The Role of Polyamines in Enteroviral Attachment

Thomas Mckenzie Kicmal

Follow this and additional works at: https://ecommons.luc.edu/luc_theses

Part of the Virology Commons

Recommended Citation
https://ecommons.luc.edu/luc_theses/3996

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2019 Thomas Mckenzie Kicmal
LOYOLA UNIVERSITY CHICAGO

THE ROLE OF POLYAMINES IN ENTEROVIRAL ATTACHMENT

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY

THOMAS M. KICMAL

CHICAGO, ILLINOIS

AUGUST 2019
ACKNOWLEDGEMENTS

I would like to thank Dr. Bryan Mounce for being a dedicated, nurturing, and supportive mentor throughout my program. He has played a central role in my scientific development and his guidance and insight has been invaluable my success. I would also like to thank the entire Mounce lab for providing a friendly and collaborative environment and for helping achieve my research goals.

I would like to thank the members of my thesis committee (Dr. Thomas Gallagher, Dr. Nina Clark, Dr. Francis Alonzo, and Dr. Adam Driks). They have provided valuable insight into my project and have challenged me in ways that have truly helped me to become a better scientist.

Finally, I would like to thank my family and my girlfriend for their love and support that has allowed me to navigate and persevere the many stresses and challenges of graduate school. They have all been vital to my success and growth as a scientist and as a person.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

LIST OF FIGURES ................................................................................................................ vi

LIST OF TABLES .................................................................................................................. vii

LIST OF ABBREVIATIONS ..................................................................................................... viii

ABSTRACT ............................................................................................................................... ix

CHAPTER ONE: BACKGROUND ......................................................................................... 1
  Literature Review .................................................................................................................. 1
    Enteroviruses and CVB3 ................................................................................................. 1
    CVB3 Structure .............................................................................................................. 1
    Viral Attachment ............................................................................................................ 2
    CVB3 Attachment to Cells ............................................................................................ 3
    CVB3 Entry into Cells ................................................................................................... 5
    Polyamines .................................................................................................................... 7
    Polyamines and Viruses .............................................................................................. 10
    Bunyaviruses ............................................................................................................... 12
    Flaviviruses ................................................................................................................ 13
    Aims and Hypothesis .................................................................................................... 14

CHAPTER TWO: MATERIALS AND METHODS ................................................................ 16
  Cell Culture ..................................................................................................................... 16
  Infection and Enumeration of Viral Titers ..................................................................... 16
  Drug Treatments ............................................................................................................ 17
  Thin-layer Chromatography for Determination of Polyamines .................................. 17
  RNA Purification and cDNA Synthesis ....................................................................... 18
  qPCR-based Attachment Assay .................................................................................. 18
  Single Cycle Replication Attachment Assay .............................................................. 19
  Plaque Formation Attachment Assay ......................................................................... 19
  Statistical Analysis ...................................................................................................... 20

CHAPTER THREE: RESULTS .......................................................................................... 21
  Verification of Polyamine Depletion ........................................................................... 21
  VP3\(^{234R}\) Mutant Virus DFMO Resistance Characterization .................................. 22
  CVB3 Attachment to Polyamine Depleted Cells ....................................................... 23
  Recapitulation of Attachment Phenotype in Alternative Cell Line .......................... 25
  The Ability of the VP3\(^{234R}\) Mutant to Recover Attachment Deficits .................... 26
  Investigation into Diverse Viruses ............................................................................. 27
The Ability of Exogenous Polyamines to Rescue Attachment Deficits

CHAPTER FOUR: SUMMARY AND DISCUSSION
Summary...........................................................................................................31
Discussion........................................................................................................32
The VP3234R Mutant ....................................................................................32
Potential Mechanisms..................................................................................33
Importance.....................................................................................................34

REFERENCE LIST.............................................................................................37

VITA..................................................................................................................52
LIST OF FIGURES

Figure 1. Structure of Enteroviruses................................. 2
Figure 2. Entry of CVB3 into Polarized Intestinal Epithelial Cells ......................... 6
Figure 3. The Mammalian Polyamines ........................................ 7
Figure 4. The Polyamine Synthesis Pathway ........................................ 9
Figure 5. Location of the VP3\textsuperscript{234R} Mutation ................................. 12
Figure 6. DFMO Treatment Depletes Polyamines ........................................ 21
Figure 7. VP3\textsuperscript{234R} Mutation Enhances CVB3 Replication in Polyamine-depleted Cells. 23
Figure 8. Polyamine Depletion Precludes CVB3 Binding to Susceptible Cells .......... 25
Figure 9. Polyamine Depletion Precludes CVB3 Binding to 293T Cells .................. 26
Figure 10. VP3\textsuperscript{234R} Mutation Recovers CVB3 Binding to Polyamine-depleted Cells ..... 27
Figure 11. Polyamine Depletion Precludes Binding of Diverse Viruses ................. 29
Figure 12. Attachment Deficits can be Rescued with Exogenous Polyamines .......... 30
Figure 13. Polyamines are Involved in the Attachment of Viruses ....................... 36
LIST OF TABLES

Table 1. List of Primers 20
LIST OF ABBREVIATIONS

AdoMetDC  S-adenosylmethionine decarboxylase
APAO      Acetylpolyamine oxidase
CAR       Coxsackie and adenovirus receptor
CVB3      Coxsackievirus B3
DAF       Decay accelerating factor
dcAdoMet  Decarboxylated S-adenosylmethionine
DFMO      α-difluoromethylornithine
HRV2      Human Rhinovirus 2
LACV      La Crosse virus
ODC       Ornithine decarboxylase
PUT       Putrescine
RD        Rhabdomyosarcoma
SMO       Spermine oxidase
SPD       Spermidine
SPM       Spermine
SSAT      Spermidine/spermine-N¹-acetyltransferase
ZIKV      Zika virus
ABSTRACT

Polyamines are small polycationic molecules with flexible carbon chains that are found in all eukaryotic cells. Polyamines are involved in the regulation of many host processes and have been shown to be implicated in viral replication. Depletion of polyamine pools in cells with FDA approved drugs restricts replication of diverse RNA viruses. Viruses can exploit host polyamines to facilitate packaging, transcription, translation, and protease activity but other mechanisms remain largely unknown.

Picornaviruses, including Coxsackievirus B3 (CVB3), are sensitive to depletion of polyamines and remain a significant public health threat. We employed CVB3 as a model system to investigate a potential pro-viral role for polyamines using a forward screen. Passaging CVB3 in polyamine depleted cells generated a mutation in capsid protein VP3 at residue 234, which is involved in receptor binding. We show this mutation confers resistance to polyamine depletion. Through attachment assays, we demonstrate that polyamines facilitate CVB3 attachment to susceptible cells, and that the capsid mutant rescues this inhibition in polyamine depleted cells. More divergent viruses also exhibited reduced attachment to polyamine depleted cells, suggesting that polyamines may facilitate attachment of diverse RNA viruses. Virus-receptor interactions are involved in the pathogenesis, transmission, and host range of viruses; thus understanding this process is crucial to combating virus infection. Further, these studies inform additional mechanisms of action for polyamine-depleting pharmaceuticals with implications for potential antiviral therapies.
CHAPTER ONE

BACKGROUND

Literature Review

Enteroviruses and CVB3

Enteroviruses are non-enveloped positive-sense RNA viruses that are members of the *Picornaviridae* family. Enteroviruses are a significant threat to public health and lead to at least 10-15 million infections per year in the US alone (1). Coxsackievirus B3 (CVB3) was the fifth most commonly reported enterovirus in the US between 2014-2016 causing 4% of reported enterovirus infections (2). A study in Yantai, China, found that CVB3 infections were widely distributed, with a seroprevalence of 52.3% (3). Further, CVB3 is a significant contributor to the development of viral myocarditis and dilated cardiomyopathy (4, 5), as well as other severe conditions such as aseptic meningitis (6). There are currently no standard antivirals or vaccines that can be used to prevent or treat infections. Thus, discovering novel aspects of CVB3 replication could uncover potential therapeutic targets that may prevent many of the pathologies associated with this and related viruses.

CVB3 Structure

Picornaviruses, including CVB3, are non-enveloped with an icosahedral capsid. The capsid itself is composed of 60 protomers with each protomer composed of one copy of each of the four viral structural proteins, VP1-4 (7, 8). VP1-3 are located on the external surface while VP4, a much smaller protein, is on the inner surface (7, 8). The
icosahedral symmetry of the capsid generates two-fold, three-fold, and five-fold axes of symmetry. Surrounding the five-fold axis of symmetry is a deep depression in the capsid termed the “canyon” (8). At the bottom of the canyon is a hydrophobic pocket filled with a “pocket factor” thought to be a fatty acid (8, 9). The release of the pocket factor is important for conformational changes in the capsid for release of the viral RNA (10–12). A receptor binding site for CVB3 is located at the bottom of the canyon and binding to this site is thought to be involved in expelling the pocket factor (11–13).

Figure 1. Structure of Enteroviruses. Illustration of the capsid structure of an enterovirus. The external surface of the capsid is composed of repeating units of proteins VP1-3. The icosahedral capsid has a five-fold, three-fold, and two-fold axis of symmetry as indicated. Surrounding the five-fold axis of symmetry is a depression termed the “canyon”. In the canyon is the “pocket factor” as well as a receptor binding site. Adapted from Combelas et al., 2017 (14).

Viral Attachment

The entry of a virus into a host cell is the crucial first step in viral replication. Viral entry begins with attachment to the surface of a cell, which is often mediated by nonspecific electrostatic interactions between virus and components of the glycocalyx,
often proteoglycans such as heparan sulfate (15). Heparan sulfates are a very promiscuous attachment factor that are utilized by many viruses. CVB3 (16, 17), Zika (18), multiple Enteroviruses including some rhinoviruses (19–24), Rift Valley Fever virus (25), and many other viruses bind heparan sulfate (15). Viruses also bind to specific cellular receptors that lead to the ultimate uptake of the virus through fusion or endocytosis, usually mediated by conformational changes to the capsid and by recruitment of cellular machinery.

Understanding mechanisms of virus-receptor interactions is critically important not only from a basic virology perspective but also because virus-receptor binding has clinical implications, as virus-receptor binding often restricts tissue tropism, affects pathogenesis, contributes to transmission mechanisms, and restricts species specificity (26–34).

**CVB3 Attachment to Cells**

CVB3 uses the coxsackie and adenovirus receptor (CAR), the decay-accelerating factor (DAF), and possibly heparan sulfate for attachment and entry into cells (16, 17, 35, 36). Attachment to these receptors are critical for downstream viral entry events.

CAR is a transmembrane protein often found in tight junctions and is expressed on many types of epithelial cells (37). CAR is found in a variety of tissues including the heart, brain, lungs, intestines, liver, and kidneys (38–42). It is primarily an adhesion protein in the Junction Adhesion Molecule (JAM) family in the immunoglobulin superfamily and plays major roles in cell-cell adhesion and embryonic development (38, 43, 44). CAR also has functions in the immune response such as leukocyte migration
across epithelial cell junctions (45). CAR is a receptor for group B coxsackieviruses as well as multiple adenoviruses (36). The binding site for CAR lies at the bottom of the “canyon” around the five-fold axis of symmetry (13). CAR binding is crucial to CVB3 entry as it leads to a conformation change in the capsid necessary for uncoating and release of the viral RNA (11–13).

DAF is a glycosylphosphatidylinositol (GPI)-anchored protein that primarily functions as a complement inhibitor by inhibiting the C3 convertase (46, 47) and is expressed on virtually all serum exposed cells (48). DAF is utilized as a receptor by a large number of enteroviruses such as coxsackievirus A21, B1, B3, B5, multiple echoviruses, and enterovirus 70 (35, 49–53). Attempts to map the DAF binding sites on these viruses have revealed differences in binding sites on both the virus and DAF itself suggesting that DAF binding may have evolved independently for multiple viruses (52). The binding of CVB3 to DAF without CAR interactions is not sufficient for infection (35). CVB3 bound to DAF does not trigger the necessary conformation change for further capsid uncoating events and structural studies have revealed that DAF-virus interactions occur on the virus surface, not in the canyon as with CAR (35, 54). There are multiple strains of CVB3, and certain strains, including clinical isolates, that bind DAF but others do not (35, 55, 56). Rhabdomyosarcoma (RD) cells express DAF but low transient levels of CAR (57). Passage of the non-DAF binding variant Nancy on RD cells results in the emergence of a DAF binding variant, CVB3-RD (58, 59). Characterization of the amino acid differences between DAF-binding and non-DAF-binding variants reveals two major residues that mediate this difference (56). Mutation of residue 234 in VP3 of Nancy from a glutamate to a glutamine (E to Q) conferred the
ability of Nancy to bind DAF (56). Another mutation, asparagine to aspartate (N to D) at residue 138 in VP2, was also shown to be required for DAF binding (56). Both of these residues, 138 in VP2 and 234 in VP3, have been shown to be in direct contact with bound DAF (54, 56).

In addition to binding DAF and CAR, CVB3 also appears to utilize the proteoglycan heparan sulfate as an entry receptor and/or attachment factor. Treatment of cells with a heparan sulfate degrading enzyme reduced CVB3 attachment to cells (16). Additionally, a variant of CVB3, CVB3-PD, can utilize the proteoglycan heparan sulfate as an entry receptor in the absence of CAR or DAF binding (17).

**CVB3 Entry into Cells**

CVB3 entry into a host cell after attachment is complex and occurs through multiple mechanisms. One of the most characterized mechanisms involves the entry of CVB3 into polarized intestinal epithelial cells. This process involves the binding of CVB3 to DAF expressed on the apical surface of cells (60). When CVB3 binds to DAF, DAF molecules cluster and activate the nonreceptor tyrosine kinases Fyn and Abl (60). Abl activation leads to RAC dependent cytoskeletal rearrangements that allow CVB3 to migrate into tight junctions where it can bind to CAR (60). The binding site for CAR on CVB3 lies at the bottom of the canyon (13). At the bottom of the canyon is the pocket factor (a fatty acid) (8, 9). When CAR binds into the canyon the pocket factor is expelled and the capsid undergoes a conformational change that leads to the release of VP4 from the virus and primes the virus for release of its RNA (10–12). The conformational change in the capsid after CAR binding generates what are known as altered particles or “A” particles (54). Shortly after CVB3 binds to CAR, CVB3 is endocytosed into the
cell via caveolin dependent endocytosis facilitated by Fyn dependent phosphorylation of caveolin-1 (60). Enteroviruses like CVB3 are thought to eject their RNA into the cytosol through a pore formed in the host membrane (61–63). This pore is generated from exposed hydrophobic residues on VP1 and from VP4 that was released during A particle formation (61–63). This process is summarized in Fig. 2.

**Figure 2. Entry of CVB3 into Polarized Intestinal Epithelial Cells.** A simplified illustration of the entry of CVB3 into polarized intestinal epithelial cells. CVB3 binds DAF which causes DAF to cluster and trigger a signaling cascade that activates the tyrosine kinases Fyn and Abl. Activation of Fyn and Abl lead to tight junction permeabilization allowing CVB3 to bind CAR as well as phosphorylation of caveolin-1. CVB3 is endocytosed via caveolin-1 mediated endocytosis (60).

CVB3 enters nonpolarized cells differently, as CVB3 does not require DAF when CAR is accessible and not sequestered in tight junctions (64). DAF-binding and non-DAF-binding have been suggested they enter non-polarized cells via the same mechanism (64). While DAF-binding was not necessary for entry into nonpolarized cells, the ability to bind DAF enhanced viral attachment to these cells (64).
Polyamines

Polyamines are small, polycationic molecules with flexible carbon chains that are found in all mammalian cells (65). They are involved in many cellular processes including protein synthesis, regulation of gene expression, regulation of ion channels, cell cycling, and many others (66–73). Knockout of key polyamine synthesis genes is embryonically lethal in mice (66, 74). Spermidine is a substrate for the hypusination of the eukaryotic initiation factor 5A (eIF5A), a translation factor, and this is necessary for its function (75–78). The amino acid hypusine is generated from the activity of two enzymes, deoxyhypusine synthase and deoxyhypusine hydroxylase and is only found in one protein, eIF5A (75–77). Polyamines also have functions in the structure of nucleic acids. They facilitate the transition of B form DNA to Z form DNA, can bend DNA structure, and are involved in stabilizing the structure of tRNA (79–84). Further, 80% of polyamines in the cell are found associated with RNA (68).

Figure 3. The Mammalian Polyamines. The biogenic polyamines putrescine, spermidine, and spermine are small polycationic molecules with flexible carbon chains.
There are three biogenic polyamines in mammals, putrescine, spermidine, and spermine. The structure of these polyamines is shown in Fig. 3. Polyamines are synthesized beginning with the decarboxylation of an ornithine molecule by ornithine decarboxylase (ODC) generating putrescine (85). Putrescine can then be converted into spermidine and subsequently converted to spermine by the addition of aminopropyl groups by the aminopropyltransferases, spermidine synthase and spermine synthase by utilizing decarboxylated S-adenosylmethionine (dcAdoMet) as an aminopropyl donor (86). S-adenosylmethionine decarboxylase (AdoMetDC) produces dcAdoMet from S-adenosylmethionine (AdoMet) and this process is important as it diverts cellular pools of AdoMet for exclusive use for polyamine biosynthesis and has implication for regulation of polyamine levels (66, 86). An overview of this process is depicted in Fig. 4.

Polyamine metabolism also involves interconversion pathways that can reverse the synthesis of the higher order polyamines (87–89). Spermine oxidase (SMO) can convert spermine back to spermidine (87). Spermine and spermidine can also be acetylated by spermidine/spermine-N<sup>1</sup>-acetyltransferase (SSAT) (89). The acetylated polyamines can be exported out of the cell or serve as substrates to acetylpolyamine oxidase (APAO) (89). APAO converts acetylated spermine to spermidine and acetylated spermidine to putrescine (66, 90–93). The action of SSAT is important for the regulation of polyamine levels in cells (89).
Figure 4. The Polyamine Synthesis Pathway. Diagram showing the major steps of polyamine metabolism. Synthesis begins with an ornithine molecule being converted to putrescine via ODC. Spermidine and spermine are synthesized by addition of aminopropyl groups. Higher order polyamines can be converted back to lower order polyamines via interconversion pathways. Adapted from Pegg 2016 with permission (94).

The synthesis of polyamines can be targeted with drugs such as α-difluoromethylornithine (DFMO) (95). DFMO specifically inhibits ODC, the rate-limiting enzyme at the beginning of the polyamine synthesis pathway, by irreversibly binding to its active site (95–97). DFMO depletes cellular pools of putrescine and spermidine but is not as effective at depleting spermine (98, 99). It has been suggested spermine levels are not effectively reduced because a small amount of putrescine is produced during
DFMO treatment that is converted to spermine. This is evidenced by an increase in the activity of AdoMet-DC during DFMO treatment and the observation that cells pulsed with radioactive ornithine accumulate radioactive spermine during DFMO treatment (98, 100). Despite the ubiquitous nature of polyamines and their many roles in cellular processes, DFMO is not typically cytotoxic (65, 101–103). However, DFMO has some cytostatic effects (65, 95, 99, 102, 104).

DFMO is FDA approved is on the WHO list of essential medicines (105). It is currently used to treat African trypanosomiasis (106–108) and hirsutism (109). DFMO is especially effective against trypanosomes because trypanosomes require spermidine for production of the critical antioxidant, trypanothione (110). DFMO is also be investigated for its use for treatment or prevention of certain cancers such as colon cancer and neuroblastoma (65, 95, 101, 111). DFMO can be taken orally, intravenously, or topically (95). Side effects are usually mild and reversible and include gastrointestinal upset, anemia, leukopenia, and thrombocytopenia, and reversible hearing loss (102, 112–114). High doses are often required as DFMO is rapidly eliminated from the blood with 80% being excreted in the urine (95, 115). The serum half-life is 1.5-5 hours (115). DFMO can be administered 400-800 mg/kg/day intravenously to treat African trypanosomiasis (95, 115, 116).

**Polyamines and Viruses**

Viruses utilize polyamines for their replication. Polyamine depleting drugs such as DFMO drastically inhibit the replication of diverse families of viruses (78, 117–119). In DNA viruses like herpes simplex viruses and poxviruses, polyamines are thought to function in neutralizing the negative charge associated with the large genome and thus
allow for more efficient packaging (120–122). In chikungunya and Zika virus polyamines function in transcription and translation of viral proteins (123). In Ebola virus, polyamines are critical to produce a viral polymerase component by functioning in the hypusination of eIF5A (124). In bunyaviruses (La Crosse and Rift Valley fever virus) as well as Zika virus, we have shown that the depletion of polyamines results in the accumulation of noninfectious particles that stimulate innate immune responses as well as interfere with viral replication (125). Polyamines have broad functions in viral infection, many of which are not fully elucidated.

CVB3 is sensitive to depletion of polyamines via DFMO. We have previously reported that passage of CVB3 in polyamine depleted conditions generated mutations in the 2A and 3C proteases (126). We reported that DFMO inhibits viral protease activity and this inhibition is rescued by the mutations that emerged from the passages (126). From the passages another mutation had emerged, Q234R, in the VP3 capsid protein (unpublished data). The function of this mutation in polyamine depleted cells is currently unknown.
Figure 5. Location of the VP3^{234R} Mutation. CVB3 capsid showing the VP3^{234R} mutation. The mutated residue is highlighted in yellow and circled in white. The VP3 protein is colored blue. The image on right is zoomed in on a five-fold axis of symmetry. The images were generated using Chimera software with the PDB accession 4GB3 for the CVB3 capsid structure.

Bunyaviruses

Bunyaviruses of the Bunyaviridae family are enveloped negative-sense, single stranded RNA viruses. Bunyaviruses include a variety of emerging and highly pathogenic viruses that cause significant mortality and remain a significant threat to public health. They include viruses such as Rift Valley fever virus (RVFV), La Crosse virus (LACV), Sin Nombre virus, and Crimean-Congo hemorrhagic fever virus (127).

RVFV is a mosquito-borne virus that infects humans and livestock in many countries throughout Africa. Infections in humans are typically mild and self-limiting but in 1-2% of cases RVFV can cause severe complications like blindness, encephalitis, fulminant hepatitis, hemorrhagic syndromes, and renal failure (128–132). Infections in cattle are often associated with abortions, gastrointestinal hemorrhage, and diffuse hepatic necrosis (128). Abortions are one of the most prominent features of RVFV
infections in livestock with abortion rates that can reach 80-100% (128). In 1977, an outbreak infected more than 200,000 people and caused more than $115 million in livestock losses (128, 133, 134). Vaccines can be utilized to prevent the spread of RVFV. The live-attenuated MP-12 vaccine has a conditional license for veterinary use from the USDA and is undergoing human clinical trials (135, 136). It was generated from 12 serial mutagenic passages that led to attenuating mutations (137). There are 23 mutations in the MP-12 genome with 9 amino acid substitutions (138, 139).

LACV is a mosquito-borne virus that is found mainly in the Midwestern and Appalachian regions of the United States (140). It infects around 300,000 people each year with most infections being mild or asymptomatic (141). In a small subset of those infected, encephalitis can occur causing seizures, disorientation, coma, and/or death (141, 142). Encephalitic infections can lead to severe neurological sequelae such as recurrent seizures, learning disabilities, and hemiparesis (141, 143).

**Flaviviruses**

Flaviviruses of the *Flaviviridae* family are enveloped positive-sense, single stranded RNA viruses. Flaviviruses are typically arthropod-borne viruses transmitted by either ticks or mosquitoes and include viruses such as Zika virus, yellow fever virus, tick-borne encephalitis virus, dengue virus, and Japanese encephalitis virus (144). Flaviviruses infect millions of people each year and can cause severe diseases such as hemorrhagic fevers and encephalitis (144). The WHO estimates that dengue virus infects 390 million people each year with up to 3.97 billion people at risk of infection (145, 146).
Zika virus is an emerging flavivirus transmitted mainly by mosquitos that has recently caused outbreaks in Yap, French Polynesia, Brazil, and the Americas (147). In Yap, Micronesia, it was estimated 73% of Yap residents over the age of three had been infected with Zika virus (148). In Salvador, Brazil, seroprevalence studies showed 63% of the population had been infected (149). It is difficult to accurately estimate the true burden of Zika in the population due to the lack of suitable diagnostic equipment in affected areas (150). Zika infections are usually asymptomatic or cause a mild and flu-like illness but in some cases can cause severe conditions such as meningitis, encephalitis, thrombocytopenia, Guillain-Barré syndrome and multi-organ failure (147, 151–154). Zika infection can also lead to lead congenital abnormalities by infecting developing fetuses. This can lead to conditions such as microcephaly, fetal demise, hearing loss, ocular abnormalities, epilepsy, and learning disabilities (147, 155–158).

Aims and Hypothesis

Polyamines are utilized by numerous viruses including CVB3. Many of the roles that polyamines play in viral replication remain to be elucidated. The goal of this project is to investigate where polyamines function in the replication of CVB3 and to use CVB3 as a model system for application to diverse viruses. The discovery of conserved mechanisms of replication across divergent viruses could have significant implications for the development of broad-spectrum antiviral therapies.

We have shown that CVB3 is sensitive to the depletion of polyamines via DFMO. To investigate potential pro-viral mechanisms of polyamines during CVB3 replication we performed successive passages of CVB3 in polyamine depleted cells with the objective of generating viruses that can replicate independently of polyamines. From the
passages arose a mutation in the capsid protein VP3 (Q234R). The site of this mutation, residue 234, is a residue that has been implicated in CVB3 receptor binding (56).

In my first aim I investigated if the VP3\textsuperscript{234R} capsid mutant confers resistance to polyamine depletion via DFMO treatment. The mutation could be an adaptation to cell culture and not affect replication in polyamine depleted cells. It is therefore imperative to investigate its potential resistance to polyamine depletion. I hypothesized that the mutant confers resistance as the mutation was derived from passage in polyamine depleted cells. Exploring resistant mutants is valuable because they can provide clues as to where polyamines are being utilized in CVB3 replication.

In my second aim I hypothesized that polyamines play a role in CVB3 attachment to cells. The VP3\textsuperscript{234R} mutation was derived from passage of CVB3 in polyamine depleted cells and is in a residue involved in receptor binding. I also investigated the ability of the VP3\textsuperscript{234R} mutant to rescue any attachment alterations observed with polyamine depletion.

In my third aim I explored the conservation of the role of polyamines in attachment across divergent viruses. Polyamines are utilized by diverse viruses and it is possible they share common mechanisms. Revealing this potential commonality between divergent viruses may illuminate novel targets for antiviral therapies.
CHAPTER TWO
MATERIALS AND METHODS

Cell Culture

Cells were maintained at 37°C in 5% CO₂, in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) with bovine serum and penicillin-streptomycin. Vero cells were obtained through BEI Resources, NIAID, NIH (NR-10385) and were supplemented with 10% new-born calf serum (NBCS; Thermo-Fischer). 293T cells were kindly provided by Dr. Ed Campbell and were supplemented with 10% fetal bovine serum (FBS; Thermo-Fischer).

Infection and Enumeration of Viral Titers

CVB3 (Nancy strain) (159) was derived from the first passage of virus in Vero cells after rescue from infectious clone. The VP₃²³₄R virus was generated via site-directed mutagenesis of the wildtype CVB3 plasmid using the primers listed in Table 1 with Phusion polymerase (Thermo-Fisher). Rift valley fever virus vaccine strain MP-12, provided by Dr. Shinji Makino at UTMB, was propagated in Huh7 cells (160). ZIKV (strain MR766, NR-50065) and LACV (NR-540) were obtained from Biodefense and Emerging Infections (BEI) Research Resources. LACV was propagated in Huh-7 cells. ZIKV was propagated in Vero cells. HRV2 was provided by Dr. William T. Jackson and was rescued from infectious clone and propagated in Vero cells. For all infections, DFMO was maintained throughout infection as designated. Viral stocks were
maintained at -80°C. For infection, virus was diluted in serum-free DMEM for a
multiplicity of infection (MOI) of 0.1 on Vero cells, unless otherwise indicated. Viral
inoculum was added to the cells and supernatants were collected at specified
timepoints. To quantify viral titers via plaque assay, dilutions of cell supernatant were
prepared in serum-free DMEM and used to inoculate confluent monolayers of Vero cells
for 10 to 15 min at 37°C. Cells were overlain with 0.8% agarose in DMEM containing 2%
NBCS. CVB3 and HRV2 samples were incubated for 2 days at 37°C. Cells were fixed
with 4% formalin and revealed with crystal violet solution (10% crystal violet; Sigma-
Aldrich). Plaques were enumerated and used to back-calculate the number of plaque
forming units (PFU) per milliliter of collected volume.

**Drug Treatments**

Difluoromethylornithine (DFMO; TargetMol) was diluted to 100 mM solution in sterile
PBS. For DFMO treatments, cells were trypsinized (Zymo Research) and reseeded with
fresh medium supplemented with 2% NBCS. Cells were treated with 500 µM DFMO
unless otherwise indicated. Cells were incubated with DFMO for 96 hours to allow for
depletion of polyamines in Vero cells. Exogenous polyamines were prepared in a 1:1:1
solution (putrescine:spermidine:spermine) and added at a concentration of 5 µM to
either the cell culture supernatant overnight or to the viral inoculum during the
experiment as indicated.

**Thin-layer Chromatography for Determination of Polyamines**

Polyamines were separated by thin-layer chromatography as previously described
(161). For all samples, cells were treated as described prior to being trypsinized and
centrifuged. Pellets were washed with PBS and then resuspended in 200 µL 2%
perchloric acid. Samples were then incubated overnight at 4 °C. 200 µL of supernatant was combined with 200 µL 5 mg/mL dansyl chloride (Sigma Aldrich) in acetone and 100 µL saturated sodium bicarbonate. Samples were incubated in the dark overnight at room temperature. Excess dansyl chloride was cleared by incubating the reaction with 100 µL 150 mg/mL proline (Sigma Aldrich). Dansylated polyamines were extracted with 50 µL toluene (Sigma Aldrich) and centrifuged. Five microliter of sample was added in small spots to the TLC plate (silica gel matrix; Sigma Aldrich) and exposed to ascending chromatography with 1:1 cyclohexane: ethyl acetate. The plate was dried and visualized via exposure to UV.

**RNA Purification and cDNA Synthesis**

Media was cleared from cells and Trizol reagent (Zymo Research) directly added. Lysate was then collected, and RNA was purified through phenol chloroform extraction. Purified RNA was subsequently used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kits (Thermo-Fischer), according to the manufacturer's protocol, with 10-100 ng of RNA and random hexamer primers.

**qPCR-based Attachment Assay**

Vero cells were seeded at 1 x 10^4 cells per well in 24 well plates in DMEM with 2% NBCS. The cells were treated for 96 hours with 500 µM DFMO. After 96 hours, the cells were placed on ice and the media was aspirated from the cells and replaced with 200 µL of serum free media containing virus. The infected cells incubated on ice for a specified amount of time. The cells were then washed 3x with PBS and then 250 µL of Trizol was added to the cells. The RNA was extracted with a phenol-chloroform extraction and genomes were quantified by quantitative real-time PCR with SYBR
Green (DotScientific) using a one-step protocol using QuantStudio 3 (ThermoFisher Scientific). Relative genomes calculated using the ΔCT method, normalized to β-actin qRT-PCR control and calculated as fraction of the unwashed samples. Primer sequences are included in Table 1. Primers were verified for linearity using eight-fold serial diluted cDNA and checked for specificity via melt curve analysis.

**Single Cycle Replication Attachment Assay**

Vero cells were seeded at 1 x 10^4 cells per well in 24 well plates in DMEM with 2% NBCS. The cells were treated for 96 hours with 500 µM DFMO. After 96 hours, the cells were placed on ice and the media was aspirated from the cells and replaced with 200 uL of serum free media containing virus. The infected cells incubated on ice for a specified amount of time. The cells were then washed 3x with PBS. 500 uL of fresh DMEM with 2% NBCS were added back to the cells. The infected cells incubated at 37°C for approximately one replication cycle (≈12-15 hours). The supernatant was collected, and virus was quantified via plaque assay as described.

**Plaque Formation Attachment Assay**

Vero cells were seeded in 6 well plates and grown to confluence in DMEM with 2% NBCS. The cells were treated for 96 hours with 500 µM DFMO. For polyamine rescue experiments, cells were treated overnight before the infection with a 10 µM mix of polyamines (putrescine, spermidine, spermine from Millipore Sigma) added directly to the media. After the 96 hour DFMO treatment, the cells were placed on ice and the media was aspirated from the cells and replaced with 1 mL serum free media containing either 1000, 5000, or 10000 PFU. The infected cells incubated on ice for a specified amount of time. After the specified time, the cells were washed 3x with PBS and then
overlaid with 0.8% agarose containing DMEM with 2% NBCS. The plates were incubated at 37°C for plaques to develop. CVB3 was incubated for 2 days; the VP3<sup>234R</sup> mutant, MP-12, and ZIKV for 3 days; and LACV for 4 days. The cells were fixed with 4% formalin, and the plaques were visualized with crystal violet staining.

**Statistical Analysis**

Prism 6 (GraphPad) was used to generate graphs and perform statistical analysis. For all analyses, two-tailed Student’s t test was used to compare groups, unless otherwise noted, with α = 0.05. For tests of sample proportions, p values were derived from calculated Z scores with two tails and α = 0.05.

**Table 1. List of Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVB3 qPCR</td>
<td>5’-AGG-GCG-AGA-TCA-ATC-ACA-TTA-G-3’</td>
<td>5’-CTC-TGC-TGT-TGC-CTC-AC-ATC-3’</td>
</tr>
<tr>
<td>ZIKV qPCR</td>
<td>5’-GAC-CTG-GGT-TGA-TGT-TGT-CT-3’</td>
<td>5’-GTC-GTC-GTG-ACC-AAC-TCT-ATG-3’</td>
</tr>
</tbody>
</table>
CHAPTER THREE

RESULTS

Verification of Polyamine Depletion

DFMO depletes cellular polyamines by inhibiting the rate-limiting enzyme at the beginning of the polyamine biosynthetic pathway (95–97). To verify that DFMO depletes cellular polyamine pools we performed thin layer chromatography (TLC) to visualize cellular polyamines. Vero cells were treated with increasing doses of DFMO. Polyamines were labelled with dansyl groups that were visualized with UV.

We find that putrescine and spermidine are effectively depleted with DFMO treatment while spermine may be slightly reduced (Fig. 6). These results agree with the literature that DFMO depletes putrescine and spermidine while not effectively depleting spermine (98, 99).

Figure 6. DFMO Treatment Depletes Polyamines. Thin layer chromatography visualizing putrescine (Put), spermidine (Spd), and spermine (Spm) performed on Vero cells treated with increasing doses of DFMO.
VP3$^{234R}$ Mutant Virus DFMO Resistance Characterization

We observed a mutation in the capsid protein VP3$^{234R}$ arose from passage of CVB3 on DFMO treated Vero cells. To test whether this mutation confers resistance to polyamine depletion or if this mutation is simply an adaptation to cell culture, we generated VP3$^{234R}$ mutant CVB3 using site-directed mutagenesis. We treated cells with increasing doses of DFMO, from 1 µM to 1 mM, and infected with WT or VP3$^{234R}$ mutant viruses at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell. At 48 hours post infection (hpi), we measured virus titers via plaque assay and observed significant resistance in the VP3$^{234R}$ mutant, while WT CVB3 exhibited sensitivity to DFMO (Fig. 7A). To ensure the resistance observed was not due to kinetic differences in the replication of the viruses, we measured viral titers in response to 500 µM DFMO treatment over a time course. Vero cells were infected at an MOI of 0.1 and viral titers were determined by plaque assay. The VP3$^{234R}$ mutant replicated to a higher titer than the WT virus in the DFMO treated cells (Fig. 7B). While the VP3$^{234R}$ mutant and the WT virus both reached similar maximum titers throughout the time course, the VP3$^{234R}$ mutant plateaued earlier.
Figure 7. VP3<sup>234R</sup> Mutation Enhances CVB3 Replication in Polyamine-depleted Cells. A) Vero cells were either untreated or treated with increasing doses of DFMO (1 µM, 10 µM, 100 µM, 500 µM, and 1 mM). The cells were infected with the either the WT or VP3234R mutant viruses. Supernatant was collected at 24 hours and titered via plaque assay. B) Vero cells were either untreated or treated with 500 µM DFMO. The cells were infected with the WT and VP3234R mutant viruses. Supernatant was collected as specified and titered via plaque assay. *p ≤ 0.05 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.

CVB3 Attachment to Polyamine Depleted Cells

Others have reported that residue 234 in VP3 is important for the binding of CVB3 to a receptor (56). Having observed that the Q234R mutation in VP3 confers resistance to polyamine depletion via DFMO we hypothesized that the attachment of CVB3 to susceptible cells may be altered with DFMO treatment.

To assess attachment of CVB3 to polyamine-depleted cells, we inoculated untreated and DFMO treated cells on ice with the same amount of CVB3. At different times the cells were washed with PBS and RNA was collected. Viral genomes were reverse-transcribed and quantified by qPCR. Compared to input, cell-associated virus genomes were reduced 10,000-fold, which gradually recovered over ten minutes (Fig. 8A). Interestingly, the polyamine-depleted cells exhibited reduced cell-bound viral
genomes compared to the untreated cells at 5 and 10 mins (Fig. 8A). To recapitulate the results shown via qPCR we measured attachment of infectious virus. We infected untreated and polyamine-depleted cells with CVB3 as before. The cells with the inoculum were washed with PBS at different timepoints, and we added back fresh media (containing polyamines) to the cells and allowed the bound virus to replicate for one replication cycle, ≈12-15 hours. The supernatant from the cells was collected and titered via plaque assay. Similar to the qPCR-based attachment assay, DFMO treated cells had a marked reduction in viral titers through the 10 min timepoint (Fig. 8B). As an additional confirmation of this phenotype, we inoculated untreated and polyamine-depleted cells as before. Ten minutes later, the cells were washed and overlaid with agarose. For two days, bound virus formed plaques in situ. When plaques were revealed by crystal violet staining, DFMO treated cells exhibited less bound virus compared to the untreated cells (Fig. 8C). Together these results indicate that CVB3 has reduced attachment in polyamine depleted conditions as measured by multiple assays.
**Figure 8. Polyamine Depletion Precludes CVB3 Binding to Susceptible Cells.** A) Vero cells untreated or treated with 500 µM DFMO were infected with CVB3 on ice. The cells were washed with PBS at different timepoints, RNA was extracted, and bound genomes quantified via qPCR, normalizing to cellular β-actin. Cells that were left unwashed are abbreviated UW. B) Vero cells untreated or treated with 500 µM DFMO were infected with CVB3 on ice. The cells were washed with PBS at different timepoints and fresh DMEM with 2% NBCS was replaced on the cells. The infected cells incubated at 37°C for approximately one replication cycle before supernatant was collected and titered via plaque assay. Cells that were left unwashed are abbreviated UW. C) Confluent Vero cells untreated or treated with 500 µM DFMO were infected with 5000 PFU CVB3 on ice. After 10 minutes, the cells were washed with PBS and overlaid with 0.8% agarose. Plaques were allowed to form. Shown are representative wells. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.

**Recapitulation of Attachment Phenotype in Alternative Cell Line**

In order to investigate if this reduced attachment phenotype is specific to the Vero cell line, we used 293T cells treated with DFMO and performed the qPCR-based attachment assay. CVB3 exhibited reduced attachment to 293Ts similarly to Vero cells (Fig. 9). This result indicates that the observed phenotype is not Vero cell specific.
Figure 9. Polyamine Depletion Precludes CVB3 Binding to 293T Cells. 293T cells were infected with CVB3 on ice. The cells were washed with PBS at different timepoints, RNA was extracted, and bound genomes quantified via qPCR, normalizing to cellular β-actin. Cells that were left unwashed are abbreviated UW. *p ≤ 0.05, **p ≤ 0.01 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.

The Ability of the VP3^{234R} Mutant to Recover Attachment Deficits

We observed that VP3^{234R} confers resistance to DFMO treatment, is in a residue involved in CVB3 receptor binding, and CVB3 exhibits diminished binding to DFMO treated cells. We next investigated whether this VP3^{234R} mutation could rescue the attachment deficiency. We treated Vero cells with DFMO and performed a qPCR attachment assay as in Fig. 8A and 9. As before, we observed that WT CVB3 exhibited reduced binding to polyamine depleted cells. However, we observed that the VP3^{234R} virus binds better than WT in nearly all the timepoints tested in the DFMO treated conditions (Fig. 10A). Interestingly, the VP3^{234R} mutant virus bound better to the untreated cells in the 0 min timepoint, though both viruses plateaued at similar genome levels. We next performed the plaque formation attachment assay to recapitulate these
findings. As before (Fig. 8C), WT CVB3 exhibited reduced plaque formation in DFMO-treated cells (Fig. 10B). However, the VP3\textsuperscript{234R} virus exhibited more plaques in both the untreated and the DFMO treated cells compared to the WT virus. These results suggest the VP3\textsuperscript{234R} mutant recovers attachment deficits in polyamine depleted treated cells.

**Figure 10. VP3\textsuperscript{234R} Mutation Recovers CVB3 Binding to Polyamine-depleted Cells.**
A) Vero cells untreated or treated with 500 µM DFMO were infected with the WT and VP3234R viruses on ice. The cells were washed with PBS at different timepoints, RNA was extracted, and bound genomes quantified via qPCR, normalizing to cellular β-actin. B) Confluent Vero cells untreated or treated with 500 µM DFMO were infected with 5000 PFU WT and VP3234R viruses on ice. The cells were washed with PBS at different timepoints and overlaid with 0.8% agarose. Plaques that formed were counted. *p ≤ 0.05, **p ≤ 0.001 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.

**Investigation into Diverse Viruses**

Polyamine function in the replication of diverse RNA viruses. To investigate the conservation of this phenotype with other viruses, we first considered another enterovirus, human rhinovirus 2 (HRV2). As with CVB3, we performed the single cycle replication attachment assay using Vero cells treated with DFMO. As observed, HRV2 bound and replicated in untreated cells, with increasing titers observed with increasing inoculation time. However, we observed a reduction in viral titers following attachment
of the DFMO treated cells to a similar degree compared to CVB3 (Fig. 11A). We next explored this phenotype in more diverse viruses. We considered Zika virus (ZIKV), a Flavivirus, using the qPCR-based attachment assay. As with the other viruses tested, we detect inhibited attachment in the DFMO treated cells (Fig. 11B). Interestingly, the attachment reduction with DFMO treatment lasted through 120 mins of incubation. To explore more viruses, we performed the plaque formation attachment assay with HRV2, ZIKV, and two Bunyaviruses, La Crosse virus (LACV) and the MP-12 strain of Rift Valley fever virus. We observed reduced plaque formation in all cases, suggesting that cellular binding by these divergent viruses relies in some way on polyamines (Fig. 11C).
**Figure 11. Polyamine Depletion Precludes Binding of Diverse Viruses.** A) Vero cells untreated or treated with 500 µM DFMO were infected with HRV2 on ice. The cells were washed with PBS at different timepoints and fresh DMEM with 2% NBCS was replaced on the cells. The infected cells incubated at 37°C for approximately one replication cycle before supernatant was collected and titered via plaque assay. Cells that were left unwashed are abbreviated UW. B) Vero cells untreated or treated with 500 µM DFMO were infected with ZIKV on ice. The cells were washed with PBS at different timepoints, RNA was extracted, and bound genomes quantified via qPCR, normalizing to cellular β-actin. Cells that were left unwashed are abbreviated UW. C) Confluent Vero cells untreated or treated with 500 µM DFMO were infected with 10000 PFU HRV2, 5000 PFU ZIKV, 1000 PFU MP-12, and 1000 PFU LACV on ice. The cells were washed with PBS after 5 mins and overlaid with 0.8% agarose. Plaques were allowed to form. Representative images are shown. *p ≤ 0.05, **p ≤ 0.01 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.
The Ability of Exogenous Polyamines to Rescue Attachment Deficits

To investigate if the addition of exogenous polyamines could rescue the attachment of virus to polyamine depleted cells, we repeated the plaque formation attachment assay with CVB3 and 5 µM polyamines (putrescine, spermine, and spermidine) added either to the cell culture supernatant overnight prior to infection or directly to the viral inoculum during the experiment. We observed a complete rescue of virus attachment between the untreated and the DFMO treated cells with the addition of polyamines overnight or to the viral inoculum (Fig. 12A and B).

Figure 12. Attachment Deficits can be Rescued with Exogenous Polyamines. A) Confluent Vero cells untreated or treated with 500 µM DFMO were infected with 15000 PFU CVB3 on ice. A 1:1:1 mix of polyamines was added either to the viral inoculum before infection or to the cell supernatant overnight before the infection at a concentration of 5 µM. Exogenous polyamines are abbreviated PA. Cells were washed with PBS at 5 mins and overlaid with 0.8% agarose. Plaques that formed were counted. B) Representative wells from the experiment that is quantified in panel A. ***p ≤ 0.001 using Student’s t test (n ≥ 3), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.
CHAPTER FOUR
SUMMARY AND DISCUSSION

Summary

Polyamines function in broad areas of virus replication and FDA-approved drugs can block diverse virus replication via depletion of polyamines. We investigated novel pro-viral roles of polyamines during virus replication by utilizing CVB3 as a model system. CVB3 is sensitive to polyamine depletion and is a public health threat due to its association with severe brain and heart pathologies. CVB3 was passaged in polyamine depleted cells in order to investigate mutations that may allow polyamine independent replication. We isolated a mutation in a capsid protein, VP3.

The mutation in VP3, Q234R, was cloned into CVB3 using site-directed mutagenesis. We first investigated the ability of the VP3^{234R} mutant virus to confer resistance to polyamine depletion due to the possibility that the mutation could solely be an adaptation to cell culture. We find the VP3^{234R} mutant CVB3 is resistant to multiple doses of DFMO as well as over a time course of infection.

The mutated residue in VP3 is involved in CVB3 receptor binding and thus we hypothesized that polyamines may be functioning in CVB3 attachment to cells. Through a variety of attachment assays we report that CVB3 has inhibited binding to polyamine depleted cells. We then show that the VP3^{234R} mutant virus has rescued attachment to polyamine depleted cells compared to the wildtype virus.
One of the goals of our study was to employ CVB3 as a model system to find broadly conserved functions of polyamines during virus replication. We first investigated a related virus, HRV2, for the possible conservation of this polyamine dependent attachment phenotype. Through multiple attachment assays we show HRV2 has inhibited binding to polyamine depleted cells. To further investigate the extent of this phenotype, we performed attachment assays with the flavivirus, Zika, and the bunyaviruses, MP-12 and La Crosse. Here we report that polyamines function in the attachment of diverse RNA viruses to cells.

**Discussion**

The binding of viruses to susceptible cells is a crucial first step in viral replication that has many implications involving tissue tropism, pathogenesis, transmission, and restriction of host range (26–34). Here we describe that diverse viruses exhibited binding deficiency to polyamine-depleted cells. Using CVB3 as our model system, we observed that polyamine depletion reduces CVB3 binding to susceptible cells and that mutation of the capsid protein VP3$^{234R}$ rescues this phenotype. Thus, we have uncovered a novel function of polyamines in the replication of RNA viruses.

**The VP3$^{234R}$ Mutant**

The mutation in CVB3 VP3234R that was found to confer DFMO resistance and rescue binding, Q234R, is in a residue that has been shown to be important for CVB3 to bind to one of its receptors, DAF (56). A potential hypothesis could be that polyamines facilitate VP3-DAF interaction; however, our data concerning other, diverse viruses suggest that this may not be the case. There is currently no evidence that the other viruses we investigated bind to DAF. Nonetheless, it is possible that the VP3234R
mutant rescues CVB3 binding by increasing the attachment to DAF, independent of the mechanism that is reducing attachment with DFMO treatment. VP3234R mutant CVB3 binds to untreated cells better than the WT virus. This hypothetical increase in the attachment between CVB3 and DAF would increase CVB3 binding in an environment where binding is rendered difficult due to polyamine depletion.

**Potential Mechanisms**

Several mechanisms could be functioning to facilitate virus-cell interactions. First, polyamines may enhance virus binding to cells due to their polycationic nature that mediates electrostatic interactions between the viral particles and the cell surface. Historically, polycationic molecules like DEAE dextran and polybrene enhance binding of certain viruses and virus like particles to cells (162–165). It is possible polyamines could be playing a similar role. Interestingly, the mutation in VP3, Q234R, generates a positive charge at this location. Polyamines are polycationic molecules and possibly the loss of the positive charges from polyamines is at least partially restored from the added positive charge in VP3, which may substitute for polyamines.

We have preliminary evidence to support this electrostatic attraction attachment hypothesis. Addition of exogenous polyamines directly to the virus inoculum during the plaque formation assay appears to fully rescue CVB3 attachment deficits in the DFMO treated cells (Fig. S1). The ability of exogenous polyamines to rescue viral attachment in such a short timeframe suggests electrostatic mechanisms may be at play.

An additional factor that may be involved is heparan sulfates. Heparan sulfates are a very promiscuous attachment factor that are utilized by many viruses. CVB3 (16, 17), Zika (18), multiple *Enteroviruses* including some rhinoviruses (19–24), Rift Valley
Fever virus (25), and many other viruses bind heparan sulfate (15). Heparan sulfates are known to be involved in polyamine uptake (166) and cells treated with DFMO have increased heparan sulfates with increased affinity for spermine, a biogenic polyamine (166). Heparan sulfates are known to be extensively modified during their production (167) and the changes that occur in heparan sulfates that increase affinity for spermine upon DFMO treatment may coincidentally preclude virus binding to cells. We currently have not elucidated the precise mechanism of the effect of polyamines on viral attachment, but this is the target of future investigation. An experiment I intend to perform to investigate this potential mechanism involves enzymes called heparinases. Heparinases can degrade heparan sulfates by breaking glycosidic linkages (168). I plan to treat both untreated and DFMO treated cells with heparinases and investigate any changes in the attachment phenotype I typically observe with DFMO treatment. If heparan sulfates are involved in the polyamine dependent attachment phenotype, I expect heparinases to be more effective at reducing attachment to untreated cells than DFMO treated cells. I would expect this because the viruses would theoretically have impaired binding to DFMO modified heparan sulfate but not to normal heparan sulfate in untreated cells.

**Importance**

The FDA approved drug DFMO has been shown to exhibit powerful antiviral activity against diverse RNA viruses (117). DFMO inhibits CVB3 replication both *in vitro* and *in vivo* (117). Our data demonstrate that DFMO inhibits the attachment of not only CVB3 but also diverse viruses, and this underscores possible clinical implications of using DFMO as an antiviral drug. Virus-receptor binding is known to contribute to the
pathogenesis, transmission, and the tissue tropism of viruses (26–34); thus if DFMO is ever used clinically as an antiviral, it may not only reduce viral titers but may also affect the course of disease in patients if the viral attachment alterations have downstream effects on the spread of virus between tissues. We also report the emergence of DFMO resistant mutant upon passage in DFMO that can rescue viral binding deficits. This finding may have implications for DFMO’s clinical use and the importance of combination therapies to preclude resistance. Understanding the precise mechanisms by which this mutant confers resistance to polyamine depletion is crucial for the development and implementation of rational antiviral therapeutics.

The role in viral attachment is a novel function of polyamines. Polyamine involvement in the basic virology of virus-receptor binding, further informs the development of antivirals that target host polyamines. Because this phenotype appears to be conserved with several other virus families, targeting host cell entry by reducing cellular polyamines may limit virus infection. The precise mechanisms by which polyamines broadly function to reduce virus-cell attachment remain to be completely understood; however, the implications could be broadly applicable to enteroviruses and other diverse viruses.
Figure 13. Polyamines are Involved in the Attachment of Viruses. A model illustrating the involvement of polyamines in viral attachment. Depletion of polyamines via DFMO abrogates viral attachment of CVB3 and other diverse RNA viruses. The binding deficits in CVB3 can be rescued by the emergence of a DFMO resistant capsid mutant.
REFERENCE LIST


105. WHO | WHO Model Lists of Essential Medicines. WHO.


Thomas Kicmal was born in Park Ridge, Illinois, on May 17, 1994 to Kevin and Margaret Kicmal. He earned his Bachelor of Arts degree with a major in Biology with a minor in Business Administration from Carthage college in Kenosha, Wisconsin in May of 2016. In the summer of 2017, Thomas matriculated to Loyola University Chicago in the Infectious Disease and Immunology Master of Science program under the mentorship of Dr. Bryan Mounce.

Thomas’s work involved exploring drug resistant virus mutants and investigating the role of a class of small molecules, polyamines, in the attachment of viruses to cells. After completion of his Master of Science degree, Thomas will begin PhD training in the Integrated Program in Biomedical Science at Loyola University Chicago.