Spring 5-18-2024

Repurposing Clinically Relevant Metabolic Inhibitor Drugs, Difluoromethylornithine (DFMO) and Orlistat, for gammaherpesvirus Replication

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By

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A project submitted in partial fulfillment of the requirements
of the Bachelor of Arts degree in Honors Liberal Arts
Seattle Pacific University
2024

Presented at SPU Honors Symposium
May 18, 2024

Abstract:

Viruses, including herpesviruses, contribute up to 15% of all human cancers. Murine gammaherpesvirus-68 (MHV-68), a pathogen commonly found in mice, is studied due to its shared homology with several human herpesviruses. Studies done in the Delgado lab via metabolomics analysis show MHV-68 infected cells increase host cell metabolism. Clinically relevant metabolic inhibitor drugs, α-Difluoromethylornithine (DFMO) and Orlistat, respectively block polyamine and lipid production demonstrated a reduction in MHV-68 viral production. Repurposing clinically relevant drugs through the exploration of a different target shows great promise in reducing oncogenic viral titer.

Introduction:

Viruses are obligate intracellular parasites, meaning that they hijack the host's metabolism to upregulate so that they can replicate. Specifically, the oncogenesis virus prefers the increased metabolites due to increased resources for viral proliferation. Kaposi sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are one of the most common types of oncogenic gammaherpesviruses (GHV). Utilizing human herpesvirus in laboratory research is impractical due to its inability to replicate and form plaques. However, previous works have focused on GHV using Murine Herpesvirus 68 (MHV-68) as a mouse model. MHV-68 can be used as a human herpesvirus model because it is 80% homologous to KSHV and EBV and can be used to study the Y-herpesvirus infection[1]. Unlike EBV and KSHV, the model virus can undergo lytic replication and form plaques, allowing researchers to determine viral titers. Previous research reveals that MHV-68 activates the host's metabolic pathways which leads to increased viral replication[1,2]. MHV-68 increases many metabolites such as nucleic acid, glucose, glutamine, as well as lipids and polyamines[1,2,3].

Lipogenesis, the creation of fats, not only benefits cellular needs like energy but is also important for viral production. During fatty acid synthesis, citrate leaves the Krebs cycle in the mitochondria and is made into acetyl CoA-A in the cytoplasm by ATP citrate lyase (ACLY). Then acetyl-CoA is converted into malonyl-CoA by the enzyme Acetyl-CoA Carboxylase (ACC). Afterward, Acetyl-CoA and malonyl Co-A are repeatedly combined by the enzyme fatty acid synthase (FASN) until it produces a 16-carbon fatty acid called Palmitic Acid, which can then be used in membrane and energy storage applications [4]. MHV-68 infected cells increase FASN protein expression compared to mock-infected cells [2]. During metabolomics detection, 177 out of 183 lipid species were seen to increase compared to the mock infected cells [1]. FASN is a crucial metabolic enzyme that contributes to the formation of long fatty acid chains that the virus can use as a resource to accelerate viral replication [1,3]. Without fatty acid synthesis, the cell will undergo apoptosis and the host cell then would be unable to provide the necessities for further viral replication. Therefore, inhibition of FASN or fatty acid synthesis can significantly reduce viral replication. Data has shown that TOFA (5-tetradecyloxy-2-furoic acid), an allosteric inhibitor of ACC (Acetyl-CoA carboxylase), inhibits virus production [1]. However, TOFA is not clinically relevant and cannot be used in patients due to its high toxicity. In contrast, Orlistat has been predominantly used as a weight loss drug because of its fatty acid synthase (FASN)

inhibiting properties [5,6]. High quantities of fatty acids and lipid production can encourage cancer cell growth and proliferation, thus Orlistat has been successful in treating various tumor cells, such as prostate cancer cells, ovarian cancer, and others [3,6]. Orlistat has also been proven to be effective in decreasing the percentage of viral infection and production [6]. Previous clinically relevant studies have also shown Orlistat is successful in decreasing the production of coxsackievirus B3 and varicella-zoster virus [7]. As a FASN inhibitor, Orlistat is able to prevent the accumulation of high levels of fatty acid and lipids needed for viral replication. This component of Orlistat is significant and key to providing further treatment to reduce GHV production and induced cancers.

Another alteration is that MHV-68 increases polyamine metabolism. Polyamines metabolite was seen to increase up to a 3 fold during MHV-68 infection [1]. Polyamines are important in the cell due to their involvement in biological mechanisms such as DNA replication and protein synthesis. Furthermore, polyamine stimulates cellular division, playing a crucial role in the promotion of oncogenic viruses [9]. All polyamines increased 8 hours after virus infection,this includes arginine, ornithine, putrescine, and spermidine[1]. Difluoromethylornithine (DFMO) is a FDA approved drug that has been used to treat African trypanosomiasis and is involved in various anticancer studies [10]. It is an inhibitor of the enzyme ornithine decarboxylase (ODC), which converts L-Ornithine to Putrescine [9]. Without this enzyme, polyamine cannot be produced to be used by the virus. By inhibiting ODC, the rest of the polyamine pathway is likewise inhibited, which hinders the synthesis of spermidine and spermine. Multiple peer-reviewed articles have shown evidence that DFMO works to reduce viral replication[11,12,13]. Researchers treated Vero-E6 cells with various doses of DFMO concentrations for a coronavirus infection and at 24 hours post infection (hpi), they observed that DFMO significantly reduced the viral genome greater than 50-fold at 5mM [11]. Their discussion reveals that polyamines are important for viral infection and that depleting the polyamines reduces viral particles. The interest for this project is whether the FDA-approved drug, Orlistat and DFMO, will be able to block MHV-68 lytic replication. Since MHV-68 infection increases lipogenesis and polyamine biosynthesis, treatment of MHV-68 infected cells with the clinically relevant metabolic inhibitors like Orlistat and DFMO is hypothesized to decrease MHV-68 production. Moreover, this mouse model is also applicable to human herpesvirus, mitigating Kaposi's Sarcoma, and hindering the replication of KSHV and EBV.

Materials & Methods:

Drug Safety Methods. The drug safety method was performed by seeding 760,000 NIH3T3 cells in 6 x 6 cm plates with three mL of NIH3T3 media. The concentration of the stock DFMO (TOCRUS No. 2761) was 71.8mM with deionized water as the control solvent. The concentration of Orlistat (Selleckchem No.S1629) was 101mM with Dimethyl Sulfoxide(DMSO) as the control solvent. After 3 hours up to 8 hours when the cells were attached, different drug concentrations were placed into 6 x 15mL conical tubes and mixed to a total of 3 mL media, vortexed, and let stand for 30 minutes before treating the NIH3T3 cells. Media from the seeding was replaced with the drug mixtures and placed in an incubator. At 24 hours and 48 hours, observations and pictures were taken using an Olympus CKX41 optical microscope equipped with an Olympus EP50 camera at 40x magnification.

Infection, Supernatant Harvest, and Cell Counts. NIH3T3 were infected with MHV-68 at a multiplicity of infection (MOI) of 0.1. Cells were trypsinized, and supernatants were harvested by spinning it at 3000 rpm for 10 min at 4 °C then storing it at -4 °C for further plaque assays. Cell counts for cell viability were done by collecting cells from the 6 cm dishes and centrifuging them at 3000 rpm. Cells were stained with Trypan blue exclusion assays with a ratio of 1:1 with live and dead cells counted using an automated Biorad T20 cell counter (Bio Rad no.1450102) by loading in 10 uL of mixture onto the cell slide (Bio-Rad no.1450003). Total, live, and percent viability of cells were automatically counted with the Bio-Rad TC20 cell counter.

Plaque Assay. 300,000 Vero cells were seeded in a 12-well plate with 3mL of DMEM media 24 hours prior infection. For infection, serial dilutions of (0, -1, -2, -3, -4, -5, -6) from viral harvest were performed. Viral dilutions were then aspirated, and cells were overlaid with DMEM–glucose–glutamine–pyruvate (Thermo Fisher no. 12800017) –1% methylcellulose(MC) (Sigma no. M0387)–sodium bicarbonate (Sigma no. S5761)–2.5 µg/mL Amphotericin B (Thermo Fisher no. 15290026) for a week. After 7-8 days, cells were fixed in 10% formalin for 30 minutes, then the overlay was removed. Cells were stained with 1% crystal violet (Sigma no. C0775) in 20% methanol and rinsed with deionized water before drying. After drying, plaques were counted, and viral titers were calculated.

Data Analysis. The numbers of countable plaques which were approximately 10 to 100 were used to perform the viral titer calculation. Plaques were counted for each dilution in duplicates, averaged, then multiplied by the dilution factor to get the plaques forming units per ml (pfu/mL). Using GraphPad, a paired t-test was determined and calculated for significance as well as standard error mean. Normalization of cells was completed by taking the pfu/ml and dividing it by the numbers of cells from the cell count.

Results & Analysis:

Clinically Approved drug shows high safety levels for NIH3T3 cells.

A concentration range was established using previous experiments and research articles as references to determine a safe dosage of DFMO and Orlistat to be applied to NIH3T3 cells. The concentrations examined ranged from 0, 10µM, 100µM, 250µM, 500µM, and 1mM for DFMO. Another trial replicated the experiment, utilizing concentrations of 0uM, 100µM, 250µM, 500µM, and 750µM. The data was analyzed subjectively for the kill curve by looking at the proliferation, which included estimates of cell confluency as well as the health of the spindles. At 48 hours, the confluence of the cells increased to 100% for the control but proliferation was altered at 750 µM indicated by the lack of cell growth and increased gaps seen in Figure 1. When comparing the 750 µM to the control, there was a decrease in confluency by 40%, accompanied by the emergence of more spider-like spindles, indicating stressed cells. Additionally, there was an increase in the presence of floaters or dead cells. The 1 mM concentration exhibited similarities to the 750 µM, albeit with a slightly higher count of floater cells for kill curve trial two. The optimal selected concentration for the safety drug trial was 750 µM for DFMO based on this study.

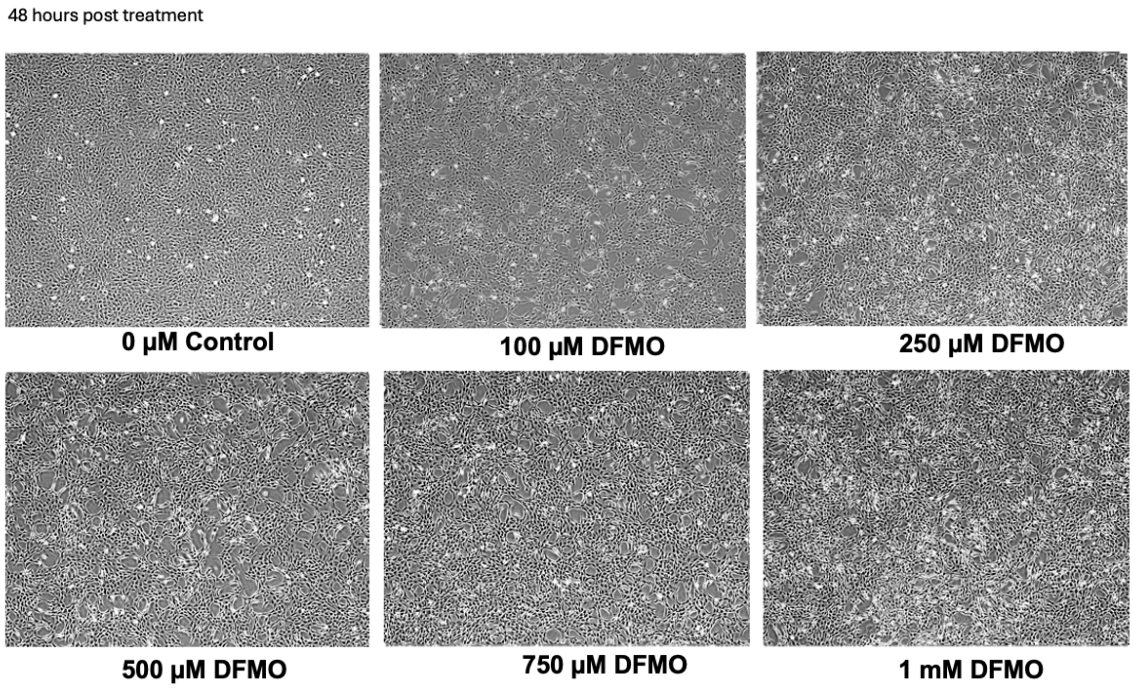


Figure 1. Assessment of cell viability and health of NIH3T3-mock infected cells with DFMO. Non-infected NIH3T3 cells were treated with different 71.8 mM stock DFMO concentrations. After 48 hours, optical microscopy images were obtained at 40x magnification.

For Orlistat, concentrations ranged from 0, 100µM, 200µM, 300µM, 400µM, and 500 µM. Various published paper indicates the start of an effective concentration in the 20-100 µM range. A similar trend of inhibition of proliferation resulted when the concentrations of Orlistat were increased. At a higher level of 200-300 µM, higher cell inhibition resulted. However, there was no difference between the 400 µM and the 500 µM. Spindles of the cells started to increase around 300 µM but by a minimal amount. Floater cells were minimal in all ranges of the drug with little difference between the concentration ranges. However, the maximum permissible concentration for Orlistat is 500 µM, constrained by the limit of the DMSO solvent. Previous experiments show exceeding a concentration of more than 0.1% of DMSO can induce cell death. The final chosen concentration from the safety drug trial was 300 µM for Orlistat based on these results.

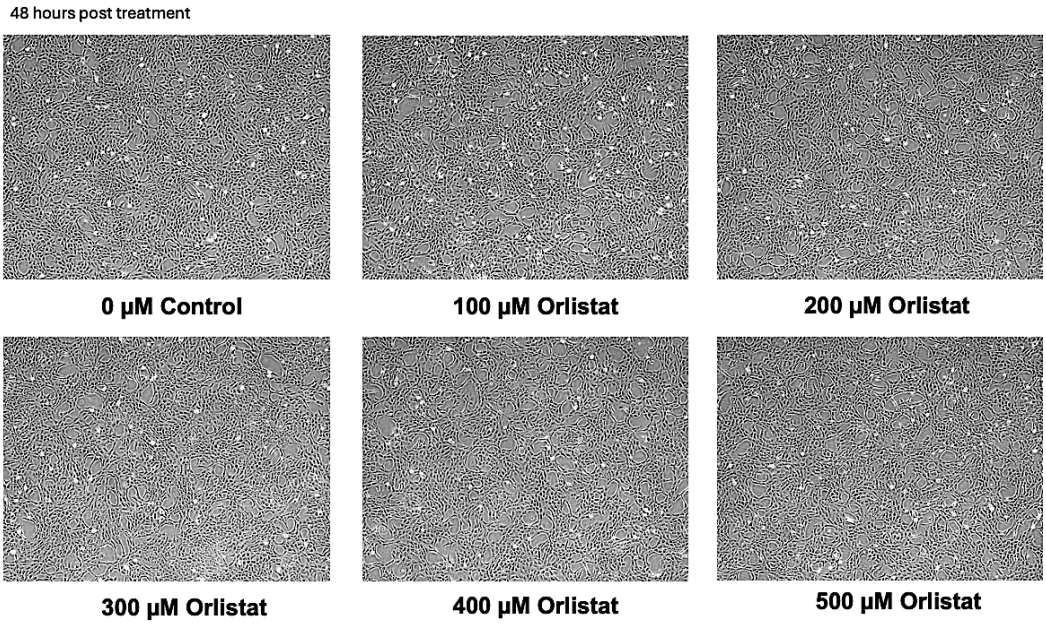


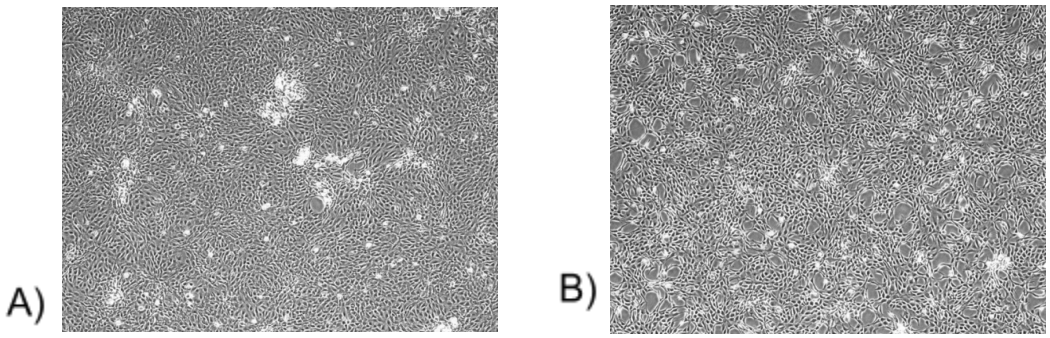
Figure 2. **Assessment of cell viability and health of NIH3T3 mock infected cells with Orlistat.** Non-infected NIH3T3 cells treated with different 101 mM stock Orlistat concentrations. After 48 hours, optical microscopy images were obtained at 40x magnification.

For the mock infected and infected NIH3T3 cells that were treated with dH_2O and drugs, similar observation strategies were made. In addition, the cytopathic effects (CPE) for the infected cells were examined for both drug-treated and control for the infection. Qualitative analysis of the cells was used to see the impact of the drug. The mock-infected cells were very confluent and do not seem to portray any stress for the dH2O exposure, however, there is decreased proliferation for the drug-treated cells from cell counts (Table 1). When infected cells were treated with DFMO, the same trend of inhibition of proliferation occurred with a decrease in 14% of cells. In contrast for Orlistat, only 3% of a decrease was seen.

Table 1. Cell counts of infected-NIH3T3 with control and specific drug concentrations. Number of live cells calculated for different conditions using an automatic cell counter.

Condition	Live Cell(#)	Live Cell(%)
MHV-68 (H2O control)	3.49 x 10^ 6	100%
MHV-68 (750 µM DFMO)	2.99 x 10^ 6	100%
MHV-68 (DMSO control)	1.90 x 10^ 6	100%
MHV-68 (300 µM Orlistat)	1.84 x 10^ 6	98%

DFMO and Orlistat show a moderate decrease in viral production.
Qualitative analysis for DFMO shows that the control infected NIH3T3 cells have many cytopathic effects (CPE) with low confluency and extremely stressed spindles (Fig. 3A). When DFMO was added to the infected cells, only proliferation was blocked, and CPE was drastically reduced. After plaque assays were conducted, the results show a decrease in viral titers. There is a decrease in viral pfu/ml with the control compared to the DFMO- treated infected cells in all trials (S1). Trial A shows a difference of 8825 pfu/mL. Trials B and C had a difference of 8,000 pfu/mL and 6,025 pfu/mL respectively. Trial D had less of a viral titer reduction of 2,420 pfu/mL. The mean for the four trials is 20% of DFMO-treated compared to the control which means that the viral titer decreases by around 80% (Figure 3A). The t-test shows that the p-value was <0.0006 and the standard mean error is 5.2. After normalizing the cell count by taking the pfu/mL divided by the cell number, the actual change was found to be 3.6x(S3).



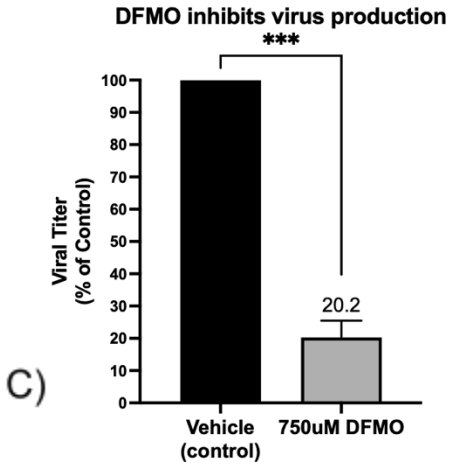


Figure 3. Quantitative and qualitative assessment of viral titer reduction for infected-NIH3T3 cells after DFMO Treatment. NIH3T3 cells were MHV-68 infected (MOI = 0.1) in 6 cm dishes. Cells were treated with dH2O control (**3A**) or 750 μM DFMO treated (**3B**) for 24 hours. Optical images were obtained at 40x using the Olympus CKX41 microscope and EP50 camera. (**3C)** Plaques were determined from plaque assays and calculated to find a reduction of viral titer (%).

Furthermore, Orlistat exhibits a similar pattern of cell inhibition and reduction in viral titer (Fig. 4, S1). Across all trials (S1), there is a consistent decrease in viral plaque-forming units (pfu/mL) in Orlistat-treated infected cells compared to controls. In Trial A, the difference is 4300 pfu/mL, while Trials B and C show differences of 2250 pfu/mL and 2700 pfu/mL, respectively. In all three trials, virus titers were significantly higher in the control group compared to the drug-treated group. On average across the trials, Orlistat-treated samples exhibit 36% of the viral titer observed in the control, indicating a reduction of approximately 64% (Fig. 4C). The t-test results indicate a p-value of <0.0001, with a standard mean error of 3.2. Following the normalization of the cell count by dividing pfu/mL by the cell number, the change is calculated to be 2.3x(S4).

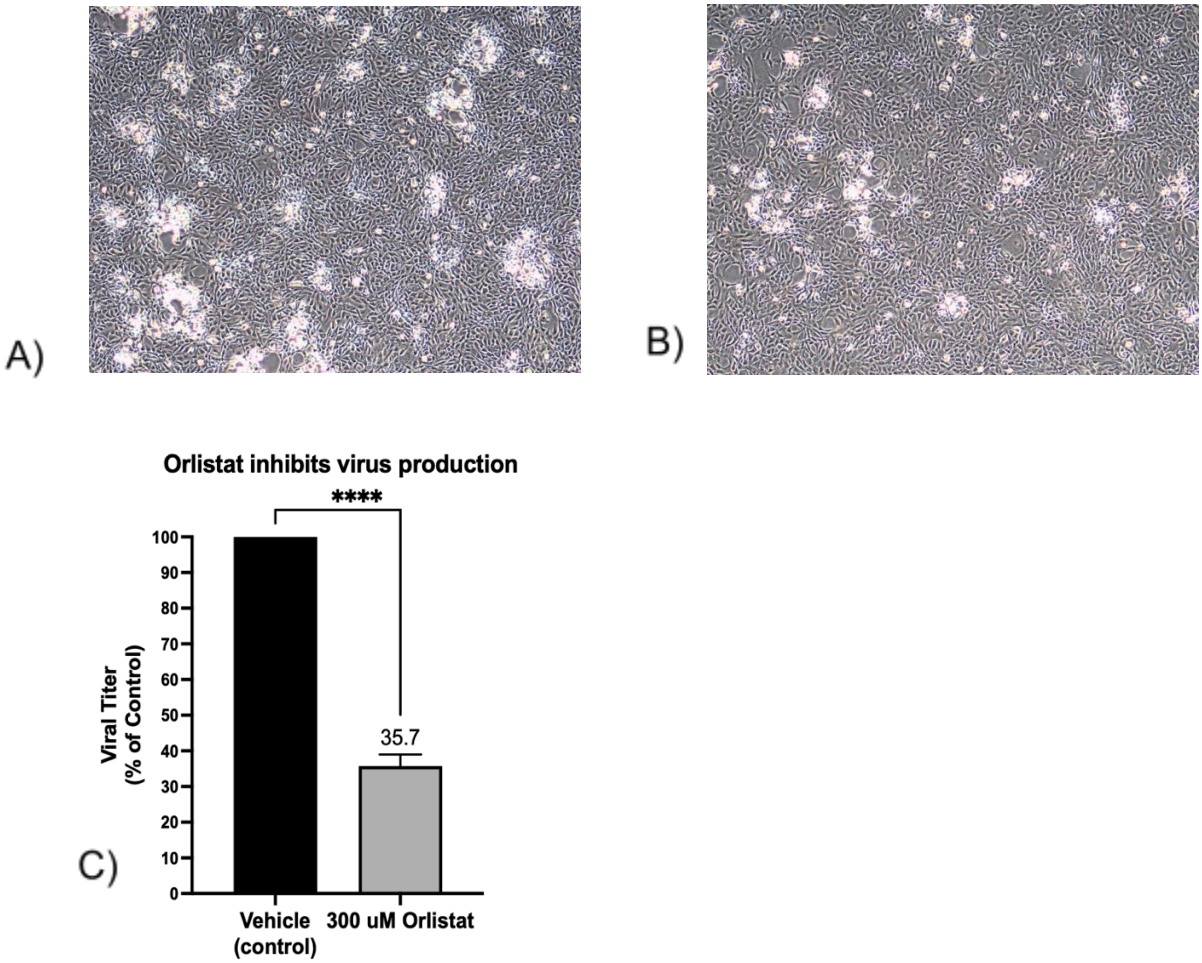


Figure 4. Quantitative and qualitative assessment of viral titer reduction for infected-NIH3T3 cells after Orlistat Treatment. NIH3T3 cells were MHV-68 infected (MOI = 0.1) in 6 cm dishes. **(4A**) Cells were treated with DMSO control or (**4B**) 300 µM Orlistat treated for 24 hours. Optical images were obtained at 40x using the Olympus CKX41 microscope and EP50 camera. (**4C)** Plaques were determined from plaque assays and calculated to find reduction of viral titer (%).

Discussion:

Increasing the drug concentrations to higher doses still maintained the integrity of the cells relatively well. Throughout the 48 hours following infection for the DFMO and Orlistat kill curve experiments, there wasn't a notable increase in the number of floating cells, suggesting minimal cell death. Both DFMO and Orlistat exhibited low levels of toxicity, suggesting that it might be feasible to administer higher doses to evaluate their efficacy in reducing viral titers. However, it's important to note that since DMSO serves as the control for Orlistat, the highest achievable concentration is limited to 500 μM. The only noticeable change was a decrease in confluency, indicating that cell proliferation is inhibited rather than the drugs being excessively cytotoxic. However, at certain concentrations, the cells began to spindle, taking on a needle-like appearance, suggesting they were under stress and lacking the necessary metabolites for normal function.

It's important to note that estimating cell floaters and confluency (live cells) is subjective, and further cell counts with automatic cell counters were used to analyze the ratio of dead and live cells accurately. For DFMO, cell confluency decreased by 14% with drug treatment, likely due to the inhibition of polyamine and putrescine formation. Cell numbers for Orlistat decreased by 3.2% with drug treatment compared to the control, which is within the normal range, with only a few cells behaving this way. As proliferation decreases, viral titers are expected to decrease since fewer cells are available for the viruses to infect.

The experiment yielded success as evidenced by the decrease in viral titer observed when infected cells were treated with the drugs. Treatment resulted in a reduction in cytopathic effects (CPE), objectively indicating a decrease in viral production, as fewer cells showed signs of stress. Viral titers varied across trials, likely due to factors such as the use of freeze-thaw vials and the concentration of virus in each vial. However, the trend of reduced viral titers was consistent across all trials. Upon normalization to account for cell inhibition, the change of viral titer decreased by 3.6x(Table S3) and 2.2x (Table S4)for DFMO and Orlistat, respectively. This suggests that both clinically approved drugs exhibit moderate virus inhibition but inhibit proliferation. Overall, DFMO and Orlistat demonstrate safety and effectiveness as inhibitors of viral production, holding promise for future therapeutic applications against viral infections.

Future experiments will aim to determine the specific stage of the viral cycle at which DFMO and Orlistat exert their inhibitory effects. Several phases of a viral infection may be investigated to determine the exact stage that DFMO and Orlistat could potentially inhibit. Real time polymerase chain reaction(qRT-PCR) will be used to detect viral gene expression at various time points during the virus life cycle. Different time points of viral gene expression are immediate early (IE), early (E), and late (L) which are associated respectively with the expression of viral proteins. A decrease in any of these proteins, will indicate which part of the viral life cycle the metabolic inhibitor drug affects. For example, if lipogenesis is blocked at a certain point in the viral life cycle, then the data would show us that lipogenesis is needed for viral replication at that specific point in its life cycle. Overall, metabolic inhibitors may offer novel treatments for gammaherpesvirus infections and potentially serve as broad-spectrum antiviral therapies.

Supplementals:

Table S1. Plaque counts and trials for DFMO and water control. Average pfu/mL for control(ddH_2O) and DFMO and percent of pfu compared to control(%).

Trial	Condition	Dilution Factor	Plaque count 1	Plaque count 2	pfu/mL	
A	Control (H2O)	100	20	26	11,500.00	100%
A	750 uM DFMO	10	54	53	2,675.00	23.26%
B	Control (H2O)	100	17	23	10,000.00	100%
B	750 uM DFMO	10	46	36	2,050.00	20.50%
C	Control (H2O)	100	15	20	8,750.00	100%
C	750 uM DFMO	10	54	55	2,725.00	31.14%
D	Control (H2O)	10	52	51	2,575.00	100%
D	750 uM DFMO	1	28	34	155.00	6.02%

Table S2. Plaque counts and trials for Orlistat and DMSO control. Average pfu/mL for control(DMSO) and Orlistat and percent of pfu compared to control(%).

Trial	Condition	Dilution Factor	Plaque Count 1	Plaque Count 2	pfu/mL	
A	Control (DMSO)	100	16	13	7250	100%
A	300 µM Orlistat	10	66	52	2950	40.69%
B	Control (DMSO)	10	65	63	3200	100%
B	300 µM Orlistat	10	21	17	950	29.69%
C	Control (DMSO)	10	88	83	4275	100%
C	300 µM Orlistat	10	30	33	1575	36.84%

Table S3.Calculation of normalized reduction in virus production for DFMO. Fold changes were calculated by dividing numbers of pfu/ml/live cells.

Condition	Total Cells (Average)	Live Cells (Average)	Viability (%)	pfu/mL (Average)	pfu/mL (Live Cell # RATIO)	Normalized reduction in virus production (fold change)
Mock (Vehicle)	1692000	1689000	99.8%			
Mock (DFMO 750uM)	1610250	1608000	99.9%			
MHV-68 (Vehicle)	1831500	1828500	99.8%	8206.25	0.00449	
MHV-68 (DFMO 750uM)	1506750	1506000	100.0%	1894.375	0.00126	**3.6**

Table S4. Calculation of normalized reduction in virus production for Orlistat. Fold changes were calculated by dividing numbers of pfu/ml/live cells.

Condition	Total Cells (Average)	Live Cells (Average)	Viability (%)	pfu/mL (Average)	pfu/mL (Live Cell # RATIO)	Normalized reduction in virus production (fold change)
Mock (Vehicle)	1459000	1459000	100%			
Mock (Orlistat 300 uM)	1099000	1097570	99.9%			
MHV-68 (Vehicle)	1121000	1117090	99.7%	4908.3	0.004393	
MHV-68 (Orlistat 300 uM)	959000	940100	98%	1825	0.001941	2.3

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Appendix 1

Panel's Title: Pathways to Innovation: Unveiling Science's Journey from Inquiry to Truth

Good afternoon everyone, my name is Lay and I will be presenting about my research opportunity in the Delgado lab at SPU, but first, I'd like to talk about where my fascination for science first arose. The first time I saw a diagram of what a virus looked like was in high school biology class and it was a bacteriophage. So as you can see, this is what the bacteriophage looks like. Something so small can be so intricate and have such a huge impact on our lives. When I got to SPU, during my sophomore year, I applied to Dr.Delgado's lab, but was rejected because I had a hard time interpreting what the research papers were saying about viruses, metabolomics, and the data. However, I persevered again and applied my junior year and got the position. I've learned so much and wanted to share my experience with you.

The realm of science is captivating for me, constantly unveiling discoveries. Whether an experiment succeeds or fails(which many times they do), the data it produces holds valuable insights for others to learn from. I'll share insights from my research journey, intertwining it with the broader scientific pathway. To begin, I'd like to present an overview of the pathway . This diagram illustrates the structure of the journey from initial inquiry to the culmination of research outcomes, which is very similar to the scientific methods. I will guide you through each step, providing insight into my journey and allowing you to experience the process of generating data. Through this exploration, I aim to foster empathy towards the tedious research experience and highlight the significance of scientific exploration.

My research project is about Identifying clinically relevant metabolic inhibitors that can therapeutically block Mouse herpes Virus(or MHV-68) production**.** In other words, using drugs that are already safe and approved and repurposing them. To do that, I would need to know a lot of background information. It would be impossible for me to start from scratch all by myself so using previous knowledge like published papers as well as Dr. Delgado, who is an expert in the field, allowed me to expand my knowledge.

Something important I've learned is that viruses contribute up to 15% of global cancer cases. Kaposi sarcoma-associated herpesvirus (also known as KSHV) and Epstein-Barr virus (which is EBV), which you might know causes mono, are oncogenic gammaherpesviruses meaning they can induce cancer. So viruses can cause cancer. However, utilizing human herpesvirus in lab research is impractical because they are not able to replicate and form plaques. Plaques are just countable particles that can be used to determine the amount of virus. Before conducting research in our lab, it's necessary to thoroughly investigate the potential model that can be utilized instead of human herpes virus. Usually, the first model that researchers turn to is the mouse. The mouse has been used as a model for many different experiments since a significant part of genes in mice share similar functions with the genes in humans. These similarities make it possible for scientists to study the physiology of mice to see information about how human

beings grow or develop diseases. This genetic similarity also means that mice and humans inherit traits in the same way. This includes susceptibility to diseases such as mouse herpesvirus that can infect mice to be used as a model.

Various works have focused on gammaherpesvirus using Murine Herpesvirus 68 (also known as MHV-68) as a mouse model. Further inspection shows that MHV-68 is 80% homologous, or similar, to EBV and KSHV, this can be used to study the Y-herpesvirus infection. Unlike the human herpesvirus, the model virus can undergo lytic, meaning active, replication and form plaques, allowing researchers to determine the amount of virus, also mouse herpesvirus will not infect humans. Furthermore, a mouse cell line for cell culturing is used so that MHV-68 can infect the cells. The cells we use are called NIH3T3, which are mouse fibroblast cells that can be cultured and passaged for experiments.

The NIH3T3 cells are then infected with the MHV-68. This is because viruses are obligate intracellular parasites, meaning that they can hijack the host metabolism for their own needs. A way to think of metabolites is that they are the nutrients or food for the cell. If you look at the figure you can see that those figures, seen in blue, white, red, represent different metabolites. Viruses increase metabolite production so they can make more of themselves. My professor conducted experiments focusing on MHV-68's impact on the host NIH3T3 metabolism, utilizing metabolomics, the measurement of metabolites during viral infection. Upon observing significant increases in different metabolites, connections were drawn between blocking these metabolite pathways to inhibiting the virus. So, if viruses increase nutrients, then blocking the upregulation of the nutrients may result in reducing the virus.

These findings formed the basis of our hypothesis. Preparations for the research also involved studying FDA-approved drugs, this included understanding the drug's mechanism of action, its previous applications, and relevant research about its efficacy against either viruses or cancer. This comprehensive learning process spanned almost a year, during which we maintained cell cultures in preparation for the actual experiment.

One of the metabolism pathways that was seen to increase was the polyamines. As you can see from this picture, all of the red color indicates an increase in that metabolite. Putrescence can be seen to increase up to a 3-fold 8 hours after virus infection. Polyamines are important in the cell because they result in protein synthesis as well as cell growth and many other functions that are crucial for cell survival. DFMO, an FDA approved drug, has been previously used for other anticancer studies in addition to African sleeping sickness, this means it can inhibit parasites. The function of DFMO blocks enzyme ornithine decarboxylase which prevents precursors from converting into putrescine, inhibiting the downstream pathway. Furthermore, it blocks the nutrients that we established the virus likes. We are basically deriving the virus from what it wants.

Another metabolic pathway seen to increase was the lipid pathway. Lipogenesis is the creation of fats, which not only benefits cellular energetic needs but is also important for viral production. Lipids or fats are high amounts of energy and supplies for the cells. Here you can see that the virus increases a lot of the lipid species. As a fatty acid synthase inhibitor, Orlistat will be able to prevent the enzyme responsible for the accumulation of high levels of fatty acid needed for viral replication. Orlistat is also used as a weight loss drug and can be bought as an over-the-counter pill. In other research, it has also proven to be effective in decreasing the percentage of viruses. So as you can see, science starts by gathering background information. It's impossible to make progress without knowing what has been discovered before.

For the actual experiment, I will just talk about the results and some procedures but not too much in detail. You can read more about that in my paper later. However, I do want to emphasize all the trials and errors that I had to process because that is what it means to be human. To do that, I do need to give you a quick introduction of the procedure and results so you can understand the struggles that I had to go through.

First, I did a drug safety test in which I determined different dosages of drugs to test for a high enough concentration to inhibit the virus, but safe enough so that it does not cause cell death. To do that, I found different concentrations of the drugs to use so that I can measure the safety level of the drug. Remember, we don’t want the drug to kill humans, we just want to reduce the virus. Then the second part after finding an optimal drug dose is to determine if the chosen concentration of drug works, this means we have to visually count the numbers of viruses. We can do this by doing a plaque assay which can allow us to determine the number of reduced viruses when a drug is being treated by counting the number of plaques. After counting the control(which has no drugs) and drug treatment(either with DFMO or Orlistat treated), I found the drug did seem to have a moderate effect on the virus to reduce viral titer. Hence, the drug does work to reduce virus titer. So since I don’t want to leave you in suspense, DFMO(FIG) you can see is able to reduce about 80% of virus titer compared to the control and Orlistat, 64% compared to the control. However, read my paper for more details and the exact fold rate at which occurred.

In the experiment part, I have in parenthesis, trial and error. In the realm of published scientific literature, only experiments yielding positive results tend to catch attention. In recent times, there has been a notable increase in the number of publications reporting successful experiments as opposed to those documenting failures. The reality of experiments is that there are so many troubleshooting and failed experiments that occur that people might not publish and talk about. However, what if we were able to have a format where we talk about our experiences and failures so that others can learn from them and avoid them? So, I will be taking this opportunity to talk about it.

My adventure at the Delgado Lab was marked by a series of recurring failures that seemed to plague every experiment. The initial blow came when the NIH3T3 cell passage reached an unexpectedly high count, around 40 passages. In cell culture, each time you split a passage of cells, the cells may result in a genetic change and become more sensitive to drugs. After three initial safety methods, I started the actual infection trial only to reveal that the FDA-approved drug we were testing was unexpectedly killing the cells. This data was shocking because we had presumed the high safety levels of the drugs we were testing. However, it became evident that the cells had become more sensitive at the higher passage, setting our project back a daunting two weeks. Just as we were recovering from this setback, our lab encountered another obstacle: we ran out of virus stock, specifically the MHV-68 required to infect the cells. It took another week to troubleshoot this and by that time, it was almost the end of the school year. Other times, I had many cells die on me because I was not fast enough to transfer them or there were times when I accidentally placed different conditions in the wrong tube, resulting in a ruined experiment that took four hours.

Reflecting on my two-year experience, it's difficult to tally the number of times I faced failure and the subsequent need for troubleshooting. Despite the numerous setbacks I encountered, each failure was a valuable learning experience that I hope the new students in the lab can learn from.

Appendix 2

Now onto the last part which is the data part. I now know that the metabolic inhibitor drugs do work to reduce viruses. Eventually the lab will be able to publish a paper about these findings and other researchers could take our data and test it in vivo and maybe use the drug to treat viral infections without having to go through the process of 10 years to get a drug to be approved. There was a time when there were no laws regarding drugs and conducted experiments, which caused many deaths and health problems for people. This is why FDA testing is an important part of the US drug approval process. However, the timeline for FDA trials can be challenging, often taking years from the initial phase of research to final approval. This long delay is due to the many phases involved in clinical trials, including preliminary studies, Phase 1, 2, and 3 clinical studies. Each level has different needs and targets. In these trials, researchers evaluate factors such as dosage, administration, side effects, and overall effectiveness in treating the target. However, repurposing of FDA approved drugs can allow us to identify new therapeutic uses for drugs currently approved for other indications. Because these drugs have passed safety testing, their repurposing could reduce the time and resources required for development and approval. Reuse makes life easier and faster for patients and pharmaceutical companies. For patients, this means faster access to new treatments tailored to their condition. Think of it like recycling: instead of starting from scratch, scientists can look at drugs already approved for one thing and see if they can be used for something else. It's almost like finding a new use for an old tool in your toolbox. It provides a good way for pharmaceutical companies to bring new drugs to

market using existing research and clinical data. This can reduce the time, effort, and money to treat a condition.

Some drugs that have been repurposed include Aspirin. It was originally developed as an antibiotic, aspirin was later repurposed for its anti-inflammatory properties and widely used to prevent heart disease and stroke. We can also use it for headaches and body pain. Another drug is Metformin which was first developed for type 2 diabetes and is now widely used. What is also interesting is that another person, Sara, in the Delgado lab is testing Metformin to see if it inhibits virus production. Another drug, Tretinoin, a derivative of Vit A can be used to treat acne and other skin conditions when applied topically while the oral form can treat leukemia. This shows we can use cosmetic products and repurpose them to solve serious health problems. This shows us that everything is connected and we can use studies from different fields to work together to produce something so novel and extraordinary. All you need to do is do some background research to connect pieces.

So I hope that my journey, trial, and tribulation, to produce this data will be meaningful for others in the future. Science is a collaborative effort to generate new inquiries and solutions. Other people's work has shaped a lot of the data I produced and I hope that I can do the same for others as well. In the science community, we all need to collaborate with others to make the most out of the data we have. Without the building blocks of others, we cannot advance anywhere, so my last message to all of you guys is that I hope that you understand what it means to be human, working together to create new knowledge as well as learn from past mistakes. Thank you for listening.