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MECHANISM OF ACTIVATION OF ANTI-HCMV NUCLEOSIDE ANALOG MBX 2168

A Major Qualifying Project Report

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ABSTRACT

Human Cytomegalovirus (HCMV) infection can progress into a persistent and life-threatening disease in infants and immune-compromised individuals, especially when the virus is resistant to available antiviral therapies. The purpose of this project was to study the cellular mechanism of activation of antiviral drug MBX 2168, a nucleoside analog that inhibits viral DNA replication. Two proposed pathways (upper and lower) were hypothesized to activate MBX 2168. This project examined both pathways, specifically utilizing de-alkylation inhibitors dCF and EHNA to test the involvement of the upper pathway, and *in vitro* kinase assays to test the mono-phosphorylation of synguanol by viral-encoded kinase UL97 (lower pathway). Through protein purification, plaque reduction assays, and *in vitro* assays, the results indicate that both pathways theoretically participate in drug activation, but the cell prefers the upper pathway.

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ACKNOWLEDGEMENTS

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BACKGROUND

The Herpesvirus Family

The *Herpesviridae* family consists of an extensive group of double-stranded DNA viruses, which includes about 100 known members. Eight of these viruses are known to infect humans, and can be further classified into three subgroups: *alpha-, beta-, and gamma-herpesviridae*. Their genomes range from 120 to 230 kb, encoding approximately 70 to 200 genes (Chee et al., 1989). The *alphaherpesviridae* group includes herpes simplex virus-1 (HSV1), herpes simplex virus-2 (HSV2), and varicella zoster virus. This subgroup is known to have a high replication efficiency, broad infectivity, and cause a latent infection in sensory nerve ganglia. In contrast, the *betaherpesviridae* include human cytomegalovirus (HCMV), human herpes virus-6 (HHV6), and human herpes virus-7 (HHV7). They have longer replication cycles, infect a smaller range of mammals, and cause latent infections in the secretory glands, kidneys, and reticuloendothelial cells. The *gammaherpesviridae* subgroup includes Epstein-Barr virus and human herpes virus-8 (HHV8 or Kaposi's sarcoma virus). They have a slow replication cycle, the most restricted host range, and typically infect lymphoid tissue (Whitley, 1996).

Human Herpesvirus-5 (HHV5/Human Cytomegalovirus/HCMV) Structure

The human cytomegalovirus (HCMV) structure consists of four main components: the envelope, capsid, tegument, and genome (**Figure-1**). The envelope consists of a fragile lipid bilayer containing glycoproteins which recognize host cell proteoglycans and aid in fusion of the virion envelope with the host cell membrane. The nucleocapsid is icosahedral-shaped consisting of 162 capsomers, which surround the double-stranded DNA genome. The tegument is the space

between the capsid and envelope, and contains viral proteins and enzymes that aid in the initiation of viral DNA replication (Hunt, 2011).

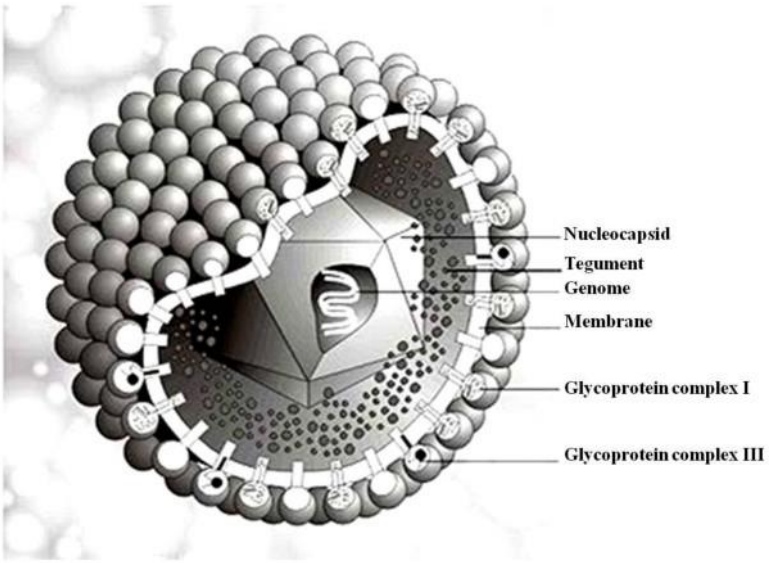

Figure-1: Structure of Human Cytomegalovirus. Shown are the main components of the HCMV virion, including the envelope, nucleocapsid, tegument, and DNA genome (Tomtishen, 2012).

HCMV has the largest genome of all the human herpesviruses (**Figure-2**). Its genome is about 235 kb long, and includes a high percentage of guanine and cytosine residues compared to other herpesviruses. The genome is organized into two main segments: unique long (U_L) and unique short (U_S). Each of the unique regions is flanked by inverted repeat sequences (Landolfo et al., 2003).

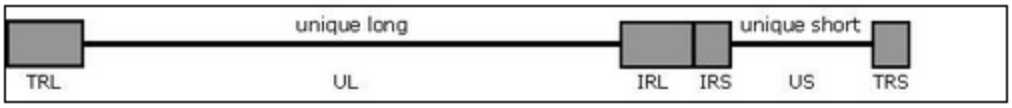

Figure-2: Map of the HCMV Genome. The HCMV DNA genome is organized into two main regions of unique sequences: the Unique Long (UL) region, and the Unique Short (US) region. The unique regions are flanked by two sets of inverted repeats (TRL/IRL and IRS/TRS). TRL, terminal repeat long; IRL, internal repeat long; TRS, terminal repeat short; IRS, internal repeat short (Kotenko et al., 2000).

HCMV Infection and Replication

HCMV infects and replicates in epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived macrophages (Landolfo et al., 2003). Viral entrance has evolved to become a quick and efficient process. The process is summarized in **Figure-3**, and involves the attachment of the virus to cell surface receptors, breakup of the viral envelop, entrance of viral DNA into the nucleus, virus gene expression, viral assembly, and viral exit from the cell (The Drug Monitor, 2002).

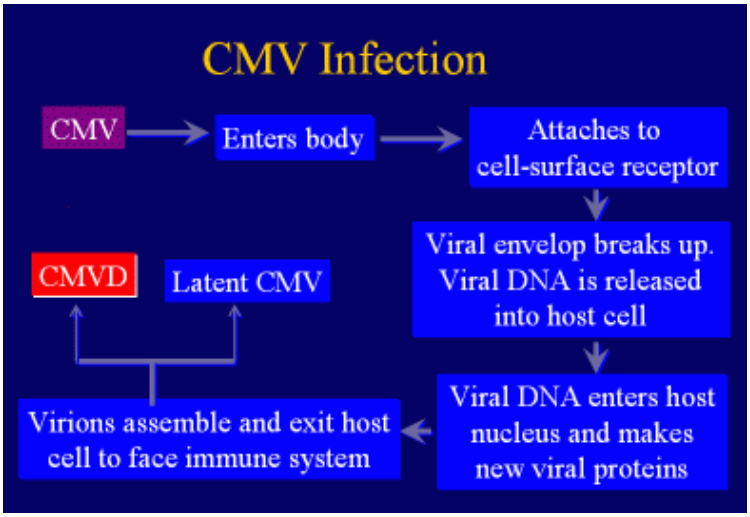

Figure-3: Summary of the HCMV Infection and Replication Processes. Shown are the main processes used by HCMV to infect cells, resulting in either acute or latent infections (The Drug Monitor, 2002).

Surface glycoproteins play a critical role in the recognition and entrance of the virion into the cell. The surface glycoprotein complex gH/gL/UL128-131 and the surface glycoprotein gB interact with each other to promote viral fusion and entry into the host cell (**Figure-4**). It is important to note that the molecular complex of gH/gL/UL128-131 is different than the molecular complex gH/gL. gH/gL/UL128-131 binds to receptors on epithelial cells, while the gH/gL complex binds to different receptors on fibroblasts. Based on current research, it is suspected that the binding of these complexes to host receptors activates glycoprotein gB which triggers viral fusion into the host cell (Wille et al., 2013).

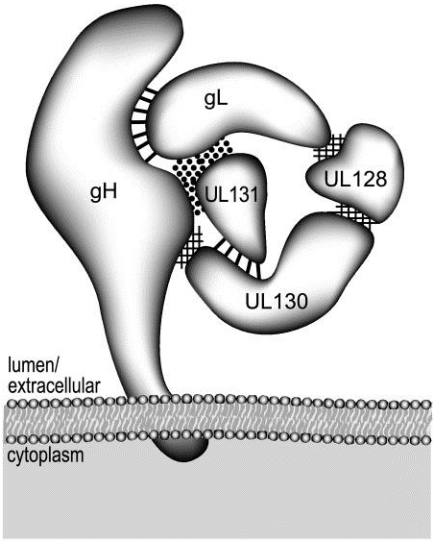

Figure-4: Structure of the Viral gH/gL/UL128-131 Complex. The straight lines represent the interaction between proteins through disulfide bonds, the crossed-lines represent protein interactions through non-covalent interactions, and the dots represent gH and gL forming a specific surface to allow for interaction with UL131 (diagram center). Protein gH is transmembrane, and the complex extends into the outer environment to interact with the host cell (Ryckman et al., 2008).

After the virus enters the cell, the virion is uncoated and the viral DNA is transported to the host cell nucleus, where viral DNA replication is initiated. Viral DNA replication is regulated in an orderly cascade of events through the expression of immediate-early (IE), early

(E), and late (L) proteins. HCMV viral gene expression typically begins one hour post-infection, with the transcription of the UL122/123 and auxiliary genes. These genes encode immediate-early proteins $IE1_{491aa}$ (IE1-72) and $IE2_{579aa}$ (IE2-86), which are essential for the early stages of the viral DNA replication process and control the transcription of a number of viral and cellular genes. For example, IE1-72 initiates the transcription of viral DNA polymerase for replicating the viral genome at around 16 hours post-infection, while IE2-86 regulates genes that help convert from early-immediate to later stages of viral replication. IE2-86 also arrests the host cell in S-phase, preventing the replication of host DNA (Landolfo et al., 2003).

The next step in HCMV DNA replication involves the transcription of β-genes, which include UL4, UL44, UL54, and UL112/113. There are two major subgroups of β-genes: β1 (E phase, 4-8 hours post infection) and β2 (E-L phase, 8-24 hours post infection). The β-genes are responsible for encoding viral replication factors, repair enzymes, and proteins responsible for immune invasion. Viral DNA is usually synthesized at 16 hours post-infection during the early-protein phase. UL54 encodes viral DNA polymerase, and UL44 keeps the DNA polymerase on the viral DNA template strand. UL112/113 encodes DNA-binding proteins that are responsible for coordinating replication centers within the host cell's nucleus, and recruiting the necessary proteins and enzymes needed for viral DNA synthesis. The newly synthesized viral genomes are then inverted, cleaved, and processed (Landolfo et al., 2003).

The final stage of virus replication involves maturation and structural development of virions. The expression of late proteins are a critical component to this process, with 75% of the HCMV open reading frames containing genes that encode for late proteins involved in the maturation of virions. The late genes are typically expressed 24 hours post infection and include γ1 (24-36 hour post infection) and γ2 (24-48 hours post infection). The newly synthesized viral

DNA leaves the nucleus and enters the cytoplasm in a temporary nuclear capsid. The DNA loses its temporary nuclear membrane, and gains its tegument and capsid. Lastly, the structure obtains it viral envelope by budding into vesicles within the Golgi apparatus, where it is then transported by exocytosis out of the cell, beginning 72 hours post infection (Landolfo et al., 2003).

HCMV Symptoms

HCMV is a worldwide epidemic, causing serious diseases in infants and immune-compromised individuals. HCMV is transmitted through bodily fluids (blood, mucous, semen, organ transplants, etc.) or placental transfer. Between 40 and 100% of the worldwide population carry the antibody against HCMV in their blood, indicating infection at some point in their lifetime. Over 90% of the US population has the HCMV antibody by the age of 80 years old. A majority of immune-competent individuals do not experience clinical symptoms, however, about 10% of HCMV cases in immune-competent patients result in mononucleosis (Public Health Agency of Canada, 2011).

HCMV can be life-threatening in infants infected congenitally. About 0.2 to 2.4% of newborns are infected with HCMV, and of this percentage, 10 to 15% experience severe symptoms at birth (Public Health Agency of Canada, 2011; Biron, 2006). Symptoms of infected newborns include low birth weight, jaundice, hepatosplenomegaly, petechiae, hepatits, myocarditis, pneumonitis, anemia, CNS abnormalities (seizures, encephalitis, etc.), retinitis, and chorioretinitis (Public Health Agency of Canada, 2011; Landolfo et al., 2003). Approximately 1 to 20% of newborns infected at birth experience organ failure resulting in death during infancy (Landolfo et al., 2003). Additionally, between 6 to 25% of those congenitally infected but

asymptomatic in infancy will develop hearing loss later in life (Public Health Agency of Canada, 2011).

HCMV can cause life-threatening disease in immune-compromised people, such as those receiving an organ transplant or with AIDS (acquired immune-deficiency syndrome). HCMV is the most frequent fatal viral infection in patients receiving an organ or hematopoietic stem cell transplant. Organ recipients who are HCMV sero-negative but receive an organ transplant from a donor who is sero-positive are at greatest risk (Biron, 2006; Landolfo et al., 2003). Common symptoms of HCMV-infected donor recipients include fever, muscle and joint pain, fatigue, low white blood cell and/or platelet cell count, and hepatitis. Pneumonitis, which is typically a result of HCMV infection during bone marrow transplants, has high mortality rate of 50 to 90% (Public Health Agency of Canada, 2011). Patients with AIDS are also at a high risk of developing HCMV infection. They often present with similar symptoms to that of organ transplant recipients, but also experience retinal damage leading to blindness (Biron, 2006). **Table 1** summarizes the common syndromes for HCMV-infected patients that are immune-compromised from either AIDS or from transplants (Landolfo et al., 2003).

Table-1: Medical Conditions Associated with HCMV-Infected Immune-Compromised Patients.

Syndromes	AIDS	Solid transplant	BMT
Esophagitis	+	+	+
Gastritis	+	+	+
Enterocolitis	+	+	+
Hepatitis	+	++	+
Pancreatitis	–	++	–
Pneumonitis	–	+	++
Retinitis	++	+	+
Encephalopathy	+	–	–
Polyradiculopathy	+	–	–

Shown are the common syndromes caused by HCMV infections in AIDS patients, or in patients receiving organ transplants or bone marrow transplants (Landolfo et al., 2003).

Nucleoside Analog Inhibition of Viral DNA Synthesis

Nucleoside analog inhibition is currently the most common method for treating HCMV infections. The synthetically made nucleosides are similar in structure to naturally occurring cellular nucleotides, but are modified to result in the termination of DNA synthesis. The synthetic nucleosides enter a cell and are phosphorylated into tri-phosphate nucleotides by viral and cellular enzymes. Typically, a viral kinase will attach the first phosphate group to the nucleoside, and human kinases facilitate the addition of the following two phosphate groups. After the nucleoside is phosphorylated, the nucleotide analogs are incorporated into the growing viral DNA strand, but their incorporation results in termination of viral DNA replication (Clercq, 2004; Biron, 2006; Deville-Bonne et al., 2010). DNA strand elongation is halted because the nucleoside analogs do not contain the 3' hydroxyl group, which would have allowed for additional nucleotide attachment (Appelboom and Flowers, 1983).

Ganciclovir Nucleoside Analog

Ganciclovir (**Figure-5A**) was the first drug approved for treatment of HCMV, and continues to be the primary method of HCMV treatment. Viral kinase UL97 phosphorylates ganciclovir to its monophosphate form, while human kinases continue to phosphorylate it to its di- and tri-phosphate forms. Ganciclovir triphosphate replaces dGTP in the growing viral DNA strand stopping DNA synthesis (Clercq, 2004; Biron, 2006).

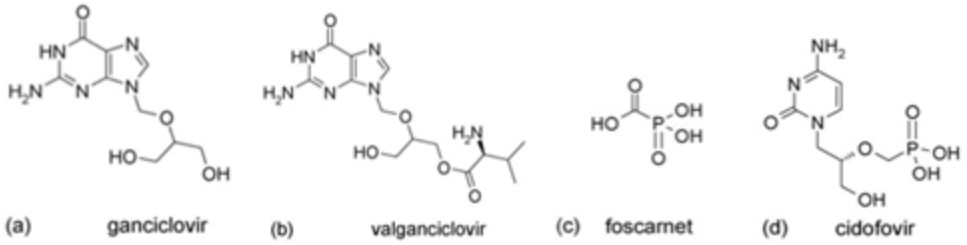

Figure-5: Structure of Several Anti-Cytomegalovirus Drugs Currently on the Market. (Biron, 2006)

Additional HCMV Antiviral Therapies

Additional HCMV therapies currently on the market include foscarnet, cidofovir, valganciclovir, and fomivirsen (**Figure-5**). Valganciclovir and cidovir are nucleoside analogs with a very similar mechanism of action to that of ganciclovir. They are both phosphorylated to their active forms within the cell, and incorporated into the growing viral DNA strand, terminating viral DNA replication. Foscarnet, a pyrophosphonate analogue, interferes with the interaction between pyrophosphate and the viral DNA polymerase (encoded by UL54 gene), inhibiting the activity of viral DNA polymerase and preventing viral DNA replication. Fomivirsen targets the mRNA of the major immediate-early transcriptional unit of HCMV, which plays a critical role in viral infection and replication (Clercq, 2004; Biron, 2006).

Several anti-viral drugs are currently in clinical development. Benzimidazole ribosides are a major area of HCMV drug development research because they do not target viral DNA polymerase but other critical viral enzymes, such as UL97. For example, Maribavir, a benzimidazole riboside, targets the UL97 viral enzyme, thus inhibiting phosphorylation of viral proteins that are critical for DNA synthesis and preventing newly synthesized viral DNA from budding and leaving the nucleus. Marbivor is currently in Phase II clinical trials. BAY 38-4766 and Letermovir are two additional non-nucleoside antiviral HCMV therapies in clinical development. These two drugs target the viral terminase complex, specifically kinases UL56 and UL89, which are responsible for viral DNA maturation (Biron, 2006; Goldner et al., 2011). **Figure-6** below shows several different HCMV drugs (either already on the market or in clinical development) and each of their targets (Marschall and Stamminger, 2009).

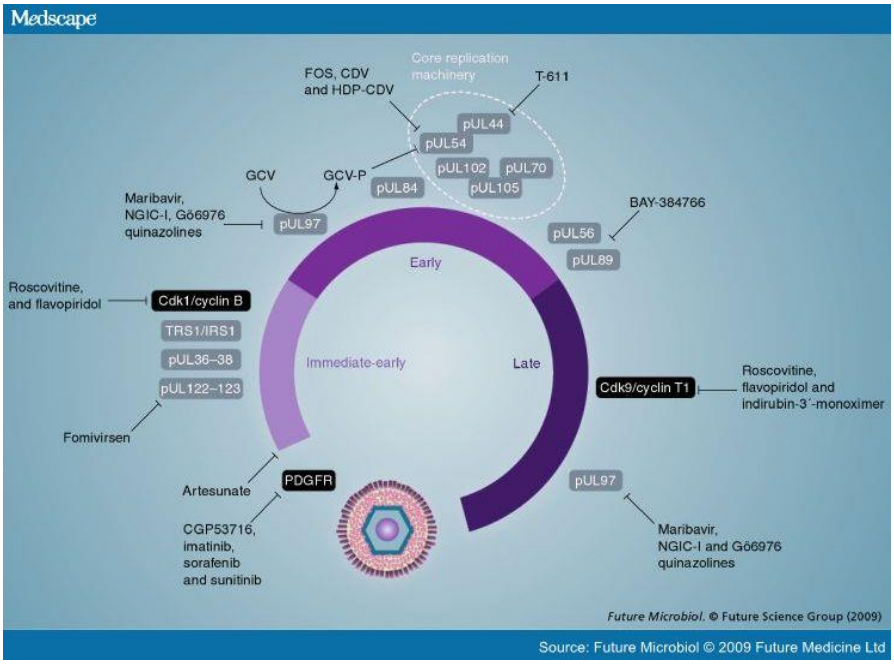

Figure-6: Diagram of Several Anti-HCMV Drugs and Their Targets. Shown is the replication cycle of HCMV relative to a variety of HCMV drugs and the proteins they affect. The drugs include those already on the market and those in clinical development (Marschall and Stamminger, 2009).

Microbiotix, Incorporated

Microbiotix, Incorporated is a biopharmaceutical company located in Worcester, Massachusetts that focuses on the research and development of small molecule anti-infective therapies. Currently, clinical research is being conducted on hepatitis C virus (HCV) and human cytomegalovirus (HCMV) drugs. Cyclopropavir is a methylenecyclopropane nucleoside (MCPN) analog against HCMV, human herpesvirus 6, and human herpesvirus 8 that is currently in phase 1b of clinical trials at Microbiotix, Inc. (Scott et al., 2011). This MQP project examined another MCPN analog very similar in structure to cyclopropavir, MBX 2168, which was first synthesized at Microbiotix and is also a nucleoside analog drug against HCMV infection (Komazin-Meredith et al., 2013). **Figure-7** shows the structures of cyclopropavir and MBX 2168. Cyclopropavir has an additional hydroxyl group but no alkyl group off of the oxygen (Chou et al., 2014).

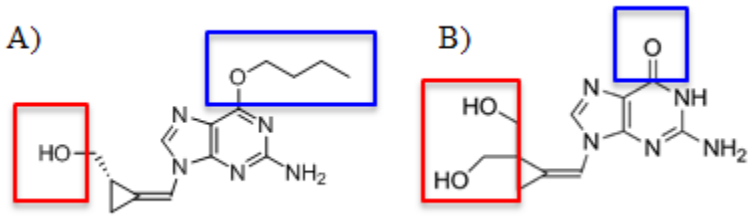

Figure-7: Molecular Structures of Drugs MBX 2168 (Panel A) and Cyclopropavir (Panel B). The red boxes show the difference in the hydroxyl groups between the two compounds: MBX 2168 has one hydroxyl group and cyclopropavir has two. The blue boxes illustrate how MBX 2168 still has its alkyl group while cyclopropavir is de-alkylated (Chou et al., 2014).

Both cyclopropavir and MBX 2168 have similar mechanisms of activation as ganciclovir. However, recent studies have shown that MBX 2168 and cyclopropavir are more broad-spectrum analogs and are active against several strains of herpes virus. Additionally, there is limited

cross-resistance between ganciclovir and MBX 2168 (Chou et al., 2014). This MQP project further examined the cellular mechanism of activation of MBX 2168, specifically the potential role of adenosine deaminase-like protein 1 (ADAL1) and viral kinase UL97 in the de-alkylation and phosphorylation, respectively, of MBX 2168.

MBX 2168 can be mono-phosphorylated and de-alkylated by two pathways, an upper and lower pathway (**Figure-8**). In the upper pathway, MBX 2168 is first mono-phosphorylated and then de-alkylated. The lower pathway is the reverse in that MBX 2168 is first de-alkylated and then mono-phosphorylated. Previous research, conducted by Gloria Komazin, has confirmed the first step in both the upper and lower pathways: viral kinase UL97 mono-phosphorylates MBX 2168 in the upper pathway, and adenosine deaminase (ADA) de-alkylates MBX 2168 in the lower pathway but with low efficiency. This MQP project examined the second step in both of these processes, specifically the potential role of ADAL1 in the de-alkylation of MBX 2168 monophosphate in the upper pathway, and the ability of viral kinase UL97 to mono-phosphorylate synguanol in the lower pathway. After MBX 2168 is mono-phosphorylated and de-alkylated, it proceeds through the remainder of the pathway to be tri-phosphorylated by host kinases and is then incorporated into the growing viral DNA strand (Komazin-Meredith, 2013).

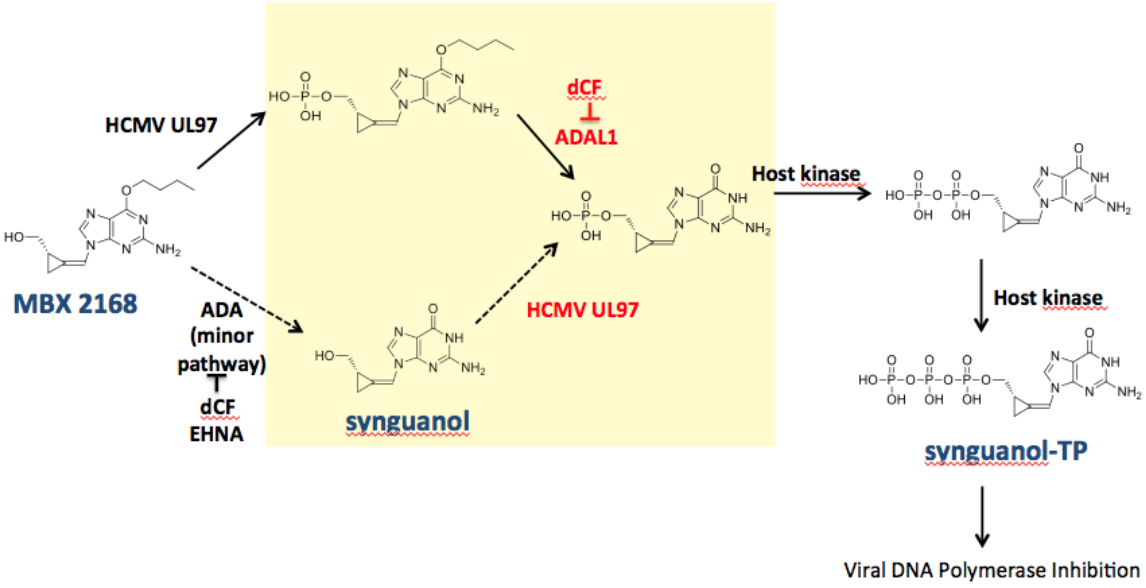

Figure-8: The Proposed Upper and Lower Pathway for MBX 2168 Activation. The portion of the pathway within the yellow box was the focus of this MQP project. The upper pathway (solid arrows) involves the initial mono-phosphorylation of MBX 2168 by viral kinase UL97 and then a de-alkylation by ADAL1. The lower pathway (dotted arrows) involves the initial de-alkylation of MBX 2168 to form synguanol, followed by the mono-phosphorylation of synguanol by UL97. dCF is an inhibitor of both ADA and ADAL1. EHNA inhibits only ADA (Komazin-Meredith, 2013).

PROJECT PURPOSE

Human Cytomegalovirus infects over 90% of the population by the age of 80 years old. Of this percentage of the population, infants and immune-compromised individuals are at the highest risk for developing life-threatening symptoms from the infection. The purpose of this project was to study the mechanism of activation of anti-viral nucleoside analog, MBX 2168, and specifically the role of viral kinase UL97 and adenosine deaminase-like protein 1 (ADAL1) in MBX 2168 activation. As discussed previously, two pathways have been proposed that activate MBX 2168. This project sought to identify which of the two pathways is the major pathway for MBX 2168 activation, and to validate the participation of two key intermediate steps in the pathways (highlighted in yellow in Figure-8). The ability of synguanol to be phosphorylated by UL97 was tested by *in vitro* kinase assays, while the ability of mono-phosphorylated MBX 2168 to be de-alkylated by ADAL1 was tested by using inhibitors dCF and EHNA in cell based assays.

METHODS

HFF Cell Culture and Maintenance

Human foreskin fibroblast (HFF) cells (ATCC, catalog number: SCRC-1041) were grown in minimum essential media (MEM) supplemented with Earl's salts and 10% fetal bovine serum (FBS) and incubated at 37°C. After three to four days of incubation, the cells were passaged. The media was removed and the cells were washed once with phosphate-buffered saline (PBS). The cells were resuspended, and agitated in trypsin-EDTA solution for two minutes. The trypsin was then removed and replaced with MEM media supplemented with Earl's salts and 10% FBS. The cells were transferred to a new flask, split by a 1:3 dilution, and given new supplemented MEM media.

Plaque Reduction Assay

HFF cells were plated at a density of 8.5×10^4 cells per well, and allowed to grow overnight at 37ºC. The cells were then infected with HCMV viral strain AD169 (ATCC, catalog number: VR-538, Biosafety Level-2) at a concentration of 80 viral PFU/mL, and incubated at 37ºC for 1 hr. A set of six MBX 2168 dilutions (100 μM, 20 μM, 4 μM, 0.8 μM, 0.16 μM, and 0 μM) was then added to the cells, and they were incubated for 9-11 days at 37ºC. Some of the wells also contained 60 μM of either EHNA or dCF inhibitors. The MBX 2168 plaque reduction assays were performed in quadruplicate.

After 9-11 days of incubation, the wells were stained with crystal violet stain (0.1% crystal violet and 20% methanol) for ten minutes, and then were rinsed with water. The number

of plaques per well were counted under a light microscope, and the data was recorded and plotted using the GraphPad Prism software.

SF21 Insect Cell Culture

SF21 insect cells (obtained from Invitrogen, catalog number: B821-01) were originally derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda*. SF21 insect cells were grown in Grace's insect media supplemented with 10% FBS at 27°C. After 3-4 days of incubation, the insect cells were passaged. The media was removed and replaced with new Grace's insect media. The cells were gently scraped off the flask and transferred to a new flask at a 1:3 dilution. New Grace's insect media supplemented with 10% FBS was added to each flask.

UL97 Expression and Purification

UL97 viral kinase was expressed in the SF21 insect cells and purified in order to perform *in vitro* kinetic assays to assess the ability of UL97 kinase to mono-phosphorylate the substrate synguanol. SF21 insect cells were infected with baculovirus containing UL97 kinase with a glutathione S-transferase (GST) tag on the N-terminus (the baculovirus strain expressing glutathione S-transferase (GST)-UL97 was donated to Microbiotix, Inc. by Donald Coen of Harvard Medical School). The infected cells were incubated at 27ºC for 72 hours. The cells and media were then harvested and spun in a swinging bucket rotor at 1000 revolutions per minute (RPM) for 5 minutes to pellet the cells. The supernatant was discarded and the cell pellet was stored at -80ºC.

Lysis buffer (100 mM Tris pH 7.5, 100 mM potassium chloride, 20% glycerol, 5.8 grams of sodium chloride, one protease inhibitor, and distilled water) was added to the frozen UL97 cell pellet. The UL97 cell solution was lysed in a French pressure cell press, centrifuged, and then filtered through a 0.2 μM HT Tuffryn Membrane (Pall Corporation). The protein solution was purified by running it through a GST affinity chromatography column, followed by an anion exchange chromatography column. After each column, a silver stained SDS-PAGE gel was used to identify fractions containing purified UL97. The fractions containing purified UL97 were pooled and concentrated using the Pierce Concentrator, PES, 10K MXCO, 0.5mL by Thermo Scientific. The purified and concentrated UL97 was stored at -80ºC for further analysis.

In Vitro Time Course UL97 Assay

In vitro kinetic assays with purified UL97 protein were performed in duplicate to determine the ability of UL97 to phosphorylate synguanol, and to examine the level of affinity of UL97 for synguanol. The time course assay involved incubating 1 mM of synguanol, 3.2 mM of ATP, and kinase buffer (50 mM Tris pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT) at 37°C. After 20 minutes, 5 ng/μL of purified GST-UL97 kinase was added to the reaction, which was then placed back in the 37°C incubator. At selected times, aliquots were taken and stopped with 2% trifluoroacetic acid (TFA). The formation of synguanol monophosphate was measured by UV absorbance (280 nm) on a reverse phase high-performance liquid chromatography (HPLC) column. Solvent A contained 0.1% TFA (Sigma Chemical, St. Louis, MO) in H_2O (EMD Chemicals Inc., Gibbstown NJ), and solvent B had 0.1% TFA in 100% acetonitrile (EMD Chemicals Inc., Gibbstown, NJ). A Grace Altima protocol (Grace Davison Discovery Sciences, Deerfield, IL) was utilized to improve sample (100 μL injections) resolution. A 150-mm by a

4.6-mm 5-µm C_{18} column was used with a gradient of 5% solvent B to 30% solvent B over a time period of 25 minutes for synguanol. Liquid-Chromatography Mass-Spectroscopy (LC/MS) (LCQ Fleet) was then used to verify the identity of synguanol monophosphate. The data generated by reverse phase HPLC for the time course and kinetics assays were plotted using GraphPad Prism software.

The kinetics assay involved introducing different concentrations of synguanol (0.25, 0.5, 1, 1.5, 2 mM) to a mixture of 3.2 mM of ATP, kinase buffer (50 mM Tris pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT) and 5 ng/µL of UL97 during the linear phase of synguanol monophosphate formation (180 minutes) at 37°C. The reaction was stopped with 2% trifluoroacetic acid (TFA), and UL97 kinetics were measured by reverse phase HPLC utilizing the same parameters as explained in the time course assay above. The results were recorded and plotted using the GraphPad Prism software.

RESULTS

HCMV is a worldwide epidemic in which over 90% of the US population will be infected at some point their lifetimes. HCMV infection is particularly life threatening in infants and immune-compromised individuals if they carry a HCMV strain that is resistant to available antiviral therapies (Public Health Agency of Canada, 2011). The purpose of this MQP project was to examine the role of two proposed upper and lower pathways in activating antiviral drug MBX 2168, a nucleoside analog that inhibits DNA replication. More specifically, this projected sought to identify which of the two pathways is the major pathway, and to validate two key intermediate steps in the pathways: 1) the ability of viral kinase UL97 to directly phosphorylate synguanol, and 2) the ability of ADAL1 to de-alkylate mono-phosphorylated MBX 2168.

The first part of the project focused on determining whether synguanol is a good substrate for viral kinase UL97, which would result in mono-phosphorylation of synguanol and validate the minor pathway. *In vitro* time course and kinetics assays were performed with purified UL97 to confirm its role in phosphorylation within the minor pathway. **Figure 9A** shows the proposed reaction by which UL97 mono-phosphorylates synguanol. **Figure 9B** shows the SDS-page gel of purified UL97 protein after affinity (column 1) and anion exchange (column 2) column chromatography. A pure protein band at the expected size of 110 kDa was obtained after the 2nd column, providing a sufficient amount of kinase to perform several kinase assays. **Panels 9C** and **9D** show the data from the *in vitro* time course and kinetics assays. A linear increase in the formation of synguanol monophosphate was observed during the first 7 hours of incubation, and the product continued to increase from seven to twenty-four hours but at a slower rate. The Michaelis-Menten plot (Panel 9D) yielded a UL97 Michaelis constant (K_m) value of 6.4 mM and a maximum reaction velocity (V_{max}) value of 6.4 pmol/minute. The increase in the formation of

synguanol monophosphate and the low K_m value indicate that synguanol is a good substrate for viral UL97.

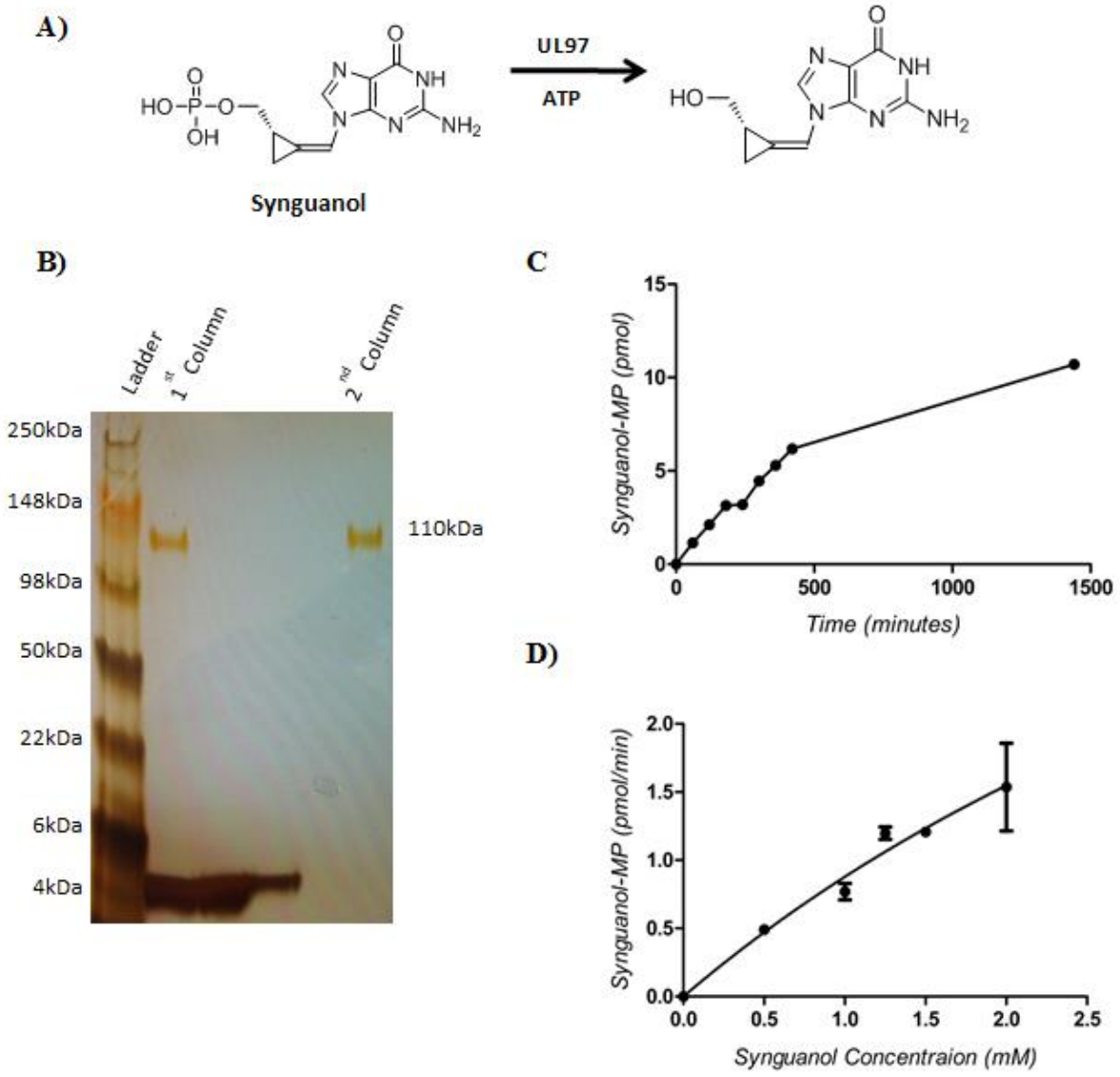

Figure 9: ***In Vitro*** **Kinase Assays: Direct Phosphorylation of Synguanol by Viral Kinase UL97. Panel A** shows a reaction by which viral kinase UL97 phosphorylates synguanol (minor pathway for drug activation). **Panel B** shows the SDS-page gel of purified UL97 kinase (110kDa) after GST affinity chromatography (column 1) and after anion exchange chromatography (column 2). **Panel C** shows the time course assay of the mono-phosphorylation of synguanol by purified UL97 kinase *in vitro*. The concentration of synguanol-PO_4 produced was determined by comparison to a non-phosphorylated synguanol standard curve (molar absorptivity was treated as equal). **Panel D** shows a Michaelis-Menten plot yielding a Km of 6.4 mM and a V_{max} of 6.5 pmol/min. This data supports the involvement of the minor pathway in MBX 2168 drug activation.

The second part of the project involved using plaque reduction assays to determine whether ADAL1 de-alkylates the monophosphate form of MBX 2168. In this assay, activation of MBX 2168 within the cell results in a decrease in the number of viral infected plaques. **Panel 10A** provides the proposed reaction by which ADAL1 de-alkylates MBX 2168 monophosphate. The HCMV viral-infected cells were incubated with MBX 2168 and either ADAL1 inhibitor dCF or ADA inhibitor EHNA (previously shown in Figure-8). **Panel 10B** provides the data from the plaque reduction assays in 3 different conditions: MBX 2168 without inhibitor, MBX 2168 in the presence of dCF (which blocks both upper and lower pathways), and MBX 2168 in the presence of EHNA (which blocks only the lower pathway). MBX 2168 with no inhibitor (black line) showed substantial plaque reduction over the drug concentration range tested, with an IC_{50} value of 0.15 μM (in this case it took only 0.15 μM of MBX 2168 to inhibit 50% of the HCMV infection). MBX 2168 in the presence of EHNA (blue line, blocking only the lower pathway) showed an IC_{50} value of about 0.16 μM, which was not significantly different than without any inhibitor. This data indicates that the lower pathway is not the preferred pathway in this type of cell. Lastly, MBX 2168 in the presence of dCF inhibitor (red line, blocking both upper and lower pathways) had significantly more plaques than MBX 2168 with no inhibitor or MBX 2168 with EHNA, with an IC_{50} value of 6.92 μM, which was 46-fold higher than MBX with no inhibitor, and 42-fold higher that MBX 2168 with EHNA. The high IC_{50} value for MBX 2168 in the presence of dCF, suggests that these two pathways together represent the main mode of drug activation in these cells.

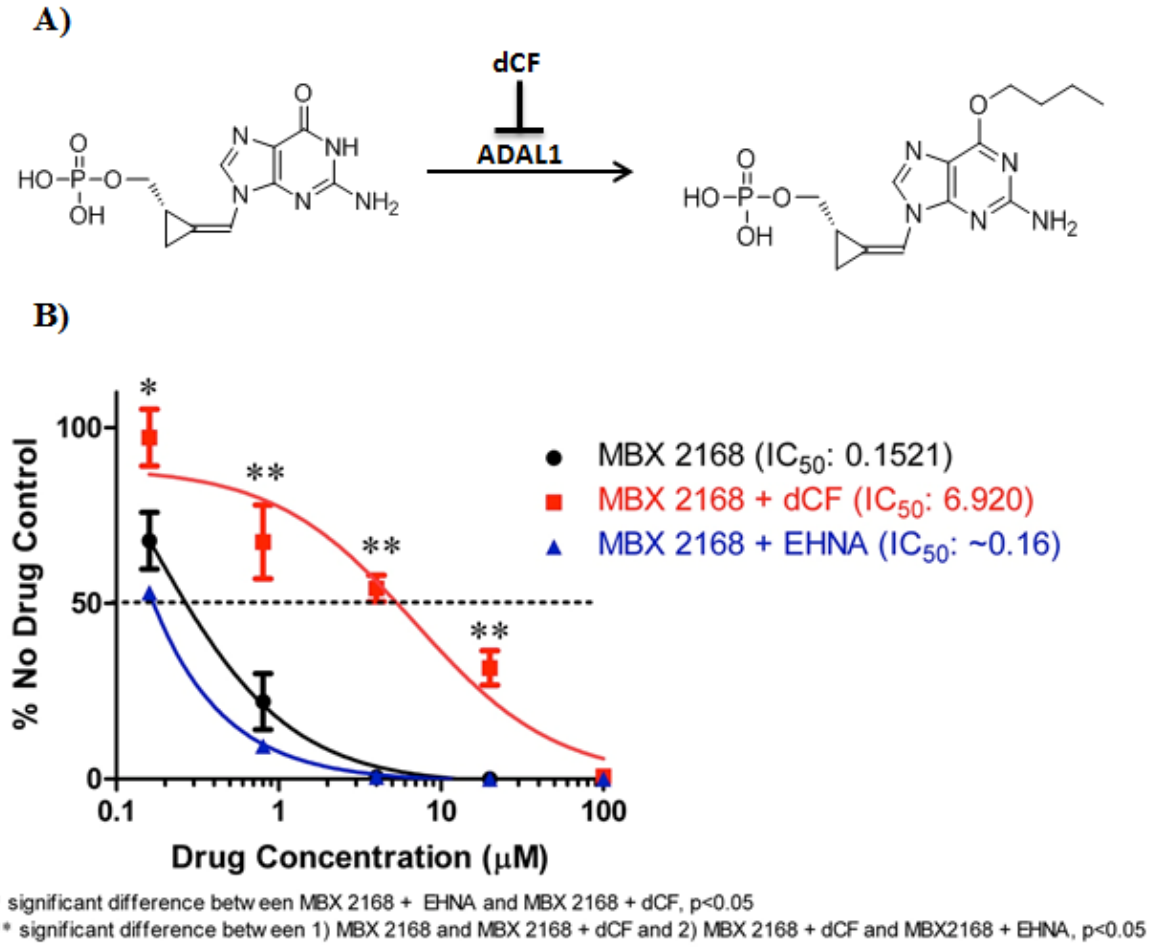

Figure 10: Plaque Reduction Assays for HCMV Virus. Panel A shows the reaction by which ADAL1 de-alkylates the mono-phosphate form of MBX 2168, which is blocked by ADAL1-inhibitor dCF. **Panel B** shows HCMV plaque reduction in the presence of increasing concentrations of MBX 2168. dCF (red) significantly reduced MBX 2168 activity (upper pathway), but it remained unchanged in the presence of ADA inhibitor EHNA (blue) (lower pathway). Together the plaque reduction data suggests that ADAL1 (upper pathway) is the cell's preferred pathway for activating MBX 2168.

DISCUSSION

Nucleoside analog drugs are a relatively effective form of HCMV therapy, in which they are tri-phosphorylated and incorporated into the growing viral DNA strand which results in inhibition of viral DNA replication (Deville-Bonne et al., 2010). Ganciclovir is the primary nucleoside analog used to treat HCMV infection (Clercq, 2004; Biron, 2006). A new analog synthesized at Microbiotix, MBX 2168, has a similar mechanism of activation as ganciclovir but is a more broad-spectrum analog inhibiting replication of multiple strains of the herpes virus (Komazin-Meredith et al., 2013). Additionally, MBX 2168 also has limited cross-resistance to ganciclovir (Chou et al, 2014). This MQP project focused on two key intermediate steps of MBX 2168 activation. The upper pathway involves the initial mono-phosphorylation of MBX 2168 followed by its de-alkylation. The lower pathway is the reverse of the upper pathway, where MBX 2168 is first de-alkylated and then mono-phosphorylated (Komazin-Meredith, 2013).

Based on the plaque reduction assay data, the upper pathway appears to be the major pathway. MBX 2168 with dCF, which inhibits both the upper and lower pathways (by inhibiting ADAL1 in the upper pathway and ADA in the lower pathway) (Komazin-Merideth, 2013) showed a significant increase in IC_{50} (reduced drug activation), indicating that the two pathways represent a major mode of drug activation in these cells. MBX 2168 with ADA inhibitor EHNA (which blocks the lower pathway) showed no significant difference from MBX 2168 without any inhibitor. If both pathways were equally utilized, it would be expected that MBX 2168 in the presence of EHNA would have an IC_{50} value between MBX 2168 without inhibitor (0.15 μM) and MBX 2168 with dCF (which blocks both pathways, 6.92 μM). However, MBX 2168 in the presence of EHNA had a low IC_{50} value of about 0.16 mM, statistically indistinguishable from

MBX 2168 without inhibitor. These results suggest that EHNA had no significant effect on inhibiting MBX 2168 activation, and that MBX 2168 was fully activated by the upper or major pathway.

The plaque reduction data also validates the involvement of the de-alkylation of mono-phosphorylated MBX 2168 reaction as an intermediate in MBX 2168 activation. dCF is known to inhibit both ADA and ADAL1 (Faletto et al., 1997). Therefore, as expected, the results showed significantly more plaque formation in the presence of dCF, indicating that dCF is most likely inhibiting ADAL1 in the upper pathway. Further plaque reduction assays need to be conducted to confirm the data presented in this report. Furthermore, it was difficult to purify a sufficient amount of ADAL1 protein to run *in vitro* assays, so further experimentation is needed to optimize ADAL1 purification. Purified ADAL1 could then be used to run *in vitro* assays to confirm the de-alkylation of the mono-phosphate form of MBX 2168 by ADAL1 and determine the protein's K_m and V_{max} values by HPLC and mass spectroscopy.

With respect to the in vitro kinase assays performed with purified UL97 kinase, the data presented in this report (**Figure-10**) suggests that synguanol is a substrate for UL97, as shown by the relatively low K_m of 6.4 mM (high enzyme-substrate affinity). The UL97 K_m for synguanol has a similar value to previous research conducted at Microbiotix, with UL97 having a K_m value of 5.3 mM for MBX 2168 and 3.4 mM for MBX 1616 (Komazin-Meredith et al., 2014). However, the literature also indicates that the UL97 K_m value is even lower for some other compounds, such as cyclopropavir (1.75 mM) (Gentry et al., 2010) and resistant strains of ganciclovir (ranging from 0.025 to 0.040 mM depending on the mutant strain) (Shannon-Lowe and Emery, 2010). Compared to the affinity of UL97 for other compounds, synguanol appears to be a sufficient substrate for UL97. Additionally, there was a linear increase in the amount of

synguanol monophosphate formed when incubated with purified UL97 over a 7 hour time period, confirming the second step in the lower pathway: the direct formation of synguanol monophosphate from synguanol. As mentioned above, this experiment was performed in duplicate and thus should be repeated to further validate the results presented and discussed in this report.

In conclusion, both the lower and upper pathways were shown in this report to theoretically lead to the activation of MBX 2168; however, based on the plaque reduction data, it appears the upper pathway predominates in these cells. Additionally, the results indicate that synguanol is a good substrate for UL97 kinase in the lower pathway, so it theoretically can occur within these cells, although at a lower rate than the preferred pathway. Further experimentation and analysis is needed to fully understand the mechanism of activation of MBX 2168 within the cell and understand its ability to combat HCMV infection.

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