock ($\sim 8 \times 10^5$ PFU/mL) was diluted to 3×10^3 PFU/mL for an MOI of 0.01, assuming 15,000 cells per well. Media was removed from both 96-well plates and equal volumes of diluted virus and diluted compound were added in triplicate per concentration. Control wells included untreated, infected cells, untreated, uninfected cells, and blank wells with no cells. The plates were incubated at 37°C for 72 hours. After 72 hours, supernatants from each triplicate well were combined and stored at -80°C for RT-PCR and plaque assays.

3.4 CELL VIABILITY ASSAYS

3.4.1 Neutral Red Assay

Neutral Red Assay was used to assess cytotoxicity of M4 and T-705. The Neutral Red Assay protocol was adapted from Repetto et.al (2008) [37]. Once supernatants were collected from the two 96 well plates, wells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS) solution. A stock of Neutral Red Solution was diluted in DPBS and incubated at 37°C for 2 hours. After a two-hour incubation, the Neutral Red solution was removed and wells were washed twice with DPBS. Neutral Red Solubilization Solution (1% Acetic Acid in 50% Ethanol) was added and absorbance at 540nm was read using a spectrophotometer.

3.4.2 CellTiterGlo Luminescent Cell Viability Assay

CellTiterGlo Luminescent Cell Viability Assay was used to measure cytotoxicity of compound A3. Vero E6 cells were suspended with 0.05% Trypsin/EDTA and diluted in D10 media. Cells were counted using a hemocytometer and Trypan Blue solution. Cells were diluted to 50,000 cells per mL and 100uL of the diluted cell suspension was transferred to each well of a 96-well opaque-walled, flat-bottom cell-culture plate and incubated for 48 hours at 37°C. Compound A3 was diluted from the 100mM stock to 100 µM. The 100 µM solution was serially diluted three-fold in D10 media with 0.1% Dimethyl Sulfoxide (DMSO) up to 0.4µM. Media was removed from wells and 100 µL of each compound dilution to the respective wells in triplicate. Cells were incubated with the compound for 24 hours at 37°C. After 24 hours, the plate was equilibrated to room temperature. 100 µL of CellTiterGlo Reagent from the

CellTiterGlo Luminescent Cell Viability Assay Kit (Promega Corporation, Product #G7571) was added to each well. The contents were mixed for two minutes on an orbital shaker. After 10 minutes, the clear bottoms of the wells were covered with white tape and a luminometer was used to measure luminescence.

3.5 PLAQUE ASSAY

Vero E6 cells were suspended with 0.05% Trypsin/EDTA, diluted in D10 media, and seeded into 6-well cell-culture plates and incubated for 24 hours at 37°C. Supernatant samples were serially diluted in D2 media. Media was removed from wells and 200µL of diluted supernatant was added to duplicate wells. Plates were incubated at 37°C for 1 hour. The plates were rocked gently every 15 minutes to prevent drying. After 1-hour incubation, the inoculum was removed. Equal volumes of nutrient overlay (2x Minimum Essential Medium, 4% FBS, 2% Penicillin/Streptomycin, and HEPES buffer) and heated 1.6% SeaKem Agarose Solution were mixed and added to the wells. Once the overlay solidified, the plates were incubated for 72 hours at 37°C and 5% CO₂. After 72 hours, the plates were fixed with 37% Formaldehyde for 2-3 hours. Once the plates were fixed, the formaldehyde and agar plugs were removed and disposed of in formaldehyde waste containers. A 0.1% Crystal Violet in 20% Ethanol Solution was added to each well for 10 minutes. Plates were rinsed with water and visible plaques were counted to determine viral titer of supernatants. A two-way ANOVA analysis with a Dunnett's Multiple Comparisons Test was performed on GraphPad Prism 6 to analyze the statistical significance of the differences in viral titers with the control.

3.6 VIRAL RNA QUANTIFICATION

3.6.1 RNA Extraction from Supernatants

RNA extraction was performed using the Invitrogen PureLink Viral RNA/DNA Kit (Invitrogen, cat. #12280-050). 100 μ L of supernatants from wells infected with virus and treated with each compound and dilution was mixed with 900 μ L of TRI Reagent Solution (Ambion, Part # AM9738) for virus inactivation. 200 μ L of Chloroform was added to each sample and inverted for 20 seconds. Samples were then centrifuged at 12,000xg for 15 minutes at 4°C. The aqueous phase was transferred to a clean tube and 500 μ L of 70% Ethanol was added. Lysates were transferred to a Viral Spin Column and centrifuged at 6,800xg for 1 minute. Columns were washed with 500 μ L of Wash Buffer with Ethanol twice and centrifuged at 6,800xg for one minute each time. 40 μ L of sterile RNase-free water was used to elute the RNA. RNA was stored at -80°C.

3.6.2 Semi-quantitative Real-Time Polymerase Chain Reaction

The semi-quantitative Real-Time Polymerase Chain Reaction of RNA extracted from supernatant samples was done using the SuperScript III Platinum One-Step qRT-PCR Kit with ROX (Invitrogen, cat.# 11745-100). 2x PCR Master Mix, forward/reverse primers, probe, SuperScript III RT/Platinum Taq Mix, and nuclease-free water were mixed as instructed with 5 μ L of extracted RNA samples in a 96-well PCR plate. 5 μ L of nuclease-free water was used as a no-template control and a standard curve was made using samples with known concentrations.

FAM fluorescence data was collected during the 55°C incubation step. The ROX passive reference was not used during analysis.

4.0 RESULTS

4.1 AIM 1: TO DETERMINE CYTOTOXICITY OF THE COMPOUNDS IN VERO E6 CELLS USING TWO CELL VIABILITY ASSAYS

Neutral Red dye is absorbed by metabolically active cells through active transport into lysosomes. After absorption, an acidic environment leads to the release of the neutral red dye back into the media. The amount of neutral red dye that is solubilized from cells is measured by absorbance at 540nm and gives a relative estimate of cell viability. An absolute quantification of cells using a standard was not performed; however, relative cell viability between treated wells and untreated control wells was used to calculate a percent survival. The cells were incubated with neutral red dye diluted in PBS for two hours without media. The lack of nutrients for the two hour duration led to cell death unrelated to the cytotoxicity of the compounds; however, cells treated with A3 were disproportionately affected by the absence of media leading to a greater cytotoxicity measurement compared to previous cytotoxicity measurements.

Data previously collected using the CellTiterGlo luminescent cell viability assay was used to estimate cytotoxicity for compound A3, as the cytotoxicity data from the neutral red assay was unreliable. CellTiterGlo luminescent cell viability assay quantifies ATP in solution using luciferase to create a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of metabolically active cells present.

Similar to the neutral red assay, relative luminescence was compared between compound-treated wells and untreated controls to calculate a percent survival. However, it is important to note that both compounds M4 and T-705 were incubated with cells for 72 hours in the neutral red assay, while cells were only incubated for 24 hours with A3 in the CellTiterGlo luminescent cell viability assay and therefore can impact the comparability of the CC_{50} and selectivity indexes.

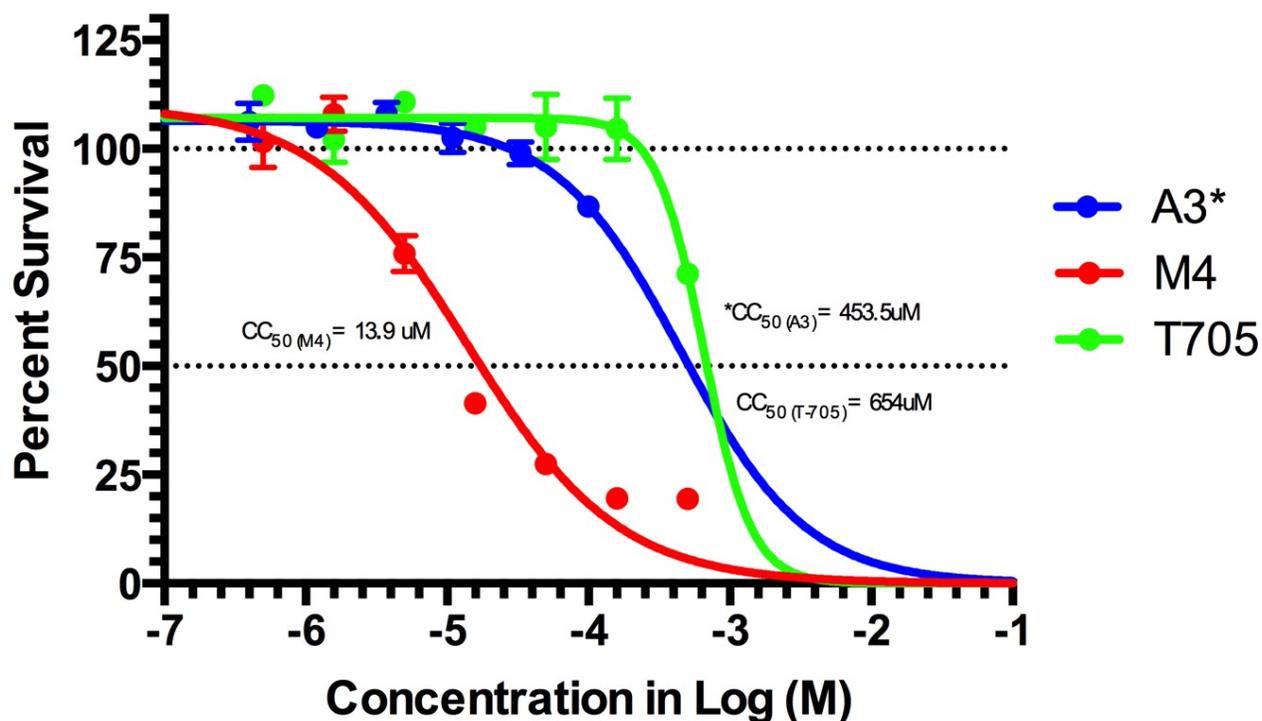


Figure 1. Percent Survival and CC_{50} Estimation based on compound concentration for A3, M4, and T-705. Cytotoxicity data for was measured using CellTiterGlo Luminescent Cell Viability Assay with A3 after a 24-hour incubation and Neutral Red Assay for M4 and T-705 after a 72-hour incubation. The maximum concentration tested for A3 was 100 μM , while the highest concentration tested for M4 and T-705 was 500 μM . CC_{50} was estimated using non-linear regression to fit a log (concentration) vs. percent survival curve on GraphPad Prism 6.

A CC_{50} , the concentration at which the compound is cytotoxic to 50% of cells, was estimated using the log (dose) vs. percent survival curve (Figure 1). The CC_{50} of A3, was 453.5 μM (95% CI: 121.3, 1695), the CC_{50} of M4 was 13.9 μM (95% CI: 2.25, 85.3), and the CC_{50} of T-705 was 654 μM (95% CI: 403.2, 1061).

4.2 AIM 2: TO DETERMINE EFFICACY OF COMPOUNDS IN INHIBITING VIRAL REPLICATION BY MEASURING REDUCTION IN VIRAL TITER AND VIRAL RNA AND TO CALCULATE SELECTIVITY INDEXES

Cells were incubated with the compound and virus simultaneously; therefore, the compounds can potentially affect the viral entry step, the post-viral entry stage, or both. Supernatants collected 72 hours after treatment and infection were used in plaque assays to estimate the viral titer. Efficacy in the compounds was measured by viral titer reduction. Real-time Polymerase Chain Reaction was also performed on viral RNA extracted from the supernatants to observe whether the trend in viral titer reduction was consistent with viral RNA reduction.

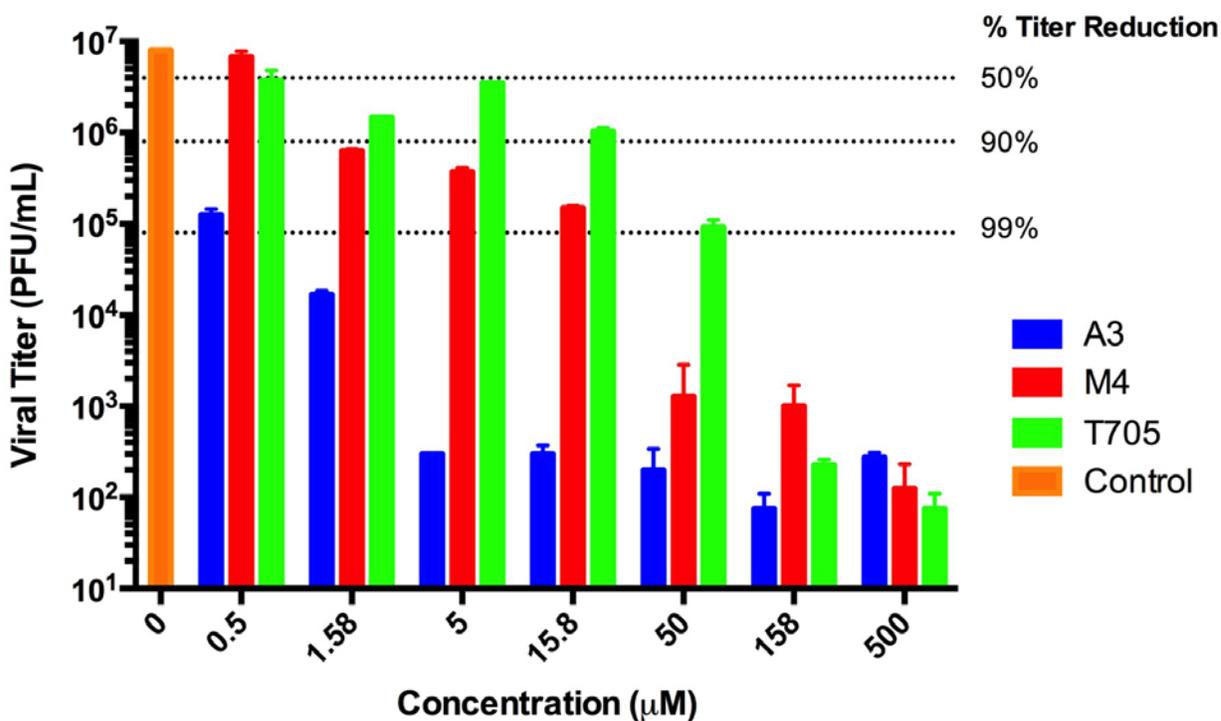


Figure 2. Viral Titer in PFU/mL of supernatants based on compound concentration measured by plaque assay.

A two-way ANOVA analysis with a Dunnett's Multiple Comparisons Test was performed on GraphPad Prism 6. Viral titer reduction of supernatants of cells treated with compounds A3, M4, and T705 was statistically significant at all concentrations ($p < 0.001$).

Changes in viral titer based on compound concentrations are shown in Figure 3. All three compounds displayed statistically significant reductions in viral titer compared to the control at all concentrations ($p < 0.001$). A 5-log viral titer reduction was seen with A3 at 158 μM , a 4-log viral titer reduction with M4 at 500 μM , and a 5-log viral titer reduction with T-705 at 500 μM (Figure 2). The trend in viral RNA reduction was comparable to the trend in viral titer reduction (Figure 3).

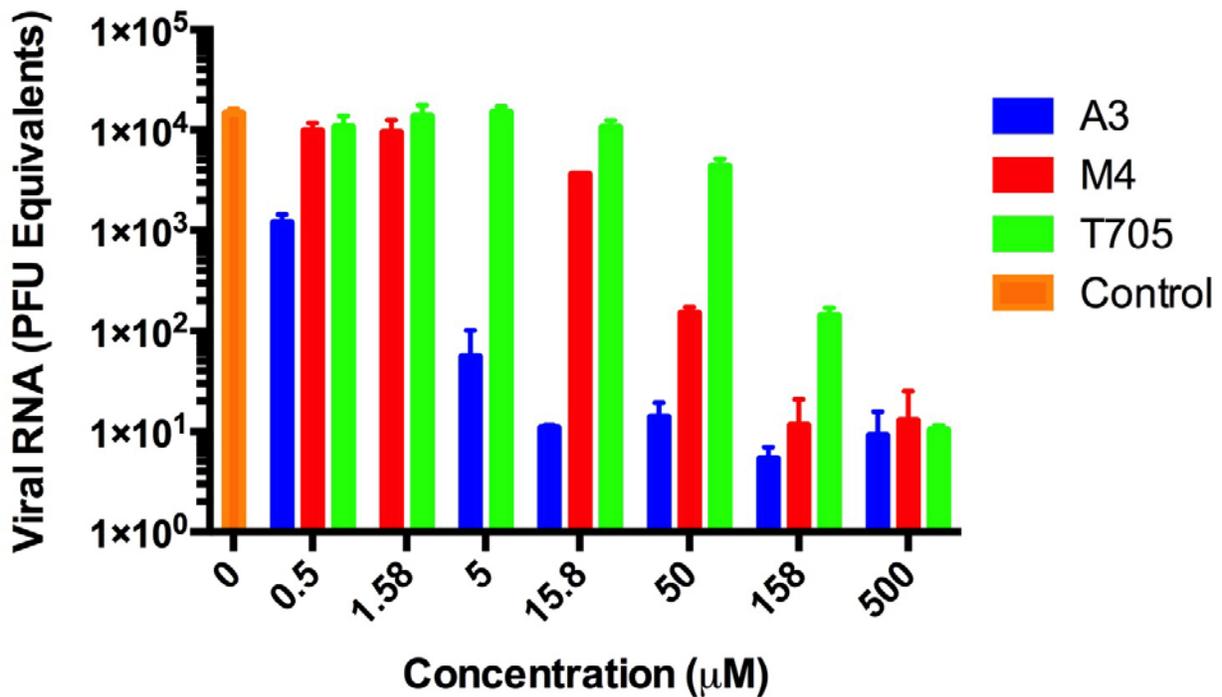


Figure 3: Viral RNA in PFU Equivalents of supernatants based on compound concentration measured by RT-PCR.

Data on viral RNA for A3 at 1.58 μM and M4 at 5 μM was not included due to an error during RNA extraction. The trend in viral RNA reduction was consistent with the viral titer reduction observed with plaque assay.

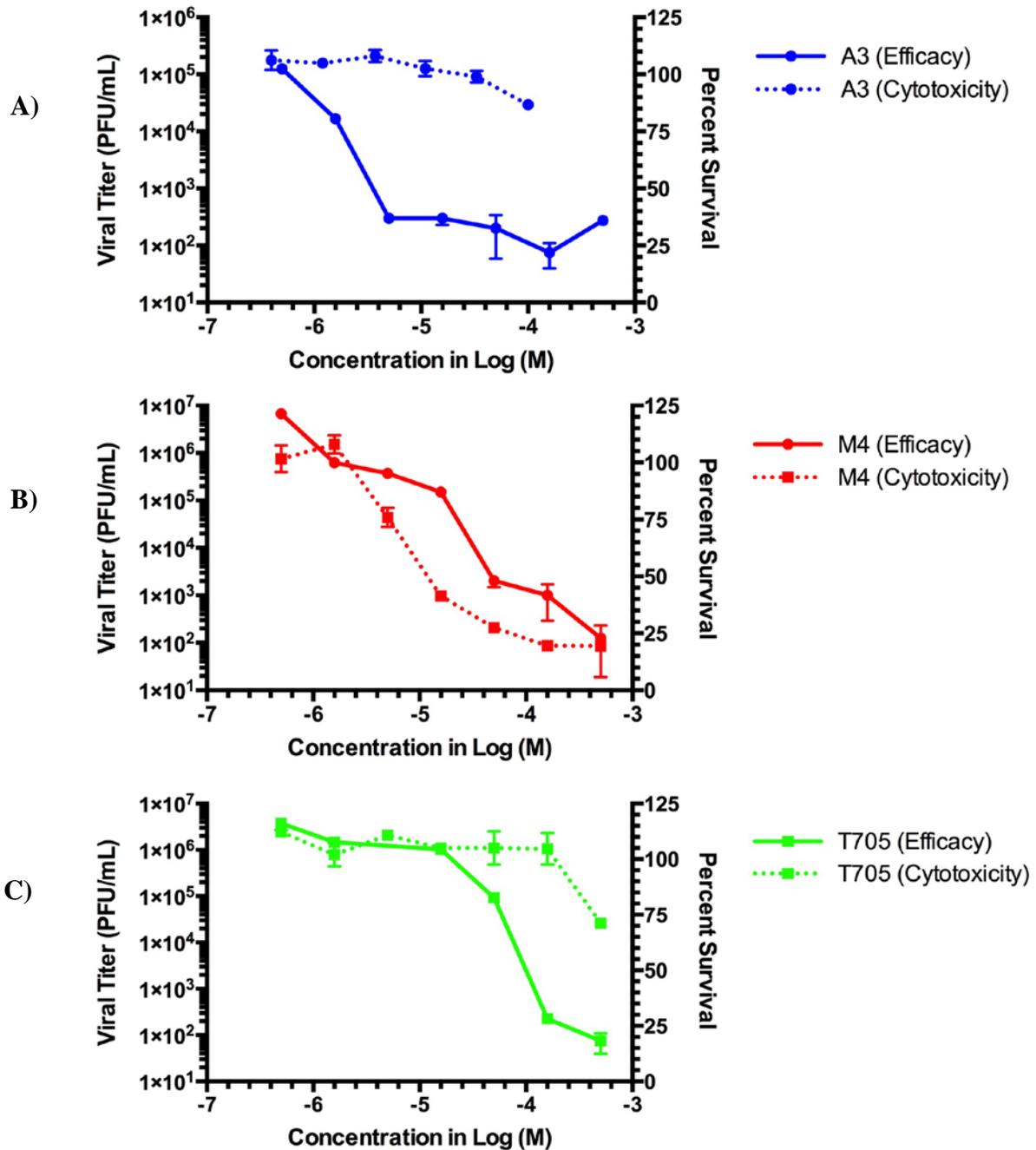


Figure 4: Comparison of trends in efficacy and cytotoxicity of A3, M4, and T-705 based on compound concentrations. This figure illustrates the potential role of cytotoxicity in the observed viral titer reduction of compound M4. The viral titer reduction that occurs with compound A3 and T-705 without a decreased percent survival suggests that the viral titer reduction is due to the antiviral activity of the compounds rather than the cytotoxicity.

In some cases, viral titer reduction can be attributed to cytotoxicity of compounds rather than their antiviral activity. In order to determine whether the observed viral titer reduction is associated with compound cytotoxicity, percent survival and viral titer were graphed in Figure 4.

Increased viral titer reduction at higher concentrations without an increased cytotoxicity indicates that viral titer reduction is due to antiviral activity of the compound whereas, a similar downward trend in both percent survival and viral titer, as seen with compound M4 (Figure 4B), suggests a role of cytotoxicity in the observed reduction in viral titer. Increasing inhibition of viral replication was observed with A3 and T-705 from low to high concentrations without an increased cytotoxicity, attributing the viral titer reduction to the compound's antiviral properties.

Viral titer was used to estimate the inhibitory concentrations, because the desired effect of the compounds is viral titer reduction. The inhibitory concentration, the concentration at which a certain percentage of viral replication is inhibited^[38], was estimated using the log (dose) vs. viral titer curve for 50%, 90% (1-log), and 99% (2-log) viral titer reduction (Figure 5).

The inhibitory concentrations for A3, M4, and T-705 are summarized in Table 1 with 95% confidence intervals.

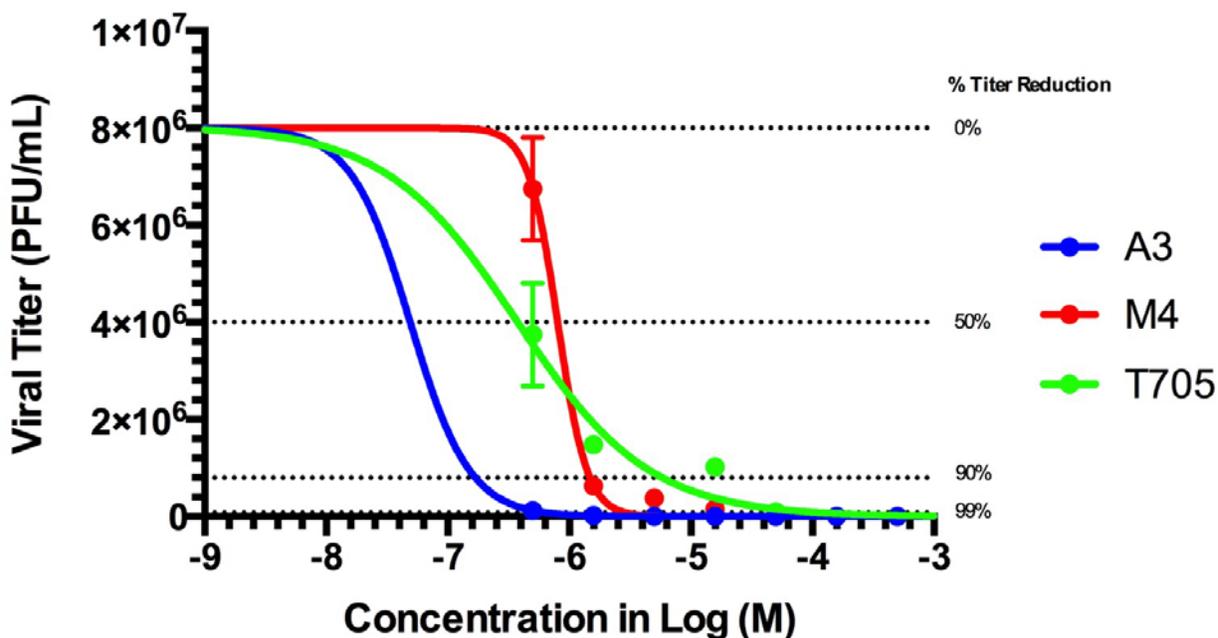


Figure 5: Viral Titer and IC Estimation based on compound concentration for A3, M4, and T-705.

IC₅₀, IC₉₀, and IC₉₉ was estimated using non-linear regression to fit a log (concentration) vs. viral titer curve on GraphPad Prism 6. The fits of the curves could be improved by testing a wider range of concentration of compounds.

Table 1: Summary Table of CC₅₀, Inhibitory Concentrations, and Selectivity Indexes of A3, M4, and T-705.

Compound:	A3*	M4	T-705
CC ₅₀ (μM)	453.5 (121.3, 1695)	13.9 (2.25, 85.3)	654 (403.2, 1061)
IC ₅₀ (μM)	0.048 (0.039, 0.059)	0.784 (0.68, 0.90)	0.423 (0.161, 1.0)
IC ₉₀ (μM)	0.168 (0.153, 0.185)	1.41 (1.11, 1.79)	3.37 (0.395, 28.8)
IC ₉₉ (μM)	0.646 (0.629, 0.663)	2.68 (1.82, 3.95)	34.3 (0.268, 4392)
SI (CC ₅₀ /IC ₅₀)	9,448	17.73	1,546
SI (CC ₅₀ /IC ₉₀)	2,699	9.86	194
SI (CC ₅₀ /IC ₉₉)	702	5.19	19.1

*Cytotoxicity data of A3 was collected after a 24-hour incubation, whereas cytotoxicity data of M4 and T-705 was collected after a 72-hour incubation. Comparison between the CC₅₀ and selectivity indexes of A3 with that of M4 and T-705 is limited. Inhibitory concentrations are comparable because efficacy testing was performed identically with all 3 compounds.

The selectivity index, or the therapeutic index, is the ratio of the CC₅₀ to the concentration at which the desired efficacy is observed ^[38]. The ideal compound should have a high selectivity index, which suggests that the compound is highly efficacious with low cytotoxicity ^[39]. Cytotoxicity of a compound can contribute to a viral titer reduction that is not attributable to the antiviral compound acting directly on the virus ^[37]. The selectivity index formula accounts for this, as a higher cytotoxicity will result in a lower selectivity index even if the inhibitory concentration is low. The calculated selectivity indexes based on desired efficacy are shown in Table 1. Compound A3 was effective at very low concentrations, while inhibitory concentrations of T705 were high with very large 95% confidence intervals. Furthermore, due to a low CC₅₀, compound M4 had a low selectivity index. Selectivity indexes can be compared as long the cytotoxicity and efficacy is measured under the same circumstances. Differences in cell lines, duration of compound exposure, stage at which the compound is introduced, and virus

strain can all impact the selectivity index. Because duration of compound exposure as well as the cell viability utilized varied between compound A3 and both M4 and T-705, comparison of selectivity indexes between them is limited.

5.0 DISCUSSION

Rift Valley Fever is a public health threat that currently affects Africa and parts of the Middle East, but has the possibility of spreading to other countries and continents across the world. Rift Valley Fever virus is considered a serious bioterrorism threat to both humans and animals. Past outbreaks have placed enormous economic burdens on the agricultural communities due to livestock mortality, abortion storms, and trade bans. The emergence of the virus in Europe or the United States can have devastating socio-economic impacts. Without any approved vaccines or therapeutics, the prevention, treatment, and control of Rift Valley Fever virus would be difficult. Discovery of effective antiviral drugs is crucial to the public health effort in combating Rift Valley Fever.

Of the three compounds tested, A3 had the highest selectivity indexes. The data suggests that A3 has low cytotoxicity and high efficacy in inhibiting Rift Valley Fever virus replication *in vitro* in Vero E6 cells. The selectivity index of A3 was greater than that of T-705, a compound previously demonstrated to be an effective RVFV replication inhibitor^[33], at all tested efficacy levels; however, the difference in duration of compound exposure during cytotoxicity testing limits comparability of CC₅₀ and selectivity indexes. However, A3 was very effective at very low concentrations compared to the higher inhibitory concentrations of T-705. Further studies must be conducted to determine A3's candidacy as a potential antiviral therapy for RVFV. Compound M4 had a high cytotoxicity and therefore a relatively low selectivity index.

A wider range of compound concentrations must be tested to estimate more accurate CC₅₀ and Inhibitory Concentrations. Vero E6 cells are ideal for *in vitro* antiviral efficacy testing because Vero E6 cells do not produce type-1 interferon and therefore lack a natural antiviral response [29]. However, other cell lines including human-derived cell lines, should also be used in testing due to variable cytotoxicity as well as species-specific efficacy. Furthermore, because the MP-12 vaccine strain is an attenuated strain of the virus with deletions in the genome, the effects of the compounds on the MP-12 virus can differ from those on the wild-type strains such as ZH501. Efficacy of A3 should be tested with a wild-type virus to confirm consistent inhibition of RVFV independent of the virus strain.

Although A3's mechanism of action against other viruses, such as influenza and Arenaviruses is known, whether the mechanism is the same with RVFV has yet to be determined [30,31]. Knowledge of the mechanism of action can be helpful in predicting toxicity and potential for viral resistance [39]. Also, combination therapies with other antiviral drugs can demonstrate improved efficacy in viral inhibition [39].

As expected, T-705 was effective in inhibiting viral replication *in vitro*; however, the data suggests that compound A3 is more effective than T-705 at low concentrations. While compound M4 has cytotoxicity levels too high for use as a therapeutic, the broad-spectrum antiviral compound A3 shows promise as a candidate for antiviral therapy against Rift Valley Fever Virus.

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