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Novel Antivirals Unveiled Against Acute and Persistent Coxsackievirus B3 Infection via Rapid Screening

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NOVEL ANTIVIRALS UNVEILED AGAINST ACUTE AND PERSISTENT COXSACKIEVIRUS B3 INFECTION VIA RAPID SCREENING

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Dedicado a mis padres,
Isidro y Maria del Mar

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Coxsackievirus A	CVA
Coxsackievirus B	CVB
Coxsackievirus B3	CVB3
Coxsackievirus B1	CVB1
Coxsackievirus B4	CVB4
Sindbis virus	SINV
La Crosse encephalitis virus	LACV
Human rhinovirus 14	HRV14
Mayaro virus	MAYV
Chikungunya virus	CHIKV
Vaccinia virus	VACV
Dulbecco's modified medium	DMEM
Newborn calf serum	NBCS
Fetal bovine serum	FBS
Phosphate-buffered saline	PBS
Developmental Therapeutics Program	DTP
National Institute of Health	NIH
Multiplicity of Infection	MOI
Plaque forming unit	PFU
Relative fluorescence units	RFU
Quantitative Real Time PCR	qRT-PCR
50% inhibitory concentration	CC50
50% cytotoxic concentration	IC50
Selectivity Index	SI
Tris-buffered saline with Tween	TBST
Persistent Coxsackievirus B3 cell line	CVB3 PANC

ABSTRACT

Enteroviruses cause 10 to 15 million infections annually in the United States, and Coxsackievirus B3 is one of the most commonly reported. Coxsackieviruses may become persistent, characterized as a viral infection that is not cleared from host cells and that generates a continuous infection. Patients who develop persistent CVB3 infection may not respond to the same antivirals as an acute infection, which may be detrimental. Therefore, there is a need for broad-range antiviral drugs to combat acute *and* persistent CVB3 infection, as there is no well-accepted treatment available. We developed a model system to study persistent CVB3 using a pancreatic ductal cell line PANC-1, and we used Vero-E6 cells to study acute infection. We maintained persistently infected cells in tissue culture for over a year and characterized the infection.

In efforts to identify novel antivirals, using the National Institutes of Health's Developmental Therapeutics Program (DTP), we screened thousands of compounds for antiviral activity against acute *and* persistent CVB3, and among the top hits was Ro 5-3335. Ro 5-3335 is a 1,4-benzodiazepine nordazepam. It acts as RUNX1-CBFB inhibitor against acute myeloid leukemia. Further, it can inhibit gene expression in HIV-1 at the transcription level through interference with Tat-mediated transactivation. We find that Ro 5-3335 potently inhibits persistent and acute CVB3 infection, likely by affecting a cellular pathway. We also show that Ro 5-3335 is broadly antiviral and inhibits a variety of other human pathogens. This work underscores the importance of targeting persistent and acute infection and highlights the potential

for Ro 5-3335 as a broad-acting antiviral molecule. Overall, Ro 5-3335 is a promising antiviral that can be used to target CVB3 at multiple stages of infection.

CHAPTER ONE:

BACKGROUND

Enteroviruses and Coxsackievirus B3

Enteroviruses are a significant public health concern as they cause 10 to 15 million symptomatic infections annually in the United States. The enterovirus genus includes Poliovirus, Coxsackieviruses, Echoviruses, Enterovirus 71, Enterovirus D68, and Rhinovirus (1). The most commonly reported enterovirus eliciting these infections are Coxsackieviruses. Specifically, they are considered one of the top viral pathogens that cause outbreaks in neonatal intensive care units across the United States yearly (1). Coxsackieviruses are enteroviruses that belong to the *Picornoviridae* family, comprised of non-enveloped viruses containing positive-sense, single-stranded RNA (1,2). They are divided into two subgroups: Coxsackievirus A (CVA) and Coxsackievirus B (CVB). Coxsackieviruses are found worldwide and have a unique ability to adapt to different environmental conditions which plays a critical role in their ability to spread and transmit disease. Coxsackieviruses have been isolated from the environment specifically in waste waters, soils, raw vegetables, shellfish, and surfaces (2,3). Therefore, similar to poliovirus, its transmission is through the fecal-oral route via contaminated hands, food and water (1-3); thus, it is considered a hand, foot, mouth disease. However, studies have shown transmission of Coxsackievirus via aerosol following a seasonal pattern (2,3,4). Coxsackieviruses infect the gastrointestinal tract and the upper respiratory tract as two replication sites, then spread to target organs via the lymphatic system and the bloodstream (4).

Coxsackievirus B3 Infection, Symptoms, and Clinical Outcomes

A principal etiologic agent of the CVB group is Coxsackievirus B3. CVB3 represents a significant source of human disease, particularly in young children, elderly adults, and immunocompromised individuals who cannot produce a sufficient immune response against these pathogens. Symptoms of infection may include sore throat, rash, fever, myalgia, extreme fatigue, headache, and chest pain (5). The severity of CVB3 disease ranges from acute febrile to gastrointestinal illness, aseptic meningitis, myocarditis, dilated cardiomyopathy, hepatitis, pneumonia, encephalitis, and unexpected sudden death (6-9). Typically, CVB3 is diagnosed through clinical examinations of symptoms verified by laboratory testing through RT-PCR to detect enterovirus RNA in respiratory secretions, urine, and serum (5). Although RT-PCR can verify the presence of RNA, viral cultures are needed to allow for typing of an isolate (5). Currently, there are no approved treatments or vaccines to protect against Coxsackievirus B3 infection. There are supportive measures recommended such as rest and hydration, or acetaminophen and topical treatments varying on severity of symptoms. However, healthcare professionals emphasize the importance of promoting infection control strategies within a hospital and home environment by maintaining routine hand hygiene (5). This also emphasizes the importance of continuing the search for novel antiviral and vaccine targets that may control Coxsackievirus infection to prevent further disease.

Coxsackievirus B3 Virus Structure and Replication Cycle

Coxsackieviruses are small (25-30nm diameter) non-enveloped viruses with an icosahedral capsid. The CVB3 genome is approximately 7.4 kilobase pairs, that serves as a template for protein translation and genome replication during the CVB3 life cycle (11,12).

Since it has a positive sense genome, viral RNA can be directly translated into viral proteins by the host cell. The genome is comprised of several structured elements including the cloverleaf structure at the 5' terminus that is 742 nucleotide long and the stem-loop structures within the viral internal ribosomal entry site (IRES), which are critical components in viral replication and protein synthesis. A large open reading frame encodes for a total of 11 viral proteins (10,11,12). The first four are capsid proteins, considered as structural proteins (VP1-VP4) that form the viral capsid icosahedral structure. The next seven proteins are non-structural proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$, $3D^{pol}$) that promote viral protein synthesis, replication, release and spread by interacting with the RNA genome and polyproteins (12). The *cis*-acting RNA element on 2C has been shown to modulate IRES efficacy and influences RNA translation (13). Within the 3' poly(A) tail there are additional stem-loop structures that are about 100 nucleotide long that play a role in forming replication complexes during RNA synthesis (12). This structure is depicted in Figure 1 (10).

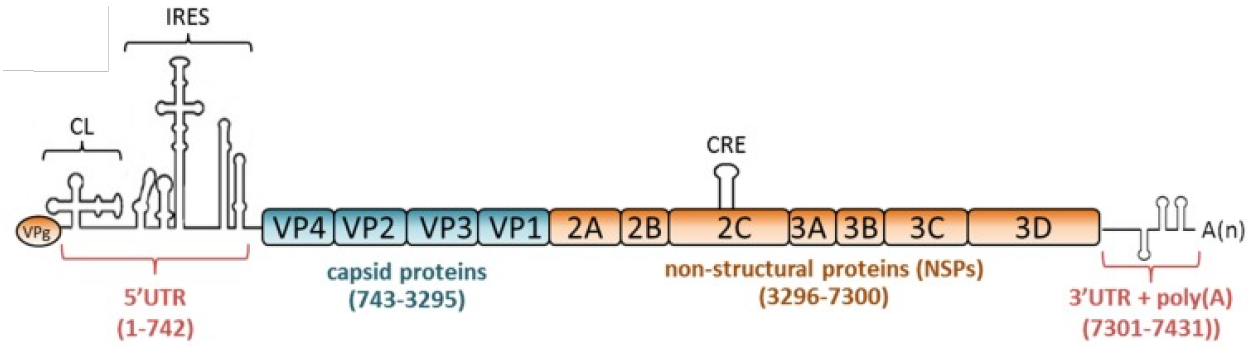


Figure 1. CVB3 Structure and Genome Diagram. The CVB3 viral genome serves as a template for protein translation and genome replication during the CVB3 viral life cycle. It consists of several structured elements including the cloverleaf structure at the 5' terminus, the stem-loop structures within the viral internal ribosomal entry site (IRES), and the large open reading frame that encode for its 11 viral proteins. The first four capsid proteins are structural proteins, and the next seven proteins are non-structural proteins that contain the *cis*-acting RNA element on 2C. Within the 3' poly(A) tail there are additional stem-loop structures. Image adapted from (10).

The CVB3 viral life cycle is relatively short, approximately 8 hours long. It begins with the viral attachment to the host cell through two host cell target receptors CAR (Coxsackievirus and adenovirus receptor) which functions as the internalization receptor, and DAF (decay-accelerating factor) which functions as the primary attachment protein or co-receptor (14). Upon internalization into the host cell through clathrin-mediated endocytosis, the viral particle undergoes a conformational change in the viral capsid that induces the uncoating of its positive-sense RNA genome into the cytoplasm (14,15). This viral genome functions as a template for the translation of the polyprotein and replication of the viral genome (14, 15, 16). Viral proteins are translated into a large polyprotein and subsequently cleaved by virus-encoded proteases, 2A, 3C, and 3CD yielding in individual structural (VP1-VP4) and nonstructural proteins ($2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$, $3D^{pol}$) (16). Replication is then catalyzed by an RNA-dependent RNA polymerase, 3D, which plays an essential role in transcription of the negative-strand viral RNA intermediate. The negative sense strand then provides as a template for the synthesis of multiple copies of a positive sense RNA strand (16). Subsequently the new virions are assembled as released as mature virions from host cell through lytic release to spread infection, catalyzed by the viral protein 2B (16). This CVB3 viral life cycle is depicted in Figure 2 (14).

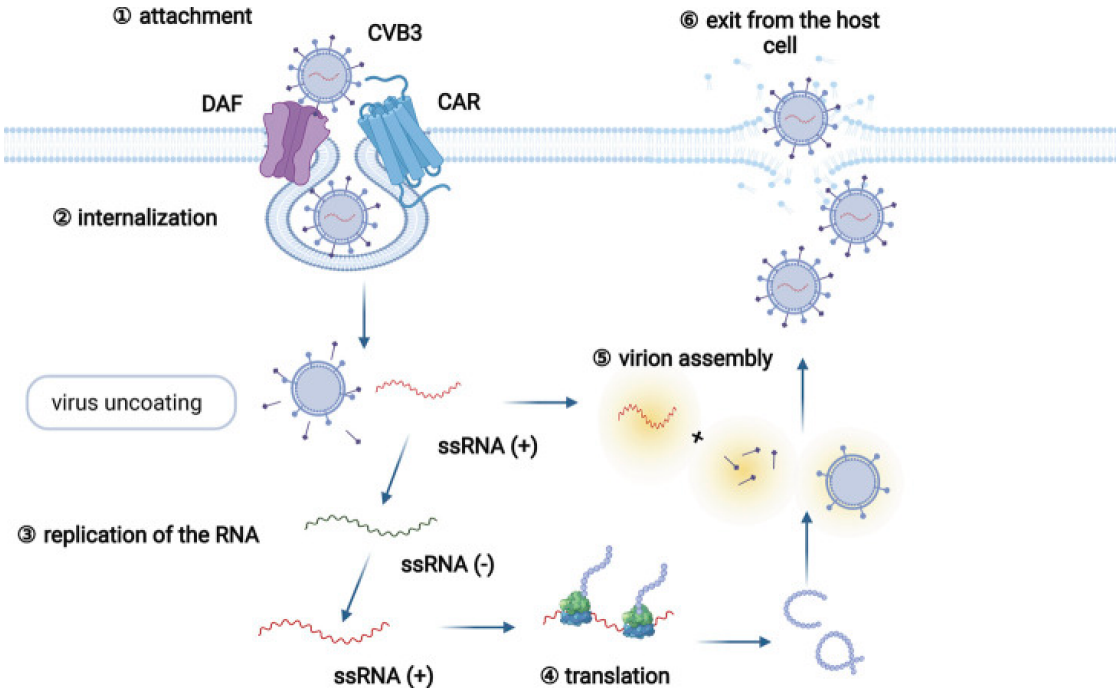


Figure 2. CVB3 Viral Life Cycle. The diagram above displays the life cycle of CVB3 once CVB3 infects a host cell. It begins with the viral attachment to the host cell through two cell receptors CAR (Coxsackievirus and adenovirus receptor) which functions as the internalization receptor, and DAF (decay-accelerating factor) which functions as the primary attachment protein or coreceptor. The virus will begin uncoating its positive-sense RNA into the cytoplasm facilitating RNA genome replication and viral protein translation since it serves as a template for the translation of the viral polyprotein. Subsequently the new virions are assembled as released as mature virions from host cell through lytic release to spread further infection. Image adapted from (14).

Persistence and Coxsackievirus B3

Although CVBs are cytolytic viruses, studies have investigated a unique phenotype of CVB3 which is the ability to establish persistence within specific tissues, including pancreatic ductal cells, *in vitro* and *in vivo*, which has been implicated in the pathogenesis of chronic diseases in humans and experimental models (17, 18, 19). Specifically, persistent CVB3 RNA has been found to be maintained in immunocompromised patients with chronic medical conditions (18).

Persistence is characterized as the establishment of viral infection with failure of viral clearance by the immune system (20). Persistent CVB3 infection is not well-described or understood; however, *in vitro* studies suggest that the mechanism of persistence is a consequence

of mutations promoting the rapid evolution of Coxsackieviruses, moderating it into a less lytic form that allows persistent infection with lower levels of viral RNA detection (18). It has been shown that persistent CVB3 infection results in viral adaptation via mutations to its capsid proteins, and maintenance of viral fidelity (19). These mutations could alter the virus's ability to adapt and survive within cellular environments that prevent the cells immune system from detecting or clearing. As displayed in Figure 3, studies suggest that CVB persistence is a result of co-evolution between viral factors, such as different mutations within the viral genome and host cellular factors, such as changes in metabolism or cell surface receptors (17, 19). Other studies suggest that CVB persistence is a result of two types of persistent viral infections: steady-state infection and carrier-state infection (21,22). Steady-state infection is characterized by global host cell infection; however, its replication cycle is altered and no longer lytic as a result of mutations or co-evolution. Carrier-state infection is characterized a state in which as a small portion of host cells carry the viral genome and are undergoing productive viral replication. These infected cells seed virus and continuously infect surrounding cells (21,22). The consensus seems to be that CVB persistence is dependent upon the multiple host-virus interactions.

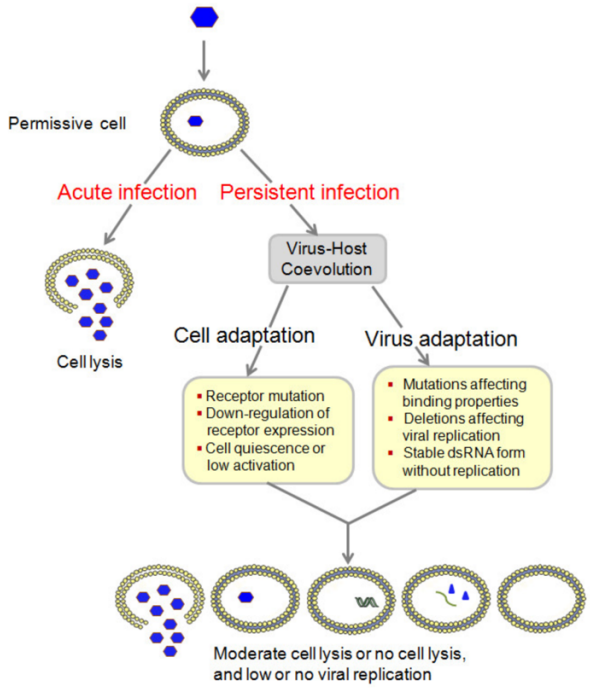


Figure 3. Possible Mechanisms for CVB3 Persistence. Coxsackievirus B has been shown to establish persistent viral infection within specific tissues, and this persistence has been implicated in pathogenesis of various chronic diseases, specifically in immunocompromised individuals maintaining CVB RNA within them. Persistent CVB3 infection is not well-described or understood; however, various studies suggest that persistence is the result of co-evolution between the virus and the host cell.
Image adapted from (22).

Antiviral Development

Despite the high prevalence of Coxsackievirus B3, currently, there are no approved antivirals or vaccines to treat the virus. Both acute and persistent infection are important disease states, and it is important to further understand their mechanism, but also to develop antivirals that will benefit the patient with either type of infection.

In the past, antivirals that demonstrated antiviral activities against CVB3 and CVB4 have been identified, such as pleconaril. Pleconaril has been shown to bind the virus capsid and inhibit CVB from attaching to the host cell receptors, preventing viral internalization (23). Unfortunately, the FDA rejected this antiviral due to the emergence of resistant viruses, and reduction in antiviral efficacy within a patient when pleconaril treatment is initiated 24 hours after onset of symptoms (23). Furthermore, studies reported that inefficacy of pleconaril against

CVB3, specifically, *in vitro* (23). Remdesivir, a nucleoside analog, has been shown to inhibit RNA synthesis in CVB3 and other enteroviruses such as EV71 replication (23). However, studies have reported that remdesivir needs to further establish a safety profile as it has been shown to cause cardiotoxic effects on the cardiovascular system in patients which is a significant concern because CVB3 is implicated in cardiovascular disease (24). There are many antiviral compounds and repurposed drugs, such as umifenovir (inhibits virus-endosome fusion), fluoxetine (inhibits viral replication by targeting 2C and 3A protein; fluoxetine-resistant CVBs have emerged), hixentra (contains IgG neutralizing antibodies), and itraconazole (targets 3A protein) that are effective against other acute and persistent CVB strains such as CVB1, CVB2, CVB4, and CVB5 (22,23). However, it seems that CVB3's high rate of evolution allows it to acquire resistance against various antiviral compounds and render them ineffective (25). Further, this rapid evolution allows the virus to rapidly adapt to changes in the cellular environment that the antiviral compounds may be imposing (25). It has been suggested that viral proteins or host factors involved in virus replication can be important targets for antiviral drugs.

Developing novel antiviral therapies is a challenge yet crucial in advancing the fight against viral infections such as CVB3. The NIH developed a novel approach that allows researchers to identify new antiviral compounds and facilitate drug development by establishing a repository of chemical substances and their biological activities through rapid drug screens (26). Rapid drug screens provide the opportunity to test an array of different compounds, known and unknown, to efficiently identify which ones could be used therapeutically against pathogens. This approach has been successful in identifying antiviral compounds against chikungunya virus (27), La Crosse virus (28), SARS-CoV-2 (29), Ebolavirus (30), monkeypox virus (31), influenza A (32), Zika virus (33), and more. Antiviral development requires substantial time and cost to

characterize the compound, address the efficacy of its biological activity, its safety among pre-clinical and clinical testing, and its potential approval by the FDA. Therefore, the approach of rapid drug screens using a library of compounds, and potentially repurposing drugs, provides a method that could aid in the rapid identification of antiviral compounds and decrease development cost and time (34).

Aims and Hypothesis

In efforts to identify novel antivirals, we performed a drug screen using a selection of compounds available from the National Institutes of Health's Developmental Therapeutics Program (DTP). We screened thousands of compounds for antiviral activity against acute and persistent CVB3, and among the top hits was Ro 5-3335. This compound acts as RUNX1-CBFB inhibitor against acute myeloid leukemia and inhibits gene expression in HIV-1 at the transcriptional level through interference with Tat-mediated transactivation (36, 37). Here, we show that Ro 5-3335 exhibits antiviral activity *in vitro* against acute and persistent Coxsackievirus B3.

In the first aim, we established an acute and persistent CVB3 *in vitro* model system to study and identify potential therapeutics for both types of infection by developing a rapid screen to identify compounds with antiviral activity. By performing a primary and secondary screen, we established several NIH compounds that overlapped in antiviral activity against acute and persistent CVB3 infection. Among the top hits we selected Ro 5-3335 as our drug of choice due to its antiviral activity and minimal cytotoxicity to cells.

In the second aim, we characterized the effects of Ro 5-3335 on acute and persistent CVB3 infection. We established that Ro 5-3335 can effectively reduce acute and persistent viral titers in a dose dependent manner with minimal toxicity. Ro 5-3335's analogs, Ro 24-720 and

WUN40378 have potent antiviral activity against both infections. Further, Ro 5-3335 is broadly antiviral against viruses. These data suggest that Ro 5-3335 is protecting the cell from viral infection potentially by modulating a cellular pathway.

In the third aim, we examined where in the viral life cycle Ro 5-3335 may be inhibiting acute and persistent infection. We established that Ro 5-3335 has broad impact on several steps of the viral life cycle during an acute infection. However, in persistent infection, it can be observed that Ro 5-3335 lowers viral egress after multiple rounds of infection. We find that Ro 5-3335 potently inhibits persistent and acute CVB3 infection, likely by affecting a cellular pathway.

These data highlight the need to understand acute CVB3 infection versus a persistence CVB3 infection; our work demonstrates the potential for rapid antiviral screens to identify potential antivirals against both persistent and acute infection, which may be applicable to other viruses.

CHAPTER TWO:

MATERIAL AND METHODS

Cell Culture

Cells were incubated at 37°C containing 5% CO_2 in Dulbecco's modified medium (DMEM; Life Technologies) containing bovine or calf serum, penicillin-streptomycin, and cipro. Vero-Luc2p and Vero-hACE2 (BEI Resources) were supplemented with 10% newborn calf serum, penicillin-streptomycin and cipro (NBCS; Thermo Fisher). PANC-1, Huh7, HeLa, 293T, and HepG were supplemented with 10% fetal bovine serum, penicillin-streptomycin and cipro (FBS; Thermo Fisher).

Establishment and Maintenance of Persistently Infected PANC-1 cells

PANC-1 cells (ATCC) were infected with Coxsackievirus B3 (Nancy Strain), CVB3 (ATCC), or CVB3 (H3) at a multiplicity of infection (MOI) of 0.1 to establish CVB3-PANC persistently infected cells. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin and cipro at 37°C containing 5% CO_2. Multiple persistently infected cell lines were established to compare infections from different strains.

Rapid Screening of Antiviral Compounds

Vero cells were seeded on 96-well plates and treated with a 1:100 dilution of each compound from the NIH DTP compound plates 4 hours prior to infection. Vero cells were then infected with CVB3 at a multiplicity of infection (MOI) of 0.1 PFU/mL and incubated for 48

hours at 37°C containing 5% CO_2. Medium was removed, cells were fixed with 4% formalin, and living cells were stained using crystal violet solution (Sigma-Aldrich). Excess stain was removed in a mild bleach solution, and cells were allowed to dry for 48 hours. Crystal violet stain was resuspended in 10% acetic acid and placed on an orbital shaker for 15 minutes. The absorbance at 590 nm was detected using a BioTek Synergy H1 plate reader. Similarly, CVB3-PANC cells were seeded on 96-well plates and treated with a 1:100 dilution of each compound from the NIH DTP compound plates incubated for 24 hours at 37°C containing 5% CO_2. Supernatant from the 96-well plate was then collected and transferred to their respective wells on Vero cells in a 96-well plate. These cells were incubated for 24 hours at 37°C containing 5% CO_2. Medium was removed, cells were fixed with 4% formalin, and living cells were stained using crystal violet solution (Sigma-Aldrich). Excess stain was removed in a mild bleach solution, and cells were allowed to dry for 48 hours. Crystal violet stain was resuspended in 10% acetic acid and placed on an orbital shaker for 15 minutes. The absorbance at 590 nm was detected using a BioTek Synergy H1 plate reader.

Drug Treatment

Standard treatment experiments were as follows. Vero- cells were seeded in 24 well plates. 24 hours later these cells were treated at increasing dose concentrations NT, 5 μM, 50 μM, 75 μM, and 125 μM with Ro 5-3335 (NIH DTP compound), Benzoic acid, 4-amino, 4-nitrophenyl ester (NIH DTP compound), Closiramine aceturate (NIH DTP compound), 3,4-diphenyl isochroman-1-one (NIH DTP compound), 1-(3-Bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea (NIH DTP compound), 2-Amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl)phenyl] acetamide (NIH DTP compound), Acetic Acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester (NIH DTP compound), Redoxal (NIH DTP compound), Pleconaril

(Cayman Chemical), and Benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide) (NIH DTP compound) for 24 hours and incubated at 37°C containing 5% CO_2. These drug-treated cells were then infected at a multiplicity of infection (MOI) of 0.1 PFU/cell with CVB3. Similarly, CVB3-PANC cells were seeded in 24 well plates. 24 hours later these cells were treated with increasing dose concentrations as listed above with the exact drugs listed above and incubated for 48 hours at 37°C containing 5% CO_2.

Viability Assay

Vero cells were seeded in a 96 well plate. 24 hours later these cells were treated with increasing dose concentrations NT, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, and 125 μM with Ro 5-3335 (NIH DTP compound) and Pleconaril (Cayman Chemical) and incubated for 24 hours at 37°C containing 5% CO_2. After the 24-hour treatment, 30 μL of CytoTox-Fluor cytotoxicity assay reagent (Promega) was added to each well and incubated at 37°C containing 5% CO_2 for 30 minutes. Fluorescence of the plate was taken using a SpectraMax iD3 fluorometer at a 495 nm excitation/ 520 nm emission. The same was done for CVB3-PANC cells.

Infection and Enumeration of Viral Titers

HRV14 (provided by Bill Johnson), CHIKV (provided by Sachetana Mukhopadhyay), CVB1(BEI Resources), CVB3 (Nancy Strain) (provided by Julie Pfeiffer), CVB3 (ATCC (provided by Chris Robinson), CVB3 (H3) (provided by Chris Robinson), MAYV (BEI Resources), LACV (BEI Resources), VACV (BEI Resources) were used in viral assays. In plaque assays, dilutions of cell supernatant that were either drug treated or infected were prepared in serum-free DMEM and used to inoculate a confluent monolayer of Vero cells for 15 minutes incubated at 37°C. Cells were then overlaid with 0.1% agarose in DMEM containing 2% NBCS. CVB3 (Nancy Strain), CVB3 (ATCC), CVB3 (H3), HRV14, CHIKV, MAYV samples

were incubated for 2 days at 37°C containing 5% CO_2, LACV samples were incubated for 4 days at 37°C containing 5% CO_2, and VACV samples were incubated for 24 hours at 37°C containing 5% CO_2. Following incubation, cells were fixed with 4% formalin and stained with crystal violet solutions (10% crystal violet; Sigma-Aldrich). Plaques were enumerated and used to back-calculate the number of PFU per milliliter. In viral infections, viral stocks were diluted in serum-free DMEM before infecting cell samples for a multiplicity of infection (MOI) of 0.1 on Vero-cells unless otherwise indicated.

Direct Incubation

CVB3 stock was combined with increasing concentrations NT, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, and 500 μM Ro 503335 and incubated at 37°C containing 5% CO_2 for 4 hours. Virus was then directly quantified via plaque assay for all samples.

RNA Extraction and qPCR

Media were cleared from cells, and cells were collected using TRIzol reagent (Zymo Research). RNA was purified, and DNase treated (Zymo Research) and used for cDNA synthesis with 5x All-In-One RT-PCR master mix (BioBasic). cDNA was analyzed by qPCR using CVB3-specific primers, control beta-actin primers, and SYBR green master mix (Thermo Fisher Scientific) by using a QuantStudio real-time PCR system (Thermo Fisher) and the $\Delta\Delta CT$ method. These values were then normalized to untreated controls to allow for direct comparison between samples.

Viral Propagation and Egress

Vero cells were seeded in 24 well plates. 24 hours later these cells were treated with a dose concentration of 25 μM of Ro 5-3335 and incubated at 37°C containing 5% CO_2. 24 hours

later the drug-treated cells were infected at an multiplicity of infection of 10 PFU/cell and incubated at 37°C containing 5% CO_2 for 8 hours to look at the 6-8 hour viral life cycle of CVB3 and see if viral egress was impacted. After 8 hours supernatant was collected to then perform a plaque assay and look at viral titers. Once the supernatant was removed from the cells, 1x PBS was added to the cells. The PBS containing cells were freeze-thawed 2x and collected to perform a plaque assay. Freezing ruptures the cell so we can see how much virus stayed in the cells. PBS indicates if cells had any internal viral particles, and supernatant indicates if viral particles were excreted into the external environment. Similarly, CVB3-PANC cells were seeded in 24 well plates. 24 hours later these cells were treated with a dose concentration of 25 μM of Ro 5-3335 and incubated at 37°C containing 5% CO_2 for 24 hours. After 24 hours, the supernatant was collected to then perform a plaque assay and look at viral titers. Once the supernatant was removed from the cells, 1x PBS was added to the cells. The PBS containing cells were freeze-thawed 2x and collected to perform a plaque assay.

Statistical Analysis

Prism 10 (GraphPad) was utilized to generate graphs and perform statistical analysis. For all analyses, analysis of variance (ANOVA) was used to compare groups.

Table 1. Top Hits in Initial Antiviral Screen Against Acute and Persistent Infection

DTP NSC No.	Acute Primary Screen Value (Absorbance)	Persistent Primary Screen Value (Absorbance)	Compound Name	Description	Prior antiviral activity against:
66020	2.17	1.824	Ro 5-3335	RUNX1-CBFB leukemia inhibitor and HIV-1 gene expression inhibitor	HIV-1
163802	1.44	1.992	Benzoic acid, 4-amino, 4-nitrophenyl ester	None known	None known
335506	1.446	3.733	Closiramine aceturate	Antihistamine drug	Influenza A
55862	2.868	1.209	3,4-diphenyl isochroman-1-one	None known	None known
215718	2.598	1.598	1-(3-Bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea	Target protein for development of contraceptive drugs	None known
140873	2.722	2.974	2-Amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl) phenyl] acetamide	RUNX1-CBFB inhibitor	HIV-1
180964	1.413	2.022	Acetic Acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester	A type of acetone	None known
73735	2.29	1.865	Redoxal	Inhibitor of de novo pyrimidine synthesis	HIV-1
207895	1.014	1.744	Benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide	Inhibition of MDM2 and MDMX in prostate cancer cells	None known

Table 2. IC50, CC50, SI Values Derived from Ro 5-3335 Treatment on Acute and Persistent CVB3

Virus	Cell Type	IC50 value (μM)	CC50 value (μM)	SI value
CVB3 (Nancy)	Vero	1.549	125<	80.69<
CVB3-PANC	CVB3-PANC	1.414	100	70.72

Table 3. IC50 Values Derived from Ro 5-3335 Treatment on Diverse Viruses

Virus	Cell Type	IC50 value (μM)
CVB3 (Nancy)	Vero	1.549
	293T	35.3429
	Panc-1	24.2856
	HeLa	34.4173
	HepG2	33.2708
	Huh7	26.2852
Pers. CVB3 (Nancy)	Vero	1.414
Pers. CVB3 (H3)	Vero	28.2405
Pers. CVB3 (ATCC)	Vero	16.4912
HRV14	Vero	17.2252
CHIKV	Vero	48.4377
CVB1	Vero	2.6743
LACV	Vero	18.8829
VACV	Vero	26.6709
MAYU	Vero	69.9443

Table 4. IC50 Values Derived from Analogs of Ro 5-3335 Treatment CVB3

Analog	Virus	Cell Type	IC50 value (μM)
Ro 24-7429	CVB3 (Nancy)	Vero	2.0963
WUN40378	CVB3 (Nancy)	Vero	6.1113
Ro 24-7429	Pers. CVB3 (Nancy)	CVB3-PANC	11.0441
WUN40378	Pers. CVB3 (Nancy)	CVB3-PANC	87.1152

CHAPTER THREE:

RESULTS

Rapid Screening of Molecules Exhibiting Antiviral Activity Against Acute and Persistent Infection.

To study persistence, we established persistent CVB3 infection in PANC-1 cells (19). To assess infection, we measured viral titers over a span of 8 months and observed that infection was maintained with no significant changes in viral titer (19). The viability of uninfected PANC-1 cells and CVB3-infected PANC-1 cells were measured at 4 months, and we observed no significant changes indicating that infection did not change cellular viability (19). Hence, we investigated if other viruses could establish persistence in PANC-1 cells. We infected PANC-1 cells with Sindbis virus (SINV) and La Crosse virus (LACV). SINV is a mosquito-borne enveloped, positive-sense RNA alphavirus and LACV is a mosquito-borne enveloped, negative-sense bunyavirus. SINV and LACV were not able to establish a persistent infection as they killed PANC-1 cells shortly after infection (19). As a control, we infected Huh7 cells with CVB3, and observed that CVB3 lytically killed the Huh7 cells (19). These data suggest that we established a persistent CVB3 infection in PANC-1 cells, which we then used in a rapid drug screen against acute and persistent CVB3 infection.

To identify novel antivirals, we performed a drug screen of screen thousands of compounds for antiviral activity against acute and persistent CVB3 from the National Institutes

of Health's Developmental Therapeutics Program (DTP). This approach allows us to efficiently identify antivirals by exposing cells and virus to a wide variety of compounds. In the primary screen against acute CVB3 infection, we plated Vero cells to confluence in 96-well plates. Four hours prior to infection, we treated the cells with a 1:100 dilution of the NIH DTP compounds, followed by an infection at a multiplicity of infection (MOI) of 0.1 plaque-forming units (PFU) per cell with CVB3 for 48 hours. After 48 hours, we fixed the cells with 4% formalin and living cells were stained with crystal violet. As controls, we had a positive control by treating cells with ribavirin, a known broad-spectrum antiviral, a negative control leaving cells untreated (CVB3 only), and another positive control where we had uninfected, untreated cells (Vero only). To quantify the signal, crystal violet stain was resuspended in 10% acetic acid. The absorbance at 590 nm was detected using a BioTek Synergy H1 plate reader. Readings were pooled for each compound and compared to the values from the ribavirin-treated cells, untreated, and uninfected cells on each plate (Figure 1A). Values that were equivalent to or higher than the ribavirin-positive control and uninfected, untreated cells (Vero only) were considered to have antiviral activity and were selected for a secondary screening if they overlapped with the same compounds as our persistent CVB3 primary screen (described below). Any compounds with cytotoxic effects or rendered ineffective against CVB3 generated a reduced absorbance indicating a readout of a cleared well or no living cells. However, any compounds that were not cytotoxic and rendered effective as a potential antiviral against CVB3 generated a higher absorbance indicating a readout of a stained well with live cells. Thus, our primary screen selected for compounds that were both nontoxic and exhibited anti-CVB3 activity (Table 1).

In the primary persistent infection screen, we plated CVB3-PANC cells to confluence in 96-well plates. 24 hours later, we treated the cells with a 1:100 dilution of the NIH DTP

compounds for 24 hours. Supernatant from the 96-well plate was then collected and transferred to their respective wells on Vero cells in a 96-well plate. 24 hours later medium was removed, cells were fixed with 4% formalin, and living cells were stained using crystal violet solution. As controls, we had a positive control by treating cells with ribavirin, a known broad-spectrum antiviral, untreated cells (CVB3-PANC only) or uninfected cells (PANC-1). To quantify the signal, we performed the same steps mentioned during the primary screen against acute CVB3 infection. Readings were pooled for each compound and compared to the values from the ribavirin-treated cells, untreated, and uninfected cells on each plate (Figure 1B). Values that were equivalent to or higher than the ribavirin-positive control and uninfected, untreated cells were considered to have antiviral activity and were selected for a secondary screening if they overlapped with the same compounds as our acute CVB3 primary screen. Thus, our primary screen selected for specific compounds that were both nontoxic and exhibited anti-CVB3 activity (Table 1). The top nine overlapping hits were selected for secondary screening.

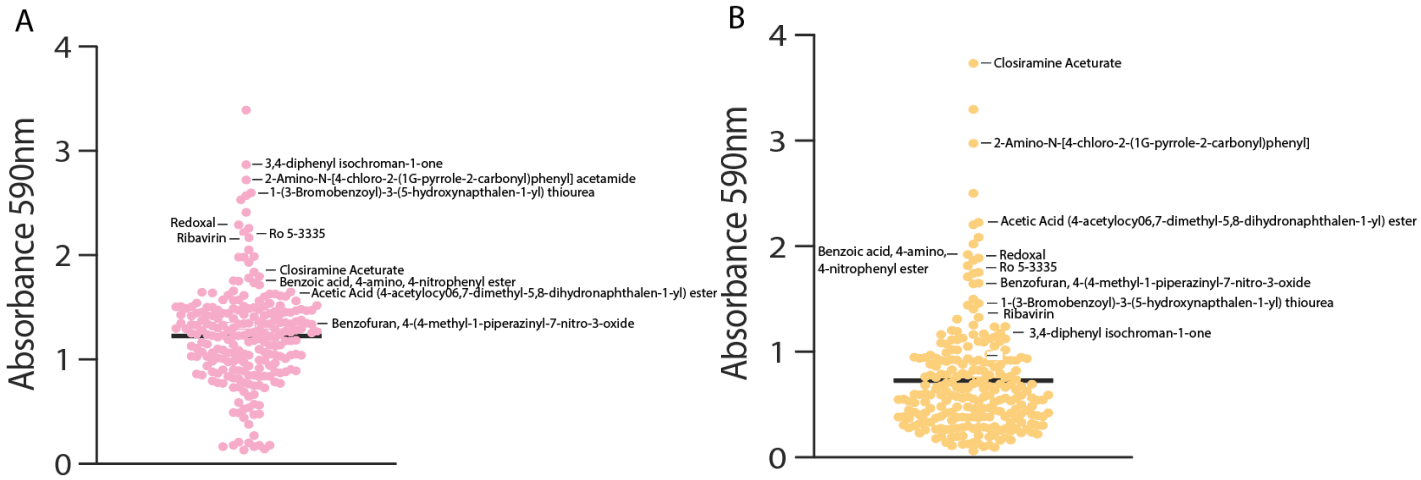


Figure 4. Potential Antiviral Compounds Identified as Top Hits in Primary Screen. Each compound is represented by a single dot, and compounds that overlapped in both acute and persistent screen that were identified as top hits were labeled, along with a positive control, ribavirin. The threshold line is indicated by the black line. Anything above the black line is considered to have antiviral activity, anything below does not. **(A)** Acute CVB3 crystal violet stain quantification, top panel. Vero cells were treated with NIH compounds 4 hours prior to acute CVB3 infection at an MOI of 0.1. After 48 hours, cells were fixed with 4% formalin, stained with crystal violet, and any signal emitted was quantified by the plate reader. **(B)** Persistent CVB3 crystal violet stain quantification, bottom

panel. CVB3-PANC cells were treated with the same NIH compounds. After 48 hours, cells were fixed with 4% formalin, stained with crystal violet, and any signal emitted was quantified by the plate reader.

In the secondary screen against acute CVB3 infection, we treated Vero cells with increasing doses, 0, 5, 50, 75, and 125 μM, of the nine compounds, 24 hours prior to infection with CVB3 at an MOI of 0.1 to allow for multiple rounds of viral replication since the virus cycle of CVB3 normally takes only 8 hours. Cells were then incubated for 24 hours. After 24 hours, we collected samples and measured viral titers via plaque assay. We observed that treatment with benzoic acid, 4-amino, 4-nitrophenyl ester (Figure 5A) did not reduce viral titers, indicating that it did not exhibit effective antiviral activity; however treatment with Ro 5-3335, closiramine aceturate, 3,4-diphenyl isochroman-1-one, 1-(3-bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea, 2-amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl)phenyl] acetamide, acetic acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester, redoxal, and benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide) produced a significant decrease in varying degrees of viral titer indicating that it did exhibit effective antiviral activity (Figure 5B-5I).

In the secondary screen against persistent CVB3 infection, we treated CVB3-PANC cells with increasing doses, 0, 5, 50, 75, and 125 μM, of the nine compounds for 24 hours. After 24 hours, we collected samples and measured viral titers via plaque assay to quantify the number of infectious particles. Similarly to the primary screen against acute CVB3 infection, we observed that treatment with benzoic acid, 4-amino, 4-nitrophenyl ester (Figure 5J) did not reduce viral titers, indicating that it did not exhibit effective antiviral activity; however treatment with Ro 5-3335, closiramine aceturate, 3,4-diphenyl isochroman-1-one, 1-(3-bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea, 2-amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl)phenyl] acetamide, acetic acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester, redoxal, and

benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide) produced a significant decrease in varying degrees of viral titer indicating that it did exhibit effective antiviral activity (Figure 5K-5R).

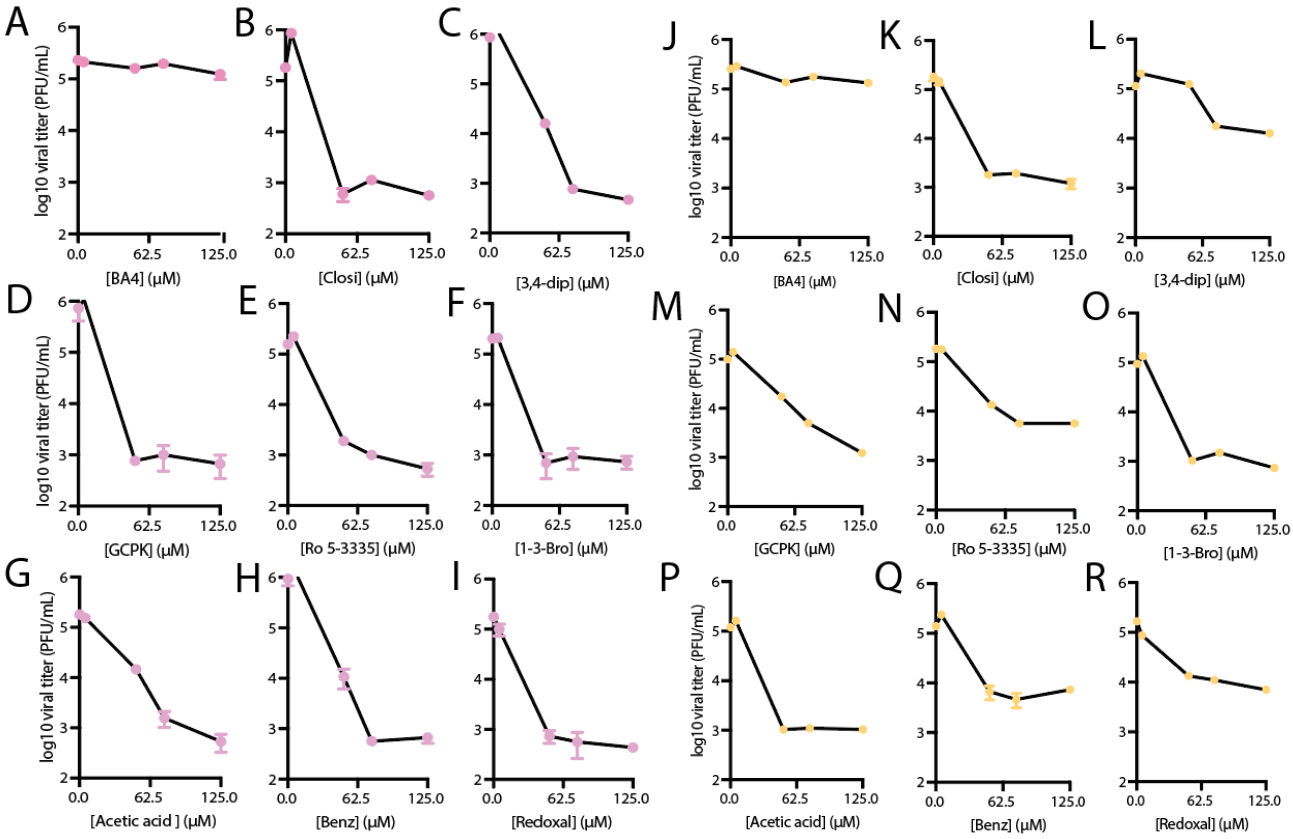


Figure 5. Secondary Screen Verified Top Hits Exhibiting Antiviral Activity. Top antiviral hits against acute CVB3 infection, left panel. The 9 compounds were used to treat Vero cells with increasing dose concentrations of **(A)** Benzoic acid, 4-amino, 4-nitrophenyl ester, **(B)** Closiramine aceturate, **(C)** 3,4-diphenyl isochroman-1-one, **(D)** 2-Amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl)phenyl] acetamide (GCPK), **(E)** Ro 5-3335, **(F)** 1-(3-Bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea, **(G)** Acetic Acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester, **(H)** Benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide), and **(I)** Redoxal and infected with CVB3 at an MOI of 0.1. After 24 hours a plaque assay was performed to determine viral titers. Top antiviral hits against persistent CVB3 infection, right panel. The 9 compounds were used to treat CVB3-PANC cells with increasing dose concentrations of **(J)** Benzoic acid, 4-amino, 4-nitrophenyl ester, **(K)** Closiramine aceturate, **(L)** 3,4-diphenyl isochroman-1-one, **(M)** 2-Amino-N-[4-chloro-2-(1G-pyrrole-2-carbonyl)phenyl] acetamide (GCPK), **(N)** Ro 5-3335, **(O)** 1-(3-Bromobenzoyl)-3-(5-hydroxynapthalen-1-yl) thiourea, **(P)** Acetic Acid (4-acetylocy06,7-dimethyl-5,8-dihydronaphthalen-1-yl) ester, **(Q)** Benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro-3-oxide), and **(R)** Redoxal. After 24 hours a plaque assay was performed to determine viral titers.

We performed a cell viability assay to determine which compounds may or may not be toxic to our cells to verify that the decrease in viral titers is due to the compound inhibiting virus replication and not toxicity. In the cell viability analysis for Vero cells (Figure 6A) and CVB3-PANC cells (Figure 6B), we observed that most compounds were effective in reducing viral titers and not cytotoxic. Ro 5-3335 was of particular interest because in both acute CVB3 and persistent CVB3 infection it exhibited strong antiviral activity, high cell viability, as well as defined properties, and its repurposed activity for a variety of diseases, such as acute myeloid leukemia and HIV-1.

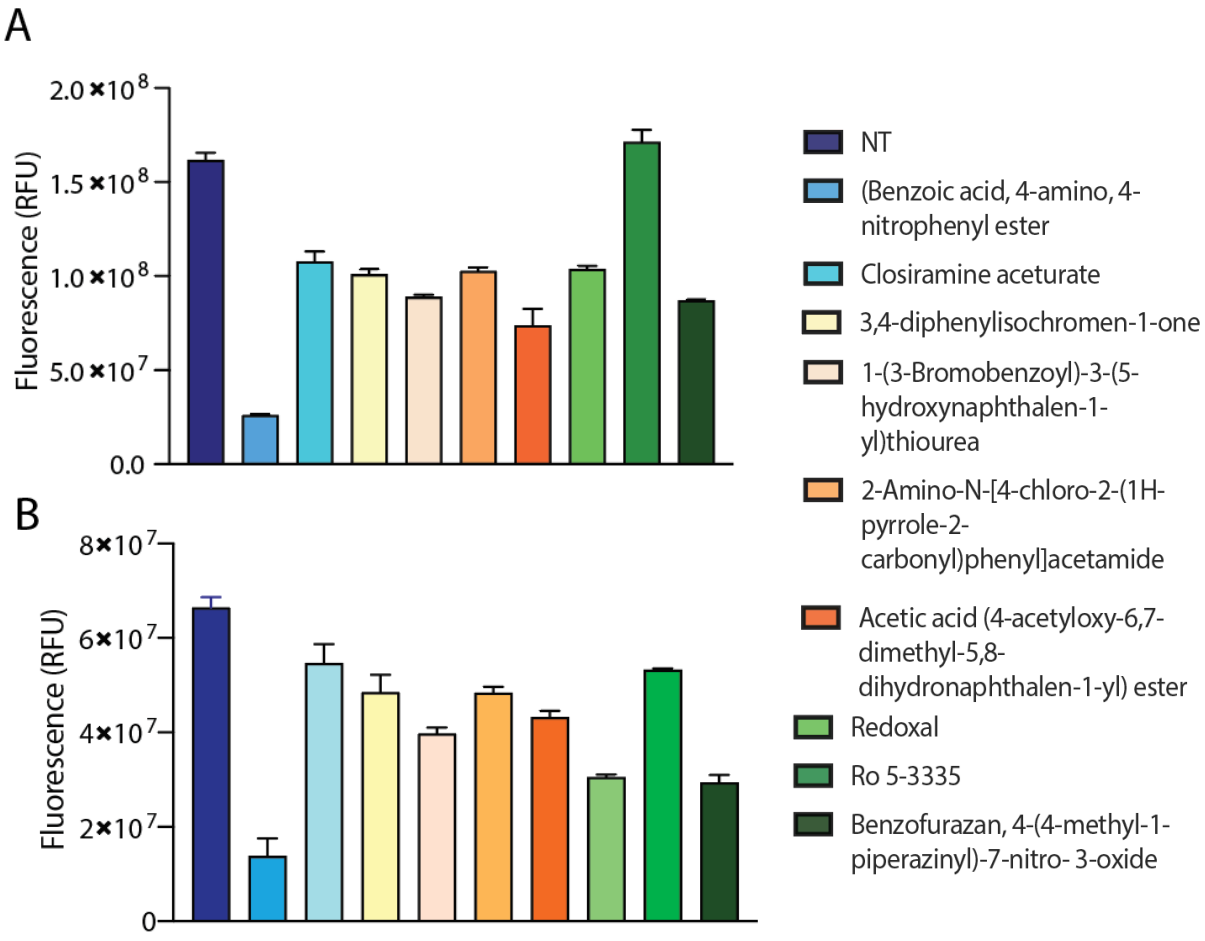


Figure 6. Cell Viability of the Compounds Identified as Top Hits. **(A)** Cell viability analysis on top antiviral hits on Vero cells. Vero cells were treated with the 9 different compounds that were considered as top hits at a high concentration of 125 µM for 24 hours prior to analysis for cell viability. **(B)** Cell viability analysis on top antiviral

hits on CVB3-PANC cells. CVB3-PANC cells were treated with the 9 different compounds that were considered as top hits at a high concentration of 125 μM for 24 hours prior to analysis for cell viability.

Ro 5-3335 Exhibits Acute and Persistent Antiviral Activity with Minimal Cytotoxicity.

We next assessed the range of concentrations at which viral titers respond to Ro 5-3335 against acute and persistent CVB3 infection. For acute CVB3 infection, we treated Vero cells with increasing doses, 0, 1, 5, 10, 25, 50, 100, and 125 μM of Ro 5-3335, 24 hours prior to infection with CVB3 at an MOI of 0.1 to allow for multiple rounds of viral replication. Cells were then incubated for 24 hours. After 24 hours, we collected samples and measured viral titers via plaque assay (Figure 7A). We observed a significant decrease in viral titers beginning at 25 μM, consistent with our findings in studies using Ro 5-3335 treatment against HIV-1 and acute myeloid leukemia, where the concentration of choice that had the high efficacy and minimal cytotoxicity was 25 μM (35, 36, 37). We measured cell viability to determine Ro 5-3335's toxicity and to verify that the decrease in viral titers is due to the compound inhibiting viral activity and not the compound killing cells due to toxicity. In the cell viability analysis for Vero cells (Figure 7E), we observed that Ro 5-3335 was not cytotoxic to our cells, as fluorescent levels were relatively unchanged with increasing doses. With these data, we calculated the 50% inhibitory concentration value (IC50) to be 1.549 μM (Table 2). We calculated the 50% concentration of cytotoxicity (CC50) to be greater than 125 μM (Table 2). Further, we calculated the selectivity index (SI) to be greater than 80.69 (Table 2), demonstrating favorable pharmacological properties of the compound. We observed dose-dependent reductions in acute CVB3 titers with Ro 5-3335 treatment, providing a wide range of concentrations with antiviral activity and minimal cytotoxicity.

For the persistent CVB3 infection, we treated CVB3-PANC cells with increasing dose concentrations, 0, 1, 5, 10, 25, 50, 100, and 125 μM of Ro 5-3335 for 24 hours, after which we

measured viral titers via plaque assay (Figure 7B). Similar to the acute CVB3 infection, we observed a significant decrease in viral titers beginning at 25 μM. We performed a cell viability assay to determine if Ro 5-3335 was toxic to our persistently infected cells. We observed that Ro 5-3335 was effective in reducing viral titers and not cytotoxic to our cells (Figure 7F). We calculated the 50% inhibitory concentration value (IC50) to be 1.414 μM (Table 2). We calculated the 50% concentration of cytotoxicity (CC50) to be 100 μM (Table 2), for a selectivity index (SI) of 70.72 (Table 2). It was of interest to us that this compound effectively worked in both acute and persistent CVB3 infection. The question then becomes how or why this compound effectively decreases viral titers in both kinds of infection that differ from each other mechanistically in viral infections.

We investigated pleconaril as a positive control, since it has established antiviral activity against acute CVB3 infection, as it binds the capsid and inhibits enterovirus entry (23). Although it was rejected by the FDA, it is an effective positive control to compare to Ro 5-3335. For acute CVB3 infection, we treated Vero cells with increasing doses of pleconaril, 0, 1, 5, 10, 25, 50, 100, and 125 μM, 24 hours prior to infection with CVB3 at an MOI of 0.1. Cells were then incubated for 24 hours, prior to measuring viral titers via plaque assay (Figure 7C). We observed a significant decrease in viral titers beginning at 50 μM. For the persistent CVB3 infection, we treated CVB3-PANC cells with increasing doses for 24 hours and measured viral titers via plaque assay (Figure 7D). Similar to the acute CVB3 infection, we observed a significant decrease in viral titers beginning at 50 μM. These data validated that our compound, Ro 5-3335, was an effective candidate in reducing viral titers in acute and persistent CVB3 infection, over a range of concentrations and with favorable pharmacological properties.

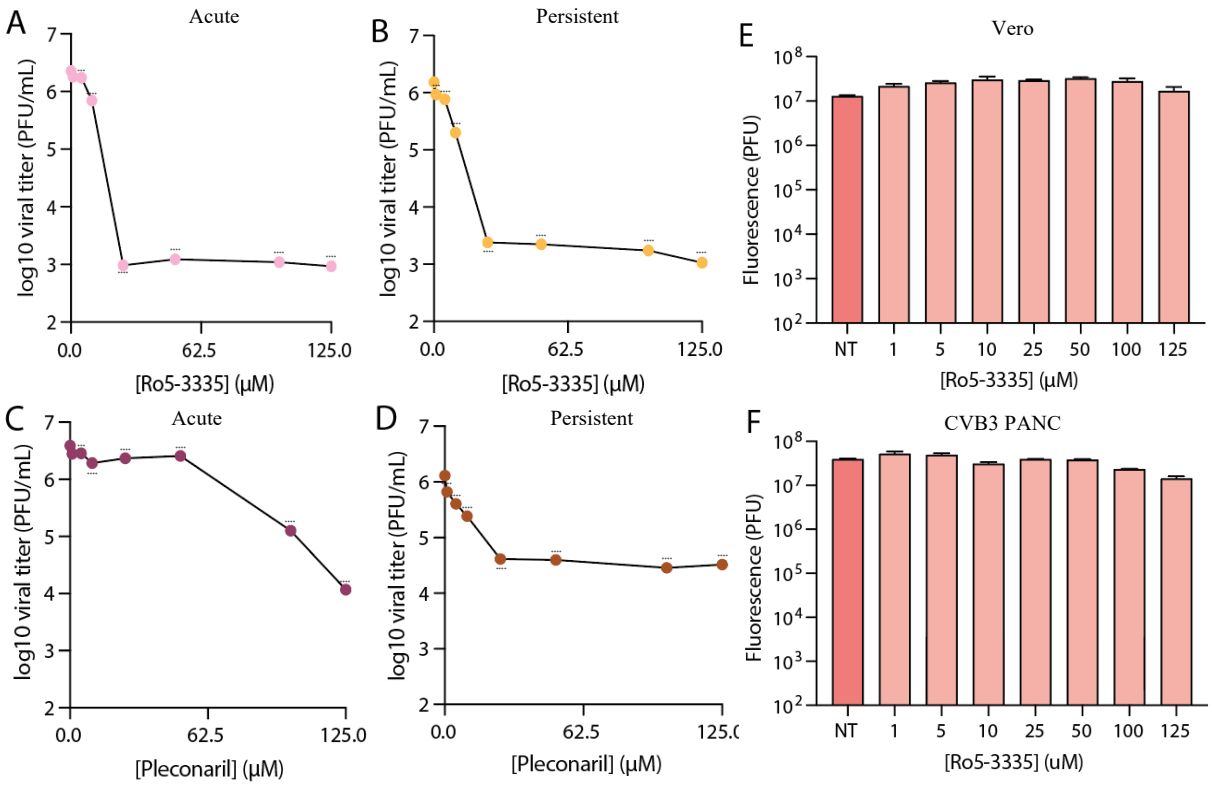


Figure 7. Ro 5-3335 Exhibits Antiviral Activity with Minimal Cytotoxicity. (A) Ro 5-3335 dose response against acute CVB3 infection. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to CVB3 infection at an MOI of 0.1. 24 hours later a plaque assay was performed to quantify viral titers. **(B)** Ro 5-3335 dose response against persistent CVB3 infection. CVB3-PANC cells were treated with increasing concentrations of Ro 5-3335 for 24 hours. 24 hours later a plaque assay was performed to quantify viral titers. **(C)** Pleconaril dose response against acute CVB3 infection. Vero cells were treated with increasing concentrations of Pleconaril 24 hours prior to CVB3 infection at an MOI of 0.1. 24 hours later a plaque assay was performed to quantify viral titers. **(D)** Pleconaril dose response against persistent CVB3 infection. CVB3-PANC cells were treated with increasing concentrations of Pleconaril for 24 hours. 24 hours later a plaque assay was performed to quantify viral titers. **(E)** Cell viability of Vero cells after dose response. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to cell viability analysis. **(F)** Cell viability of CVB3-PANCs after dose response. CVB3-PANC cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to cell viability analysis.

We next considered Ro 5-3335's activity in several cell types different from Vero and persistently infected PANC-1 cells. We confirmed Ro 5-3335's antiviral phenotype by measuring viral titers after Ro 5-3335 treatment and infection of CVB3 in Huh7 cells, PANC-1 cells (without persistent infection), 293T cells, HeLa cells, and HepG2 cells. We treated these cells at increasing concentrations of Ro 5-3335, 24 hours prior to infection with CVB3 at an

MOI of 0.1. Cells were then incubated for 24 hours prior to measuring viral titers by plaque assay.

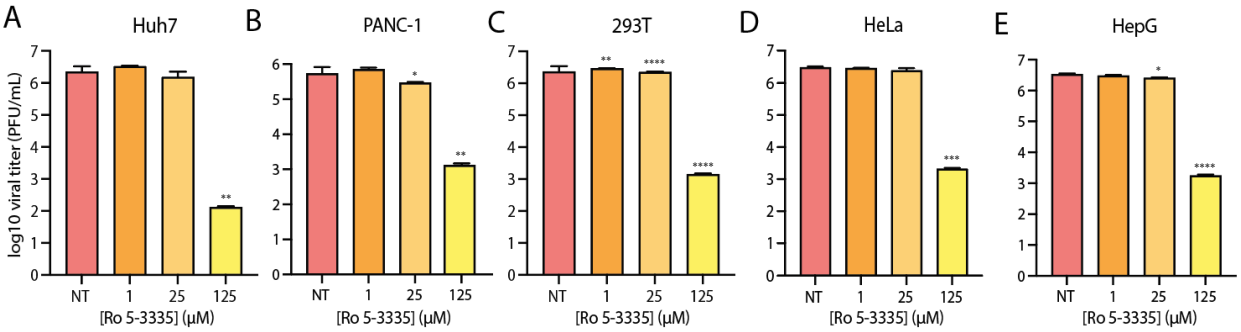


Figure 8. Ro 5-3335 Exhibits Antiviral Activity in Various Cell Types. (A) Huh7, **(B)** PANC-1**, (C)** 293T**, (D)** HeLa**, (E)** HepG, were treated with increasing concentrations of Ro 5-3335 24 hours prior to CVB3 infection at an MOI of 0.1. 24 hours later a plaque assay was performed to determine viral titers.

We observed that Ro 5-3335 reduced viral titers in all cell types as we increased drug concentration (Figure 8). We further observed that Ro 5-3335 demonstrated better antiviral activity in Vero cells and CVB3-PANC cells compared to the other cell types. We calculated the IC50 values (Table 3), which were higher than for Vero or PANC-1 cells. Thus, Ro 5-3335 exhibits antiviral activity in several cell types.

Ro 5-3335 Exhibits Broad Antiviral Activity Against Other RNA Viruses.

We next assessed Ro 5-3335's antiviral activity against other viruses. We treated Vero cells at increasing concentrations 24 hours prior to infection with human rhinovirus 14 (HRV14; Figure 9A), Coxsackievirus B1 (CVB1; Figure 9B), vaccinia (VACV; Figure 9C), chikungunya virus (CHIKV; Figure 9D), La Crosse virus (LACV; Figure 9E), Mayaro virus (MAYV; Figure 9F) at an MOI of 0.1 to allow for multiple rounds of viral replication. After 24 hours, we collected samples and measured viral titers via plaque assay (Figure 12A-F). HRV14 is a rhinovirus, of the *Picornaviridae* family (such as CVB3), and is a non-enveloped, positive-sense RNA virus (38). CVB1 is an enterovirus, of the *Picornaviridae* family (such as CVB3), and is a

non-enveloped, positive-sense RNA virus (5). VACV is an orthopoxvirus, of the *Poxiviridae* family, and is an enveloped, double-stranded DNA virus (39). CHIKV is a mosquito-borne alphavirus, of the *Togaviridae* family, and is an enveloped, positive sense RNA virus (40). LACV is a mosquito- borne orthobunyavirus, of the *Peribunyaviridae* family, and is an enveloped, negative-sense RNA virus (41). MAYV is a mosquito-borne alphavirus, of the *Togaviridae* family, and is an enveloped, positive-sense RNA virus (42). We observed that Ro 5-3335 exhibited significant antiviral activity against all RNA viruses, except for VACV which is a DNA virus. These viruses are all very different from one another and have no known conserved features; thus, it seems likely that Ro 5-3335 is not directly inhibiting the virus but targeting a cellular factor that, in turn, inhibits viral infection. Interestingly, VACV, and perhaps other DNA viruses, are an exception. Further, we calculated the IC50 values (see Table 3 for IC50 values) to determine the 50% concentration of inhibition. We observed dose-dependent reductions in viral titers with Ro 5-3335 treatment in other RNA viruses, providing a wide range of concentrations with antiviral activity against other viruses.

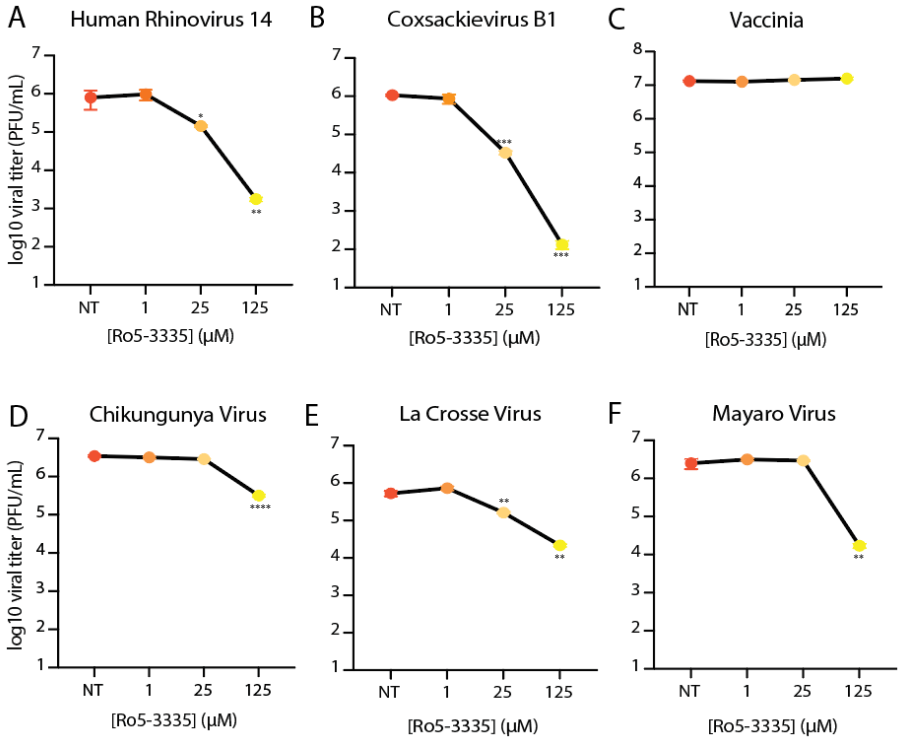


Figure 9. Ro 5-3335 Exhibits Broad Antiviral Activity Against Other RNA Viruses. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to respective infections of **(A)** Human Rhinovirus 14 (HRV14), **(B)** Coxsackievirus B1 (CVB1), **(C)** Vaccinia (VACV), **(D)** Chikungunya Virus (CHIKV), **(E)** La Crosse Virus (LACV), **(F)** Mayaro Virus (MAYV) at an MOI of 0.1. 24 hours later a plaque assay was performed to determine viral titers.

Ro 5-3335 Exhibits Antiviral Activity Over Several Rounds of Viral Replication.

To characterize the effect of Ro 5-3335 over multiple rounds of replication against acute and persistent CVB3 infection, we performed a time course assay. To assess Ro 5-3335 against acute CVB3 infection, we seeded Vero cells and treated with 25 μM Ro 5-3335 24 hours prior to infection with CVB3 at an MOI of 0.1. CVB3 replication is 8 hours, and to measure viral replication over several rounds of replication, we collected supernatant samples at regular intervals after infection and measured viral titers via a plaque assay (Figure 10A). We observed that Ro 5-3335 maintained reduced viral titers over the full course of infection, compared to the untreated Vero cells that had higher viral titers. This suggests that treatment of Ro 5-3335 was maintained over several rounds of acute CVB3 viral infection.

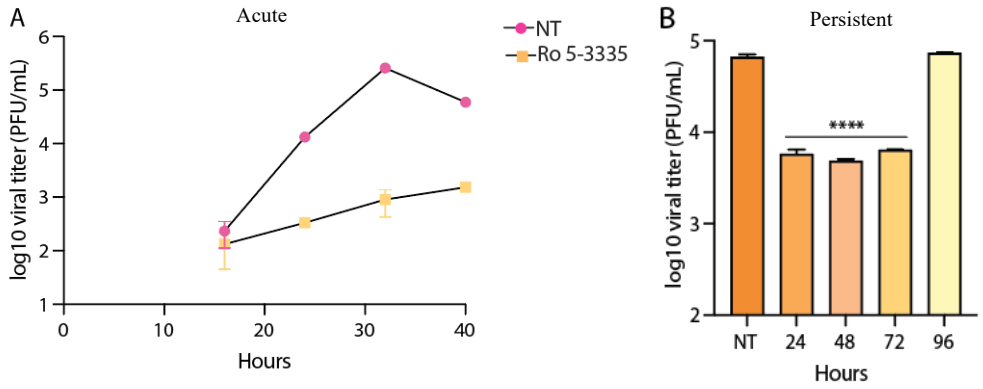


Figure 10. Ro 5-3335 Exhibits Antiviral Activity Over a Period of Time. (A) Acute CVB3 infection time course. Vero cells were treated with 25 μM 24 hours prior to CVB3 infection at an MOI of 0.1. Supernatant was then collected at indicated times, 8, 16, 24, 32, and 40 hours post-infection, and a plaque assay was performed to measure viral titers. **(B)** Persistent CVB3 infection time course, drug treatment once. CVB3-PANC cells were seeded in 24-well. Supernatant containing any viral particles that may have been released during growth was removed and fresh media was added. At this point, CVB3-PANC cells were treated with 25 μM of Ro 5-3335. Supernatant was then collected at indicated times, 24, 48, 72, and 96 hours post-treatment, and a plaque assay was performed to measure viral titers.

To assess Ro 5-3335 against persistent CVB3 infection, we seeded CVB3 PANC cells and removed supernatant before addition of Ro 5-3335 to remove any viral particles that may have been released into the media during growth. We added fresh media and treated the CVB3 PANC cells with 25 μM Ro 5-3335. We collected supernatant samples 24, 48, 72, 96, hours post treatment. We then measured viral titers via a plaque assay (Figure 10B). We observed that Ro 5-3335 maintained reduced viral titers, compared to the untreated CVB3 PANC cells. However, at 96 hours we see an increase in viral titers, indicating that perhaps Ro 5-3335 lost its effect or was metabolized. This suggests that treatment of Ro 5-3335 was maintained over several rounds of persistent CVB3 viral infection, up until 96 hours. An alternative method could be to increase the dose of Ro 5-3335 for treatment or add interval doses of Ro 5-3335 at each time point to prevent the loss of activity.

Ro 5-3335 Exhibits Broad Antiviral Activity at Multiple Steps of the Acute Viral Life Cycle.

We next investigated the antiviral mechanism(s) of Ro 5-3335 on acute CVB3 infection. First, determined if Ro 5-3335 antiviral activity was a result of Ro 5-3335 directly inactivating the virus. We hypothesized that it was not directly inactivating the virus given the effect of Ro 5-3335 on different RNA viruses, in which have no conserved features, and the only common denominator was that they infected Vero cells (Figure 9). We directly incubated CVB3 with increasing concentrations of Ro 5-3335 for 8 hours. After 8 hours we measured viral titers via plaque assay (Figure 11A). We observed no significant changes in viral titers, confirming our hypothesis that Ro 5-3335 is not directly inactivating the virus. We next investigated the stage in the viral life cycle of CVB3 that was inhibited by Ro 5-3335. The viral life cycle of CVB3 is 8 hours long and subsequently follows these steps: 0 to 2 hours is viral attachment by binding to cellular receptors, 2 to 4 hours is protein translation, 4 to 6 hours is viral replication, and 6 to 8 hours is viral egress. We treated Vero cells with 25 μM Ro 5-3335 at the indicated times before and after CVB3 infection at an MOI of 5. We then performed a plaque assay to measure viral titers at 8 hours post infection (Figure 11B). We observed that Ro 5-3335 has antiviral activity at every point of the life cycle.

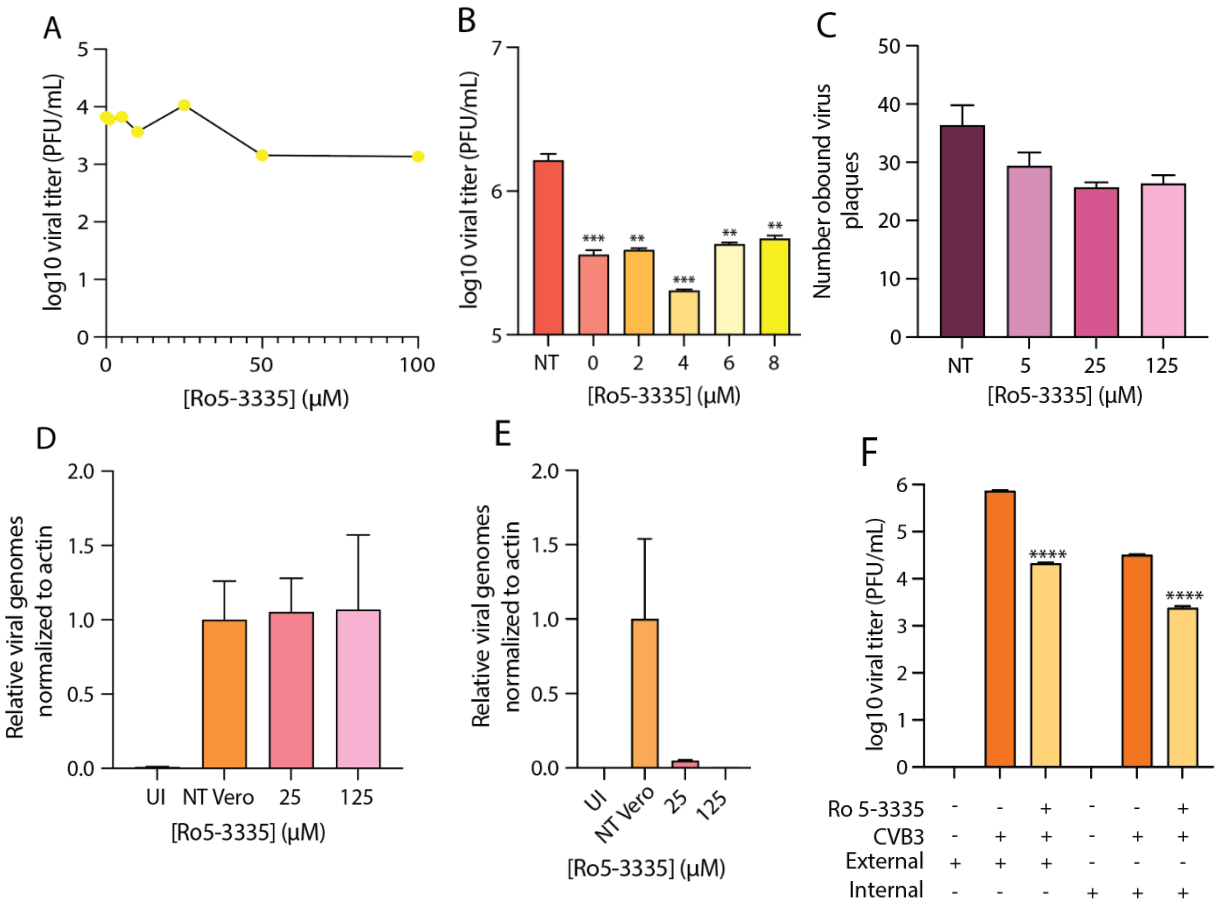


Figure 11. Ro 5-3335 Exhibits Broad Antiviral Activity at Multiple Steps of the Viral Life Cycle in Acute CVB3 Infection. (A) Direct viral incubation with Ro 5-3335. Stock CVB3 was treated with increasing concentrations of Ro 5-3335. After 8-hour incubation, a plaque assay was performed to measure viral titers. **(B)** Ro 5-3335 time of addition. Vero cells were treated with 25 μM of Ro 5-3335 at indicated times before and after CVB3 infection at an MOI of 5. 24 hours post infection a plaque assay was performed to measure viral titers. **(C)** CVB3 binding assay. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to a 5-minute infection of CVB3 on ice. Ice allows the virus to bind to cells but prevents the virus from entering the cells. Vero cells were then washed with 1x PBS to remove any unbound virus. Plaques were measured 48 hours by crystal violet staining where plaque formation indicated attachment of virus to the cell receptor. **(D)** 4–6-hour CVB3 replication. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to infection by CVB3 at an MOI of 10. 4-6 hours later, the supernatant was removed, and cells were collected in TRIzol. The viral genomes collected from the cells were determined by qPCR using CVB3 specific primers. **(E)** 24-hour CVB3 replication. Vero cells were treated with increasing concentrations of Ro 5-3335 24 hours prior to infection by CVB3 at an MOI of 0.1. 24 hours later, the supernatant was removed, and cells were collected in TRIzol. The viral genomes collected from the cells were determined by qPCR using CVB3 specific primers. **(F)** Viral egress. Vero cells were treated with 25 μM of Ro 5-3335 24 hours prior to an 8-hour infection by CVB3 at an MOI of 5. Supernatant was collected to quantify released viral particles via plaque assay (extracellular virus). 1x PBS was added to the cells within the well after supernatant was removed, freeze-thawed twice to rupture the cells to quantify how much viral particles were inside the cell via plaque assay (intracellular virus).

Interestingly, we see a lower reduction in viral titer at the 4-hour time point which is an indication that viral replication may be affected by Ro 5-3335. We decided to further investigate

the effects of Ro 5-3335 at different points of the CVB3 life cycle by performing stage-specific assays. First, we investigated if Ro 5-3335 was inhibiting acute CVB3 attachment by performing a binding assay. We treated Vero cells with increasing concentrations of Ro 5-3335 24 hours prior to a 5-minute infection incubation of CVB3 on ice. Ice allows the virus to bind to cell but prevents the virus from entering the cells. Vero cells, untreated and treated, were then washed with 1x PBS to remove any unbound virus, as well as residual Ro 5-3335. Plaques were revealed 48 hours later by crystal violet staining where plaque formation indicated attachment of virus to the cell receptor. We observed a modest reduction of viral binding as the dose of Ro 5-3335 increased (Figure 11C). However, it was not a strong response of Ro 5-3335 antiviral activity and does not fully explain the multi-log drop in viral titer that we observe. To determine if Ro 5-3335 was inhibiting acute CVB3 replication, we analyzed Vero cells for viral genomes via qPCR. We treated the Vero cells with increasing concentrations of Ro 5-3335 24 hours prior to a 4 to 6 hours CVB3 infection of an MOI of 10. We collected our untreated and treated CVB3 infected Vero cells with TRIzol for RNA purification. RNA was purified, reverse transcribed to produce cDNA, and analyzed by qPCR using CVB3 specific primers (Figure 11D). Samples were normalized to cellular actin using the ΔΔCT method, then normalized to the untreated control cells, set to 1, to compare untreated versus treated samples. We observed that there was no significant change in viral genome levels after increasing concentrations of Ro 5-3335, which was unexpected given the antiviral activity of the drug. We decided to measure viral genome levels, allowing for multiple rounds of viral infection. We treated the Vero cells with increasing concentrations of Ro 5-3335 24 hours prior CVB3 infection of an MOI of 10. We collected our untreated and treated CVB3 infected Vero cells with TRIzol for RNA purification 24 hours later. RNA was purified, reverse transcribed to produce cDNA, and analyzed by qPCR using CVB3

specific primers (Figure 11E). We observed a reduction in viral genome levels in a dose-dependent manner. This suggests that Ro 5-3335 elicits a reduction in viral genome levels over multiple rounds of CVB3 infection. We next considered that if viral genomes were not significantly impacted multiple rounds of infection after Ro 5-3335 treatment, then we may observe aberrant viral packaging and egress. Vero cells were treated with 25 μM of Ro 5-3335 24 hours prior to an 8-hour infection by CVB3 at an MOI of 5. Supernatant was collected to quantify released viral particles via plaque assay (extracellular virus). 1x PBS was added to the cells within the well after supernatant was removed, freeze-thawed twice to rupture the cells to quantify viral particles inside the cell via plaque assay (intracellular virus) (Figure 11F). We observed that Ro 5-3335 reduced viral titers in both the intracellular and extracellular samples. This suggests that Ro 5-3335 is impacting viral replication and viral production within the cell, thus causing a reduction of viral particles being released via viral egress into the environment to cause further infection. Therefore, we find that Ro 5-3335 potently inhibits acute CVB3 infection, likely by affecting a cellular pathway that in turns reduces viral titers at multiple stages of viral infection.

Ro 5-3335 Inhibits Persistent CVB3 Replication and Viral Egress.

We next investigated the antiviral mechanism of Ro 5-3335 on persistent CVB3 infection. As we observed from the time course infection, Ro 5-3335 reduced viral titers up until 96 hours. This suggests to us that Ro 5-3335 is impacting viral replication and viral egress since after collecting the supernatant at each time point there was a significant reduction in viral titers compared to the untreated group (Figure 12B). To determine if Ro 5-3335 was inhibiting persistent CVB3 replication, we analyzed CVB3 PANC cells for viral genomes via qPCR. We treated CVB3 PANC cells with increasing concentrations of Ro 5-3335 for 24 hours. We

collected our untreated and treated CVB3 PANC cells with TRIzol for RNA purification. RNA was purified, reverse transcribed to produce cDNA, and analyzed by qPCR using CVB3 specific primers (Figure 12A). Samples were normalized to cellular actin using the ΔΔCT method, then normalized to the untreated control cells, set to 1, to compare untreated versus treated samples. We observed that there was a change in viral genome levels in dose-dependent manner compared to the NT levels. We next considered that if viral replication was impacted by Ro 5-3335, then we should observe a reduction of packaging and egress. CVB3 PANC cells were treated with increasing concentrations of Ro 5-3335 for 24 hours. We analyzed internal versus external virus as in acute infection. We observed that Ro 5-3335 reduced viral titers in both the intracellular and extracellular samples in a dose dependent manner. This suggests that Ro 5-3335 is impacting viral production within the cell, thus causing a reduction of viral particles being released into the environment to cause further infection. Further, in our untreated cells, we expected to see higher viral titers in our intracellular samples as it is a hypothesized characteristic of CVB3 PANC to cells to have an accumulation of viral particles inside the cells. Therefore, we find that Ro 5-3335 potently inhibits persistent CVB3 infection, likely by affecting a cellular pathway that in turns reduces viral egress.

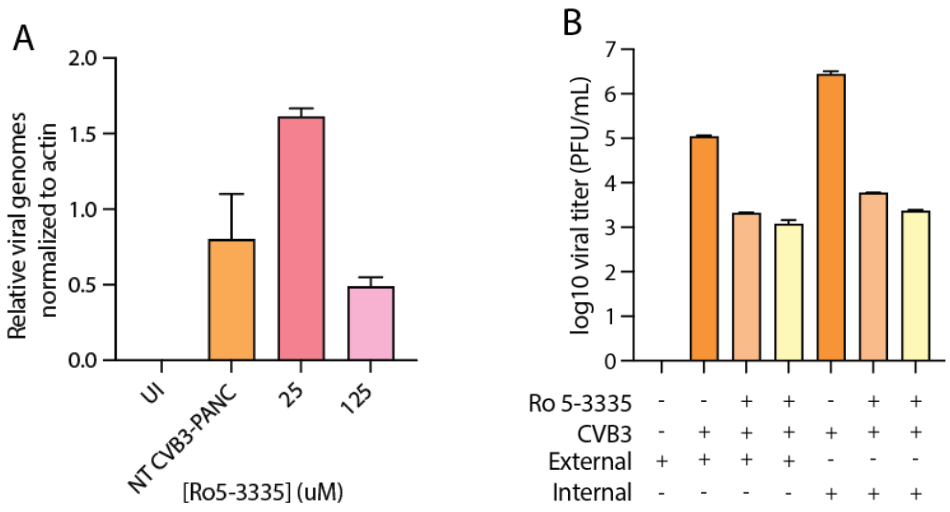


Figure 12. Ro 5-3335 Inhibits Persistent CVB3 Replication and Viral Egress. (A) 24-hour CVB3 replication. CVB3 PANC cells were treated with increasing concentrations of Ro 5-3335 for 24 hours. 24 hours later, the supernatant was removed, and cells were collected in TRIzol. The viral genomes collected from the cells were determined by qPCR using CVB3 specific primers. **(B)** Viral egress. CVB3 PANC cells were treated with increasing concentrations of Ro 5-3335 for 24 hours. Supernatant was collected to quantify released viral particles via plaque assay (extracellular virus). 1x PBS was added to the cells within the well after supernatant was removed, freeze-thawed twice to rupture the cells to quantify how much viral particles were inside the cell via plaque assay (intracellular virus).

Analogs of Ro 5-3335, Ro 24-729 and WUN40378, Are Antiviral Against CVB3 Infection.

We obtained Ro 5-3335 analogs, Ro 24-7429 and WUN40378, to determine the effects on acute and persistent CVB3 infection, and further investigate if the structural differences impact CVB3. For acute CVB3 infection, we treated Vero cells with increasing concentrations of Ro 24-7429 and WUN40378, respectively, 24 hours prior to infection with CVB3 at an MOI of 0.1. Cells were then incubated for 24 hours, and viral titers were measured via plaque assay. When treated with Ro 24-7429 (Figure 13D), we observed a significant decrease in viral titers beginning at 25 μM and maintained reduce viral titers. When treated with WUN40378 (Figure 13E), we saw a significant decrease in viral titers at 125 μM. This indicated to us that Ro 24-7429 was more effective in reducing viral titers, and at a lower concentration, than WUN40378 for acute CVB3 infection. Further, we calculated the IC50 values (see Table 4 for IC50 values) to determine the 50% concentration of inhibition.

For persistent infection, we treated CVB3-PANC cells with increasing doses for 24 hours. Cells were then incubated for 24 hours, and viral titers were measured via plaque assay. When treated with Ro 24-7429 (Figure 13F), we observed a significant decrease in viral titers beginning at 25 μM, similar to the acute CVB3 infection. When treated with WUN40378 (Figure 13G), we saw a significant decrease in viral titers beginning at 25 μM. This indicated to us that Ro 24-7429 was more effective in reducing viral titers, and at a lower concentration, than WUN40378 for acute and persistent CVB3 infection. However, we also observed that WUN40378 was more effective in reducing viral titers against persistent infection versus acute infection. We observed dose-dependent reductions in CVB3 titers with Ro 24-7429 and WUN40378 treatment, providing a wide range of concentrations with antiviral activity.

Hence the question, what about the different structures determines the efficacy of Ro 5-3335 and its analogs in reducing viral titers against acute and persistent CVB3 infection. The chemical formula of Ro 5-3335 is $C_{13}H_{10}ClN_3O$ (Figure 13A), the chemical formula of Ro 24-7429 is $C_{14}H_{13}ClN_4$ (Figure 13B), and the chemical formula of WUN40378 is $C_{13}H_{10}FN_3O$ (Figure 13C); and they are all made up of aromatic rings. WUN40378 is very similar to Ro 5-335, however it has a fluorobenzene (F) instead of a chlorobenzene (Cl). This difference in fluorine to chlorine could be an explanation as to why Ro 5-3335 is more effective in reducing acute CVB3 viral titers compared to WUN4038. However, both Ro 5-3335 and WUN40378 can effectively reduce persistent CVB3 viral titers, indicating the chlorine on the chlorobenzene is a strong antiviral agent within the compound. Whereas Ro 24-7429 is slightly different than Ro 5-3335, as it has one more carbon, 3 more hydrogens, one more nitrogen, and no oxide in its structure. Chlorobenzene is also present within both Ro 24-7429 and Ro 5-3335, and they both effectively reduce acute and persistent CVB3 indicating that chlorine is a strong antiviral agent.

It could also be suggested that the presence of the oxide could be reducing persistent viral titers, as seen in both Ro 5-3335 and WUN4038; however, it is absent in Ro 24-7429, and we still see a significant reduction in viral titers.

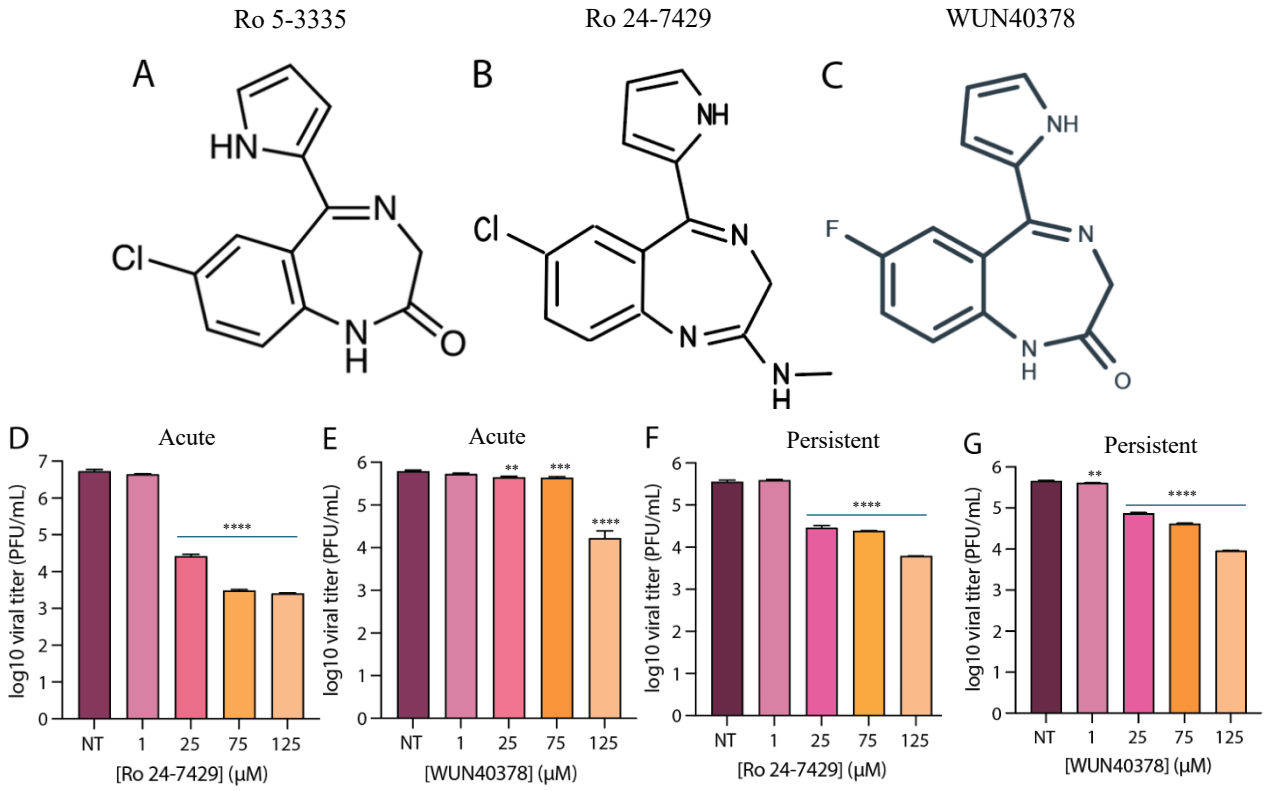
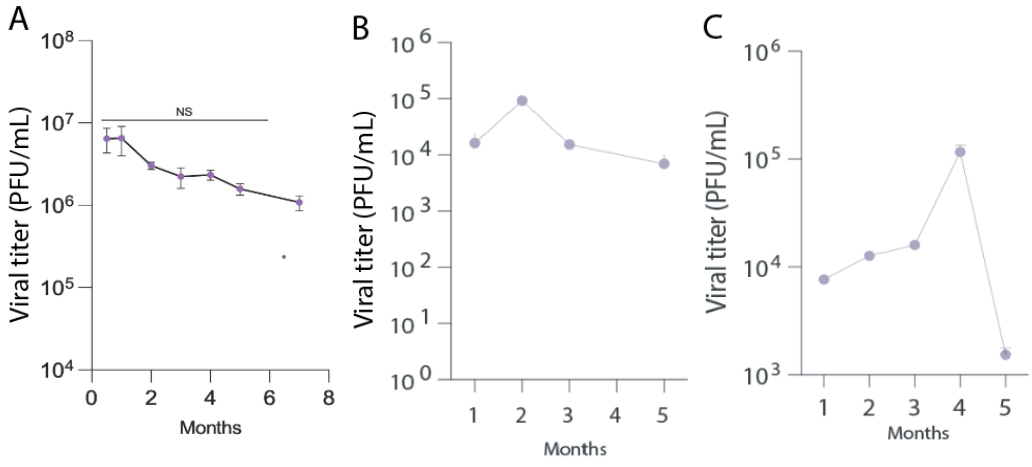


Figure 13. Ro 5-3335 Analogs, Ro 24-7429 and WUN403778, Are Antiviral. (A) Ro 5-3335 structure **(B)** Ro 24-7429 structure. **(C)** WUN40378 structure. **(D)** Ro 24-7429 dose response against acute CVB3 infection. Vero cells were treated with increasing concentrations of Ro 24-7429 24 hours prior to CVB3 infection at an MOI of 0.1. 24 hours later a plaque assay was performed to quantify viral titers. **(E)** WUN40378 dose response against acute CVB3 infection. Vero cells were treated with increasing concentrations of WUN40378 24 hours prior to CVB3 infection at an MOI of 0.1. 24 hours later a plaque assay was performed to quantify viral titers. **(F)** Ro 24-7429 dose response against persistent CVB3 infection. CVB3-PANC cells were treated with increasing concentrations of Ro 24-7429 for 24 hours. 24 hours later a plaque assay was performed to quantify viral titers. **(G)** WUN40378 dose response against persistent CVB3 infection. CVB3-PANC cells were treated with increasing concentrations of WUN 40378 for 24 hours. 24 hours later a plaque assay was performed to quantify viral titers.

Ro 5-3335 Exhibits Broad Antiviral Activity Against Different Persistent Strains of CVB3.

Finally, we investigated the antiviral activity of Ro 5-3335 on different strains of persistent CVB3, as strains of CVB3 exhibit distinct phenotypes during persistent infection.

Therefore, an antiviral may not be effective in different strains of viruses, as viruses evolve and mutate. Persistently infected CVB3 from the Nancy strain is the strain we used throughout this study. We compared our CVB3 PANC Nancy to CVB3 H3 and CVB3 ATCC strains, which differ by several amino acids. To assess that our cells were persistently infected, we measured viral titers over a span of 5 months and observed that infection was maintained with no significant changes in viral titer (Nancy CVB3 PANC; Figure 14A, H3 CVB3 PANC; Figure 14B, ATCC CVB3 PANC; Figure 14C). We assessed if Ro 5-3335 exhibited antiviral activity in all three persistent strains, so we treated our respective CVB3 PANC cells (Nancy, H3, and ATCC) with increasing doses of Ro 5-3335 for 24 hours and measured viral titers via plaque assay (Figure 14 D-F). We observed a significant decrease in viral titers beginning at 25 μM for all three strains. These data suggest that Ro 5-3335 can effectively reduce persistent CVB3 infection of multiple strains of the virus.



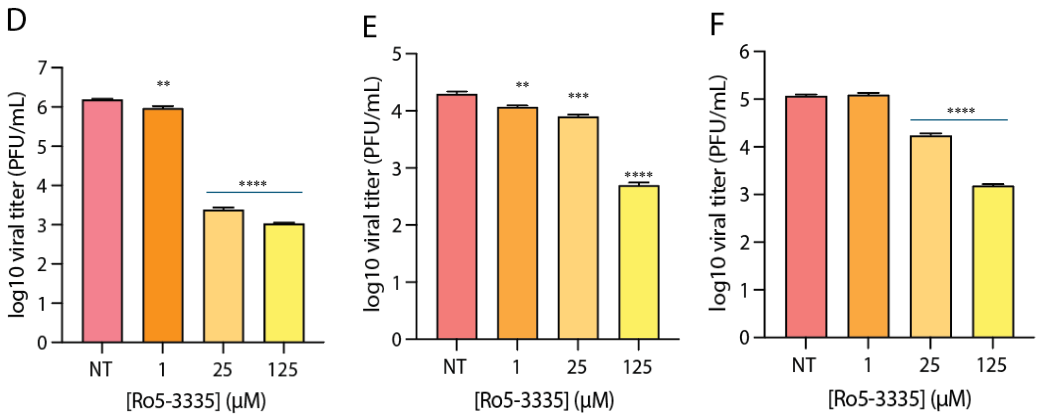


Figure 14. Ro 5-3335 Exhibits Antiviral Activity Against Different Persistent Strains of CVB3. (A) Persistent CVB3 Nancy. A persistently infected PANC-1 cell line was established with the CVB3 Nancy strain. Supernatant was collected at each month and viral titers were quantified via plaque assay. **(B)** Persistent CVB3 H3. A persistently infected PANC-1 cell line was established with the CVB3 H3 strain. Supernatant was collected at each month and viral titers were quantified via plaque assay. **(C)** Persistent CVB3 ATCC. A persistently infected PANC-1 cell line was established with the CVB3 ATCC strain. Supernatant was collected at each month and viral titers were quantified via plaque assay. **(D)** Ro 5-3335 dose response against persistent CVB3 Nancy. Nancy CVB3-PANC cells were treated with increasing concentrations of Ro 5-3335. After 24 hours, supernatant was collected, and viral titers were measured via plaque assay. **(E)** Ro 5-3335 dose response against persistent CVB3 H3. H3 CVB3-PANC cells were treated with increasing concentrations of Ro 5-3335. After 24 hours, supernatant was collected, and viral titers were measured via plaque assay. **(F)** Ro 5-3335 dose response against persistent CVB3 ATCC. ATCC CVB3-PANC cells were treated with increasing concentrations of Ro 5-3335. After 24 hours, supernatant was collected, and viral titers were measured via plaque assay.

CHAPTER FOUR:

DISCUSSION

In this modern era of emerging zoonotic diseases, antiviral and vaccine development are crucial for effective control of viral diseases (43). Antivirals play a significant role in targeting diverse viruses, including helping the immune system minimize the spread and replication of the virus within the body. Antiviral therapies may impart antiviral activity by directly affecting the virus, such as inhibiting the biological activities of viral structural proteins or the replicative enzymes (virus-directed therapeutic). Antivirals can also directly interact with host factors such as a cell receptor or cellular pathways to inhibit virus-host interaction (host-directed therapeutic), and more (44). Studies suggest that developing and identifying novel antivirals that target host factors rather than viral factors increases the threshold to viral resistance and provides broad-spectrum antiviral activity against different viruses since a significant concern of antivirals is the emergence of resistant viruses (44).

Although developing novel antiviral therapies is a significant challenge, it remains crucial in the fight against viral infections. An alternative strategy to developing novel antivirals *de novo* is to test known compounds, including ones that are FDA-approved, for antiviral activity against different viruses. Rapid drug screens provide the opportunity to test an array of different FDA-approved compounds to efficiently identify potential therapeutics against different pathogens. The purpose is not solely to identify novel antivirals, but to introduce the idea of using known FDA-approved compounds or repurposed drugs as an alternative therapeutic approach to rapidly discover new antiviral agents in light of the rapid emergence of pathogens.

Through our primary and secondary screening, we identified several compounds of interest that exhibited significant antiviral activity against acute and persistent CVB3 (Table 1).

Many of the compounds had previously shown antiviral activity, such as 2-amino-n-[4-chloro-2-(1g-pyrrole-2-carbonyl) phenyl] acetamide, a RUNX1-CBFB inhibitor (similarly to Ro 5-3335), against HIV-1 infection (45), redoxal, an inhibitor of de novo pyrimidine synthesis, against HIV-1 infection (46), and closiramine aceturate, a known antihistamine, against influenza A (47). Interestingly, Benzofuran, 4-(4-methyl-1-piperazinyl-7-nitro, 3-oxide did not have known antiviral activity; however, it has a known function as an inhibitor of MDM2 and MDM4 in cancer cells (48). MDM2 and MDM4 play a significant role in the progression of cancer as it inhibits and degrades the tumor suppressor protein, p53. Interestingly, studies suggest that p53 may play a significant role in the antiviral immune response by inducing cell apoptosis as it senses cell stress and enforcing type I IFN response (49). p53-dependent apoptosis has been demonstrated to control and inhibit virus infection in VSV, influenza A, herpes simplex virus, and poliovirus (49). Further, p53 has been shown to directly upregulate transcription of several target genes that influence the type I interferon pathway during an HIV, HCV, and influenza A viral infection, which results in impaired viral replication (49). Many viruses have evolved different strategies to inactivate p53 to prevent early apoptosis, allowing for effective viral replication, including CVB3 (50). Interestingly, it has been found that p53 plays a critical role in the control of CVB3 replication; however, studies suggest that CVB3 may have evolved strategies to facilitate the downregulation or degradation of p53, or perhaps encoding for p53 antagonistic proteins (50). If a patient has been diagnosed with cancer and is infected with CVB3 or other viruses that downregulate or degrade p53 within host cells, this could rapidly progress the status of the cancer and lead to detrimental effects.

Ro 5-3335 is considered a benzodiazepine compound, which is a class of depressant drug that is normally prescribed to treat and manage various conditions such as insomnia, anxiety, and

seizures. Benzodiazepines can be administered via intramuscular, oral, intravenous, intranasal, or rectal gel forms (51). However, it has been shown that benzodiazepine is usually well absorbed from the gastrointestinal tract which has important implications for antiviral development against CVB3, and other enterovirus infections, since the GI tract (fecal-oral route) is the main route of transmission. Ro 5-3335 has been found to have antiviral activity against HIV-1 and anti-inflammatory activity against forms of leukemia and sepsis by interacting with RUNX1 and CBFβ (35, 36, 37). RUNX1 and CBFβ are host transcriptional regulators that form a heterodimer important for DNA binding, gene expression regulation, and play a key role in hematopoiesis which is the formation of circulating blood cellular components, such as white blood cells, red blood cells, and platelets (35). Adult acute myeloid leukemia (AML) and pediatric acute lymphocytic leukemia (ALL) are considered core binding factor leukemias that contain mutations within the transcription factor genes RUNX1 (T-cell specific transcription factor) and CBFβ (35). These mutations are involved in the development on leukemia, known as leukemogenesis, that result in recurrent chromosome abnormalities, hyperproliferation of hematopoietic precursor cells, and genetic changes in oncogenes and tumor suppressor genes (35). Ro 5-3335 was identified through a rapid drug screen to have inhibitory activity against the RUNX1- CBFβ interaction. It has been demonstrated that Ro 5-3335 directly binds to RUNX1 and CBFβ, inhibiting their transcriptional function, decreasing leukemia burden and leukemia progression in a mouse model, thus increasing life expectancy, and attenuated RUNX1-dependent hemopoiesis in zebrafish embryos (35). Mice were treated with 300 mg/kg/d orally for 30 days.

Further, Ro 5-3335 has also been found to inhibit gene expression in HIV-1 at the transcriptional level through interference with Tat-mediated transactivation (36, 37). HIV-1 is a

human immunodeficiency virus that attacks the human body's immune system and has a complex life cycle that involves a unique transcriptional interaction between the viral Tat protein and its target RNA element, TAR (36,37). Tat binds to TAR to regulate the transcription of the HIV genome, cellular gene expression, and generates a cellular environment suitable for HIV viral replication by altering the immune response (52). The rate of HIV-1 transcription decreased significantly in the presence of Ro 5-3335 indicating an effect on Tat-mediated transactivation (36,37). Interestingly, it was found that Ro 5-3335 did not directly inhibit the Tat-Tar interaction, but that RUNX1 binds Tat with high affinity, thus Ro 5-3335 inhibits Tat-mediated transcription together with CBFβ by interacting with RUNX1 (36,37).

Additionally, it was found that Ro 5-3335 prevented LPS-induced septic shock *in vivo*, which is systemic inflammatory response to severe bacterial infection that results in high mortality, by inhibiting RUNX1, attenuating IL-6 production (52). An appropriate inflammatory response is critical for pathogen elimination, however excessive inflammatory response can become fatal and cause tissue damage (52). It was found that RUNX1 regulates TLR4, a receptor that normally plays a role in initiating the innate immune response, and unfortunately a target of bacterial endotoxins which activate TLR4 to cause chronic and acute inflammatory disorders (53). RUNX2 has also been found to potentially act as a transcriptional coactivator for the production of IL-6 in macrophages. IL-6 is associated with the development of severe sepsis and organ dysfunction (52). Ro 5-3335 was able to maintain excessive inflammation (or septic shock) levels and protect LPS-induced endotoxic shock within mice by inhibiting RUNX1 (52). In pre-clinical trials mice received 5 mg/kg of Ro 5-3335 via the intraperitoneal route which attenuated expression of RUNX1 to control acute excessive inflammation (54). Overall, it can be observed that RUNX1 and CBFβ play a role in many viral, bacterial, and cancer associated

diseases. Ro 5-3335 inhibition activity against these transcription factors results in the decrease of disease progression.

Here, we show that Ro 5-3335 exhibits antiviral activity *in vitro* against acute and persistent Coxsackievirus B3. Ro 5-3335 has been shown to impart antiviral activity that make it a potential candidate against CVB3 infection. There would still need to be further pharmacological characterization of Ro 5-3335's molecular mechanism to fully understand its antiviral activity against acute and persistent CVB3 infection, including *in vivo* studies. We believe the compound interferes through a host cellular pathway that results in the reduction of viral genome levels and viral egress for both acute and persistent infection. We have seen that it decreases viral titers in a dose dependent manner, and exhibits nontoxic effects, both *in vitro* in our studies and other studies against HIV-1 and *in vivo* during pre-clinical trials in mice to control overexpression of RUNX1 during an inflammatory response. Additionally, it exhibits broad-spectrum antiviral activity *in vitro* in different cell types and against distinct viruses, suggesting that the drug may be repurposed for the treatment of a variety of viruses infecting different tissues. The relationship between CVB3 infection and RUNX1- CBFβ has not yet been studied, however, it would be interesting to see if RUNX1- CBFβ interacts with the viral genome of CVB3 or contributes to the viral progression within a host cell by interacting with the host genome, or even mediates the interaction between the viral and host genome, attenuating the antiviral immune response, providing a possible mechanism of how Ro 5-3335 lowers CVB3 infection if it is binding to RUNX1. Interestingly, it was found that RUNX1 and RUNX3 play a role in enhancing the proapoptotic activity of p53 (55). As we know CVB3 has evolved different strategies to inactivate p53 to prevent early apoptosis, allowing for effective viral replication (50). Therefore, a possible strategy is perhaps CVB3 is using RUNX1 as a way to regulate the

proapoptotic activity of p53, which allows it to downregulate or degrade p53 within host cells and continue CVB3 infection. Or Ro 5-3335 could be inhibiting RUNX2 along with RUNX1, as RUNX2 prohibits p53-dependent apoptotic cell death, allowing for p53 to induce virus-induced apoptosis, thus resulting in a decrease in viral infection in the cells (55). Overall, the interaction of RUNX1- CBFβ could be the cellular pathway in which Ro 5-3335 is inhibiting to reduce viral activity within acute and persistent infection.

Persistence has been observed in CVB3-infected PANC-1 cells, yet it is not well-described or understood; however, *in vitro* studies suggest that the mechanism of CVB persistence is dependent upon the multiple host-virus interactions. By studying acute versus persistent infection *in vitro*, we can study how viral infection differs. By studying the differences, we can gain insight on the mechanism of persistence and how the virus has evolved or adapted to this type of infection. Studies have suggested that CVB3 persistence is similar to a steady-state infection, where its replication cycle is altered and no longer lytic as a result of mutations or co-evolution. Our studies have shown us that a characteristic of persistence is that it has higher intracellular viral particles versus what is excreted extracellularly compared to the acute infection where it is the opposite. This supports a hypothesis of CVB3 persistence being implicated in having an altered replication cycle, that differs it from its normal lytic function, retaining higher viral particles within the cell than the outside environment. Further, studies have suggested that persistent virus infection is less robust compared to acute infection (56, 57). An intriguing question would be what triggers CVB3 to transition from a lytic state to a persistent state? Would there be a way to prevent this transition from occurring, thus preventing the prevalence of persistent infections? We know very little about persistence and how it occurs *in vivo*; however, persistent viral forms of CVB3 and other group B enteroviruses have been found

to persistently infect cardiac cells, which can be a precursor of dilated cardiomyopathy and aseptic myocarditis. Specifically, persistence of group B enteroviruses, including CVB3, have been linked to the deletion of 17 to 50 nucleotides in the 5' terminal of the virus (56,57). The 5' terminal is crucial for initiation of the replication and translation of the viral genome, however a region of the 5' known as stem-loop "d" remains intact which contains a binding site for the RNA polymerase precursor required for viral genomic RNA replication (56). This deletion has been verified within mice, human patients, and isolated viral variants. This deletion could explain the difference in the mechanism of viral replication of persistence versus acute infection. However, the exact mechanism of viral persistent infection within cardiac cells remains largely unknown. Regardless, these mutational differences in acute and persistent infection may be better understood to develop antivirals that target either or both types of infection.

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VITA

Maria del Mar Villanueva Guzman was born in Tucson, Arizona, on July 16th, 2000, to Isidro Villanueva Torrecillas and Maria del Mar Guzman Bueno. Her parents' eagerness led to her childhood experience of moving around the world, from Arizona to Belgium, Connecticut to California, Illinois, and soon New York. She attended Loyola University Chicago where she received a Bachelor of Science, *cum laude,* in Molecular Biology with a concentration in Bio Ethics and Global International Studies in May 2022. During her undergraduate studies at Loyola, she studied with Dr. Yoel Stuart and performed lake-to-lake morphological and parasitological variation research across 50 lakes in three-spined stickleback. At RUSH, she studied with Dr. Sarah Sansom and Dr. Micheal Lin and took part in the multicenter evaluation of *Candida auris* contamination in healthcare environments and patients.

Maria del Mar Villanueva Guzman remained at Loyola University Chicago in the Infectious Disease and Immunology Research Institute studying a Master of Science in the Infectious Disease and Immunology program. She joined Dr. Bryan Mounce’s lab to study enterovirus host-viral interactions and novel antiviral therapies. After completion of her degree, she will be attending Icahn School of Medicine in Mount Sinai’s Biomedical Science PhD Program in NYC to continue her studies as a PhD student in Microbiology.