IMATINIB MESYLATE AS AN EFFECTIVE ANTI-VIRAL TREATMENT FOR ALPHAVIRUS INFECTIONS

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ABSTRACT

Alphaviruses are plus-strand RNA viruses that are transmitted by mosquitoes. There are very limited vaccines and treatment options available to those infected with alphaviruses, resulting in significant human and animal morbidity and mortality each year. Viruses are parasites of host cell metabolism and alphaviruses have been shown to increase glycolytic flux during infection to aid viral replication. Imatinib mesylate is an FDA-approved tyrosine kinase inhibitor that is used to treat several types of cancers. A hallmark of tumorous cells is an elevated metabolic rate and Imatinib successfully slows metabolism by inhibiting tyrosine kinases that are required to activate metabolic enzymes, such as hexokinase in the glycolytic pathway. It was hypothesized that Imatinib could be used to slow metabolism in virally-infected cells and reduce viral replication. Alphavirus-infected cells were treated with various concentrations of Imatinib and at a concentration of 6 μM, viral replication was reduced by more than 40% while cell viability was still at 100%. The efficacy of Imatinib treatment at inhibiting alphavirus replication was confirmed at different times post infection (6, 12, 18, and 24 hours post infection), different levels of infection (multiplicities of infection= 0.1, 1, and 10), and within different cell lines (BHK, Huh7 and HEK). Further analysis in mouse or other animal models is needed to confirm the utility of Imatinib as a therapeutic option for treating alphavirus infection, but the data are promising and shows a significant reduction in viral replication and may represent a novel treatment option for alphavirus infections.

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KEYWORDS

- Alphavirus
- Anti-virus
- Imatinib

INTRODUCTION

Alphaviruses are enveloped, plus-strand RNA viruses, that are transmitted *via* mosquito vectors. Alphaviruses, including Eastern, Western, and Venezuelan Equine Encephalitis Virus, Chikungunya Virus, Sindbis Virus, and Semliki Forest Virus, are responsible for millions of infections each year (15, 22). Chikungunya virus recently spread to North, South, and CentralAmerica and resulted in more than 2.9 million confirmed and suspected cases, with nearly 300 deaths as of summer 2016 (11, 26). Alphavirus infection results in a wide range of symptoms including rash, malaise, chronic arthralgia, and can even lead to fatal encephalitis (2, 23, 30). There are few effective treatment options available for those infected with alphaviruses and a novel method to treat and inhibit alphavirus infections is imperative to public health.

Traditionally, antiviral therapies have been designed and engineered to target viral proteins, instead of host cell proteins in order to prevent cytotoxicity to the host cells (17). However, due to the rapid rate of mutation and diversity within viral species, viral specific drug treatments are limited in efficacy and burdened with the rapid development of drug resistance. New strategies involve altering or limiting cellular pathways that the virus relies on and thereby reducing the viral replication capacity of the host cell (4). All viruses are parasites of the host cell metabolic pathways and depend on the host cell for all biomolecule and energy synthesis requirements. Many viruses, including alphaviruses, have been shown to manipulate cellular metabolic pathways to enhance the cellular environment and make it optimal for viral replication (8, 9, 19, 29). Sindbis virus (SINV) is the prototype alphavirus and is commonly used as the model virus for other alphaviruses. For Sindbis virus, an elevated glycolytic rate appears to benefit the virus replication(6). It was hypothesized that drugs that inhibit or reduce metabolic rates may effectively be used to slow and inhibit viral replication.

Metabolic inhibitors have been studied extensively in the cancer research fields. Cancer is essentially a metabolic/cell division disorder where cells grow uncontrollably with an elevated metabolic rate. Many cancer drugs target this elevated metabolism and reduce or slow down metabolism to normal levels, thereby inhibiting cancer growth and metastasis (27). Due to the widespread burden of cancer, there has been much research in metabolic inhibitor drugs and there are several options that are FDA-approved and are used clinically to treat cancers (5, 10, 16). Viral infections induce a similar elevated metabolic phenotype in host cells and some of these metabolic inhibitor cancer drugs have been repurposed as novel treatment options for viral infections.

Imatinib mesylate, or Gleevec®, is a tyrosine kinase inhibitor that prevents the phosphorylation and activation of key enzymes in metabolic pathways (1). Specifically, Imatinib has been shown to reduce the activity of hexokinase, which is a key enzyme in the glycolysis pathway (3, 18). Sindbis virus is dependent on glycolysis and it was predicted that inhibiting the glycolytic pathway would result in less viral replication. We hypothesized that Imatinib mesylate, which is already used clinically, may be repurposed and effectively used to treat alphavirus infection. Here we present our findings that Imatinib mesylate successfully reduces Sindbis virus replication in cell culture at low enough concentrations that do not affect host cell viability. Investigating different Imatinib concentrations, viral titers, infection times, treatment addition times, and various cell lines have all shown Imatinib to be efficacious at inhibiting Sindbis virus infection.

METHODS

CELLS AND CELL CULTURE

Baby Hamster Kidney (BHK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with streptomycin/penicillin and 10% FBS. Cells were incubated in T75 flasks or 24-well culture plates and kept in an incubator at 37°C and 5% CO_2. Cells were passaged every 3 days once 85% confluent using PBS and 0.25% Trypsin. Cells were counted and seeded into well plates (24-well) 24 hours before an experiment so

that the cells would be about 70% confluent at the time of infection/treatment (75,000 BHK cells/ml= ~37,500 BHK cells/well). Human hepatoma (Huh7) cells and Human embryonic kidney (HEK) cells were maintained in similar conditions but were passaged every 4 days.

VIRUS AND VIRAL INFECTION

The 100 +/- 10 mg of fresh rind and curd saSindbis virus, which belongs to *Togaviridae* and is an old world alphavirus, was used as the model alphavirus for all experiments. A Green Fluorescent Protein (GFP) reporter was previously inserted into the viral genome *via* a duplicated subgenomic promoter at the 3' end of the viral genome. This GFP is concurrently expressed with the viral proteins and the relative GFP fluorescence serves as a direct indicator for viral replication in infected cells. These double subgenomic alphavirus reporters are well established and used widely in the alphavirology field(21, 23, 28). Prior to infection, a sample well was used to count cells. Cells were trypsinized, resuspended, and counted using Trypan Blue staining and a hemocytometer. Once an accurate count of cells/well was available, the amount of virus could be calculated and added to the specified wells. Cells were infected with a multiplicity of infection (MOI) of 1, unless otherwise noted. MOI is the ratio of infectious virus particles (plaque forming units (PFU)) to number of cells and an MOI=1 will infect a majority of cells in the well. The corresponding amount of virus was added to the total volume needed and then was added to each well being infected. The plates were incubated for 1 hour at 37ºC to allow the virus to infect and enter the host cells. Following 1 hour of infection, the media containing the virus was removed, the cells were washed with PBS, and fresh media (with or without the treatment) was added to the cells. The infected cells were put back in the incubator and allowed to infect for the specified time.

IMATINIB TREATMENT

Imatinib mesylate was purchased (Sigma) and resuspended in H_2O to make a 100mM stock. This 100mM stock was then serially diluted with two-fold dilutions to acquire stock concentrations at 50mM, 25mM, 12.5mM, 6.25mM, 3.125mM, and 1.56mM. To treat the cells, the mM stocks were diluted 1:1000 in cell culture media to provide media with 100μM, 50μM, 25μM, 12.5μM, 6.25μM, 3.125μM, and 1.56μM concentrations of Imatinib in cell culture media that could be added directly to infected cells. A control treatment was prepared by diluting H_2O solvent 1:1000 in media following the protocol as the drug dilutions. The dilutions of Imatinib in cell culture media were always prepared fresh for experiments. For the experiments with different times of Imatinib addition, the media was removed at the specified time and replaced with fresh DMEM containing the corresponding dilution of Imatinib or the solvent control.

CYTOTOXICITY OF IMATINIB AND PLATE READER

Cell viability was assessed using the alamarBlue Cell Viability Assay® from ThermoFisher according to manufacturer's protocols. Briefly, Imatinib treated cells were stained with a concentration of 100μM Resazurin/alamarBlue and allowed to incubate for 1 hour at 37ºC. Following the one hour incubation, the plates were analyzed on an EnSpire Multimode Plate Reader ® from PerkinElmer for fluorescence at an excitation of 540–570nm and an emission of 580–610nm to measure the viable cells. The mock/control treated cells were set to 100% and then the viability readings from the treated wells were calculated as a percentage of the control. All experiments were performed and analyzed multiple times in triplicate.

QUANTIFYING VIRUS REPLICATION AND FLOW CYTOMETRY

Virus replication was quantified based on the GFP reporter inserted into the viral genome. GFP was measured in infected/treated cells using the plate reader (see above) and an excitation of 488nm and an emission filter of 509nm. Additionally, cells were trypsinized, resuspended, and run over a Guava easyCyte® flow cytometer from Millipore to assess the percent of cells infected (expressing GFP) and the relative levels of GFP fluorescence per cell. 5,000 cells were analyzed for each sample and each condition was run in triplicate. The same parameters, regions, thresholds, and gating was used for all analysis to successfully measure GFP positive cells.

STATISTICAL ANALYSIS

All data graphs were generated with Microsoft Excel. Data and statistical analysis was performed using R Software. Student's t-tests were performed and a *p-value* <0.05 indicated statistically significant differences. Error bars indicate the Standard error of the mean unless noted otherwise.

RESULTS

QUANTIFYING VIRUS REPLICATION AND FLOW CYTOMETRY

Baby Hamster Kidney (BHK) cells were infected with a double subgenomic Sindbis virus (SINV) containing a fluorescent reporter (GFP) protein inserted in the viral genome(21). Virus was added to the cells to achieve a multiplicity of infection (MOI) of one and after one hour of infection, Imatinib

was added to specified wells at various low concentrations of Imatinib (0–100μM). After 24 hours of infection, the cells were analyzed for GFP fluorescence as an indicator for SINV replication either with a plate reader or with the flow cytometer. Data from the flow cytometer accurately matched the data from the plate reader, but the flow cytometer allowed more specifics to be analyzed (fluorescence per cell and percent infected). Treatment of SINV-infected BHK cells with Imatinib showed a dose-dependent decrease in viral replication (Figure 1). Cytotoxicity of the Imatinib concentrations was also measured on the BHK cells using alamarBlue Cell Viability Assay. Imatinib started reducing cell viability at concentrations above 12μM, with a dose-dependent curve of cytotoxicity at higher concentrations of Imatinib. Treatment with concentrations less than 12μM resulted in greater than 80% cell viability. However, at 6μM Imatinib treatment, viral replication was significantly reduced by more than 50%. The IC50 and CC50 were calculated to be 4.8μM and 28.1μM, respectively, giving a therapeutic index of 5.85 for using Imatinib as an antiviral for alphaviruses. Ribavirin, which is a well-established antiviral therapy that is used in severe viral infections, has a therapeutic index of around 5–8 for Sindbis virus, indicating that Imatinib treatment is equally effective at treating alphavirus infection(13, 20, 24).

IMATINIB'S EFFECT OVER TIME AND DIFFERENT LEVELS OF SINV INFECTION

Imatinib's effects on SINV virus replication over time was investigated next. Cells were infected with dsSINV-GFP at an MOI=1 and then half of the cells were left untreated and half of the cells were treated with 10μM Imatinib. Cells were analyzed every 6 hours for a 24 hour period to detect GFP fluorescence

and calculate the percentage of cells infected using flow cytometry. Significant inhibition of virus replication (Student's t–test $p<0.05$) was seen in all time points past 12 hours that were treated with Imatinib (Figure 2A). Additionally, BHK cells were infected with an increasing amount of virus and then treated with mock solvent or 10uM Imatinib and allowed to infect for 24 hours. Cells were analyzed on flow cytometry to determine the percentage of cells infected (Figure 2B). Increasing the level of infection (multiplicity of infection= MOI) by up to 10 fold still showed a reduction in virus infection in cells treated with 10μM Imatinib (Figure 2B).

TIME OF TREATMENT AFFECTS IMATINIB INHIBITION OF SINV

We sought to validate Imatinib as a treatment option by investigating different times of treatment and at a higher infection level (MOI=10). BHK cells were treated with 10μM Imatinib at 6 or 3 hours pre infection, at the same time as infection (0hrs), or 3, 6, or 12 hours post infection. The virus was added at time 0 at an MOI=10 and then the cells were allowed to infect for 24 hours before being analyzed on flow cytometry. Each time point was analyzed and the treatment was set as a percentage of the untreated control (Figure 3).

IMATINIB INHIBITION OF SINV WORKS IN MULTIPLE CELL TYPES

To confirm the anti–viral effect of Imatinib on infected cells, the study was expanded to include human cultured cells. Human Hepatoma cells (Huh7) and Human Embryonic Kidney cells (HEK) were utilized by being plated and infected with dsSINV–GFP at an MOI=10 similar to the BHK cells. 10μM of Imatinib treatment successfully reduced SINV replication

in both the Huh7 and the HEK cells at 24 hours post infection with an MOI=1(Figure 4).

DISCUSSION

Imatinib mesylate, or Gleevec®, is already FDA–approved and is used regularly in cancer treatments. Clinically, Imatinib is administered orally at low dosages (400mg p.o. b.i.d=.800mg/day)(5). We report that at similarly low concentrations (10 μM), Imatinib reduced SINV replication in cultured cells. This novel finding suggests that metabolic inhibitors used in cancer chemotherapy may be repurposed and used for treating viral infections. Imatinib mesylate works by inhibiting tyrosine kinases within the host cell(5, 18). Many metabolic enzymes require phosphorylation from tyrosine kinases in order to be activated. The general inhibition of tyrosine kinases reduces the ability of the cell to have elevated metabolic rates. This limitation on cellular pathways prevents the virus from manipulating host cell metabolism and hijacking certain pathways for optimal viral replication. During Imatinib treatment, the cell is maintained at lower metabolic rates and therefore limits the ability of the virus to replicate. This hypothesis has been validated and it has been shown that Sindbis virus is significantly inhibited in cells treated with 10μM Imatinib. This concentration of Imatinib was not toxic to the cells and presents a therapeutic window where Imatinib may be used effectively to limit virus replication, while maintaining host cell viability (Figure 1). Imatinib's therapeutic index was similar to Ribavirin, which is a clinical antiviral used to treat severe cases of Respiratory Syncytia virus (RSV)(12). Although it would be more advantageous if Imatinib had a higher therapeutic index, the proof–of–principle is still validated that anti–cancer and anti–metabolism compounds may be repurposed and used for antivirals. Imatinib is effective against Sindbis virus and investigation of other
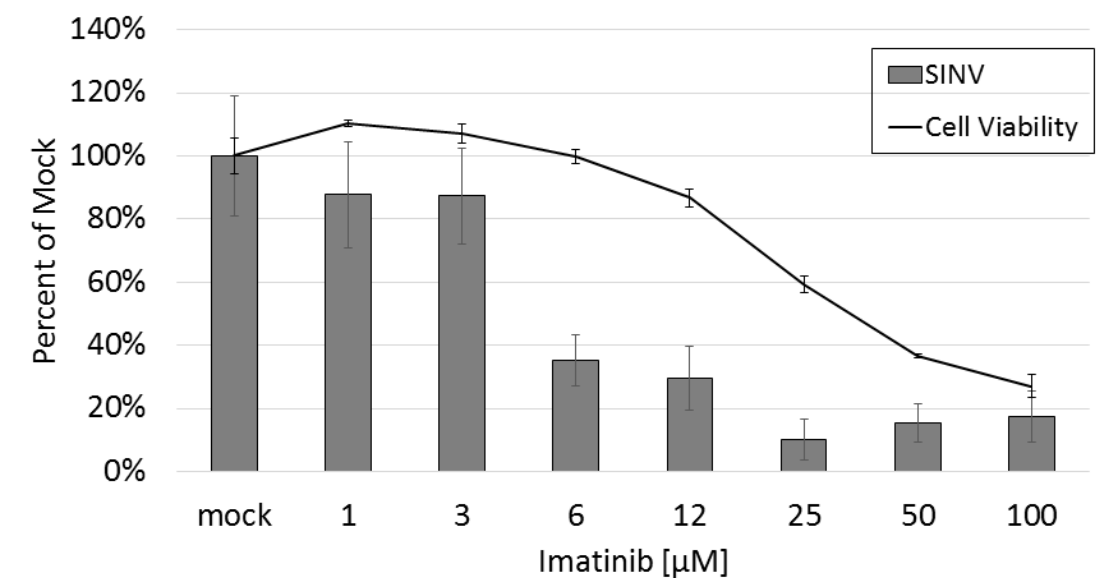

Figure 1: Imatinib inhibition of Sindbis virus and cell viability
Flow cytometry analysis of BHK cells infected with dsSINV-GFP at an MOI=1 and treated with dilutions of Imatinib SINV replication (Gray bars) and cell viability (Black line) are shown with increasing concentrations of Imatinib. Both the mock/untreated samples were set to 100% and the different treatment conditions were calculated as a percentage of the mock. SINV was significantly inhibited at 6 μM of Imatinib compared to mock treated (*p*<0.05). Error bars represent standard deviation n=3.

anti–metabolism compounds may lead to the discovery of an even more effective drug that can be used to treat viral infections.

This Imatinib anti–viral effect was confirmed at different times during the virus replication cycle and indicates a general overall reduction in virus infection as compared to a step/cycle specific inhibition effect. Over the course of 24 hours, untreated cells showed a gradual increase in percent of SINV– infected cells eventually reaching about 25% infected, which is statistically significant when compared to the Imatinib treated cells that only reached about 5% of cells infected at 24 hours. Significant reduction in virus replication was seen in a wide variety of infection levels, as indicated by different infection MOI's (Figure 2). Interestingly, the plates that were examined at different time points showed higher infection levels (up to 25%) compared to the MOI plates (only up to about 17%). This may be due to the fact that the

time course plates were physically moved every 6 hours, which would result in a disruption of the media and may have redistributed the virus supernatant throughout the culture. These results imply that Imatinib may be successfully used to reduce viral loads at different times and levels of infection.

To further assess the efficacy of Imatinib to lower viral replication, the impact of treatment timing on viral infection was investigated. Understanding the opportune timing for treatment is a critical component for antivirals to discover if the compound is more effective if given prophylactically prior to exposure or if post–infection treatment is more effective. The greatest inhibition of virus replication was seen in cells that received the Imatinib treatment 3 or 6 hours prior to infection. Adding the Imatinib at the same time as infection or up to 3 hours post infection still achieved about a 20% reduction in virus replication, but adding
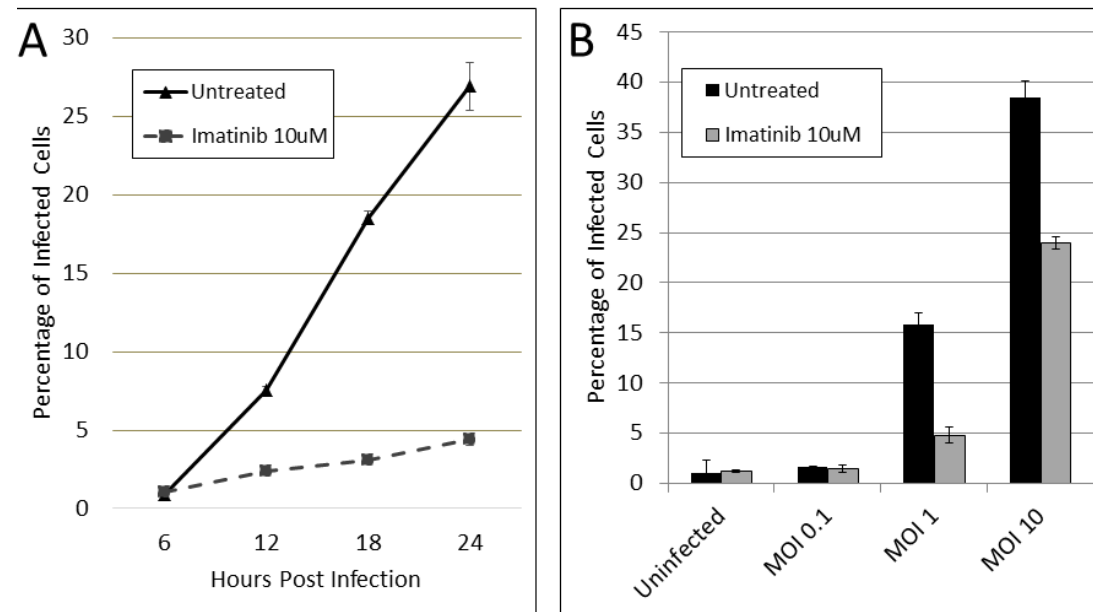
Figure 2: Imatinib treatment at different times post-infection and SINV concentrations
A) SINV-infected BHK cells (MOI=1) were treated with 10μM Imatinib (dotted line) or left untreated (black line) and analyzed every 6 hours for the percentage of cells infected using flow cytometry. B) BHK cells infected with increasing amounts of virus and treated with 10 μM Imatinib (gray) or left untreated (black). The percentage of cells infected was determined with flow cytometry at 24 hours. Significant reductions (Students T-test P<0.05) in dsSINV-GFP replication are indicated with an asterisk (*).
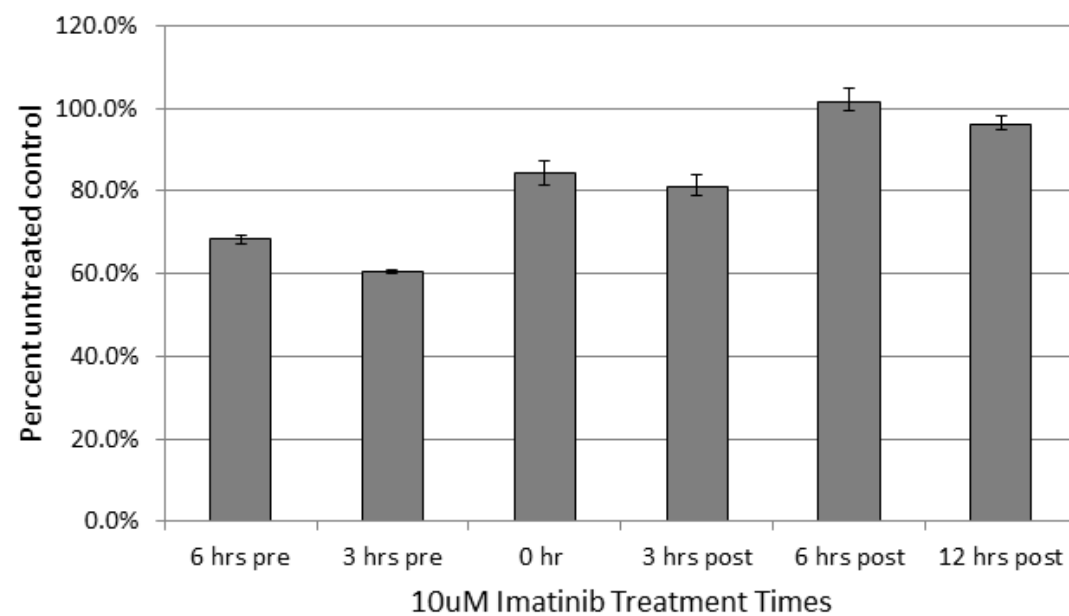

Figure 3: Treatment with Imatinib at different treatment times
BHK cells were treated with 10 μM Imatinib or solvent control at the times indicated and dsSINV-GFP (MOI=10) was added at time 0. At 24 hours post infection, the cells were analyzed for dsSINV-GFP *via* flow cytometry. The untreated sample for each condition was set to 100%. The greatest difference was seen with a single treatment.

the Imatinib 6 hours or later after infection did not result in a significant inhibition of virus infection (Figure 3). The 20% reduction is not as significant as what was seen in previous experiments, but that is likely due to the higher MOI and also greater movement of the plate during the infection incubation. Pre-infection treatment with Imatinib reduced the virus replication more effectively than treatments that were added concurrently or post infection. This indicates that Imatinib may be most effective as a prophylactic drug, given prior to high risk exposure situations. However, due to the inherent side effects of anti-cancer and anti-metabolic drugs, prophylactic treatment might not be feasible in a clinical setting, but post-infection treatment may be effective. Although not as significant as a pre-infection treatment, even treating with Imatinib at 3 hours post-infection successfully reduced virus infection and confirms the potential utility of using Imatinib as a post-exposure/infection treatment option (Figure 3).

The Imatinib inhibition of SINV infection

was further confirmed in human cultured cells, both liver (Huh7) and kidney cells (HEK), which both showed a similar reduction in virus replication as was observed in the BHK cells (Figure 4). Huh7 and HEK cells are common cell lines used in virology to assess virus replication in human tissue and it is promising that both cell lines show an effective reduction in SINV replication when treated with 10μM Imatinib. Variation in viral reduction is likely due to specific differences in the cell type, but the overall trend of viral inhibition with Imatinib treatment is maintained in the different human cell lines. This validates the potential efficacy of using Imatinib as a therapeutic anti-viral treatment option in human alphavirus infections.

In this study, a single metabolic inhibiting cancer drug known as Imatinib mesylate was investigated. Imatinib has shown very promising inhibition of Sindbis virus replication at different times, levels/MOI's, and host cells. Further work will be done
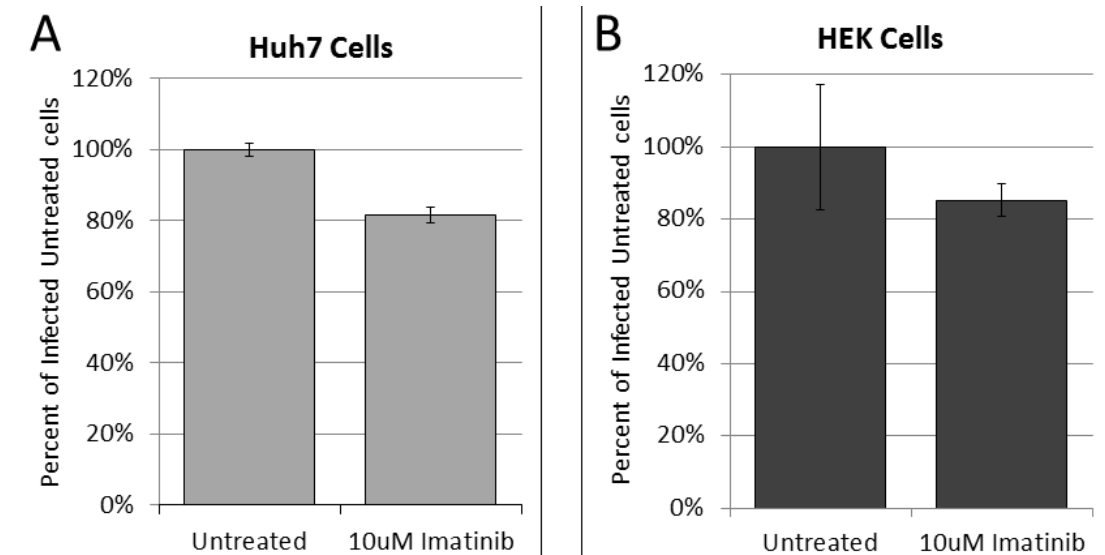

Figure 4: A) Human hepatoma (Huh7) and B) Human embryonic kidney (HEK) cells were infected with dsSINV-GFP at an MOI=10 and then treated with 10 μM Imatinib or solvent control (untreated). At 24 hours post infection, the cells were analyzed for dsSINV-GFP *via* flow cytometry. All data is displayed as a percentage of the untreated samples. (n=6).

to validate and confirm these findings with Imatinib in model organisms and in primary cells. Additionally, there are many other FDA-approved cancer drugs that specifically target and slow cellular metabolism(7, 14, 25, 27). Further work will be performed to screen other metabolic inhibitor compounds as effective antivirals. By studying clinically used and already approved compounds, it may be possible to discover a novel antiviral treatment option that can rapidly be used to treat and bring aid to the millions of alphavirus infections that are occurring globally.

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