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LOYOLA UNIVERSITY CHICAGO

CHARACTERIZING A NOVEL COCKSACKIEVIRUS B3 PROTEASE MUTANT AND ITS RESPONSE TO POLYAMINE DEPLETION

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

BRIDGET M HULSEBOSCH

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ABSTRACT

Enteroviruses, including Coxsackievirus B3 (CVB3), are pervasive pathogens that cause significant disease, including cardiomyopathies. Unfortunately, no treatments or vaccines are available for infected individuals. We identified the host polyamine pathway as a potential drug target, as inhibiting polyamine biosynthesis significantly reduces enterovirus replication in vitro and in vivo. Here, we show that CVB3 is sensitive to polyamine depletion through the polyamine analog diethylnorspermidine (DENSpm) which enhances polyamine catabolism through induction of polyamine acetylation. We demonstrate that CVB3 acquires resistance to DENSpm via mutation of the 2A protease, which enhances proteolytic activity in the presence of DENSpm. Resistance to DENSpm occurred via mutation of a non-catalytic site mutation and results in decreased fitness. These data demonstrate the potential for targeting polyamine catabolism as an antiviral therapy as well as highlight a potential mechanism of resistance.
CHAPTER ONE

BACKGROUND

Review of Literature

Coxsackievirus B3

Enterovirus infections are one of the most abundant viral infections in the United States and cause a wide range of diseases, ranging from mild to severe (5–7). These viruses include poliovirus, enterovirus, human rhino viruses, echo viruses and coxsackieviruses. All of these viruses are within the Picornaviridae family and are non-enveloped. Enteroviruses are transmitted through many routes, including the fecal-oral route, respiratory route, and even through some bodily fluids (8, 9). Coxsackieviruses are positive-sense RNA enteroviruses, and cause a wide range of diseases and symptoms ranging from mild to severe (10). Coxsackievirus B3 (CVB3) specifically is spread through the fecal-oral route, i.e. when feces contaminated with the virus are inadvertently consumed (8, 9). Incidences of non-polio enteroviruses, including CVB3, have increased in recent years and can cause up to 10 to 15 million infections per year in the United States (5).

Clinical Outcomes of CVB3 Infection

CVB3 infection has a wide range of symptoms from mild to severe. CVB3 was first identified as a cause of a non-paralysis poliomyelitis syndrome, specifically causing aseptic meningitis (6, 11). Aseptic meningitis occurs when CVB3 infects the central nervous system (CNS) and causes major inflammation in the meninges, brain parenchyma, and cranial nerves.
CVB3 primarily will infect cardiac tissue and can persist in cardiac tissue causing viral myocarditis (5, 8, 10, 11, 13), which is inflammation of the heart muscle (11, 13). This inflammation can cause both acute and chronic heart failure (13, 14). Viral myocarditis from CVB3 infection can eventually progress to dilated cardiomyopathy, which is a leading reason for heart transplantation across the globe (13). CVB3 infection can also lead to the degradation of brain, pancreas, and muscle tissue (11).

Although CVB3 infection can lead to severe disease, most symptoms are mild. Symptoms can include mild fever and rash (7). There is also a chance of gastrointestinal distress from infection as well (7). There is no vaccine or antivirals currently available for this virus so most severe and mild symptoms are treated non-specifically (13). This emphasizes the importance of identifying novel antiviral targets to control infection and prevent severe disease and death.

**CVB3 Replication Cycle**

The replication cycle of CVB3 is outlined in Figure 1 (1) and begins when the virus interacts with the decay-accelerating factor (DAF) protein on the surface of the host cell. This interaction facilitates the attachment of the virus to the coxsackievirus and adenovirus receptor (CAR) and then internalized through clathrin-mediated endocytosis (15, 16). This endocytosis of CVB3 requires dynamin (15). The virus undergoes clathrin uncoating for fusion with early endosomes and the RNA genome is released (15).

The virus’s positive-sense RNA is then translated into a large polyprotein, and since the viral genome does not have a 5’ 7-methyl guanosine cap, translation is mediated through ribosomes binding to the genome’s internal ribosome entry site (IRES) at the 5’ end (16). The
polyprotein is then processed and cleaved to yield both the viral structural proteins and the nonstructural proteins (16).

Figure 1. CVB3 Replication Cycle
Replication of CVB3 begins when the virus is taken up by the cell. The viral genome is then translated into a large polyprotein by host proteins and is processed and cleaved into individual viral proteins. A negative sense RNA genome template is transcribed to create copies of positive sense RNA which is then packaged with the viral proteins. The viral progeny are then released from the cell through lytic release. Image adapted from (1).

Replication of CVB3’s viral genome is catalyzed by its RNA polymerase, 3D\textsuperscript{Pol}. A negative sense RNA strand is first synthesized by 3D\textsuperscript{Pol}, and then this negative sense strand acts as a template for multiple copies of a positive sense RNA strand (16, 17). These viral genome copies are then packaged with structural proteins creating infectious progeny (16). These virions are then released from the cell through lytic release, catalyzed by the viral protein 2B (16).
CVB3 Proteases and Proteolytic Processing

Viral proteases have diverse roles during infection, including enterovirus proteases. CVB3’s viral genome, shown in Figure 2 (2), encodes two distinct proteases, 2A and 3C. These two proteases catalyze the majority of cleavage of the viral polyprotein once the viral genome is translated during infection (18–20).

Upon entry into the cell, CVB3’s RNA genome is translated by the host cell’s machinery, synthesizing the polyprotein (18–20). 2A performs the primary cleavage of the polyprotein and cleaves out 3 individual polyproteins, P1, P2, and P3 (16). 3C then catalyzes subsequent polyprotein cleavage (16, 18, 19). This 2A protease also has many cellular targets, most notably eIF4G, an important cap-dependent translation factor, and its cleavage shuts off host protein translation, thereby enhancing IRES-mediated CVB3 translation (18–24).

The viral protease 3C will catalyze the cleavage of all the individual viral proteins from the three smaller polyproteins. The 3C protease also has important host cleavage targets, including transcription factors, and Bax and Bid proteins (16, 24–26). These cleavage events

Figure 2. CVB3 RNA Genome
The CVB3 viral genome consists of a large open reading frame that encodes for its 11 viral proteins. The first 4 viral proteins are structural proteins, and seven next proteins are all non-structural proteins. The open reading frame is flanked by two highly structured non-coding regions, including its IRES. Image adapted from (2).
will aid in the inhibition of host protein synthesis and activate the apoptotic pathway (16, 24–26).

Due to the importance and necessity of both the 2A and 3C proteases for CVB3 infection, protease inhibitors are an effective antiviral strategy against CVB3 infection (27–29).

**Cellular Functions of Polyamines**

Polyamines are small, flexible, positively charged carbon chains crucial for mammalian cell function (3, 30, 31). All three biogenic polyamines, putrescine, spermidine, and spermine, are found in all mammalian cells, although at differing concentrations (30).

Polyamines are known to be crucial for many different cellular functions. Polyamines are involved in protein synthesis, membrane interactions, protein-RNA interactions, and even gene expression. Polyamines are able to bind to both DNA and RNA due to their positive charge altering the shape and conformation of the molecules (3, 30). It has also been shown that polyamines are able to stimulate protein activity, especially DNA polymerases (32, 33).

Their biosynthesis pathway is outlined in Figure 3 (3) and begins with the polyamine precursor molecule, ornithine, which is synthesized from arginine. Ornithine is then converted to the first polyamine, putrescine (PUT), via the rate-limiting enzyme ornithine decarboxylase 1 (ODC1) (3, 30). Putrescine is then further converted to spermidine (SPD) via spermidine synthase (SRM). Spermidine is then converted to the final polyamine spermine (SPM) via spermine synthase (SMS). Both SPD and SPM can be catabolized back to PUT or marked for degradation and export via the enzyme spermidine/spermine acetyltransferase 1 (SAT1) (3, 30). These two enzymes, ODC1 and SAT1, are critical in the synthesis and degradation of polyamines and can be critical drug targets for many ailments (34–36).
Function of Polyamines During Viral Infection

Polyamines are essential for some viruses and their successful replication. One such function of polyamines in infection is through viral packaging. Many viruses, both RNA and DNA viruses have been shown to be utilized for viral packaging, specifically to neutralize RNA/DNA genomes (37). They have also been shown to be incorporated into the virions by some DNA and RNA viruses (31, 38–40). Similar to their function in host cells, polyamines are necessary to stimulate viral proteins in both RNA viruses and DNA viruses, like viral polymerases or kinases (41–44). Host polyamines have also been shown to be modulated during infection with DNA viruses and they have also been shown to be critical for replication of many
RNA viruses as well (30, 38, 39, 45, 46). The polyamine biosynthesis pathway has been effectively targeted for parasite and cancer treatments (47, 48), and because of their necessity for viral infections, the inhibition of host polyamines have shown to be a potential target for novel antiviral therapies (4, 38, 45, 46).

**Drug Inhibitors of Polyamines**

Recent studies showed that targeting polyamine metabolism with compounds like difluoromethylornithine (DFMO), a suicide inhibitor of the ODC1 enzyme, effectively inhibits polyamine synthesis (36, 49). This can be an effective treatment for many ailments, specifically viral infections. Because these drugs degrade cells of polyamines, which are necessary for viral infection, it has been shown to be an effective antiviral therapy in vitro and in vivo for multiple RNA viruses (4, 38, 45). Figure 4 (3) outlines the biosynthesis of polyamines and the different compounds that are effective at inhibiting polyamine synthesis. Both DFMO and another compound, diethylnorspermidine (DENSpm), have been shown to be effective at inhibiting RNA viral replication (4, 45, 46). DENSpm is a polyamine analog that activates SAT1, promoting the catabolism of spermidine and spermine back to putrescine or leading to their degradation in peroxisomes (48, 50). Although it targets another enzyme in the polyamine synthesis pathway, it still degrades cells of polyamines and has also been shown to be a potential antiviral against RNA viruses, including CVB3 (4, 46). Another compound that has shown to be effective at inhibiting viral replication is ribavirin which will deplete cells of polyamines as well through nucleotide pool depletion and SAT1 induction (35).

Developing novel antiviral therapies, like through polyamine depletion, is critical for fighting viral infections. RNA viruses are able to mutate their viral genome with ease; however,
this can be detrimental when treating an infection with an antiviral. Viruses and other pathogens can quickly adapt to therapies and be able to replicate in the presence of these antivirals (51–53). It is crucial to understand this potential for resistance to treatment when developing antivirals and be prepared for them. This knowledge can aid in future treatment of viral infections and also gives a greater understand into the biology of a virus and its life cycle.

**Figure 4. Inhibitory Compounds of Polyamine Synthesis**

There are multiple compounds that can inhibit the polyamine synthesis pathway. Two primary compounds are DFMO, a suicide inhibitor of ODC1, the rate-limiting enzyme, and DENSpm, a polyamine analog that activates the enzyme SAT1 which will then degrade cells of polyamines. Image adapted from (3).

**Aims and Hypothesis**

Polyamines facilitate CVB3 infection and depleting cells of polyamines with DFMO inhibits CVB3 replication (4); however, recent studies identified escape mutants after multiple rounds of replication in the presence of DFMO that allowed the virus to overcome this depletion through enhanced 2A and 3C protease activity (4, 38). In order to determine if CVB3 is able to overcome this depletion from another polyamine-depleting compound besides DFMO, wildtype
CVB3 was passaged repeatedly in cells pre-treated with DENSpm. A potential escape mutant was discovered in the 2A viral protease, S35G, and did not show a sensitivity to DENSpm treatment. Therefore, the hypothesis of this work is that enterovirus proteases are sensitive to polyamine depletion and the novel S35G mutant enterovirus overcomes this sensitivity by enhanced proteolytic activity. The goal of this project is to establish the potential fitness and stability of enterovirus mutations that do not rely on cellular polyamines for replication and give future direction in the development of antivirals through the following aims.

In the first aim, we measured the effect of polyamine depletion on both the wildtype CVB3 and a novel 2A$^{S35G}$ mutant that was rescued after multiple replication cycles in cells depleted of polyamines from DENSpm treatment. The goal was to establish that DENSpm effectively inhibits CVB3 wildtype replication, but the 2A$^{S35G}$ mutant is resistant to polyamine depletion through rescued 2A protease activity.

In the second aim, we characterize replication and fitness of the novel viral protease mutant S35G. The goal was to establish the stability of the mutant over multiple replication cycles and determine the fitness of the mutant virus compared to wildtype CVB3.

In the third aim, new S35 mutants were cloned into the CVB3 2A protease and their resistance to DENSpm was measured and compared to the S35G mutant. The goal of this aim was to establish if different mutations in the 2A protease would establish resistance to polyamine depletion as well.

This work will highlight the need for specific antivirals against CVB3 infection, but also the importance of proper treatment against these infections by physicians and other healthcare
professionals in order to prevent antiviral resistance. This work also gives further insight into the replication of CVB3 and its interactions with different host molecules to enhance infection.
CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Cells were maintained at 37 °C in 5% CO2, in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Waltham, MA) with bovine serum and penicillin–streptomycin. Vero (BEI Resources) and HeLa cells were supplemented with 10% new-born calf serum (NBCS; Thermo-Fischer, Waltham, MA). 293T and Huh7 cells, kindly provided by Susan Uprichard, were supplemented with 10% fetal bovine serum (FBS; Thermo-Fischer).

Generation of 2A\textsuperscript{S35} Mutants

CVB3 (Nancy strain) (54) was derived from the first passage of the virus in Vero cells after rescue from the infectious clone. Briefly, the CVB3 infectious clone (55) was linearized with SapI (New England Biolabs (NEB)) and used to generate RNA in vitro. This RNA was transfected into Vero cells to recover virus. The 2A\textsuperscript{S35} mutant viruses were generated via site-directed mutagenesis of the wildtype plasmid using primers 5’-C TGG CAA AAC TGT GTG TGG GAA GGT TAC AAC AGA GAC CTC-3’ (forward) and 5’-GAG GTC TCT GTT GTA TGG GAA GGT TAC AAC AGA GAC CTC-3’ (reverse).

Infection and Enumeration of Viral Titers

CVB3 was derived from the first passage of virus in Vero cells, which were obtained through BEI Resources, National Institutes of Allergy and Infectious Diseases, National Institutes of
Health (NR-10385). For all infections, DENSpm and DFMO were maintained throughout infection as designated. Viral stocks were maintained at \(-80^\circ\text{C}\). For infection, virus was diluted in serum-free DMEM for a multiplicity of infection (MOI) of 0.1 on Vero cells, unless otherwise indicated. The viral inoculum was overlain on cells for 10 to 30 min, and the cells were washed with PBS before replenishment of media. Supernatants were collected from CVB3 24 hpi and 48 hpi, or as indicated. Dilutions of 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 overlaid with 0.8% agarose in DMEM containing 2% NBCS. Samples were incubated for 2 days at 37°C. Following incubation, cells were fixed with 4% formalin and revealed with crystal violet solution (10% crystal violet; Sigma-Aldrich, St. Louis, MO). Plaques were enumerated and used to back-calculate the number of plaque-forming units (pfu) per milliliter of collected volume.

**CVB3 Serial Passage**

Vero cells were treated with DENSpm 16 h before infection with CVB3 at MOI of 0.1. After 24h, 1/10th of cell culture volume was used to inoculate the next passage. This process was continued for ten passages, at which time viral RNA was purified from the cellular supernatant, reverse transcribed, amplified using CVB3 specific primers (4), and Sanger sequenced. Sequences were aligned to CVB3 parental genome, and mutants were confirmed by manual chromatogram inspection.

**Drug Treatments**

N1,N11-Diethylnorspermine (DENSpm; Santa Cruz Biotechnology, Santa Cruz, CA) and Difluoromethylornithine (DFMO; TargetMol, Boston, MA) were diluted to 100x solution (10 mM and 100 mM, respectively) in sterile water. For DENSpm treatment, cells were trypsinized
and reseeded with fresh medium supplemented with 2% serum. Following overnight attachment, cells were treated with DENSpm as indicated for 16 h to allow for complete depletion of polyamines. For DFMO treatment, cells were treated 96 h prior to infection. During infection, media were cleared and saved from cells. The same media containing DENSpm and DFMO were then used to replenish the cells following infection. Cells were incubated at the appropriate temperature for the duration of infection.

**RNA Purification and cDNA Synthesis**

Media were cleared from cells, and Trizol reagent (Zymo Research, Irvine, CA, USA) was directly added to cells or supernatant. The lysate was then collected, and RNA was purified according to the manufacturer’s protocol utilizing the Direct-zol RNA Miniprep Plus Kit (Zymo Research, Irvine, CA, USA). 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.

**DENSpm and DFMO Sensitivity Assays**

Vero cells were treated with either 500 nM to 100 μM DENSpm for 16 h or 100 μM to 1mM DFMO for 4 days prior to infection with CVB3 at an MOI of 0.1. At 24 hpi, supernatant was collected and titers were determined. Titers measured after drug treatment were divided by titers without treatment to obtain percent replication compared to control untreated conditions.

**Stability and Competition Assays**

To measure the stability of the mutations, Vero cells were infected at an MOI of 0.1 with the viral mutant for 24 h. The virus was then passed to new cells by transferring 50 μL supernatant. After five passages, RNA was extracted and purified from supernatants and reverse
transcribed. Sanger sequencing was used to determine whether mutations were stable over the passages by looking at the chromatograms and determining the presence or absence of the mutant nucleotide as previously described (4). Competition assays were similarly performed, but Vero cells were infected at an MOI of 0.1 with an equal combination of wildtype and mutant CVB3 and passaged five times. Fitness was determined via Sanger sequencing and analysis of the chromatogram to determine if the wildtype or mutant nucleotide was present in the sample.

**Protease Plasmid Cloning**

Primers were designed to target the wildtype 2A protease as previously described (4). CVB3 plasmids containing the 2A protease were used to clone the mutant protease. To target the 2A and 2A mutant protease, the primers included NotI and XbaI recognition sites. Protease sequences were amplified via PCR and cloned into the pFLAG-CMV vector. Clones were verified for sequencing (GenScript, Piscataway, NJ). Oligonucleotide sequences corresponding to the amino acid sequence for the wildtype and mutant 2A and 3C protease targets were designed and cloned into the pGlo-3F vector and verified by sequencing (GenScript) as previously described (4).

**Transfections**

293T or Vero cells were plated at 80%–90% confluency and either treated with 10 μM, 50 μM, or 100 μM DENSpm for 16 h or left untreated. The plasmids were then transfected in the combinations described in the figures, according to the manufacturer’s protocol, using LipoD293 (SignaGen Laboratories). The transfection was incubated at 37 °C for 24 h.
Luciferase Protease Assay

Veros were treated with 10μM, 50μM, or 100μM for 16 h or left untreated. They were then transfected using LipoD293 (SignaGen Laboratories, Rockville, MD) with the 2A substrate alone, 2A substrate plus the 2A WT protease or 2A mutant protease, and a TK Renilla transfection control plasmid. For luciferase assays, cells were combined with firefly substrate followed by subsequent Renilla (Stop and Glo; Promega, Madison, WI) luciferase substrate 24 h post-transfection. Luciferase assays were performed according to the manufacturer’s recommendations (Promega), and results were measured via the Veritas Microplate Luminometer (Turner BioSystems, Promega). Protease activity was determined by diving firefly luciferase activity by the Renilla luciferase activity control and normalizing to untreated samples.

Western Blots

Samples were collected with Bolt LDS Buffer and Bolt Reducing Agent (Invitrogen, Waltham, MA, USA) and run on polyacrylamide gels. Gels were transferred using the iBlot 2 Gel Transfer Device (Invitrogen). Membranes were probed with primary anti-bodies for eIF4G, (1:1000, Santa Cruz Biotechnology), actin (1:2000, ProteinTech, Rosemont, IL), SAT1 (1:100, Santa Cruz Biotechnology), and GAPDH (1:1000, Santa Cruz Biotechnology). Membranes were treated with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and visualized on FluorChem E imager (Protein Simple, San Jose, CA).

Plaque Size Measurement

Vero cells were seeded in 10 cm dishes and grown to confluence. Approximately 30 plaque-forming units (PFU) of each mutant was diluted in a 2.5 mL inoculum of serum-free DMEM. The media on the Vero cells were aspirated and replaced with the 2.5 mL inoculum
containing the virus. The inoculum was incubated on the cells for approximately 30 min at 37°C. After 30 min, an overlay of 8 mL 0.8% agarose was added to each dish. The dishes were incubated at 37 °C for 2 days to allow plaque formation. The cells were fixed with 4% formalin and the agarose plugs removed. The fixed cells were stained with crystal violet. Plaque size was determined using ImageJ software (Version 1.51k) (56).

**Thin Layer Chromatography Determination of Polyamines**

Polyamines were separated by thin-layer chromatography as previously described (57). 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. Supernatant (200 μL) 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 microliters 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.

**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 a \( p = 0.05 \). For tests of sample proportions, p values were derived from calculated Z scores with two
tails and \( a = 0.05 \). Half-maximal inhibitory concentration (IC50) values were calculated using Prism 6 using the built-in analysis tool.
CHAPTER 3

RESULTS

DENSpm Induces Polyamine Depletion

DENSpm induces polyamine depletion via the upregulation of SAT1 and concomitant acetylation, interconversion, and degradation of the polyamines spermidine and spermine. To confirm that DENSpm induces these changes, we treated Vero-E6 cells with increasing doses of DENSpm and measured translation of SAT1 by Western blot. We observed increasing signal for SAT1 with increasing doses, from 10 μM to 100 μM (Figure 1S). Additionally, we measured cellular polyamines by thin layer chromatography. As expected, we found that DENSpm treatment correlated with depletion of spermidine and spermine and an increase in putrescine (Figure 2S).

DENSpm Limits CVB3 Infection

To determine if this polyamine depletion affected CVB3 infection, cells treated with 100 μM DENSpm were infected with wildtype CVB3 at a multiplicity of infection (MOI) of 0.01 and viral titers were measured over 72 h. We observed that viral titers were significantly reduced throughout infection, though viral titers nearly reached untreated levels by 72 h (Figure 5A). Finally, cells treated with increasing concentrations of DENSpm were infected with CVB3 at MOI 0.01, measuring viral titers at 24 h. We observed that viral titers decreased (Figure 5B), suggesting that DENSpm restricts virus replication both in a dose-dependent manner and over several rounds of replication.
After establishing that CVB3 is sensitive to depletion of cellular polyamines by DENSpm, we wished to determine the evolution of CVB3 after multiple replication cycles in cells depleted of cellular polyamines after DENSpm treatment. Previous work showed that CVB3 gains resistance to cellular polyamine depletion from DFMO treatment through both a 2A protease mutation and a 3C protease mutation (4). To determine if the same or different mutations emerged with passage in DENSpm treatment, CVB3 was passaged ten times in Vero-E6 cells treated with 100 μM DENSpm 16 hours pre-infection or left untreated. The cells were then infected with CVB3 at an MOI of 0.1. After 24 hours post infection (hpi), 1/10 of the supernatant was passaged and used to inoculate the next set of cells. Viral titers were determined per passage.
and multiple passages (Figure 6A). After 10 passages, the virus that was passaged in cells treated with DENSpm exhibited higher titers compared to that of virus passaged in untreated cells. This difference in titers suggested that this virus from DENSpm-treated cells gained resistance to DENSpm.

![Graph showing viral titers](A)

**Figure 6. CVB3 Gains Resistance to DENSpm after Passaging**

(A) Vero-E6 cells were left untreated or treated with 100 μM DENSpm for 16 hours prior to infection with CVB3 at an MOI 0.1. Virus was collected at 24 hpi and used to inoculate the next passage. Viral titers were determined via plaque assay for the passages shown.

(B) CVB3 passaged 10 times over Vero-E6 cells, either treated with 100 μM or untreated, were used to infect Vero cells treated with increasing doses of DENSpm for 24 hpi. Viral titers were determined by plaque assay.

(C) Partial 2A protease sequence of CVB3 compared to other enteroviruses. Red arrows indicate amino acid residues of the protease active site, and the black arrow indicates mutated amino acid residue of 2AS35 mutants.

* 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.
Supernatant from the 10th passage of virus passaged in DENSpm-treated cells and untreated cells was then used to measure resistance to DENSpm, infecting cells with increasing doses of the drug. We found that CVB3 that was passaged in cells treated with DENSpm did not appear to be sensitive to DENSpm treatment at 50 μM and 100 μM compared to virus that was repeatedly passaged in untreated cells, which had a significant decrease in titers (Figure 6B).

Viral RNA of the 10th passage was extracted, purified, reverse transcribed, and Sanger sequenced. When aligning with the parental genome, an S35G mutation was found in the 2A protease (black arrow) (Figure 6C). It is important to note that this S35G mutation does not occur in the active site (red arrows) of the 2A protease, and no mutations were found in virus that was passaged in untreated cells. While the serine at position 35 of the 2A protease is conserved between CVB3 and the distantly related human rhinovirus 1A (HRV1A), other enteroviruses exhibit distinct amino acids at this site, and it is not strictly conserved. We also repeated these passages under similar conditions at a later time and found the same S35G mutation in the 2A protease again showing that this mutation seems to be specific and consistent.

**CVB3 Resistant to Polyamine Depletion via S35G Mutation in 2A Protease**

This 2A<sup>S35G</sup> mutation was cloned in the CVB3 parental strain using mutagenic PCR on the CVB3 infectious clone. The presence of the mutation was verified, and infectious virus was made by transfecting the plasmid with the mutant into 293T cells expressing the T7 polymerase. After successfully recovering virus, we measured the replication kinetics of the mutant compared to the wildtype virus. To this end, untreated Vero cells were infected with either wildtype CVB3 or the 2A<sup>S35G</sup> CVB3 at an MOI of 0.01. Supernatant was collected from the cells at different
points of infection, and viral titers were determined (Figure 7A). The mutant virus showed similar replication kinetics to the wildtype virus although at slightly lower titers.

Because this mutant was isolated after passage in DENSpm, it was anticipated that the 2A$^{S35G}$ mutant may resist DENSpm-mediated viral restriction. To determine resistance to DENSpm, cells were treated with increasing doses of DENSpm 16 hours prior to infection or left untreated. Cells were then infected with either wildtype or mutant CVB3 for 24 hours. Supernatant was collected and titers were determined. Wildtype virus replication significantly reduced with DENSpm treatment compared to the untreated control; however, replication of the 2A$^{S35G}$ mutant did not significantly reduce with DENSpm treatment compared to the untreated control suggesting the mutant gained resistance (Figure 7B). We confirmed that cellular polyamines were reduced with DENSpm treatment (Figure 7C).

Because DENSpm and DFMO both reduce cellular polyamine levels, we investigated whether the mutant was resistant to DFMO treatment as well. Vero cells were treated with increasing doses of DFMO 96 hours prior to an infection or left untreated. The cells were then infected with either CVB3 wildtype or the mutant CVB3 for 24 hours. The supernatant was collected, and viral titers were determined via plaque assay. As with DENSpm, the replication of wildtype virus decreased significantly with DFMO treatment compared to control (Figure 8A). The 2A$^{S35G}$ mutant virus did not have a significant decrease in replication with DFMO treatment compared to the untreated control, suggesting a partial resistance to DFMO as well. Again, we confirmed polyamine depletion by thin layer chromatography (Figure 8B). Thus, resistance to DENSpm via 2A$^{S35G}$ confers resistance to DFMO-mediated polyamine depletion.
Vero cells lack an intact antiviral interferon (IFN) response, making them highly susceptible to viral infection (58). Although these cells demonstrate both wildtype CVB3 sensitivity and 2A<sup>S35G</sup> mutant resistance to DENSpm treatment, the impact of polyamine depletion on wildtype CVB3 in immunocompetent cells is yet to be determined. This immunocompetence may also have an impact on the 2A<sup>S35G</sup> mutant’s ability to overcome this resistance.
this sensitivity as well. To address this, we performed additional dose responses with two cell lines that both contain intact IFN signaling, Huh7 and HeLa cells (59, 60). Huh7 and HeLa cells were treated with increasing concentrations DENSpm 16 h pre-infection with either wildtype CVB3 or the 2A mutant at an MOI of 0.1. We found that in both Huh7 (Figure 9A) and HeLa (Figure 9B), wildtype virus titers

![Graph showing normalized titers of CVB3 and CVB3 2A S35G mutants](image)

**Figure 8. CVB3 2A S35G Mutant Gains Resistance to DFMO**

(A) Vero cells were treated with increasing doses of DFMO, from 100 μM to 1 mM, for 4 days prior to infection with wildtype CVB3 or 2A protease mutant. Viral titers were determined via plaque assay at 48 hpi.

(B) Thin layer chromatograms resolving the polyamines Put, Spm, and Spm after treatment with DFMO.

* p ≤ 0.05, ** p ≤ 0.01 using Student’s t-test (n ≥ 2), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.
Next, we wanted to compare the fitness and replication characteristics of the $2A^{S35G}$ mutant to wildtype virus. Passaging CVB3 in cells can potentially result in mutations with a greater or equal replication advantage, as previously observed (4, 28). To determine if this mutant had a change in fitness manifesting as a change in replicating virus, we measured titer and replication differences between the $2A^{S35G}$ mutant and wildtype virus. Vero cells were plated and treated with 100 μM DENSpm for 16 h or left untreated, and then infected with either the mutant or wildtype virus at an MOI of 0.1 for 24 h. The mutant virus exhibited lower titers compared to wildtype virus after a 24 h replication period (Figure 10A). However, DENSpm treatment had a smaller relative effect on viral replication for the mutant virus compared to the wildtype virus, with a 3.5-fold decrease in viral titers compared to 12.6-fold decrease for wildtype CVB3.
Importantly, we wanted to establish the stability of the mutant and consider its fitness. To ascertain the stability of the mutant, Vero cells were left untreated and infected with the CVB3 \(2A^{S35G}\) mutant at an MOI of 0.1. After 24 hours, 1/10th of the supernatant from the infected cells was passaged into the next set of wells for 24 hours. This was done for five passages, and the supernatant was collected. Viral RNA was extracted, purified, reverse transcribed, and sequenced. The samples were aligned with the parental genome, and we found that after multiple replication cycles the mutation remained and was stable (Figure 10B, above). We then performed a competition assay to look at the fitness of the mutant after multiple replication cycles. Vero cells were infected with an equal amount (PFU) of CVB3 wildtype and the protease mutant at an MOI of 0.1; 1/10th of the supernatant from the cells was passaged into the next set of cells for five passages. Viral RNA from the fifth passage was extracted, purified, reverse transcribed, and sequenced. The sequenced samples were aligned to the parental genome, and the wildtype CVB3 was the only virus present, suggesting that compared to the wildtype, the protease mutant had a reduced fitness (Figure 10B, lower).

To establish if the protease mutant produced the same amount of infectious particles, the ratio of viral genomes to infectious particles was measured (Figure 10C). Cells were left untreated and infected with wildtype or protease mutant virus at an MOI of 0.1. Viral titers were determined by plaque assay, and viral RNA was extracted, purified from the supernatant, and reverse transcribed. The amount of viral genomes was quantified by qPCR, and the ratio of genomes to infectious virus (PFU) was measured. No significant difference was found between the wildtype and protease mutant.
Additionally, we measured the plaque sizes of both wildtype and mutant virus (Figure 11A,B). We observed a small yet significant decrease in plaque size of the protease mutant, suggesting that this mutant has a decreased fitness.
Prior work demonstrated that polyamines, specifically spermidine, enhances chymotrypsin activity (61); however, whether polyamines affect enterovirus proteases, like 2A and 3C of CVB3, has only recently been explored (4). To determine if polyamines are necessary for protease activity, we treated Vero cells with varying concentrations of DENSpm for 16 h or left them untreated. We then used a dual luciferase reporter system previously described and shown in Figure 12 (4) to measure both 2A and 3C protease activity without polyamines present (Figure 13). We observed a significant decrease in protease activity at all concentrations of DENSpm; thus, both viral wildtype proteases are dependent on polyamines for robust activity.
Mutant has rescued protease activity

Because this 2A<sup>S35G</sup> protease mutation confers resistance to polyamine depletion, and we have seen altered 2A protease activity in mutants previously (4), we hypothesized that the 2A<sup>S35G</sup> mutant has modulated protease activity to overcome CVB3’s sensitivity to polyamine depletion. To investigate this hypothesis, we used our luciferase protease assay and cloned the 2A<sup>S35G</sup> mutant protease into a pCMV expression plasmid. Firefly luciferase activity was measured 24 hours after transfection and normalized to a transfection control (Renilla luciferase). We found that 2A<sup>S35G</sup> protease activity did not significantly decrease with DENSpm treatment (Figure 14A), suggesting that this mutant confers protease resistance to polyamine depletion.

To determine if the 2A<sup>S35G</sup> mutant protease has enhanced proteolytic cleavage of cellular targets during infection, cells were treated with DENSpm 16 hours prior to infection or left untreated. Cells were then infected with wildtype or protease mutant CVB3 at an MOI of 5 for 24 hours. Cellular lysates were collected, and eIF4G cleavage was analyzed via Western blot.

**Figure 12. Dual Luciferase Reporter System**
Cleavage of target sequence in reporter system allows for robust firefly luciferase activity which can be measured and normalized to a renilla luciferase. This reporter was cloned and co-transfected as stated. Image adapted from (5).
(Figure 14B). We observed the appearance of three bands at approximately 95 kDa, corresponding to cleaved eIF4G, specifically during viral infection, not present in uninfected (UI) sample. This eIF4G signal was decreased with increasing DENSpm concentration with wildtype CVB3 infection. In contrast, cleaved eIF4G was observed uniformly with mutant CVB3 infection, suggesting that the 2A\textsuperscript{S35G} mutation maintains the ability to cleave eIF4G despite DENSpm-mediated polyamine depletion.

![Figure 13. Polyamine Depletion Inhibits 2A and 3C Wildtype Protease Activity](image)

**Figure 13. Polyamine Depletion Inhibits 2A and 3C Wildtype Protease Activity**

Dual luciferase protease activity reporter systems were cloned and co-transfected with (A) wildtype 2A or (B) wildtype 3C protease into Vero cells left untreated or treated with increasing doses of DENSpm. Firefly luciferase activity was measured 24 h later and normalized to renilla luciferase transfection efficiency control and subsequently normalized to untreated cell transfection.

* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 using Student’s t-test (n ≥ 3), comparing treated samples to un-treated controls. Error bars represent ± 1 SEM.

**S35 Varients Confer Resistance to Polyamine Depletion**

Finally, we wanted to determine if different mutations in the 2A protease could cause the same type of resistance to polyamines as 2A\textsuperscript{S35G}. Two different amino acids were chosen for the new mutations, lysine, a positively charged amino acid, and alanine, another neutral amino acid.
Lysine was chosen for its positive charge that may replace the positive charge polyamines provide to the viral protease and may cause an even greater resistance to polyamine depletion than the S35G mutation. Alanine was chosen for its neutrality to see if another neutral amino acid similar to glycine may cause the same resistance to polyamine depletion. Both the 2A$^{S35\text{A}}$ and 2A$^{S35\text{K}}$ mutations were cloned in the CVB3 parental strain using mutagenic PCR on the CVB3 infectious clone. The presence of the mutation was verified, and infectious virus was made by transfecting the plasmid with the mutant into 293T cells expressing the T7 polymerase. Both viral mutants were recovered. To measure the replication kinetics of these new mutants compared to the wildtype virus, Vero cells were infected with either the S35A mutant, S35K mutant, and wildtype virus at an MOI of 0.01. Supernatant was then collected at different points of infection and titers were determined via plaque assay (Figure 15A). Replication of the mutants over 72 hours was very similar to the wildtype virus.

Next, we wanted to determine if these new mutants were resistant to DENSpm treatment similarly to 2A$^{S35\text{G}}$ mutant. Cells were treated with increasing doses of DENSpm 16 hours prior to infection or left untreated. Cells were then infected with either wildtype, 2A$^{S35\text{A}}$, or 2A$^{S35\text{K}}$ CVB3 for 24 hours. Supernatant was collected and titers were determined. Similarly to the 2A$^{S35\text{G}}$ mutant, both 2A$^{S35\text{A}}$ and 2A$^{S35\text{K}}$ showed resistance to DENSpm treatment and polyamine depletion (Figure 15B). However, the S35K mutant did not show as much resistance as the S35G mutant even with the added positive charge to replace the charge of the polyamines.
Figure 14. 2A\textsuperscript{S35G} Mutant has Rescued Protease Activity without Polyamines Present

(A) Dual luciferase protease activity reporter systems were cloned and co-transfected with 2A\textsuperscript{S35G} mutant into Vero cells left untreated or treated with increasing doses of DENSpm. Firefly luciferase activity was measured 24 h later and normalized to renilla luciferase transfection efficiency control and subsequently normalized to untreated cell transfection.

(B) Vero cells were left untreated or treated with increasing doses of DENSpm for 16 h prior to infection with wildtype CVB3 or 2A mutant CVB3. Total cellular protein was collected 24 hpi and analyzed via Western blot for eIF4G and β-actin.

** p ≤ 0.01, *** p ≤ 0.001 using Student’s t-test (n = 2), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.
Figure 15. S35 Mutants Experience Resistance to DENSpm Treatment

(A) Vero cells were left untreated and were infected with CVB3 and 2A mutant proteases at an MOI of 0.01. Samples were collected every 24h and titered via plaque assay.

(B) Vero cells were treated with increasing doses of DENSpm, from 10 μM to 100 μM, for 16 hours prior to infection with wildtype CVB3 or 2A protease mutants. Viral titers were determined via plaque assay at 48 hpi. Viral titers were used to calculate the percent replication in DENSpm, by dividing the titer of the virus after infection of DENSpm-treated cells by the titer of the virus after infection of untreated cells at 48 hpi.

p ≤ 0.05, ** p ≤ 0.01 using Student’s t-test (n ≥ 2), comparing treated samples to untreated controls. Error bars represent ± 1 SEM.
CHAPTER FOUR

DISCUSSION

Role of Polyamines for Viral Proteases During Infection

Polyamines have diverse and important roles during infection, and here we highlight a critical role for polyamines in protease activity during enterovirus infection. As summarized in Figure 16, CVB3 protease 2A activity relies on cellular polyamines (Figure 16A). Thus, when polyamines are depleted, through compounds like DENSpm or DFMO, protease activity is inhibited, and viral replication is reduced (Figure 16B). However, escape mutants isolated after replicating CVB3 in cells depleted of their polyamines, like 2A$^{53G}$, overcome this sensitivity to polyamine depletion allowing for protease activity along with subsequent viral replication (Figure 16C). Our data not only show CVB3 and viral proteins’ reliance on cellular molecules for infection, but also the potential risk of antiviral resistance.

Previous work has also found many other functions of polyamines during RNA viral infections besides protease activity. Polyamines have been shown to be packaged in viral progeny, specifically spermidine, in bunyaviruses (39). Inhibiting polyamines during infection has also shown to limit viral binding (45, 46), and escape mutants have been identified that can bind to cells without polaymines present (46). Other pathways have also shown to affect polyamine levels which in turn will inhibit viral infection as well. For example, depletion of nucleotide pools via ribavirin, an FDA approved antiviral therapy, will activate SAT1 and deplete polyamines (35).
Polyamines show significant promise as an antiviral target, and this and several other studies show the importance of polyamines in viral infections (3, 30). Repurposing drugs is an efficient way to accelerate antiviral development, as drugs with extensive characterization are better understood in terms of their pharmacokinetics and toxicity profile. DENSpm has undergone clinical trials, and DFMO is FDA approved. DFMO has already been shown to be effective at limiting viral titers in vitro and in vivo (4, 38, 45), and DENSpm is well tolerated in patients, though not yet approved (34). Important questions remain concerning the viability of polyamine depletion as an antiviral therapy, including drug dosage and delivery mechanisms.

**Figure 16. Role of Polyamines for CVB3 Proteases During Infection**

(A) During wildtype infection polyamines are necessary for 2A protease activity for a robust infection. However, when polyamines are depleted by DENSpm (B), virus output is reduced. With an adapted CVB3 mutant, there is a rescue of protease activity even in the presence of DENSpm and viral output increases.
These studies further highlight that even if polyamine depletion is a viable antiviral strategy, viruses mutate to overcome treatment, and additional precautions would be necessary in successfully treating infected patients, mitigating the emergence of resistant mutants.

**Viral proteases as an Antiviral Target**

Targeting proteases to combat viral infection shows significant promise. Recent studies have shown that targeting CVB3 proteases are a potential effective antiviral therapy and significantly decrease viral replication, though they are not used clinically (27, 28). Protease inhibitors have not only been effective antiviral therapies for CVB3 but also for other viruses, including HIV, hepatitis C virus, and noroviruses (62, 63). Our data suggest that polyamines contribute to protease activity in vitro during enterovirus infection, and this highlights an opportunity for targeting protease activity indirectly, by targeting host polyamines. Because there are no antiviral therapies currently available for CVB3 infection, targeting CVB3 proteases, potentially through polyamine depletion, may hold significant promise.

**Drug Resistance and Viral Escape Mutants**

Drug resistance and viral escape from antiviral therapy is a rising threat to health and patient care. The 2A\textsuperscript{S35} escape mutant we identified in this study provides insight into both viral evolution and how potential antivirals (in this case, DENSpm) work during infection. However, they also show how quickly a virus gains resistance to treatment. Others have described the genetic pliability of picornaviruses, like rhinoviruses, and the subsequent ability to overcome antiviral treatments (64). Depending on the stability and fitness of the escape mutants, antiviral resistance can be devastating to a patient where not only is the virus resistant to treatment, but it is also stable after multiple replication cycles similar to this 2A\textsuperscript{S35G} escape mutant has shown to
be in vitro. However, further studies need to be performed to determine if these results can be translated in vivo and this mutant and other similar mutants maintain this resistance and stability. Further, additional studies in cells pertinent to CVB3 replication, such as cardiac cells, will be necessary to test the function of polyamine inhibitors in more physiologically relevant cells.

We previously showed that other escape mutants in the CVB3 proteases exhibit stability and no loss in fitness compared to wildtype virus (4). In contrast, the mutant described here, 2A^{S35G}, exhibits reduced fitness, suggesting that the serine at position 35 is important for viral fitness that may be sacrificed to promote replication in polyamine-depleted cells. Because resistance to polyamine-depleting drugs is possible, combination therapy may be an effective solution against these infections, and our work highlights the need for combination therapy (or other mitigation techniques) to prevent the emergence of the mutants we have identified.
APPENDIX A

SUPPLEMENTAL FIGURES
Figure 1S. DENSpm Treatment Activates SAT1
Vero-E6 cells were treated with 10, 50, and 100 μM DENSpm for 16 h prior to collection for Western blot for SAT1.

Figure 2S. DENSpm Effectively Depletes Cells of Polyamines
Vero-E6 cells were treated with 10, 50, and 100 μM DENSpm for 16 h prior to collection for thin layer chromatography for polyamines measuring the presence of putrescine (Put), spermidine (Spm), and spermine (Spm).
REFERENCE LIST


VITA

Bridget Hulsebosch was born in Palatine, Illinois, on October 12th, 1996, to Thomas and Nancy Hulsebosch. She attended Loyola University Chicago where she received a Bachelor of Science in Environmental Science with a concentration in Public Health. There she studied with Dr. Justin Harbison and performed vector-control and ecology research on local mosquito populations and vector-borne disease.

Hulsebosch remained at Loyola University Chicago in the Infectious Disease and Immunology Research Institute in the Master of Science in Infectious Disease and Immunology program. She joined Dr. Bryan Mounce’s lab to study host-viral interactions and novel antiviral therapies. After completion of her degree, she will continue her training with Dr. Bryan Mounce in Stritch School of Medicine’s IPBS Ph.D. program.