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CHARACTERIZATION OF NOVEL NUCLEOSIDE ANALOG DRUGS AGAINST HSV-1 INFECTION

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ABSTRACT

Infection with Herpes Simplex Virus-1 (HSV-1) is common in humans, and can lead to severe symptoms if drug resistance occurs. The goal of this project was to characterize antiviral activity against HSV-1 and toxicity levels of various novel nucleoside analogs developed at Microbiotix, Inc., to identify a potential nucleoside analog candidate for treatment of HSV-1 infection, and to better understand its mechanism of action. Using plaque reduction assays and an MTS assay, compound MBX 2168 was identified as a potential candidate, showing both strong antiviral activity and low toxicity. Mutations in three of the the viral kinases showed minimal effect on the IC_{50} of MBX 2168, suggesting that the initial phosphorylation of the nucleoside drug is not performed by a viral kinase. Kinetic studies of one human kinase of the salvage pathway found that 2-deoxyguanosine kinase is also not likely to initiate phosphorylation of MBX 2168, suggesting that MBX 2168 may be first phosphorylated by a cellular kinase other than deoxyguanosine kinase. Finally, sequence analysis of an HSV-1 strain resistant to MBX 2168 revealed a point mutation in the viral DNA polymerase enzyme at residue 814, replacing a glycine residue with serine. Molecular modeling of this mutant polymerase suggest that the mutation is not directly involved in the enzyme's active site, but does result in a slight shift in other active site residues. The results of these studies did not identify the exact mechanism of phosphorylation of MBX 2168, but suggest that the drug inhibits HSV-1 by inhibiting DNA synthesis.

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BACKGROUND

The Herpesvirus Family

The herpesvirus family is a large group of enveloped DNA viruses that cause a variety of diseases in humans and vertebrates. There are over 30 sequenced herpesviruses, which have been further classified into three subfamilies: alpha herpesvirus, beta herpesvirus, and gamma herpesvirus (McGeoch et al., 2006). The alpha herpesviruses include herpes simplex virus-1, herpes simplex virus-2, and varicella zoster virus. In general, these have a short reproduction cycle, and cause latent infections in nervous tissues. The beta herpesviruses include cytomegalovirus, human herpes virus-6 and human herpes virus-7. These have longer life cycles, and cause latent infection in lymphatic tissues. The gamma herpesviruses include the Epstein-Barr virus and human herpes virus-8, also known as Kaposi's sarcoma virus (Gesser, 1997). This project investigated herpes simplex virus-1 (HSV-1).

Prevalence and Symptoms of HSV-1 Infection

Studies have indicated that between 50% and 90% of Americans test seropositive for HSV-1, making it a very common infection in the United States (Gesser, 1997). In other countries, this value can reach up to 95% (Cowan et al., 2003). HSV-1 is easily transmitted through contact with infected lesions or other body fluids. Common symptoms of infection include recurrent lesions of the mouth, nose, or eyes. Less common symptoms include lesions of the genitals (Williams et al., 2011). These HSV-1 symptoms result from the cytopathic effects of cell lysis or death that occur when the virus replicates or the host immune system recognizes an infected cell (Motamedifar and Noorafshan, 2008). *In vivo,* these effects commonly result in characteristic lesions of the oral mucosa. In cell culture, HSV-1 infection is characterized by the

formation of viral plaques in a cell monolayer. These plaques result from the infection and replication of a virus inside a cell followed by cell lysis or death (Hunt, 2010).

One challenge that this virus presents is the establishment of latent infections. HSV-1 has the ability to set up long-term infections in the sensory neurons of the host by maintaining parts of its genome as extra-chromosomal molecules (Gesser, 1997). Because only a small number of the viral genes are expressed at this stage, the virus does not kill the cell and the host is also unable to recognize the foreign viral particles needed to destroy the infected cell. Upon external stimulation, the viral genome can become reactivated, allowing the disease to persist for a long time and possibly spread (Gesser, 1997).

Immunodeficiency presents another challenge to managing HSV-1 infection. In patients with a weakened immune system, such as cancer patients, HIV patients, or organ transplant patients, common symptoms can become severe, leading to secondary infections such as pneumonia or meningoencephalitis (Piret and Boivin, 2011). In HIV patients, herpetic lesions spread and recurrences are frequent (Piret and Boivin, 2011). Since the number of immune-compromised patients in the United States is estimated to be around 10 million, managing HSV-1 infection within these patients can be a significant problem (Kemper et al., 2002).

HSV-1 Structure

In its mature form, HSV-1 is comprised of three basic structures known as the capsid, the envelope, and the tegument (**Figure-1**). The capsid is an icosahedral structure that houses the viral DNA. The envelope is a lipid-bilayer structure embedded with various glycoproteins that surround the capsid and aids in viral infection. The tegument is a proteinaceous matrix that links the capsid with the envelope (McGeoch et al., 2006).

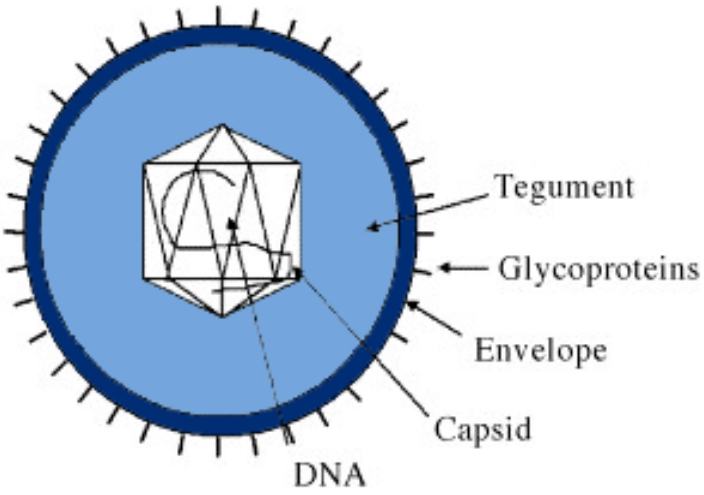


Figure-1: Structure of an HSV-1 Virion (Hughes et al., 2002).

Mechanism of HSV-1 Infection

HSV-1 typically infects cells of the mucosal tissues by interaction of viral glycoproteins of the envelope with mucosal cell surface receptors (Mettenleiter et al., 2009). The interaction between viral glycoproteins and receptors causes the viral envelope to fuse with the cell membrane, bringing the virus into the cytoplasm of the cell. Once inside the host cell, the viral DNA travels to the nucleus of the cell in order to take advantage of the host's transcriptional machinery to catalyze the transcription of early HSV-1 genes. These early genes are required for the transcription of the rest of the viral genes and the eventual replication of the entire genome (Boehmer and Lehman, 1997).

HSV-1 Replication

After transcription of the early viral proteins, replication of the HSV-1 genome proceeds within the host nucleus (Boehmer & Lehman, 1997). The synthesis of viral proteins required for

replication peaks between 5 and 7 hours (Boehmer & Lehman, 1997). It is likely that replication is initiated by the interaction of a viral DNA binding protein with the DNA origin of replication site. This protein is believed to then recruit a number of other viral proteins that eventually make up a helicase/primase complex. Primase initiates the elongation of primers, while viral DNA polymerase (UL30), adds the complementary nucleotide sequence of four deoxynucleotides (dTTP, dATP, dCTP, and dGTP) (containing the bases thymine, adenine, cytosine, and guanine, respectively) to the existing DNA strands (Boehmer & Lehman, 1997). Before DNA replication begins, the viral genome circularizes, and replication moves outward from one or more replication forks (Boehmer & Lehman, 1997). HSV-1 DNA is replicated as a concatemer, a continuous series of interlocked DNA structures consisting of multiple copies of the genome. Finally, the concatameric DNA is then cleaved and packaged into new viral capsids that leave the cell by exocytosis and continue infecting other cells (Boehmer & Lehman, 1997).

Inhibition of Viral DNA Synthesis by Nucleoside Analogs

The most common treatment for HSV-1 infection is with nucleoside analogs. These chemicals have structures similar to the natural nucleosides used to synthesize new strands of DNA, but instead they prevent DNA elongation. **Figure 2** demonstrates the mechanism by which these analogs inhibit DNA synthesis. Nucleoside analogs target HSV-1 replication by competing with the natural nucleoside substrates for the active site of the viral DNA polymerase (Deville-Bonne et al., 2010). Like normal nucleosides, they are converted by viral or cellular kinases to mono-, di-, and tri-phosphate forms, and become incorporated into growing DNA strands. In order for them to be effective against HSV-1 infection, these drugs must be tri-phosphorylated (Deville-Bonne et al., 2010). However, their chemical modifications do not

allow them to further extend the DNA strand. In the presence of these drugs, the viral DNA polymerase attaches these analogs to the growing chain of viral DNA, resulting in a halt in DNA chain elongation (Bacon et al., 2003).

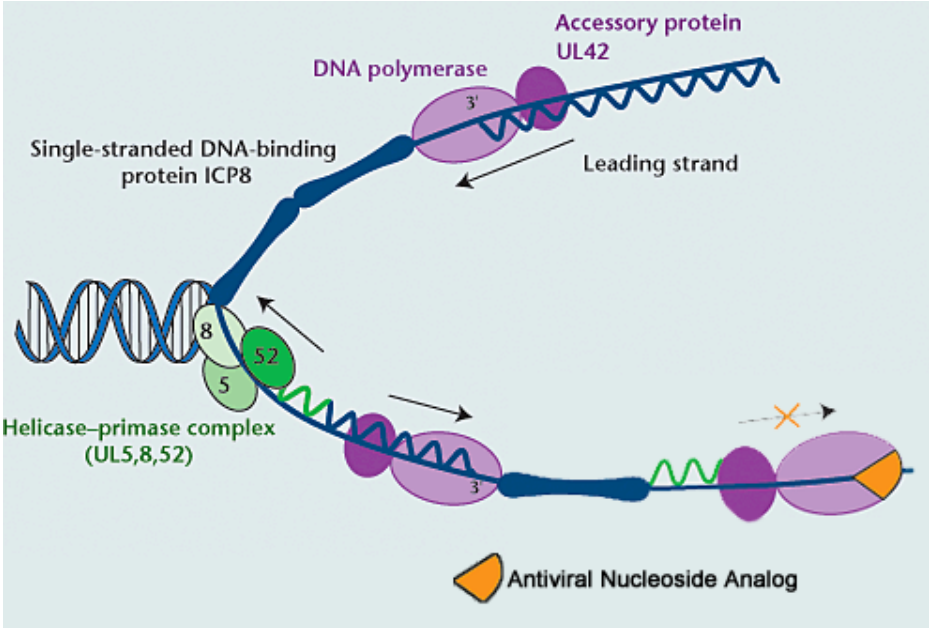


Figure 2: Mechanism of Action of Nucleoside Analogs. The diagram above shows the incorporation of nucleoside analogs in their triphosphate form (yellow) into a growing DNA chain. Because these analogs cannot be linked to additional DNA precursors, chain elongation stops. (Crumpacker and Schaffer, 2002)

Once nucleoside analogs have been incorporated into a DNA strand, chain elongation stops because these analogs lack the 3' hydroxyl group necessary for additional nucleotide attachment (Appelboom and Flowers, 1983).

Acyclovir

One of the most commonly available HSV-1-targeting nucleoside analogs is acyclovir (ACV). This antiviral drug is an acyclic analog of 2-deoxyguanosine, the purine nucleoside used

in cellular DNA replication (Appelboom and Flowers, 1983). Studies have shown that acyclovir is an effective antiviral drug both *in vitro* and *in vivo* (Appelboom and Flowers, 1983). The measured half maximal inhibitory concentration (IC_{50}) of ACV against various HSV-1 isolates typically falls between 0.07 and 0.97 μg/mL (Sangdara and Bhattarakosol, 2008). *In vivo* toxicity studies of the drug show that it is relatively non-toxic (Brigden and Whiteman, 1983). **Figure 3** compares the structure of a natural GTP nucleoside in its nucleotide form (right panel) to an analog known as acyclovir also in its nucleotide form (left panel). The important hydroxyl group is demonstrated.

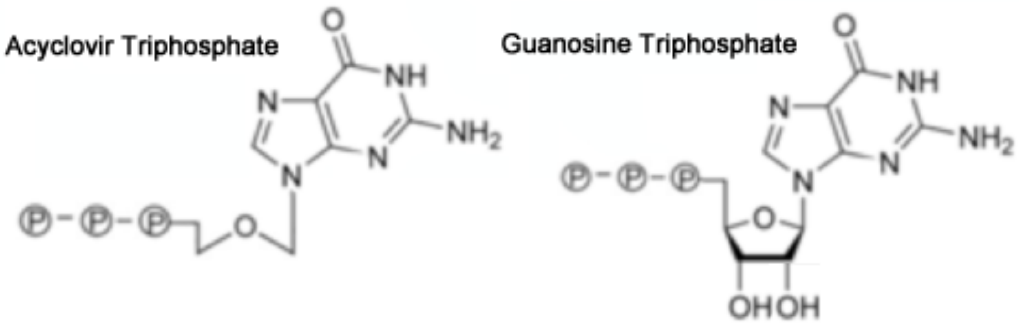


Figure 3: Comparison of the Chemical Structure of Normal Guanosine Triphosphate with Acyclovir Triphosphate. Note the absence of most of the cyclic sugar component and deoxy groups in the Acyclovir structure. (Lin and Lu, 1997; Santner et al., 2012).

The pro-drug form of acyclovir known as valacyclovir uses the same mechanism of viral inhibition, but is more orally bioavailable (Lebrun-Vignes, 2002). The combination of high potency and minimal toxicity make acyclovir a highly successful treatment for HSV-1 infection (Appelboom and Flowers, 1983).

Other HSV-1 Antiviral Therapies

There are several other known antiviral treatments for HSV-1 infection such as Foscarnet, Ganciclovir, Penciclovir, Famciclovir, and Docosanol (Safrin and Phan, 1993). Penciclovir and its pro-drug Famiciclovir are both deoxyguanosine analogs that inhibit viral DNA synthesis. Ganciclovir and Foscarnet work by similar methods (Safrin and Phan, 1993; Tomicic et al., 2002). Docosonal is a topical drug that blocks the fusion of the HSV-1 envelope with the plasma membrane of a host cell (Leung and Sacks, 2004).

Activation of Natural Nucleosides and their Analogs

In their active triphosphate form, most nucleosides analogs are too polar to pass through the membrane of an HSV-1 infected cell. Therefore, most of the available antivirals are administered as pro-drugs and are subsequently phosphorylated within the infected cells (Deville-Bonne et al., 2010). Many HSV-1 antivirals likely share similar mechanisms of activation as natural nucleosides do (Deville-Bonne et al., 2010). **Figure 4** depicts the salvage pathway by which natural nucleosides are phosphorylated. The first phosphorylation step in this pathway converts the nucleoside to its monophosphate form using host deoxynucleoside kinases. The second and third phosphorylation steps are completed by various other cellular kinases, producing nucleoside diphosphates and nucleoside triphosphates (Deville-Bonne et al., 2010).

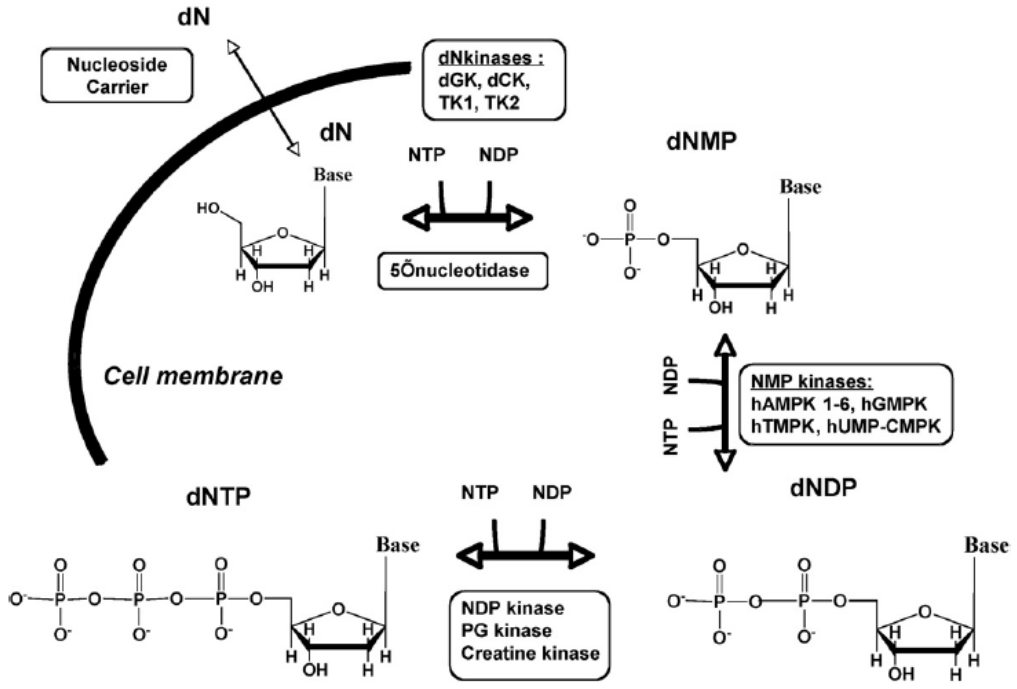


Figure 4: Mechanism of Activation of Natural Nucleosides by a Host Cell Salvage Pathway. This figure depicts the various kinases involved in phosphorylating deoxynucleosides to their triphosphate form. This pathway is suspected to be involved in the phosphorylation of nucleoside analogs also (Deville-Bonne et al., 2010).

Viral Kinases

In addition to host kinases, viral kinases are also involved in the phosphorylation of nucleosides and their analogs (Deville-Bonne et al., 2010). HSV-1 encodes a 40 kDa thymidine kinase (TK) that has shown to be important to the process of phosphorylating some nucleoside analogs. In infected cells, the substrates of this kinase include deoxythymidine (dT), deoxycytidine (dC), and deoxyguanosine (dG). TK transfers a phosphate to these substrates from ATP to produce nucleoside monophosphates (Deville-Bonne et al., 2010). TK has broader activity than human thymidine kinases, and is able to phosphorylate antivirals such as Acyclovir and Ganciclovir to their monophosphate form. Because of its broad activity, viral TK is often

exploited in the activation of antiviral nucleoside analogs (Deville-Bonne et al., 2010). Drugs can be developed that will not become phosphorylated in uninfected cells because they lack viral TK (Deville-Bonne et al., 2010).

Cytomegalovirus encodes a kinase that is analogous to HSV-1 TK from its UL97 gene. This kinase plays a role in viral morphogenesis, and also interacts with tumor suppressor factors in host cells (Deville-Bonne et al., 2010). Interestingly, the UL97 kinase can use antivirals such as ganciclovir as a substrate, but shows little activity with natural nucleosides (Deville-Bonne et al., 2010). Epstein-Barr Virus also encodes its own TK with narrower substrate activity than HSV-1. This kinase is unable to phosphorylate deoxycytidine, acyclovir, or ganciclovir (Deville-Bonne et al., 2010).

The HSV-1 genome also encodes two other kinases from viral genes UL13 and US3. The UL13 kinase is a serine/threonine kinase that is located in the HSV-1 tegument. Its exact function is unclear, but UL13 kinase has been shown to be important to viral replication (Kato et al., 2006). The function of US3 Kinase has also not been determined, but it may play a role in regulating cell apoptosis (Kato et al., 2006).

Human Kinases

There are over 500 identified human kinases (Manning, 2012), and four of these have been shown to be involved in the nucleoside salvage pathway (Kato et al., 2006). Human thymidine kinase 1 (hTK1) and human deoxycytidine kinase (hdCK) are found within the cytosol of cells (Kato et al., 2006). Within the mitochondria of cells are human thymidine kinase 2 (hTK2) and human deoxyguanosine kinase (hdGK) (Kato et al., 2006). hdCK, hdGK, and hTK2 exist as dimers, while hTK1 exists as a tetramer. The four kinases have slightly different

substrate specificities (Kato et al., 2006). **Table 1** lists some of the characteristics of the human and viral kinases mentioned above that are known to participate in nucleoside phosphorylation.

Abbreviation	Name	Natural substrates	Sub-cellular location	length	Molecular weight (Da)
hdCK	Human deoxycytidine kinase EC 2.7.1.74	dC, dG, dA	Cytosol	260	30,519
hdGK	Human deoxyguanosine kinase EC 2.7.1.113	dG, dA,	Mitochondria	277	32,054
hTK1	Human thymidine kinase 1 EC 2.7.1.21	dT, dU	Cytosol	234	25,397
hTK2	Human thymidine kinase 2 EC 2.7.1.21	dT, dU, dC	Mitochondria	266	31,142
HSV1-TK	Herpes simple virus-1 thymidine kinase EC 2.7.1.21	dT, dC, dTMP	–	376	40,913

Table 1: Characteristics of Some of the Kinases Involved in Nucleotide Phosphorylation. In column-1, an "h" denotes a human cellular kinase. Also listed are the natural substrates, sub-cellular location, kinase length and molecular weight (Kato et al., 2006).

Challenges with HSV-1 Antiviral Therapies

Although antiviral therapies can be effective at inhibiting HSV-1 infection, resistant strains of HSV-1 to these antivirals can become problematic, particularly among the immune-compromised individuals (Piret and Boivin, 2011). Mutations in the viral genome sometimes result in a strain of virus that is able to tolerate the presence of the antiviral drug. In HIV positive patients, the prevalence of infections harboring strains of HSV-1 resistant to acyclovir has ranged from 3.5% to 7%. In patients with organ transplant, resistant strains were found in 2.5%-10% of infections (Piret and Boivin, 2011).

Resistance by HSV-1 to acyclovir is typically due to mutations affecting either the viral thymidine kinase or DNA polymerase. The most common mutations involve decreased activity

or a change in specificity of TK (Gilbert et al., 2002). About 50% of acyclovir-resistant strains have demonstrated an addition or deletion mutation in their TK gene. Most other resistant strains show point mutations within the TK gene (Gilbert et al., 2002).

The second form of resistance to acyclovir is due to mutations in the DNA polymerase. These mutations are more rare because unlike TK, the function of the viral DNA polymerase is necessary for its survival (Gilbert et al., 2002). Any change that significantly alters the active site of DNA polymerase may result in the inability of that virus to proliferate. Therefore, any mutation in the DNA polymerase that confers resistance to an antiviral drug (including the one isolated in this project) must be slight enough to maintain polymerase functionality (Gilbert et al., 2002).

Microbiotix, Inc.

Located in Worcester, Massachusetts, Microbiotix Inc. is a research company that is involved in the discovery and development of small molecule anti-infective drugs. Their goal for the topic of HSV-1 is to develop broad-spectrum antiviral herpes drugs. The company is currently developing cylcopropavir, a nucleoside analog that is currently in Phase 1 clinical trial. Cyclopropavir is active against HCMV, HHV-6, HHV-8 and EBV but has no activity against alpha herpesviruses HSV-1 and VZV. In order to increase spectrum of activity a number of cyclopropavir analogues were synthesized at Microbiotix. Their hope is that among these newly synthesized analogs, they can identify some that are both potent and have low toxicity against all human herpes viruses.

PROJECT PURPOSE

Herpes Simplex Virus-1 (HSV-1) causes widespread disease, infecting between 50% and 90% of the US population. Several antiviral nucleoside analogs have been shown to decrease the infection of HSV-1, however, in patients with a compromised immune system, HSV-1 can develop resistance to these antivirals. The purpose of this project was to characterize novel nucleoside analogs developed by Microbiotix Inc, and to identify among them a potential HSV-1 antiviral drug demonstrating strong activity against HSV-1 and low toxicity to cells. Once a potential antiviral drug was identified, this project sought to investigate the mechanism by which it inhibits HSV-1 infection by analyzing potential kinases that activate the drug and isolating drug resistant virus.

METHODS

Cell Culture and Maintenance

The Vero cell line (ATCC), originally derived from African Green Monkey kidney epithelial cells, is permissive for HSV-1 and was used in tests of antiviral drug activity against HSV-1. Vero cells were grown in Minimum Essential Medium (MEM) containing Earle's salts and L-glutamine supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Human Foreskin Fibroblast (HFF) cells (ATCC) were used in studies of antiviral toxicity. HFF cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin, and streptomycin.

Both cell lines were passaged after 2 to 3 days of growth, or once confluence had been reached. To do this, media was removed and cells were washed with 10 mL of a 1X phosphate buffer saline solution (Mediatech, Inc). Cells were removed by adding 1mL of 0.25% trypsin (Mediatech, Inc) and once detached, were suspended in new media and split no more than one flask into 4 new T-150 flasks. Fresh media was then added, and cells were grown in a 37°C incubator in the presence of 5% carbon dioxide.

Plaque Reduction Assay

The plaque reduction assay was used to determine the efficacy of antiviral drugs against wild type HSV-1. It was also used to evaluate the resistance of mutant strains of HSV-1 to antiviral drugs. Wild type HSV-1 strain F was obtained from ATCC and kinase deletion mutant viruses were a gift from Bernard Roizman (University of Chicago). Vero cells were plated in 6-well plates at a concentration of 1×10^6 cells/well, and were incubated for 24 hours at 37°C in

5% CO_2. After incubation, growth media was removed and replaced with 0.5 mL media containing virus at concentrations of 140 viral PFU/mL. Virus-containing media was allowed to infect cells for 1 hour, while antiviral drug media was prepared.

Drug-containing media contained equal parts of 1% Methocel (Sigma) and DMEM plus 10% fetal bovine serum. It also contained decreasing concentrations of acyclovir, cyclopropavir, and other analogs developed at Microbiotix. With the exception of Acyclovir, drugs were added to this media at a concentration of 100 μM, 25 μM, 12.5 μM, 6.25 μM, 1.56 μM, and 0.4 μM. Because of Acylcovir's high potency, 10-fold dilutions were used to produce media containing 100 μM, 10 μM, 1 μM, 0.1 μM, and 0.1 μM drug. Each drug dilution was tested in duplicate.

After infection for 1 hour, virus media was aspirated off of cells and replaced with 3 mL of drug media. Each plate contained one control well that received DMEM and methocel mixture with no added drug. Plates were incubated in drug and control media for 72 hours, after which the media was aspirated off, and cells were stained with 1% Crystal Violet. Plaques were counted by visual inspection and the IC_{50} of the drug was calculated using GraphPad Prism software.

Cytotoxicity Assay

To measure the toxicity of antiviral drugs to HFF cells, the Promega Cell Titer 96 AQ One cell proliferation assay was used. In a 96 well plate, HFF cells were plated at a concentration of 2.5×10^4 cells/well in MEM supplemented with 10% FBS. Plates were incubated for 24 hours at 37°C in 5% CO_2. In a separate 96-well plate, antiviral drugs were diluted 3-fold from 1 mM drug to 1.37 μM drug in MEM supplemented with 10% FBS. Media was removed from the HFF cells after 24 hours and replaced with 200 μL of drug media. Control

wells contained no antiviral drugs, and each drug dilution was tested in duplicate. Plates were incubated for 72 hours at 37°C in 5% CO_2.

After 72 hours incubation, developing media was prepared by mixing 20 μL CellTiter 96® AQueous One Solution Reagent with 100 μL un-supplemented MEM per well. Drug media was removed from cells and replaced with 120 μL/well developing media. Plates were allowed to develop for 2 hours before measuring the absorbance of each well at 490 nm.

Time of Addition Assay

To investigate the time at which nucleoside analog MBX 2168 is able to inhibit infection with HSV-1, a time of addition assay was used. Vero cells were plated in a 96-well plate at a concentration of 2.5×10^4 cells per well. After 24 hours, media containing 50 μM MBX 2168 was prepared. Cells were infected with HSV-1 at an MOI of 5 PFU/cell for 1 hour, after which the media was replaced with normal growth media. Drug media was added to certain wells at the initial time of infection (time 0), as well as at 2 hours, 4 hours, 6 hours, 8 hours, and 24 hours post infection. Each treatment was done in duplicate.

To determine viral titer in these samples, 100 μL of media from each well was used to infect one well of a new 96-well plate of Vero cells. Each well was then diluted 3-fold down 12 rows of the plate. Dilution plates were incubated for 72 hours and wells were visually inspected to find a single plaque. This dilution was used to calculate the concentration of virus present at each of the drug-addition time points.

Protein Cloning and Purification

Human deoxyguanosine kinase (dGK) was cloned by Genscript into a pUC57 vector, and was amplified with PCR using primers containing BamHI and NdeI restriction sites. After purification with the QIAquick PCR Purification Kit (Qiagen), DNA was digested with BamHI and NdeI restriction enzymes, and ligated into a pET19b vector (Novagen) that was cut with BamHI and NdeI and dephosphorylated using Calf Intestinal Phosphatase for 1 hour. After 24 hours ligation at 16°C, the vector was transformed into DH5α cells (Invitrogen) and plated on agar plates containing ampicillin for 24 hours at 37°C.

The dGK- pET19b plasmid was then transformed into high expression BL21 DE3 pLysS cells (Novagen) and plated on ampicillin agar plates. A single colony was picked and grown in 50 ml LB Miller media overnight. The overnight culture was then added to 1-liter of LB media and grown until O.D. reached 0.6, before inducing protein expression with IPTG. After IPTG induction, cells were grown for 20 hours at 16°C. Cells were lysed using a French Press and sonication, and the extracted protein was purified with a nickel column (Qiagen) followed by anion exchange column (GE Healthcare). Protein expression was confirmed using SDS-PAGE.

Measuring Substrate Phosphorylation by Human Deoxyguanosine Kinase

In order to test the ability of human deoxyguanosine kinase to phosphorylate various substrates, a pyruvate kinase lactate dehydrogenase-coupled assay was employed. Reaction buffer was prepared containing 100 mM Tris pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 2 mM DTT, 10 mM ATP, 2 mM PEP, 0.2 mM NADH, and 10 μL of Pyruvate Kinase Lactate Dehydrogenase enzyme mixture 900-1400 U/ml. In a 384 microwell plate, 2-fold dilutions of substrates deoxyguanosine (dG) and MBX 2168 were prepared. Each dilution was done in duplicate.

Before initiating the reaction, 10 μL of reaction buffer was combined with 6 μL of water, and 2 μL of the substrate dilutions. In addition, control wells were prepared without substrate present. In the same plate, 2-fold dilutions of NADH were made, in duplicate. After equilibrating the plate for 15 minutes at 37°C, 2 μL of dGK was added to the wells containing reaction mixture. Immediately after adding enzyme, fluorescence at 460 nm was measured by a plate reader. Plates were then incubated at 37°C and fluorescence measurements were made after 20 minutes, 40 minutes, 60 minutes, 90 minutes, and 120 minutes.

This assay measured the phosphorylation of a substrate by monitoring the disappearance of NADH in solution. In the presence of a substrate with high affinity, dGK converts the substrate to its monophosphate form by removing a phosphate from ATP. The Pyruvate kinase enzyme in the reaction then uses ADP to convert PEP into pyruvate and regenerates ATP. Lactate dehydrogenase then converts pyruvate to lactate by removing hydrogen from NADH. Therefore, the disappearance of NADH can be used to measure the amount of substrate that is phosphorylated by dGK. The amount of of NADH disappearance should equal the amount of dGK substrate that is converted to its monophosphate form.

HPLC was done to confirm the level of phosphorylation by dGK. Reactions of dGK with MBX 2168 and ATP in reaction buffer incubated over night at 37°C were run on C18 HPLC column under conditions which would separate MBX 2168-MP from ATP, ADP, and MBX 2168.

Generation of Resistant Virus

A strain of HSV-1 resistant to MBX 2168 was produced to investigate the mechanism of action of this antiviral nucleoside analog. Vero cells were grown in media containing 10 μM

MBX 2186, and infected at an MOI of 0.01 PFU/cell HSV-1. After 3 days incubation at 37°C, supernatant was collected from the infected flasks, and 2 μL was used to infect another flask of Vero cells. This flask was also treated with double the concentration (20 μM) of drug in the first flask. This process was repeated twice more, increasing the concentration of antiviral drug in the media to 40 μM and then 80 μM. After the 80 μM drug flask became fully infected with virus, the contents of the flask were centrifuged and infected cells were collected.

To isolate one virus strain, plaque purification was performed. Vero cells were plated in a 96-well plate at a concentration of 2.5×10^4cells/well. In a separate 96-well plate, the collected supernatant containing viral particles was diluted 3-fold from well A1 to well A8 and then 3-fold from column 1 to column 12. Media was removed from the Vero cells, replaced with the virus dilutions, and allowed to infect cells for 1 hour. After 1 hour, virus media was removed and replaced with media containing 80 μM MBX 2168.

Plates were incubated for 72 hours, and a well containing a single plaque was identified using a microscope. Contents from this well were collected, diluted in MEM, and used to infect another 96-well plate of Vero cells in 80 μM MBX 2168 media. This process of selecting a single plaque was completed once more to ensure that the population of virus was homogenous.

To investigate possible mutations in the viral polymerase of this apparent MBX 2168 resistant virus, sequencing of the gene UL30 was performed. PCR was used to amplify the gene, and sequencing was performed by UMass Medical School. Molecular modeling by Schrödinger software was used to evaluate the effect of this mutation on the structure of the DNA polymerase.

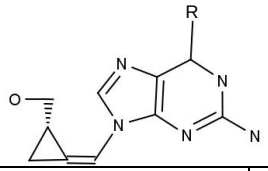
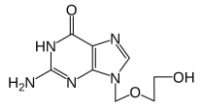
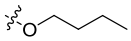
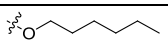
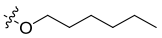
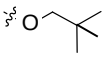
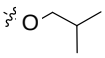
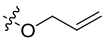
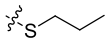
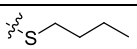
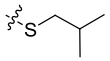
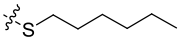
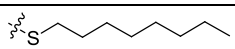
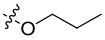
RESULTS

In the US, the prevalence of HSV-1 infection in humans is estimated between 50% and 90% of the population. In cases where patients have weakened immune systems, the antiviral therapies available for treatment may become ineffective as the virus develops resistance. This project aimed to screen a number of novel anti-HSV-1 nucleoside analogs developed by Microbiotix, Inc, based on their antiviral activity and toxicity, and to identify among them a potential novel anti-HSV-1 drug. Additionally, this project sought to investigate the mechanism by which the selected nucleoside analog inhibits HSV-1 infection.

A total of 15 nucleoside analogs previously developed at Microbiotix, Inc. were tested for their ability to inhibit HSV-1 plaque formation in a Vero cell monolayer, which is permissive for HSV-1 infection. Plaque formation in the presence of the nucleoside analogs was compared to a control that was treated with no nucleoside analogs. **Table 2** shows the average IC_{50} values (the concentration resulting in 50% reduction in viral plaques) calculated for each of the compounds tested. The well characterized nucleoside analog Acyclovir was also tested to make comparisons to a current HSV-1 therapy. The average IC_{50} values of compounds MBX 2168, MBX 2169, and MBX 2172 ranged between 5 and 6 μM and were the lowest of the 15 compounds tested .

To determine whether these compounds are toxic to HFF cells, a cell proliferation assay was performed to measure the concentration necessary to cause cell death (**Table 2**). All of the compounds tested showed very low toxicity except for MBX 2179, which appeared to be toxic to HFF cells, with a CC_{50} value (50% cytotoxicity concentration) of around 50 μM. Also shown in this table are the structures of the nucleoside analogs. All compounds except acyclovir share the same general structure but differ in the R group.

Table 2: Comparison of the Structure, Efficacy, and Toxicity of Various Nucleoside Analogs

General Structure[a]: 			
Compound	**R[a]**	**Average IC_{50} (µM)[b]**	**Average CC_{50} (µM)[c]**
Acyclovir		1.26 ± 1.10	>1000
MBX 1616		105.25 ± 104.57	>1000
MBX 2167		10.53 ± 8.64	>1000
MBX 2168		5.29 ± 3.29	>1000
MBX 2169		6.14 ± 0.18	>1000
MBX 2170		12.78 ± 9.12	>1000
MBX 2171		14.47 ± 4.33	>1000
MBX 2172		5.50 ± 0.47	>1000
MBX 2174		9.38 ± 1.80	>1000
MBX 2175		15.38 ± 7.95	>1000
MBX 2176		59.38 ± 13.74	>1000
MBX 2177		7.35 ± 1.35	>1000
MBX 2178		101.36 ± 109.23	>1000
MBX 2179		n/a	~50
MBX 2180		13.05 ± 1.56	>1000
MBX 2182		11.85 ± 0.83	>1000

a: Excludes the structure of Acyclovir, which is depicted fully in row 3.
b: IC_{50} values were collected from data from 2-10 trials.
c: CC_{50} values were collected from tests done in duplicate.

Based on its low average IC_{50} (the lowest of the 15 tested) and high CC_{50} values, MBX 2168 was chosen for further studies. To determine the time at which MBX 2168 inhibits infection by HSV-1, a time of addition assay measured the viral titer after treatment with the drug at various time points. **Figure 5** shows that the viral titer remained around 3 x 10^3 PFU/mL

if drug was added within the first 4 hours of infection. Adding MBX 2168 between 4 and 6 hours post-infection produced around a 10-fold increase compared to the time 0 addition of drug. Between 6 and 8 hours post-infection, addition of drug allowed the titer of the virus to increase almost 1000-fold compared to the time 0 addition of drug. When drug was not added until 24 hours post infection, the viral titer reached to 5.7 x 10^8 PFU/mL. These data suggest that MBX 2168 works at the time when viral DNA is being synthesized. If added after viral DNA synthesis the drug loses its activity.

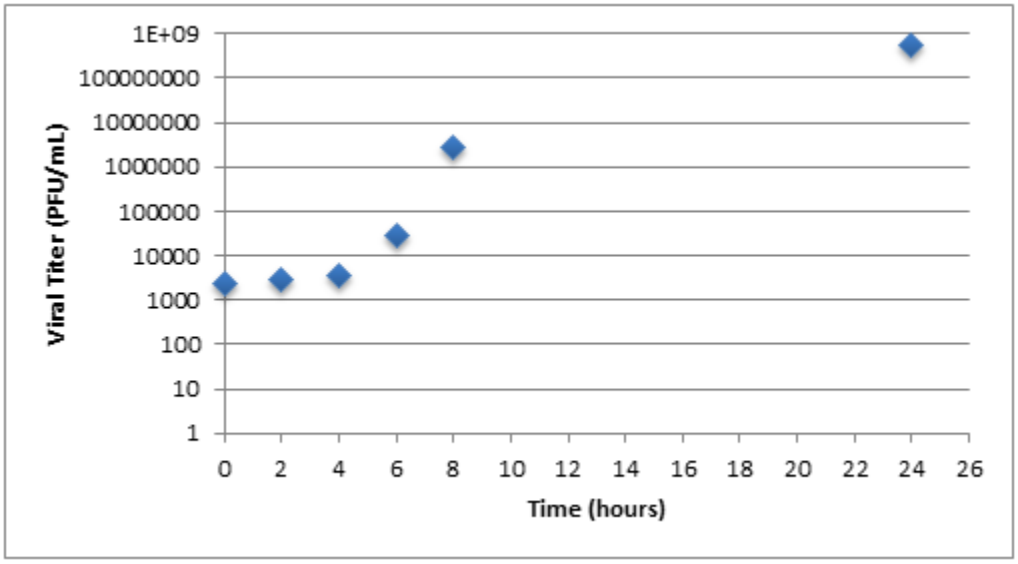


Figure 5: Viral Titers After Treatment with MBX 2168 at Varying Time Points.

To determine whether viral kinases played a role in the first phosphorylation step of MBX 2168, plaque reduction assays were used to analyze the effectiveness of the drug against mutant HSV-1 viruses. Mark Prichard and his colleagues at the University of Alabama, in collaboration with Microbiotix Inc, obtained a mutant strain of HSV-1 with a frameshift mutation in the Thymidine Kinase (TK) gene. Using the plaque reduction assay with this mutant strain, Prichard and colleagues observed a 3-fold increase in the IC_{50} of MBX 2168.

Comparatively, this same mutation produced a 100-fold increase in the IC_{50} of Acyclovir (unpublished data), suggesting that there may be another mechanism by which MBX 2168 becomes phosphorylated.

Mutant strains of HSV-1 containing deletions of the UL13 kinase or the US3 kinase were also tested using MBX 2168 and the plaque reduction assay. **Figure 6** shows the plaque reduction of these viruses in the presence of decreasing concentration of drug. Both mutant viral strains showed a reduction in plaques similar to WT, and their average IC_{50} values were less than 1-fold different than WT, indicating that UL13 and US3 kinases might not be important for 2168 activation.

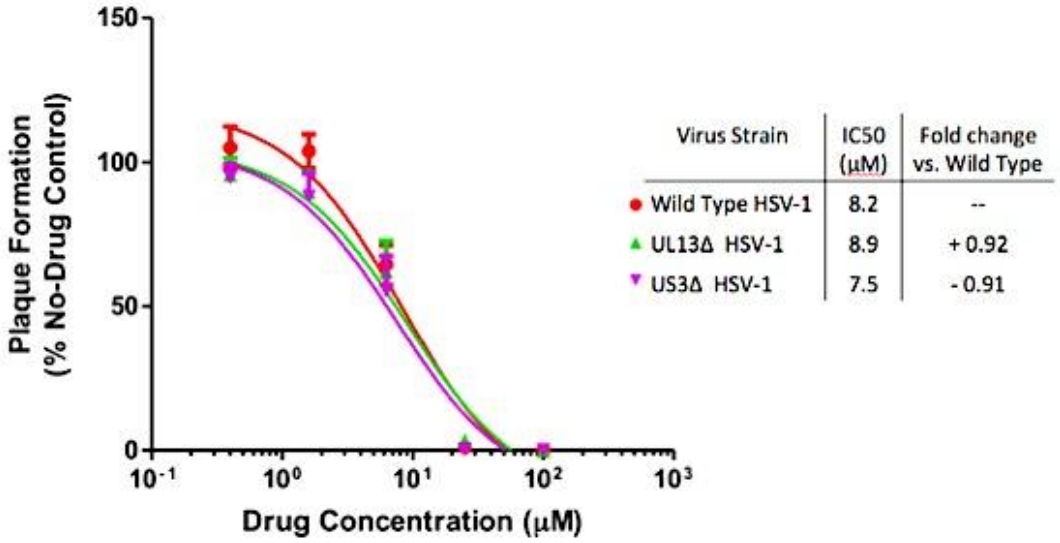


Figure 6: Plaque Reduction Assay for MBX 2168 Using HSV-1 Mutants.

To test the potential phosphorylation of MBX 2168 by human deoxyguanosine kinase (dGK), the dGK gene was cloned and expressed in *E. coli* cells. **Figure 7 (Panel A),** shows the results of an SDS-PAGE, confirming the correct expression of the 25 kDa dGK protein. After purifying the dGK protein, a pyruvate kinase lactase dehydrogenase-coupled assay was used to

indirectly monitor the *in vitro* phosphorylation of deoxyguanosine and MBX 2168 by dGK (**Figure 7, Panel B**). The results indicate that MBX 2168 is not significantly phosphorylated by human dGK. When monitoring the rate of phosphorylation measured at various substrate concentrations, deoxyguanosine (dG) (red) was quickly phosphorylated by dGK, even at low concentrations of substrate, while MBX 2168 (blue) does not become phosphorylated by dGK, even at high substrate concentrations.

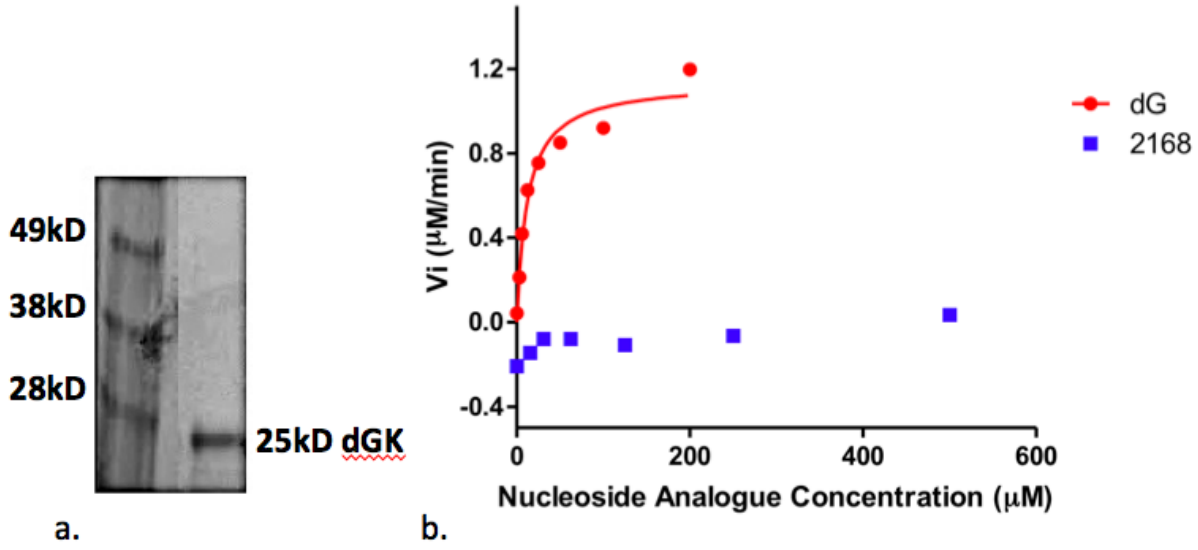


Figure 7: Phosphorylation of Deoxyguanosine Substrate and 2168 Analog by Human Deoxyguanosine Kinase (dGK) In Vitro. The left panel shows an SDS-PAGE gel of *E. coli* expressed dGK. The enzyme was purified and used in an *in vitro* reaction (right panel) to phosphorylate deoxyguanosine or 2168. The Y-axis (Vi) denotes the initial velocity of the reaction in µM per minute. The X-axis shows the concentration of the various substrates tested.

To further investigate the method by which MBX 2168 inhibits HSV-1, a strain of HSV-1 was generated that is resistant to MBX 2168 by serially passaging the virus in the presence of increasing concentrations of the drug. To confirm that this mutant virus was resistant to MBX

2168, a plaque reduction assay was performed (**Figure 8**). As expected, the mutant virus demonstrated almost complete resistance to MBX 2168 (orange curve) compared to WT virus (blue curve). The calculated IC_{50} of 2168 against WT virus in this experiment was close to the average IC_{50} determined previously. Using the mutant virus, even at high concentrations of MBX 2168 the number of plaques formed did not significantly decrease.

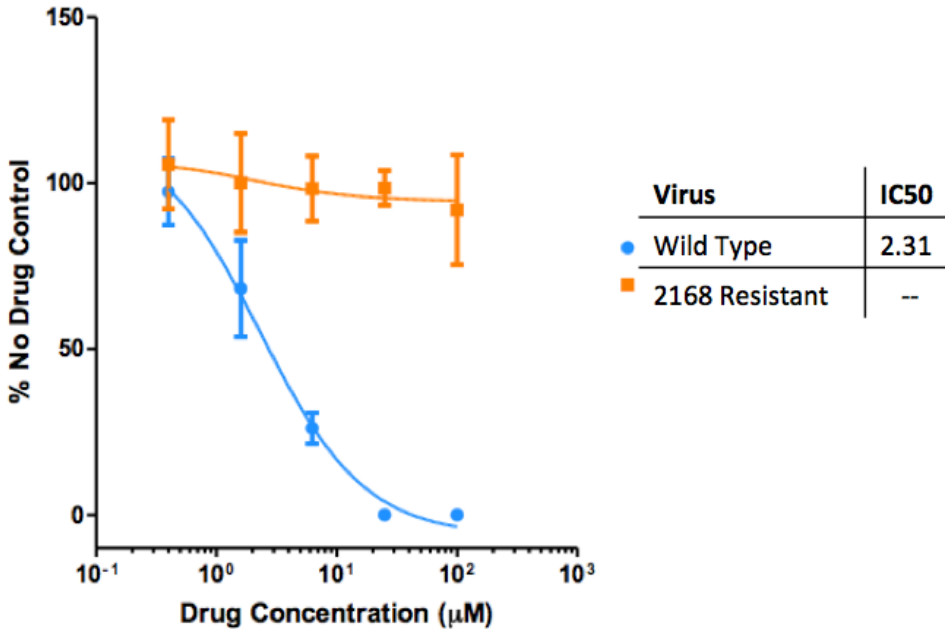


Figure 8: Plaque Reduction by MBX 2168-Resistant HSV-1.

To attempt to understand the mechanism of 2168 resistance, the virally encoded UL30 DNA polymerase gene was sequenced. This specific gene was chosen for sequence analysis because of its critical role in viral replication and its role in the mechanism of several other nucleoside analogs. The UL30 sequence analysis identified a point mutation at residue 841 changing a glycine to a serine. Molecular modeling of the UL30 crystal structure (**Figure 9**) showed that the point mutation G841S could result in a significant shift in position of two active site tyrosine residues, Tyr818 and Tyr722, compared to the wild type positioning.

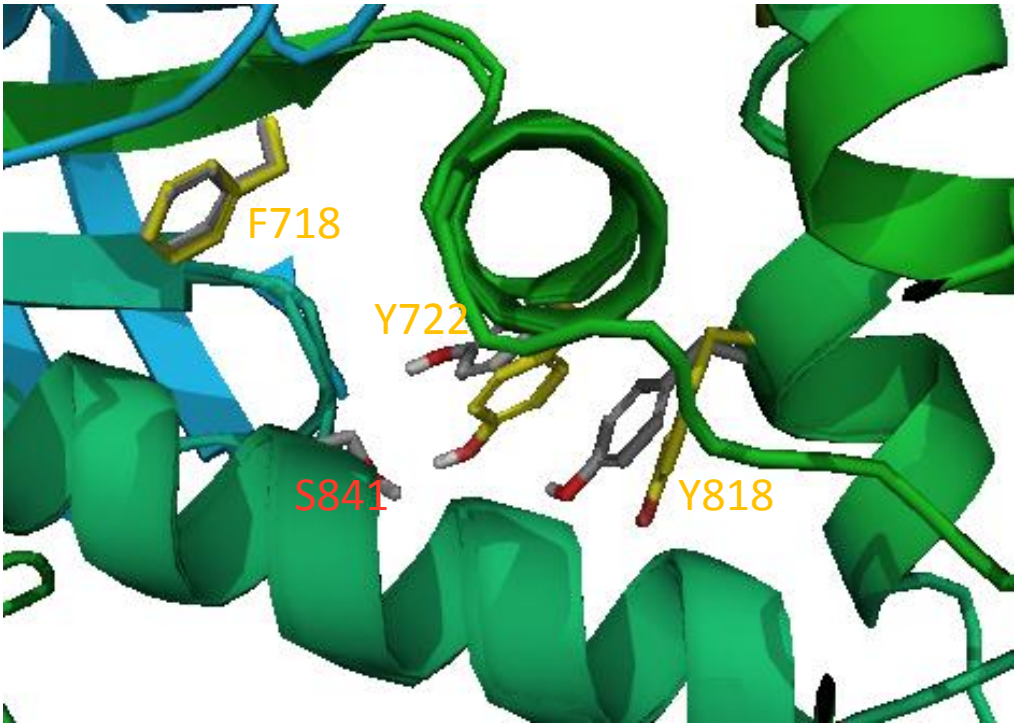


Figure 9: Molecular Model of Secondary Structure of HSV-1 DNA Polymerase. DNA Polymerase from wild type and G841S mutant strains are superimposed, and the position of the mutation is labeled in red (S841). Changes in the position of two active site tyrosine residues (Y722 and Y818) can also be noted in the wild type and mutant strains. Wild type residues are shown in yellow and mutant residues are shown in gray. Also shown is active site residue F718 which does not appear to be significantly shifted in the mutant polymerase.

DISCUSSION

This project aimed to select a novel nucleoside analog showing potential as an HSV-1 antiviral therapy, and to begin to characterize its mechanism of action. Through the use of plaque reduction assays and cytotoxicity assays, nucleoside analog MBX 2168 was found to be a strong inhibitor of HSV-1 while also being relatively non-toxic to a line of human cells. These results suggest that MBX 2168 shows potential as a future therapy for HSV-1.

In order for nucleoside analogs to inhibit DNA synthesis, they must be phosphorylated three times to the mono-, di-, and tri-phosphate forms to be incorporated into the growing DNA chain (Deville-Bonne et al., 2010). MBX 2168 shares a similar structure with other known antiviral nucleoside analogs, and is derived from the nucleoside analog cyclopropavir, so it likely that this drug must also become tri-phosphorylated to be active. Experiments were initiated to attempt to identify the enzyme responsible for 2168 phosphorylation by analyzing HSV-1 viruses containing mutations in known kinase genes. Plaque reduction assays of HSV-1 strains with a mutation in the viral thymidine kinase, which is known to phosphorylate a majority of other nucleoside analogs, showed only a 3-fold increase in the IC_{50} of MBX 2168 (Unpublished data). A typical standard for determining resistance to a drug is the observation of an increase of IC_{50} of more than 3 to 5-fold (Piret and Boivin, 2011). Since this same mutation produced a 100-fold increase in the IC_{50} of acyclovir (a nucleoside with comparable activity), it appeared that this kinase was not fully responsible for the initial phosphorylation of MBX 2168.

The same plaque reduction assay was used to test HSV-1 strains with deletions of the UL13 or US3 kinase genes. These deletions produced no significant increase in the IC_{50} of MBX 2168, which suggested that these viral kinases also do not play a large role in phosphorylation of

the drug. *In vitro* kinetic studies performed with purified human deoxyguanosine kinase also suggested that although this kinase plays a role in phosphorylation pathways of natural nucleosides, it is not responsible for the initial phosphorylation of MBX 2168.

Although the mechanism of phosphorylation of MBX 2168 was not precisely determined, the results of time of addition studies and resistance studies shed light on a possible mechanism of action of the drug. Before 4 hours of infection, treatment with the drug successfully kept viral titers relatively low, around 3×10^3 PFU/mL. However, if drug treatment was applied after 4 hours of infection, the viral titer increased more than 10-fold, and after 6 hours of infection, drug treatment allowed viral titers to increase more than 1000-fold. These results suggested that whatever process MBX 2168 interferes with probably occurs between 4 and 6 hours post infection. Since proteins involved in DNA synthesis are highly expressed between 5 and 7 hours (Boehmer & Lehman, 1997), it seems possible that MBX 2168 affects DNA synthesis.

A study of an isolated HSV-1 that was resistant to MBX 2168 supports this hypothesis. Sequence analysis of the DNA polymerase of this strain identified a point mutation G841S. This mutation is not within the known active site of the protein (Liu et al., 2006), but according to our molecular modeling, it may contribute to changes in the active site by shifting two tyrosine residues. These two tyrosine residues have been suggested to interact with acyclovir when it binds in the active site of HSV-1 polymerase. This G841S mutation suggests that the mechanism of action of the drug MBX 2168 is likely related to viral DNA synthesis.

Together, the results of this project indicate that MBX 2168 be could be used in treating HSV-1 infections in which resistance to known antivirals such as acyclovir have developed. The main therapy for HSV-1 infection is acyclovir, a drug that relies heavily on phosphorylation by the virally encoded thymidine kinase (Appelboom and Flowers, 1983). Interestingly, and

importantly, it appears that MBX 2168 does not depend as much on thymidine kinase for phosphorylation. Since the majority of strains resistant to acyclovir show mutations in the thymidine kinase (Gilbert et al., 2002), MBX 2168 is a desirable alternative because it could still be useful against viruses with these TK mutations.

In general, this project encountered only few problems. One difficulty was in adjusting the titer used in the plaque reduction assay. In order to be able to analyze results from this assay, the titer of virus used to infect the cells needed to be large enough to be able to visualize viral plaques, but small enough to allow plaque counting. It took many trials of adjusting the titer before the results of the plaque reduction assays could be interpreted. Other difficulties included successfully cloning and expressing human dGK, but successful cloning was eventually achieved by de-phosphorylating the edges of the vector prior to ligation.

Unfortunately, the exact method of phosphorylation of MBX 2168 was not elucidated in this project. For this reason, future studies should continue to attempt to identify this mechanism of phosphorylation, as this will be important for understanding how to potentially use MBX 2168 with TK-dependent nucleoside analogs to treat resistant HSV-1 strains. It is possible that the drug relies on other cellular kinases for metabolic activation, so kinetic studies of these enzymes should be performed. Although its structure as a nucleoside analog suggests that it must be phosphorylated to be active, it is possible that MBX2168 does not require phosphorylation. Other tests should be done to compare the effectiveness of the phosphorylated forms of the drug to the pro-drug form, and to discover whether this metabolism of the pro drug is necessary for antiviral activity. Additionally, future tests should sequence the viral thymidine kinase and other important genes of the MBX 2186 resistant strain to confirm that the resistance to the drug is due only to the observed mutation in the DNA polymerase.

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