Oregon Health & Science University
School of Medicine

Scholarly Projects Final Report

Title *(Must match poster title; include key words in the title to improve electronic search capabilities.)*

Characterizing a Novel, Broad Spectrum Antiviral Small Molecule

Student Investigator's Name

Kathleen Pommert

Date of Submission *(mm/dd/yyyy)*

03/17/2022

Graduation Year

2022

Project Course *(Indicate whether the project was conducted in the Scholarly Projects Curriculum; Physician Scientist Experience; Combined Degree Program [MD/MPH, MD/PhD]; or other course.)*

Scholarly Projects

Co-Investigators *(Names, departments; institution if not OHSU)*

Timothy Alexander, VGTI; Nicole Haese, VGTI

Mentor's Name

Daniel Streblow, PhD

Mentor's Department

Vaccine and Gene Therapy Institute

Scholarly Project Final Report

Concentration Lead's Name

Peter Mayinger

Project/Research Question

What are the antiviral mechanisms and molecular target of SR-33001 analogs?

Type of Project *(Best description of your project; e.g., research study, quality improvement project, engineering project, etc.)*

Basic science research

Key words *(4-10 words describing key aspects of your project)*

Antiviral, drug, broad spectrum, mechanism, HCMV, stress response, co-precipitation, Hsp27

Meeting Presentations

If your project was presented at a meeting besides the OHSU Capstone, please provide the meeting(s) name, location, date, and presentation format below (poster vs. podium presentation or other).

N/A

Publications *(Abstract, article, other)*

If your project was published, please provide reference(s) below in JAMA style.

N/A

Submission to Archive

Final reports will be archived in a central library to benefit other students and colleagues. Describe any restrictions below (e.g., hold until publication of article on a specific date).

Please hold archival of final report until desire to patent the compound can be confirmed with the institution on 06/01/2022.

Scholarly Project Final Report

Next Steps

What are possible next steps that would build upon the results of this project? Could any data or tools resulting from the project have the potential to be used to answer new research questions by future medical students?

- Confirm Hsp27 as molecular target by knockout cell line activity and thermal shift assay.
- Determine pharmacokinetic activity profile for SR-33001 analogs and perform antiviral efficacy studies in animals.

Please follow the link below and complete the archival process for your Project in addition to submitting your final report.

https://ohsu.ca1.qualtrics.com/jfe/form/SV_3ls2z8V0goKiHZP

Student's Signature/Date *(Electronic signatures on this form are acceptable.)*
This report describes work that I conducted in the Scholarly Projects Curriculum or alternative academic program at the OHSU School of Medicine. By typing my signature below, I attest to its authenticity and originality and agree to submit it to the Archive.

X _____

Student's full name

Mentor's Approval *(Signature/date)*

X _____

Mentor Name

Scholarly Project Final Report

Report*: Information in the report should be consistent with the poster, but could include additional material. Insert text in the following sections targeting 1500-3000 words overall; include key figures and tables. Use Calibri 11-point font, single spaced and 1-inch margin; follow JAMA style conventions as detailed in the full instructions.*

Introduction *(≥250 words)*

Viral infections cause a significant burden of morbidity and mortality across the world and have caused numerous infectious epidemics and pandemics in modern history, the COVID-19 pandemic being the current relevant example. In general, there is a paucity of effective antiviral agents to treat viruses that infect humans and animals. Herpes viruses are a family of viruses of particular interest to the medical community because of their widespread seroprevalence and impact on vulnerable populations. Human cytomegalovirus (HCMV) is a beta herpes virus that typically causes asymptomatic infections in humans. However, severe disease and congenital defects are observed in the immunocompromised and neonates. HCMV is a ubiquitous pathogen; adults in the United States and similarly developed countries are 50-70% seropositive[1]. Latent HCMV can reactivate under specific conditions of immunosuppression, especially in patients undergoing immunosuppressive therapy following organ and allogeneic hematopoietic stem cell transplantation. Leukopenia caused by reactivation puts many transplant patients at risk of life-threatening secondary infections[2]. In AIDS patients, HCMV causes life-threatening, disseminated visceral disease such as gastroenteritis, retinitis, and pneumonia. Furthermore, 0.1% of all infants born worldwide will be symptomatically affected by congenital HCMV infection, which can cause low birth weight, anemia, microcephaly, and chorioretinitis[1]. HCMV represents a significant disease burden among these populations and warrants study and therapeutic development.

Viruses like HCMV manipulate the host environment to favor their own transcription and translation relative to cellular genes, increasing replication efficiency and avoiding anti-viral immunity. Induction of cellular stress response by viruses can occur through factors like Protein Kinase R and the unfolded protein response (UPR)[3]. While the stress response pathway ultimately alters host translation, shifting it towards cell survival mode, viruses have evolved mechanisms to evade translational repression through mechanisms such as utilizing internal ribosomal entry sites (e.g. picornaviruses), development of other cap structures as well as by compartmentalizing viral transcription and translation[4]. In addition, many virus including alphaviruses, herpes simplex, and poxviruses cause host transcription/translational shutoff as if to promote stress response translational pathways specifically to ensure that the host cells nucleic acid and protein precursors and machinery are co-opted solely for virus replication. While there have been many studies aimed at characterizing how viruses deal with host translational inhibition, we still do not fully understand the underlying mechanisms nor do we know how to explicitly develop broad spectrum antivirals that exploit this complicated relationship.

Through high throughput screen campaigns for the identification of small molecule antivirals directed against Chikungunya virus (CHIKV), the Streblow lab in collaboration with Southern Research (SR) have identified a unique small molecule series (SR-33001) with broad spectrum antiviral activity that inhibits the replication of HCMV and other viruses in human fibroblasts. Extensive structure-activity relationship studies performed at SR and OHSU have identified highly active analogs of SR-33001 that retain activity and have better drug-like properties. We propose to characterize the molecular mechanisms involved in SR-33001 inhibition of human cytomegalovirus (HCMV) and to identify the molecular target.

Methods *(≥250 words)*

Antiviral Activity: Fibroblasts were simultaneously treated with varying concentrations of compound and infected with a predetermined MOI of viral strains maintained by the Streblow lab or other VGTI labs. This is done in triplicate. The infected and treated cells are incubated for 24-72 hours depending on viral strain. The cultures are then titered in Vero cells by diluting 10-fold repeatedly, then fixing and staining and

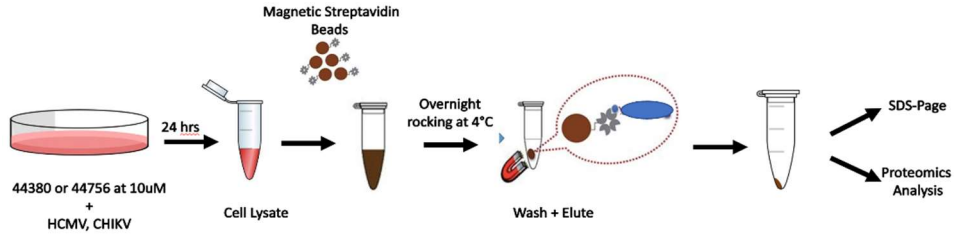
counting viral plaques. The average plaque forming units per milliliter of the triplicates is calculated for each compound dilution which is then graphically plotted so that the 90% inhibitory concentration can be calculated using Prism software.

Effect on viral transcription: Cell cultures infected with HCMV and treated with SR-43799, Foscarnet, or DMSO (control) at 24 and 48 hours post infection were harvested, lysed and RNA was isolated. Using an RT-PCR kit from ABI, and primers recognizing the HCMV UL83 gene, RNA copies of this gene were quantified for both time points of compound addition.

Effect on viral translation: Fibroblast cell cultures were infected with HCMV and subsequently treated with SR-43799, Foscarnet, or DMSO at 1, 2, or 3 days post infection. Cultures were harvested after treatment and lysed. Cell lysates were run on SDS-PAGE gel which was transferred to a western blot membrane using standard techniques. Membranes were blotted for HCMV proteins using purchased primary antibodies and secondary antibodies conjugated to HRP, which was then developed and imaged.

Proteomics and transcriptomics: Cellular lysates were obtained in the same manner as described above. Transcriptomic measurements were performed by the OHSU Massively Parallel Sequencing Resource. Quantitative proteomic measurements were performed by the Fred Hutchinson Proteomics Core (Seattle, WA). Analysis of 2-fold up or down regulated host proteins after treatment with SR-43799 was performed using STRING database (https://string-db.org/).

Co-precipitation pulldown assay: SR-44380 is the biotinylated SR-33001 analog, SR-44756 is the non-biotinylated version of this analog. Assays were performed with NHDF cell lysates and streptavidin beads from ThermoScientific. Mass spectrometry was performed by Fred Hutchinson Proteomics Core. Assay schematic shown below.



Results *(≥500 words)*

Antiviral Activity.

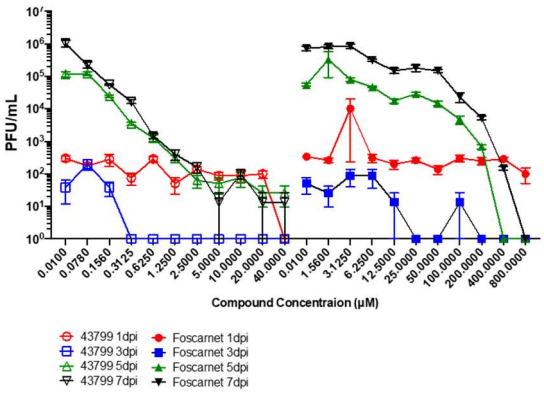
SR-33001 analog SR-43799 was tested *in vitro* for IC_{90} against several viruses across the Alphavirus and Flavivirus families. It shows potent nanomolar inhibitory activity. The results are displayed in Table 1. It was also tested *in vitro* against Herpesviruses. It was tested against HCMV at varying times of addition (1, 3, 5, and 7 days post infection), alongside Foscarnet. The compound shows potent inhibition of the virus at all times of addition, indicating that the compound is active even at later stages of infection. The IC_{90} of SR-43799 for HCMV is 0.88 µM. The results are shown in Figure 1. The compound is also active against MouseCMV, RatCMV, and HSV-2, the IC_{90}s for which are 1.98, 0.8, and 0.97 µM respectively. Thus, the SR-33001 analog SR-43799 demonstrates broad spectrum antiviral activity across multiple viral families. These findings indicate that the target of this molecule may be a host protein, as there is no expected homology of proteins across these families.

Table 1. SR-33001 Analog IC_{90} for Alpha and Flaviviruses

	CHIKV	VEEV	DENV	WNV	ZIKV
SR-43799 IC_{90} (µM)	0.05	0.02	0.3	0.15	0.15

CHIKV= Chikungunya; VEEV= Venezuelan equine encephalitis virus;
DENV= Dengue virus; WNV= West Nile virus; ZIKV= Zika virus.

Figure 1. Viral Titer Reduction (Plaque Forming Units/milliliter) of HCMV by SR-43799 and Foscarnet at Varying Times of Addition



Effects on Transcription and Translation.

To investigate the effects of SR-33001 analogs on viral transcription and genome replication, we performed qPCR for several HCMV gene transcripts and the whole viral genome and compared treatment with SR-43799 against Foscarnet, a known HCMV DNA polymerase inhibitor. SR-43799 had no effect on gene transcription or genome replication compared to controls, whereas Foscarnet demonstrated inhibition as expected. An example of qRT-PCR for an HCMV gene transcript is shown in Figure 2. Next, we investigated the effects of SR-43799 on viral protein translation. Western blots of HCMV immediate early, early, and late gene products were performed comparing SR-43799 treatment to control and Foscarnet at several times of treatment (1, 2, 3 days post infection) (Figure 3). IE1 and IE2 are immediate early expressed proteins that act as transcription regulators, gB is an early expressed surface glycoprotein, and pp28 is a late expressed tegument phosphoprotein. SR-43799 has minimal effect on the expression of the immediate early gene products, but significantly inhibits the early and late gene products. This pattern was consistent when the experiment was performed with other HCMV proteins as well.

Next, we studied the effect of SR-43799 treatment on the viral and host transcriptome and proteome. Figure 4 is a plot of the fold change of RNA transcript quantity with SR-43799 treatment compared to no treatment on the x-axis with the same comparison of protein expression on the y-axis. Human gene transcripts and proteins are identified with red dots, which as a population cluster over 0-fold change on both axes. HCMV viral gene transcripts and proteins are identified with blue dots, which cluster in the lower, right quadrant indicating an increase in the viral transcriptome and decrease in the viral proteome with SR-43799 treatment compared to control.

Because of our suspicion that the target of the SR-33001 series may be a host protein, we identified proteins which were differentially expressed (2-fold either up or down) with SR-43799 treatment compared to none. To characterize the function of these proteins whose quantity is affected during viral infection by treatment with SR-43799, we used the STRING database to group the proteins by their association with KEGG pathways. The top five KEGG pathways in which the differentially expressed proteins are enriched are shown in Figure 5. They are: PI3K-Akt signaling pathway, pathways in cancer, Rap1 signaling pathway, focal adhesion, and Ras signaling pathway.

Figure 2. qRT-PCR of HCMV UL83

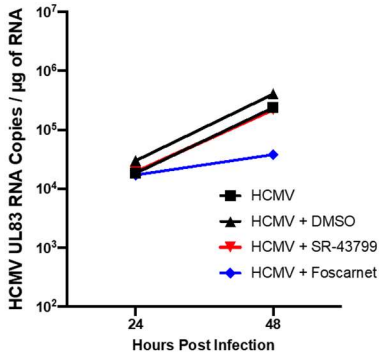


Figure 3. Western Blots of HCMV Proteins

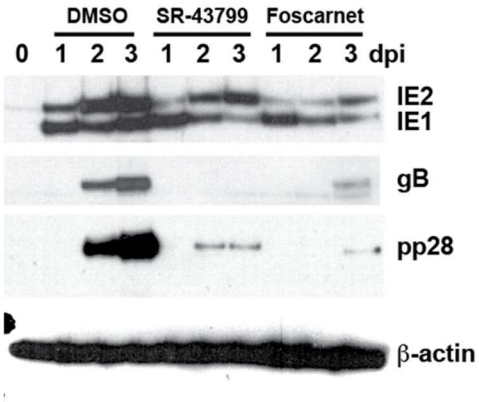


Figure 4. Fold Change of Infected Cell Transcriptome Versus Proteome with SR-43799 Treatment

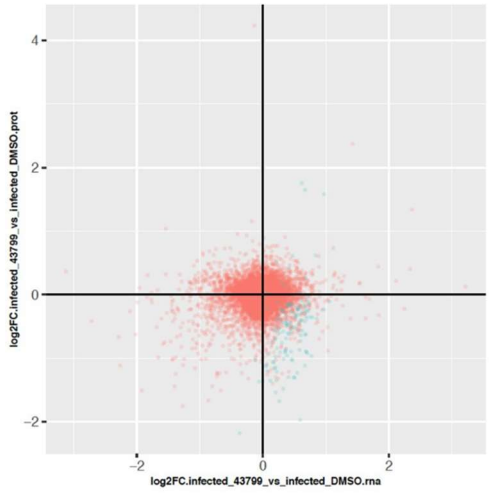
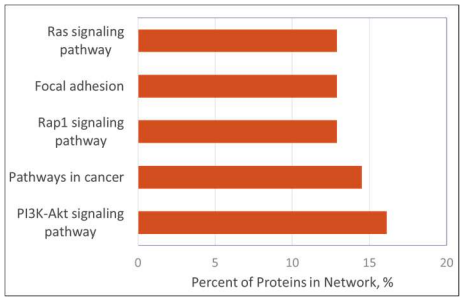


Figure 5. KEGG Pathway Involvement of Differentially Expressed Host Proteins



Target Identification.

To identify the target of SR-33001 series antiviral action we performed a co-precipitation pulldown assay using a biotinylated analog in culture with both HCMV and CHIKV. The eluate from the assay was run on SDS-PAGE, which is shown in Figure 6. A band at approximately 25kDa (arrow in figure) was present for both culture eluates in the biotinylated compound treated sample but not the non-biotinylated compound treated sample. These bands were excised and sent for mass spectrometry. The results of the mass spectrometry are shown in Table 2. The protein that was most identified by percent sequence coverage and number of peptide specific matches was heat shock protein beta-1 (also known as Hsp27). The pulldown assay was then repeated and a western blot was also performed for Hsp27, which was present only in the biotinylated compound sample, the results are shown in Figure 7.

Figure 6. SDS-PAGE of Pulldown Eluate

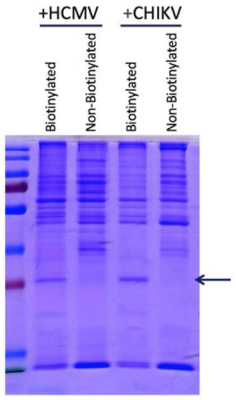
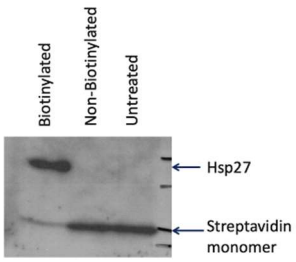


Table 2. Mass Spectrometry of Excised SDS-PAGE Bands

Gene	Name	% Coverage	#PSMs
HSPB1	Heat shock protein beta-1 (Hsp27)	74.15	214
CHCHD3	MICOS complex subunit MIC19	38.77	14
RPS8	40S ribosomal protein S8	41.35	12
RTRAF	RNA transcription, translation, and transport factor	24.59	11
RBPMS	RNA-binding protein with multiple splicing	38.27	9

Figure 7. Western Blot for Hsp27 in Pulldown Eluate



Discussion *(≥500 words)*

As the COVID-19 pandemic has demonstrated, there is a significant need for novel antiviral therapeutics, and small molecules have the advantage over other forms of therapies as being easy to produce and distribute at large scale. SR-33001 analogs show broad spectrum *in vitro* antiviral activity against Alpha, Flavi, and Herpes viruses. Of note, they were tested and are not active against Coronaviruses. The broad spectrum of activity of these small molecules points to a cellular host protein as the target of their action. The compounds have no effect on viral nucleic acid transcription or replication, especially when compared to Foscarnet, a known inhibitor of these processes. They do, however, appear to inhibit translation of certain viral proteins. HCMV's large genome is transcribed and translated in a staged viral replication cycle: immediate early, early, and late. SR-43799 inhibits the translation of early and late proteins, but not immediate early. We think this may be because SR-43799 is interfering with the virus's ability to use the cellular stress response to its advantage to produce these later stage proteins. This is significant because many different viruses exploit this phenomenon, thus any inhibitors that are developed are highly likely to be of broad spectrum against a number of clinically relevant human pathogens, many of which have no other antiviral treatment options or vaccines to reduce or prevent infection.

On the proteomic and transcriptomic level, SR-33001 analogs have no regulatory effect on host cellular proteins or nucleic acid transcripts as a whole population. There were 42 host proteins which were differentially expressed, either 2-fold up or down regulated, during viral infection and treatment with SR-43799. Analysis of known protein-protein interaction networks using the STRING database found that these proteins are enriched in several KEGG pathways, predominantly involving cellular signaling. Given our hypothesis that the compound is interfering with cellular stress response, which is a state enacted by cell signaling pathways, it may be that the target of the compound is a participant in a signaling pathway that mediates the cellular stress response.

This hypothesis is consistent with our finding of HSPB1 as the possible target identified by pulldown assay. This co-precipitation was confirmed by replication and western blot. HSPB1 has been shown to have a multifaceted function including as a protein-folding chaperone, an apoptotic regulator, and cell-signaling mediator[5]. The role of small heat shock proteins in viral infection is an emerging field of study, but studies have shown that several HSP isoforms play important roles with few showing pro-viral activity whereas others seem to have an anti-viral role[6]. In proteomic analysis of infected cells treated with SR-43799, we saw no significant difference in HSPB1 expression with infection or treatment, so if this is indeed the target, the effect of the compound must be enacted by inhibiting function rather than expression. Further work is needed to confirm that HSPB1 is the target of the SR-33001 series and to investigate the mechanism of its antiviral action. Identifying this host:virus relationship and novel inhibitors that block these interactions are potentially paradigm shifting and highly relevant for the development of new antiviral regimens.

Conclusions *(2-3 summary sentences)*

- SR-33001 analogs have antiviral activity across multiple viral families, indicating the target is likely a host protein.

- The compound does not affect viral transcription but does inhibit translation of early and late HCMV structural proteins while immediate early proteins are less affected.
- Host proteomic expression is not significantly affected, however those proteins that are differentially expressed primarily belong to cellular signaling pathways.
- HSPB1 (Hsp27) may be the cellular target for this small molecule.

References *(JAMA style format)*

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2. Crawford LB, Caposio P, Kreklywich C, et al. Human Cytomegalovirus US28 Ligand Binding Activity Is Required for Latency in CD34(+) Hematopoietic Progenitor Cells and Humanized NSG Mice. *mBio* 2019;10(4) doi: 10.1128/mBio.01889-19[published Online First: Epub Date]|.

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6. Iyer K, Chand K, Mitra A, Trivedi J, Mitra D. Diversity in heat shock protein families: functional implications in virus infection with a comprehensive insight of their role in the HIV-1 life cycle. *Cell Stress Chaperones*. 2021;26(5):743-768. doi:10.1007/s12192-021-01223-3