ANTIVIRAL MECHANISM(S) OF THE EXPERIMENTAL IMMUNOSUPPRESSIVE AGENT LEFLUNOMIDE AGAINST HUMAN CYTOMEGALOVIRUS AND POLYOMAVIRUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

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ABSTRACT

Leflunomide is an experimental immunosuppressive agent that has shown efficacy as an antiviral agent against human cytomegalovirus (HCMV) and polyomavirus strain BK (BKV). An antiviral regimen has been approved for immunosuppressed patients suffering complications from HCMV infection, whereas a good treatment option for patients infected with BKV does not exist. Unfortunately, the antiviral treatment options for patients infected with HCMV have helped promote the propagation of multidrug resistant HCMV strains. New antiviral treatment options must be developed to ensure the health of immunosuppressed patients suffering from HCMV and BKV infection. This body of work illustrates the possible antiviral mechanisms associated with Leflunomide using an in vitro model system. We have tested the hypothesis that the antiviral activity of A77 1726, the active metabolite of Leflunomide, is a result of its inhibition of phosphorylation of one or more viral structural proteins. Western blot, Southern (Dot Blot) blot, and CMV gene array analysis demonstrated that Leflunomide does not inhibit HCMV DNA synthesis, the translation of essential viral proteins, or the transcription of viral mRNA. $^{32}$P-orthophosphate labeling experiments confirm a reduction in the phosphorylation of more than one of the HCMV tegument proteins. In addition, immunohistochemical staining showed discrete changes in localization of these tegument proteins in Leflunomide-treated cells. Co-immunoprecipitation experiments
confirm that Leflunomide disrupts the interaction of viral tegument proteins suggesting that Leflunomide may inhibit complete infectious virion assembly by altering the phosphorylation states of one or more viral structural proteins. The second aspect of this work was to determine if Leflunomide would inhibit the replication of BKV, a polyoma virus unrelated to HCMV. We tested the hypothesis that A77 1726 would inhibit the production of infectious BKV particles without inhibiting DNA synthesis or large T antigen translation. Plaque assay data demonstrated a log decrease in viral titers when infected cells were treated with A77 1726. Western blot and Southern blot data confirmed the inhibition was not due to a block in protein translation of the large T antigen or a viral DNA synthesis. Immunohistochemistry confirmed there was no reduction of the large T antigen protein when infected cells were treated with A77 1726. When the phosphorylation of the large T antigen was assessed, no reductions in phosphorylation could be detected. Further studies must be completed to determine a definitive antiviral mechanism in respect to BKV, but our data supports the hypothesis that Leflunomide inhibits the replication of BKV in vitro.
This work is dedicated to my wonderful wife, Holly, for her unfailing encouragement, support, and love.
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1.1. Rationale

Cytomegalovirus is a ubiquitous pathogen that infects 40-80% of children in developed countries, and an estimated 90-100% of children in underdeveloped countries, before the age of puberty. With these percentages scientists can estimate that 50-80% of the world's population is infected with HCMV. Based on the economic cost and years of disability HCMV may cause throughout life, the Institute of Medicine has named vaccine development for human cytomegalovirus (HCMV) its top priority. Primary infection can lead to mononucleosis, but seldom do symptoms arise in healthy immunocompetent hosts. However, neonates, patients pharmacologically immunosuppressed following transplantation, and patients suffering from acquired immuno-deficiency syndrome may suffer long term, sometimes life threatening, complications due to HCMV infection.

Advancements in the development of anti-HCMV treatments such as ganciclovir, phosphoformate (foscarnet), and cidofovir have given hope to patients suffering the complications arising from HCMV infection. Unfortunately mutations in the viral genome, selective pressure, and the fact that each of these
agents inhibit viral replication at the same point in the replication cycle are all factors that have helped promote the propagation of multiple drug resistant isolates of HCMV.\textsuperscript{11,12} Ongoing work has been aimed at understanding the factors essential for HCMV replication. New treatment options must be sought to help patients suffering from the complications of HCMV infection.

Polyomaviruses are most commonly known as oncogenic viruses or small DNA tumor viruses.\textsuperscript{13} The first polyomavirus was discovered in 1953, mouse polyomavirus, and it induced salivary gland tumors in newborn mice.\textsuperscript{14} BKV, a human polyomavirus strain, was first isolated from the urine of a renal allograft recipient in 1971.\textsuperscript{15} BKV has been a source of headache for clinicians trying to increase graft retention in kidney allograft recipients.\textsuperscript{16} BKV nephropathy has been documented as a leading factor in graft loss rates in adult renal transplant populations and has been recently been credited with increased graft loss rates in pediatric kidney transplant patients.\textsuperscript{17} The only current treatment option is cidofovir, a nucleoside analogue that inhibits HCMV DNA synthesis. Cidofovir treatment has yet to be proven to show efficacy and sometimes undesirable side effects ensue.\textsuperscript{18} With no other treatment options available new therapies must be developed to deter BKV nephropathy in transplant patients.

Leflunomide, an experimental immunosuppressive agent, has recently been used as an antiviral therapy to help thwart the effects of human cytomegalovirus in renal allograft recipients\textsuperscript{19} and patients suffering from nephropathy due to polyomavirus BK infection.\textsuperscript{18} Previous work in our lab has shown the ability of
Leflunomide to inhibit the replication of HCMV in vivo and in vitro. This study is an extension of those findings and preliminary data illustrating the possible antiviral mechanism(s) of Leflunomide against BKV.

1.2. Objectives

It has been documented that A77 1726, the active metabolite of Leflunomide, can inhibit HCMV in vitro and in vivo. It is proposed that A77 1726 inhibits the production of infectious HCMV particles through the inhibition of the phosphorylation of one or more structural phosphoproteins. Recent data has also demonstrated the efficacy of Leflunomide as a treatment option for BK nephropathy.

This study was designed to test the following specific hypotheses:

I. The antiviral activity of A77 1726 is not due to an inhibition of viral mRNA transcription, DNA synthesis, or protein translation, but a result of its inhibition of phosphorylation of one or more viral structural phosphoproteins thus leading to an inhibition of the assembly of the mature infectious virion. We have utilized a CMV gene array, Southern (dot) blot analysis, and western blot analysis to determine the effect of A77 1726 on HCMV mRNA transcription, DNA synthesis, and protein translation. $^{32}$P-orthophosphate labeling experiments were incorporated into the study to test the phosphorylation of a multitude of HCMV structural proteins.
Immunohistochemical staining and co-immunoprecipitation assays helped determine if A77 1726 had an affect on the localization and function of the viral structural proteins.

II. A77 1726 will inhibit the production of BKV in vitro without inhibiting viral DNA synthesis or protein translation. A model infection system was developed to observe the cytopathic effects of BKV in human fibroblasts (MRC-5 cells). Once an infection system was established, plaque assays were used to quantitate the effect of A77 1726 on virus yield. Southern (Dot) blot and western analysis were employed to determine the impact of A77 1726 on the synthesis of viral DNA or the translation of the large T antigen. A new method of detecting protein phosphorylation, Pro-Q Phosphoprotein staining, was incorporated to test the phosphorylation of the BKV large T antigen.

1.3. Overview

Chapter 1 has given insight into the rationale and the specific hypotheses tested in this study. Chapter II is a review of all the pertinent literature related to HCMV with respect to this study. The experimental design and results of HCMV specific investigations are found in chapters III and IV. A brief review of the literature pertaining to polyomaviruses and BKV as well as experimental design and
results are contained within chapter V. Chapter VI discusses the work completed in this study and contains suggestions for future work with Leflunomide as an antiviral agent.
CHAPTER 2
LITERATURE REVIEW OF HUMAN CYTOMEGALOVIRUS

2. 1. Overview

Human viral pathogens have evolved into efficient replicating machines. The more work done unveiling the processes that afford a virus to work so efficiently, the more scientists find how complicated such a small entity can be. This chapter will review the literature that is relevant to the human cytomegalovirus portion of this investigation, and explain the rationale of the work done investigating the antiviral mechanisms of the experimental immunosuppressive agent, Leflunomide. Following a review of the taxonomy, pathogenesis, and patient populations affected by HCMV, the replication cycle will be explained. Gene regulation, the importance of viral DNA synthesis, and a detailed explanation of assembly of the virion will precede a section describing the current therapeutics prescribed to fight HCMV infection and disease. The rationale of this investigation will be illustrated as multi-drug resistant mutant viruses are discussed. Finally a section reviewing the work done by other investigators utilizing Leflunomide as an experimental immunosuppressive agent will be followed by a review of the work done by Dr. W. James Waldman that lead to this investigation.
2. 2. Human Herpesviruses

Herpesviruses are members of the family *Herpesviridae* based on the architecture of their virion. Over 130 herpesviruses have been identified to date. Of these 130 nine herpesviruses have been isolated from humans. These viruses include *Human herpesviruses (HHV)* 1 (Herpes simplex virus-1), *HHV*-2 (herpes simplex virus-2), *HHV*-3 (Varicella-zoster virus), *HHV*-4 (Epstein-Barr virus), *HHV*-5 (Cytomegalovirus), *HHV*-6A, *HHV*-6B, *HHV*-7, and *HHV*-8 (Kaposi’s sarcoma-associated virus). Human Cytomgalovirus, HCMV, falls into the subfamily beta-herpervirus.21

2. 3. Human Cytomegalovirus Structure

2. 3. 1. The Genome

Human cytomegalovirus has a linear genome consisting of 235,000 base pairs. There are two distinct components of the HCMV genome, the unique long (UL) and the unique short (US) components. Each unique component is flanked by inverted repeat sequences \( b \) and \( c \). Inverted repeat \( b \) is comprised of TRL/IRL, and inverted repeat \( c \) is comprised of IRS/TRS. Repeat sequence \( a \) can be found at the genome termini and in an inverted orientation between the UL/US junction allowing for genome inversion.22 No scientific evidence has uncovered a reason for inversion.

HCMV strain AD169, which has been fully sequenced, contains 208 open reading frames (ORFs).23 HCMV has one of the most complex genomes of known animal viruses. Most open reading frames are named according to the region of the
genome in which it can be found, for example UL82, which encodes a protein named pp71, can be found in the U1 region of the genome. To date 70 ORFs have been shown to be dispensable for growth in cultured fibroblasts.

Genome replication follows a productive replication scheme. HCMV DNA replication has mainly been defined through transient DNA replication assays. The viral genes associated with viral origin-dependent DNA replication consist of six CMV replication fork proteins, a DNA polymerase (protein encoded by UL54) and its processivity factor (phosphoprotein encoded by UL44), a DNA binding protein (phosphoprotein encoded by UL57), and a three sub-unit helicase-primase complex (made up of proteins encoded by UL70, UL102, and UL105). Several thousand copies of the viral genome are synthesized per infected cell over a 48 hour period. Viral DNA synthesis produces concatemers and most viral DNA produced is longer than unit length lacking terminal fragments, suggesting a directional rolling cycle mode of replication.

2.3.2. The Virion

The HCMV virion consists of one copy of the linear genome encapsulated within the nucleocapsid. Surrounding the nucleocapsid is protein matrix termed the tegument, which is surrounded by a lipid bi-layer covered with virally encoded glycoproteins. The nucleocapsid of HCMV is comprised mainly of the Major Capsid Protein (MCP) which makes up the majority of the pentamers and hexamers. Two other less abundant proteins, the minor Capsid Protein (mCP) and the minor
Capsid-Binding Protein (mC-BP) associate with each other to form triplexes that can be found between hexons and pentons. The formation of the nucleocapsid occurs in the nucleus of an infected cell. The synthesized viral genome is then packaged into the pre-formed nucleocapsid with the aid of the HCMV terminase and possibly pUL97. Upon packaging the nucleocapsid moves to the inner nuclear membrane and buds into the perinuclear cisternae.

The tegument of the HCMV virion is proposed to contain more than 20 virally encoded proteins, many of these being phosphoproteins. The two most abundant proteins found in the tegument are phosphoprotein 65 (pp65) and phosphoprotein 150 (pp150). The functions of each protein are not well defined but pp65 is a major phosphate acceptor and makes up the majority protein mass in viral dense bodies, enveloped viral protein masses found in the cytoplasm of HCMV infected cells. In mutagenesis studies, pp65 was found to be nonessential for the production of wild type viral titers, but kinetics of replication was altered. Both pp65 and pp150 are highly immunogenic, with pp65 being the hallmark in antigenemia assays to diagnose HCMV clinically.

Other viral proteins found in the tegument include, but are not limited to phosphoprotein 71 (pp71), phosphoprotein 28 (pp28), and ppUL69. All of these proteins are phosphoproteins, much like the vast majority of the other tegument proteins. Pp71 has been shown to transactivate gene expression through transient transfection experiments. Mutagenesis studies have shown that a virus lacking the pp71 ORF has a multiplicity of infection growth restriction due to a lack in
transcription of immediate early genes. Pp 28 is another essential viral protein that is phosphorylated. Recent work has suggested that pp28 is important in the later stages of viral assembly, whereas pp71 seems to play a major role in early replication events. In the absence of pp28 the HCMV virion lacks a complete tegument and an envelope. The protein product encoded by ORF UL69 has three isoforms, only one of which, the phosphorylated form, is found in the HCMV virion. ppUL69 has also been shown to transactivate gene expression, and a mutant virus lacking ppUL69 can grow to wild type viral titers, but it takes much longer to reach wild type viral titers.

Surrounding the tegument is a lipid bi-layer, derived from intracellular membranes. The bi-layer, the viral envelope, has 60 or more viral glycoproteins embedded in it. The most prominent glycoprotein embedded in the viral envelope is glycoprotein B (gB). HCMV gB influences not only viral entry into cells but also plays a role in cell-to-cell transmission. In recent work, Sanchez et al. has shown that gB localizes to compartments near the golgi, suggesting that gB is embedded in cellular membranes of these compartments and may help facilitate the maturation of the HCMV virion.

2.4. HCMV Replication

2.4.1. Entry

HCMV replication begins as the viral particle binds to the cell surface and ends as many new progeny virus mature in the cytoplasm of infected cells and egress to the cell surface in order to find new cells to infect. Viral attachment and
penetration occurs at the cell surface most likely involving gB. Recent work has demonstrated that gB and a complex of two other virally encoded glycoproteins – gH and gL – are necessities for HCMV membrane fusion. It must be noted that homologues to gB and other virally encoded glycoproteins are essential for the attachment and penetration in other herpes viruses. Once bound to the cell surface, the HCMV envelope fuses to the cell membrane and the tegument and nucleocapsid are released into the cell. The viral nucleocapsid migrates to the nucleus of the infected cell and the viral DNA enters the nucleus, thus beginning viral gene expression.

2.4.2. Regulation of Gene Transcription

The transcription of HCMV genes is temporally regulated. Three distinct classes of viral genes exist – immediate early, early, and late. Immediate early genes, also known as α-genes, are the first viral genes to be expressed, sometime between 1-8 hours post infection. The transcription of this set of genes is regulated mainly through the Major Immediate Early (IE) locus, which contains the major immediate promoter. IE1 and IE2 are the major immediate early products produced. IE1 and IE2 function as regulators of early and late gene expression. Other functions of immediate early genes include inhibition of apoptosis and modulation of MHC class I expression. Upon translation of immediate early genes, early genes, or β-genes are beginning to be transcribed. Early genes are transcribed around 4-12 hours post infection. Early genes function as regulators of viral DNA synthesis, as in the case of
UL54 (viral DNA polymerase) and UL44 (polymerase processivity factor). US11 is another early gene product that encodes a protein that resides in the endoplasmic reticulum and has been shown to help in the degradation of MHC class I protein, thus helping in immune evasion. UL112-113 encoded proteins regulate the expression of core DNA replication genes. Late viral genes, also known as \( \gamma \)-genes, can be put into two categories – \( \gamma_1 \) (leaky late) and \( \gamma_2 \) (true late). Late viral proteins are mainly virion structural proteins that make up the nucleocapsid, tegument, and are found in the envelope. Leaky late genes can be transcribed in the presence of DNA synthesis inhibitors like PFA, ganciclovir, and cidofovir (see Antiviral agents section), whereas true late genes are not transcribed when DNA synthesis is blocked. The majority of late genes are structural proteins and are transcribed as early as 24 hours post infection. Proteins translated from late gene ORF’s can be found in the tegument of the HCMV virion and may also have regulatory functions, as in the case of pp71. Phosphoprotein 28 is an example of a true late gene product since the inhibition of DNA synthesis inhibits the transcription and subsequent production of pp28.

2.4.3 Nucleocapsid Assembly

Once DNA has been synthesized and late proteins are translated the assembly process begins. Very early in assembly the capsid proteins that have been translated in the cytoplasm must translocate to the nucleus and begin forming the capsid. All the major capsid proteins contain nuclear localization signals (NLSs) except for the
major capsid protein (MCP). With the help of the assembly protein precursor (pAP), which contains a NLS, the MCP/pAP complex is able to pass through the nuclear pore.\textsuperscript{56} pAP can also interact with itself, possibly contributing to a more efficient translocation process. The interaction with pAP and itself and with MCP has been shown to be phosphorylation dependent. Recent work has shown that phosphorylation events at Thr231 and Ser235 are contributing factors in the interaction between pAP and itself and the interaction between pAP and MCP.\textsuperscript{57} More recent work has also demonstrated that the small Capsid protein (SCP) interacts with the MCP in the cytoplasm of transfected cells. This interaction seems to be dependent on two linear sequences in the SCP.\textsuperscript{58} In either case the MCP has “help” getting into the nucleus to begin formation of a capsid.

The efficient translocation of MCP into the nucleus leads to interactions between the four main capsid proteins – the minor capsid protein (mCP), minor capsid-binding protein mC-BP, the small capsid protein SCP, and MCP – and two precursor proteins. This interaction leads to a preB-capsid, and the proteolytic cleavage of the precursor proteins allows for the preB-capsid to transform into a B-capsid.\textsuperscript{27} DNA packaging occurs in conjunction with these maturation events, and if DNA is not packaged into the maturing capsids there is a accumulation of empty B-capsids.\textsuperscript{59} If DNA is packaged into the maturing capsids then the B-capsids mature into virions, if DNA is not packaged into the maturing capsid it is thought that A capsids arise, capsids lacking a dense DNA core.\textsuperscript{60} Mutational analysis has shown
that HCMV TRS1 protein is essential for the efficient assembly of DNA-containing capsids. A mutant virus, ADsubTRS1, allowed for cleavage of DNA in nucleus, but DNA containing C-capsids, which mature into virions, could not be found. 61

Viral genome insertion into the maturing capsid is essential for the production of an infectious viral particle. As mentioned above the synthesis of the HCMV genome produces concatemers, genomes linked together, most likely through a rolling circle mode of replication, as shown with a recombinant HCMV containing two closely spaced PAC-1 sites. 26 A follow up study showed that there were pac2 sites at specific ends of the concatemers giving rise to a directionality to the packaging of the genome into the capsids. Cis-acting elements cleaved the complex contatemers into single genome copies allowing the genomes to be packaged into the newly formed capsid. 62 Further understanding of how viral DNA is packaged was gained while testing Benzimidazole D-ribonucleosides 2,5,6-trichloro-1-beta-D-riborfuranosyl benzimidazole riboside (TCRB) and 2-bromo-5,6-dichloro-1-beta-D-riborfuranosyl benzimidazole riboside (BDCRB), two compounds shown to inhibit HMCV replication. TCRB and BDCRB resistant viral strains have been shown to have mutations in genes UL89 and UL56, two genes whose protein products are subunits of the HCMV terminase. Within these viral strains, mutation in the UL104 ORF, the gene encoding the portal protein, was also found. These finding demonstrate a necessity for the HCMV terminase and the portal protein in the packaging of HMCV genomes into the capsids. 30 Once the viral genome is packed into the viral capsid it becomes nuclease resistant and the capsid moves to cytoplasm
of the infected cell. A study performed to test the *in vitro* affects of 2-isopropylamino-5,6-dichloro-1-(beta-l-ribofuranosyl)benzimidazole (1263W94, or maribavir)\(^3\) showed that maribavir inhibited DNA synthesis and nucleocapsid egress, suggesting that the inhibition of pUL97, a virally encoded kinase, caused these phenotypic effects. Another study using a UL97 null mutant and pharmacologically inhibited (maribavir) wild type HCMV showed that UL97 was actually involved in nucleocapsid egress into the cytoplasm.\(^6\)

2.4.4. Tegumentation, Envelopment, and Egress

The late stages of cytomegalovirus assembly, including the acquisition of a tegument, envelopment, and virion egress, have not been studied in detail. Just recently a small number of studies have tried to shed light onto the functional importance of certain viral gene products during this point in HCMV replication. The following section, though limited, will supply some of the rationale for the body of work presented in the following two chapters.

Acquisition of the viral tegument and envelopment of the HCMV virion was once thought to be a single process beginning as the virion budded through the inner nuclear membrane and ending as the virion acquired its envelope from the outer nuclear membrane. The single phase of viral assembly was hypothesized when immuno-flourescent studies showed a nuclear staining pattern for pp150 and pp65, two viral proteins that make up the majority of the tegument, suggesting a site of assembly within the nucleus of infected cells.\(^6^4\) The protein product of UL32, pp150,
has been shown to strongly interact with the nucleocapsid, by binding \textit{in vitro} to the capsid through its amino one-third end. When the HCMV virions were subjected to conditions that would dissociate all other tegument proteins, pp150 could still be found in the nucleocapsid containing fractions isolated through centrifugation.\textsuperscript{65}

A series of studies that supported an alternative to the single phase assembly process showed that pp150 actually localized to the cytoplasm of infected cells late in the replication cycle in a juxtanuclear structure that did not colocalize with the endoplasmic reticulum (ER), the endoplasmic reticulum-Golgi-intermediate compartment, or the Golgi apparatus. Immuno-flourescent studies showed that other tegument structural proteins, pp28 and pp65, and three glycoproteins could also be found localizing to this region of the infected cell. The work done in this study also argued a strictly nuclear localization pattern for pp150.\textsuperscript{43} In a study that extended the findings of the previous work, the author demonstrated that a non-myristilated pp28 localized to a compartment that co-localized with the endoplasmic reticulum-Golgi-intermediate compartment (ERGIC). This work suggested that wild type pp28, which was found localizing to the juxtanuclear structure in the presence of other viral proteins, could not traffic to this structure in the absence of other tegument proteins.\textsuperscript{66}

More recent studies have shown that a mutant virus lacking pp28 does not acquire an envelope. This particular study showed that DNA synthesis is not interrupted, and the mutant virus was able to produce immediate early, early, and late proteins, but there was a defect in the late stages of assembly, which caused a block in the production of infectious virus.\textsuperscript{38} Another group has determined that an acidic
cluster found in pp28 determines the trafficking, localization, and function of pp28. If this acidic cluster is altered then pp28 no longer displays a punctuate cytoplasmic staining pattern, and when the C-terminal two thirds of pp28 was deleted there was in major attenuation in envelopment and viral growth.\textsuperscript{67}

Though limited work has been done to detail the exact functions of late viral structural proteins and the maturation of a non-tegumented capsid into a complete enveloped virion, the evidence reviewed above suggests that late structural proteins play a vital role in the assembly of the HMCV virion. The work completed by the Waldman lab over the last three years will support the hypothesized localization and possible functions of the late viral proteins in the assembly of the HCMV virion. With the aid of Leflunomide, an experiment immunosuppressive drug with antiviral activities, we demonstrate that the phosphorylation of the tegument proteins is an important process in the assembly of infectious HCMV particles.

2.5. HCMV Pathogenesis

Human cytomegalovirus can infect, re-infect, or reactivate in permissive hosts. Upon infection, HCMV can either continue in a productive (lytic) infection or establish lifelong latency. A productive infection leads to the expression of immediate early, early, and late genes and their protein products. The results of a productive infection are infectious viral particles exiting the infected cell searching for more permissive cells to infect and continue on the productive infection cycle. Latency occurs when the virus is held in check, i.e. the complete array of gene
products is not produced and infectious virus particles cannot be detected through laboratory means.\textsuperscript{46} Cells of myeloid lineage have been shown to be important reservoirs of latent HCMV. The establishment of latency is determined by the differentiation state of the meyloid cells. Differentiated macrophages have been shown to be permissive to HCMV infection, whereas non-differentiated monocytes seem to harbor latent virus.\textsuperscript{68,69}

The immunocompetent adult rarely shows signs of a primary infection. In very few cases mononucleosis can result from a primary infection.\textsuperscript{3} One study suggested an association between the smooth muscle proliferation occurring from previous cytomegalovirus infection and restenosis following coronary angioplasty.\textsuperscript{70} HCMV, for the most part, once infecting an immunocompetent adult has no adverse effects.

In the case of immunosuppressed transplant patients, patients suffering from acquired immunodeficiency syndrome (AIDS), and children infected with HCMV during pregnancy the effects of an HCMV infection can be life threatening. Interstitial pneumonia, diffuse gastrointestinal mucosal ulceration, hepatitis, retinitis, and destructive inflammatory lesions in a variety of locations, are serious and often life-threatening complications that can arise from CMV infection or reactivation.\textsuperscript{54}

Neonates infected with HCMV during pregnancy show one of the most important clinical manifestations of HCMV infection.\textsuperscript{71} A mother may have protective antibodies against the intrauterine transmission of HCMV, but the antibodies are not protective against the development of symptoms once infection has
occurred. The highest risk to the infant is therefore during the first trimester.\textsuperscript{72,73} Five to ten percent of congenitally infected neonates show symptoms of irreversible CNS involvement. In these cases, the neonates show signs of microcephaly, encephalitis, seizures, deafness, upper motor neuron disorders, psychomotor retardation, and myopathy and choroidoretinitis.\textsuperscript{74,75} It has been stated by a review panel from the Institute of Medicine that a vaccine against HCMV was secondary in need only to a vaccine for HIV.\textsuperscript{76}

The ability of HCMV to establish latency makes it a potentially dangerous entity to immunocompromised patients. A normal human immune system is able to establish long term immunity to HCMV after initial infection, but long term immunosuppression could allow an opportunistic pathogen like HCMV to reek havoc on an immunosuppressed patient. Pharmacologic immunosuppression following organ transplantation or the adverse effects of AIDS can cause serious often life threatening complications in these patient populations when HCMV reactivates.

In solid organ transplants, the serologically negative patient who receives an organ from a serologically positive donor has the highest risk for HCMV-associated disease.\textsuperscript{77} Some studies have shown that CMV-associated disease initially localizes to the transplanted organ, but can subsequently spread systematically causing pneumonitis, enteritis, hepatitis, and retinitis. Before the use of ganciclovir (GCV), an inhibitor of HCMV replication (discussed later), pneumonia developed in 17\% of bone marrow transplant patients with a mortality due to HCMV pneumonia as high as 85\%.\textsuperscript{78} Serious complications occurred mainly in HCMV seropositive patients, with
graft-versus-host disease being the most important risk factor.\textsuperscript{79} Since GCV treatment along with immunoglobulin therapy began mortality has dropped to 30-50%.\textsuperscript{80,81}

Many immunocompromised patients treated with anti-HCMV therapies have been patients suffering from AIDS; that was until the introduction of highly active antiretroviral combination therapies (HAART), which correlated with a decrease in the number of HCMV infections in AIDS patients.\textsuperscript{82} Although there has been a decrease in the incidence of HCMV infection due to new therapeutics available to immunosuppressed patients, the emergence of drug resistant strains of HCMV still plagues clinicians as they try to combat the complications arising from HCMV infections (See section II. 7.).

2. 6. Antiviral Strategies

The current approved therapeutic options for treatment of CMV disease, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir), valganciclovir (the valine ester of gacilclovir), phosphoformic acid (foscarnet), (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir), ISIS 2922 (fomiviren), and anti-CMV hyper immune globulin (CytoGam\textsuperscript{®}), with the exception of fomiviren and CytoGam\textsuperscript{®}, act in a similar manner by inhibiting viral DNA synthesis, either through the inhibition of chain elongation or by direct binding of the viral DNA polymerase. Ganciclovir (GCV) is initially phosphorylated by HCMV pUL97, followed by preferential subsequent phosphorylations by cellular kinases. Once in the
triphosphate forms the compound competes with dGTP and dCTP for substrate binding sites on the DNA polymerase. If the ganciclovir triphosphate is incorporated into the viral DNA being synthesized then chain elongation cannot continue, thus inhibiting viral DNA replication. Cidofovir (CDV), a formulated monophosphate, is phosphorylated twice by cellular kinases upon entering the cell allowing for it to compete for substrate binding sites of the DNA polymerase. Once incorporated into the growing DNA chain, cidofovir severely inhibits the progression of DNA synthesis. The mechanism of inhibition for foscarnet (PFA) is noncompetitive inhibition of CMV DNA polymerase activity. Foscarnet utilizes the pyrophosphate binding sites on the polymerase rendering it functionally inactive. Fomivirsen is a novel antisense therapy that hybridizes to the mRNA of immediate early proteins, thus blocking translation of the immediate early proteins. Each of these compounds were promising antiviral therapies that showed efficacy in fighting HCMV infection and subsequent disease, but in the next section it will be clear that there is a need for new options in anti-CMV treatment.

2. 7. Drug Resistance

Each of the commonly prescribed medications to inhibit HCMV replication do so through the inhibition viral DNA synthesis, either through inhibiting chain elongation or inhibiting the viral DNA polymerase. The virus is an efficient, not perfect, replicating machine. Mutations arise and under selective pressure viruses have the ability to evade the effects of compounds created to derail them. Multiple
studies have demonstrated the ability to isolate HCMV mutants that continue replicating in the presence of compounds that inhibit viral DNA synthesis. A recent study demonstrated that mutations in UL97 conferred resistance to GCV. This study showed that the M460V, methionine to valine substitution at codon 460, mutation was present in HCMV isolated from kidney transplant patients who were undergoing GCV therapy. Another study documented the emergence of a HCMV isolate resistant to multiple CMV antiviral therapies. A clinical isolate was collected from a heart transplant recipient, and two strains were isolated P8 and D16. D16 was shown not only to be GCV resistant but was also resistant to PFA. As therapy regimens continue to include GCV, CDV, and PFA more drug resistant strains of HCMV are going to arise and clinicians will be faced with the continual problem of trying to fight HCMV infection in immunosuppressed patient populations.

2.8. The Novel Immunosuppressive Agent Leflunomide

Leflunomide [N-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486] is an experimental immunosuppressive agent with demonstrated effectiveness against acute and chronic allograft rejection, and is currently in Phase I clinical trials in human transplant recipients. Leflunomide was first demonstrated to have anti-inflammatory and immunosuppressive effects using rodent models of autoimmune disease. A77 1726, [N-(4-trifluoromethylphenyl)-2-cyano-
3-hydroxycrotoamide], the active metabolite of Leflunomide, has two known mechanisms of action, inhibition of protein kinase activity and inhibition of pyrimidine synthesis.\textsuperscript{93,94,95,96}

The exact protein kinases whose activity is inhibited is not completely understood but studies have shown an inhibition in the tyrosine phosphorylation of Jak1 and Jak3 via signaling through the interleukin 2 receptor (IL2R).\textsuperscript{97} Further work demonstrated an inhibition in the tyrosine phosphorylation of interleukin 4 receptor.\textsuperscript{98} Manna et al. determined that Leflunomide was able to inhibit the TNF-induced phosphorylation of I-κBα as well as TNF-induced activation of AP-1 and the c-JUN N-terminal protein kinase activation.\textsuperscript{99} The inhibition of pyrimidine biosynthesis is accomplished through the inhibition of dihydroorotate dehydrogenase.\textsuperscript{100} Pyrimidine NTP pools are depleted in the presence of Leflunomide, but this depletion in NTPs can be restored by adding exogenous uridine.\textsuperscript{101}

Leflunomide has been well documented as an immunosuppressive agent and an FDA-approved therapeutic agent prescribed to treat rheumatoid arthritis. A group in India recently reported the novel use of Leflunomide to fight HCMV disease in renal allograft recipients. In this particular study, once Leflunomide was administered, the patients viral load, detected through nested PCR, dropped to undetectable levels. No patients displayed adverse drug reactions and the authors explain the benefits of Leflunomide when comparing its cost to the cost of intravenous GCV treatment.\textsuperscript{19}
2. 9. Inhibition of HCMV by Leflunomide *in vitro*

The original study that lead to this body of work began with a simple experiment to determine if A77 1726, the active metabolite of Leflunomide, would show any characteristic effects of inhibiting the replication of HCMV in endothelial cells. Human cytomegalovirus, so named due to the increase in size and extreme cytopathology a HCMV infected cell displays, was indeed inhibited by A77 1726. Phase contrast micrographs showed that A77 1726 inhibited the spread of CMV throughout the monolayer, whereas untreated infected control cells formed infected foci that increased in size over time (figure 1). Plaque assay data quantified this visual observation, demonstrating that A77 1726 inhibited the production of infectious HCMV in a dose dependent manner over a pharmacologically relevant range of concentrations, with an IC$_{50}$ of 40-60µM (figure 2). Subsequent studies demonstrated that the inhibition of HCMV replication was not due to a depletion in pyrimidine nucleotide triphosphates (Figure 3). Dot blot analysis confirmed that viral DNA synthesis was proceeding in the presence of A77 1726 (figure 4), but transmission electron micrographs showed the accumulation of complete cytoplasmic virions was inhibited. Closer observation revealed that in A77 1726-treated cells, viral nucleocapsids did not acquire tegument of envelope (figure 5).$^{20}$ Because the majority of HCMV tegument proteins are phosphoproteins, and A77 1726 has been shown to inhibit phosphorylation of some cellular proteins, we hypothesized that the
antiviral activity of A77 1726 is a result of its inhibition of phosphorylation of one or more of the viral tegument proteins, leading to an inhibition of the assembly of the mature infectious virion.

Figure 1. A77 1726 (Leflunomide) restricts dissemination of CMV. Phase contrast micrographs of HUVEC monolayers inoculated with CMV VHL/E (0.1 PFU/cell) and incubated for 3-6 days in the presence or absence of A77 1726 (200 µM). Bar=100 µm. (Waldman et al\textsuperscript{20} and by permission from Lippincott Williams and Wilkins)
Figure 2. A77 1726 (Leflunomide) inhibits production of infectious CMV in a dose-dependent manner. Plaque assay of CMV strain P8 in HFF or strain VHL/E in HUVEC in the presence of various concentrations of A77 1726. Data points represent mean plaque number in three to four culture wells, normalized to untreated controls, ±1 SD. (Waldman et al\textsuperscript{20} and by permission from Lippincott Williams and Wilkins)
Figure 3. A77 1726 (Leflunomide) does not significantly reduce intracellular pyrimidine nucleotide triphosphate levels in CMV-infected cells and the addition of exogenous uridine does not reconstitute viral activity in A77 1726-treated CMV-infected cells. (A) High-performance liquid chromatography analysis of extracts derived from uninfected HUVEC, CMV VHL/E-infected HUVEC (CMV), or infected HUVEC treated with 200 µM uridine (CMV+Ur), 200 µM A77 1726 (CMV+A77), or both (CMV+A77+Ur). Values represent means of four replicate measurements ± SEM. (B) Plaque assay of infectious virus production in samples from which high-performance liquid chromatography extracts were prepared. Values represent mean plaque number in three culture wells, normalized to untreated controls, ±1 SD. (Waldman et al20 and by permission from Lippincott Williams and Wilkins)
Figure 4. A77 1726 (Leflunomide) does not inhibit the accumulation of viral DNA in CMV-infected cells. Dot blots of serial dilutions of DNA extracted from CMV P8-infected (or uninfected) HFF that were incubated for 48 hr postinoculation in the presence or absence of 100 µM A77 1726 (A77), or 1 mM PFA. Blots were hybridized to α-[32P]dCTP-labeled CMV-specific cDNA probe and visualized by autoradiography. (Waldman et al.20 and by permission from Lippincott Williams and Wilkins)
Figure 5. A77 1726 (Leflunomide) impairs the acquisition of tegument by viral nucleocapsids. Transmission electron micrographs of CMV VHL/E-infected HUVEC incubated for 4 days in the presence or absence of 200 µM A77 1726. Viral nucleocapsids (black arrows); cytoplasmic virions (white arrows); viral dense bodies (black arrowheads); bar=500 nm. (Waldman et al. and by permission from Lippincott Williams and Wilkins)
2. 10. Summary

Human cytomegalovirus is an opportunistic pathogen causing a wide variety of serious, often life threatening complications in immunosuppressed populations. The current approved chemo-therapeutics prescribed to control CMV infection and disease all impede viral DNA synthesis, resulting in many single and some multi-drug resistant variants that acquire resistance to traditional methods of treatment. Understanding how the virus replicates and the processes necessary to produce infectious progeny may be the only way scientists will be able to establish new methods in order to thwart the threat of drug resistant strains of CMV. In this study we have sought to determine the mechanism of a novel antiviral agent, the experimental immunosuppressive agent Leflunomide. In doing so, we have demonstrated that phosphorylation is likely essential in the late stages of HCMV assembly, the specific mechanisms of which remain to be elucidated.
3. 1. Abstract

Despite progress in antiviral chemotherapy, cytomegalovirus (CMV) continues to complicate the clinical course of many allograft recipients. Leflunomide is an experimental immunosuppressive agent currently in Phase I clinical trials. Its active metabolite, A77 1726, inhibits protein kinase activity and pyrimidine synthesis. We have previously demonstrated that this agent exhibits antiviral activity against CMV in vitro and in vivo. A77 does not inhibit viral DNA synthesis; rather it appears to disrupt virion assembly by inhibiting nucleocapsid tegumentation. In the current study we have tested the hypothesis that the antiviral activity of A77 1726 is a result of its inhibition of phosphorylation of one or more viral structural proteins. To verify that the antiviral mechanism was not related to viral gene transcription, mRNA isolated from A77 treated (or untreated) CMV-infected fibroblasts was hybridized to a CMV gene array containing all 208 viral open reading frames. No significant difference in levels of viral mRNA between A77-treated and untreated infected cells was detected. Western blot analysis of protein extracts prepared from A77-treated (or untreated) CMV-infected fibroblasts at intervals spanning 4-96 hours post-inoculation
showed no quantitative effect on production of immediate early, early, or late viral proteins. $^{32}$P-orthophosphate labeling experiments demonstrated an inhibition in the phosphorylation of tegument phosphoprotein pp150. In addition, immunohistochemical staining showed discrete changes in localization of pp150 in A77-treated cells. Co-immunoprecipitation experiments suggest that A77 disrupts the interaction of pp150 with pp28. Results of these studies suggest that Leflunomide may inhibit complete infectious virion assembly by altering the phosphorylation states of one or more viral structural proteins, and suggest a novel class of molecular targets for further antiviral drug development.

3.2. Introduction

Despite progress in antiviral chemotherapy, cytomegalovirus (CMV) continues to complicate the clinical course of many allograft recipients. HIV infection and pharmacological immunosuppression contribute to the reactivation of latent CMV or allow for primary infection in these patient populations. Interstitial pneumonia, diffuse gastrointestinal mucosal ulceration, hepatitis, retinitis, and destructive inflammatory lesions in a variety of locations, are serious and often life-threatening complications that can arise from CMV infection or reactivation. Current approved therapeutic options for treatment of CMV disease include ganciclovir, valganciclovir (oral ganciclovir), phosphoformate (foscarnet), cidofovir, and anti-CMV hyper immune globulin (CytoGam®). With the exception of CytoGam®, all of these agents act in a similar manner by inhibiting viral DNA synthesis, either through the
inhibition of chain elongation\textsuperscript{102,84} or by direct binding of the viral DNA polymerase\textsuperscript{103}; hence viral variants are emerging which exhibit multi-drug resistance.\textsuperscript{11,12,88} Leflunomide \[N-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486\] is an experimental immunosuppressive agent with demonstrated effectiveness against acute\textsuperscript{89} and chronic rejection\textsuperscript{90}, and is currently in Phase I clinical trials in human transplant recipients.\textsuperscript{91} A77 1726, \[N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotoamide\], the active metabolite of Leflunomide, has two known mechanisms of action, inhibition of protein kinase activity and inhibition of pyrimidine synthesis.\textsuperscript{93,94,95,96,97} We have previously demonstrated that this agent inhibits production of multiple isolates of CMV \textit{in vitro}, including multi-drug resistant variants, and reduces viral load by 4-6 orders of magnitude in an animal model. This antiviral activity is not accountable to A77-mediated pyrimidine depletion since addition of exogenous uridine, which restores intracellular pyrimidine levels to normal, does not reconstitute viral activity. Unlike currently prescribed anti-CMV drugs, A77 does not inhibit viral DNA synthesis. Rather, as demonstrated by transmission electron microscopy, it appears to act at a late stage of assembly by inhibiting formation of the virion tegument, the major components of which are viral phosphoproteins.\textsuperscript{20,104}

The tegumentation of CMV nucleocapsids has been proposed to occur in the nucleus of infected cells, with envelopment occurring as the virion leaves the nuclear membrane.\textsuperscript{64} In contrast, recent studies have shown that multiple HCMV tegument proteins, UL99(\textit{pp28}), UL32(\textit{pp150})\textsuperscript{43}, and pUL25\textsuperscript{105} localize exclusively to the
cytoplasm. Evidence supporting an alternate process of tegumentation suggests that the acquisition of a tegument may occur at specific sites of viral assembly within the cytoplasm of an infected cell. Sanchez et. al. has shown that pp150, pUL83(pp65), a tegument phosphoprotein, gB, a virally encoded glycoprotein, and pp28, a true late viral protein, all localize to what is hypothesized to be a cytoplasmic site of assembly in a juxtanuclear sturcture. pp28 has been shown to localize, dependent upon its myristoylation, to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. More recent studies have shown that a mutant virus lacking pp28 does not acquire an envelope. This particular study showed that DNA synthesis is not interrupted, and the mutant virus was able to produce immediate early, early, and late proteins, but there was a defect in the late stages of assembly, which caused a block in the production of infectious virus. These data suggest there may be specific locations in the cytoplasm of infected cells where late structural viral proteins may localize, in order to allow for the complete assembly of an infectious virus particle.

It has been demonstrated that pp150, a tegument phosphoprotein, binds through its amino one-third end to the nucleocapsid of CMV virions. It is hypothesized that pp150 binds to the nucleocapsid as the newly formed nucleocapsid exits the nucleus of an infected cell. Along with other tegument proteins, pp65 and pp28, pp150 localizes to specific sites of the cytoplasm - a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. Once localized to the correct region of the cytoplasm, the tegument proteins may interact
with one another. Post-translational modifications of the tegument proteins, such as phosphorylation, could play a very important role in allowing the tegument proteins to interact with one another. We hypothesize that the localization and interaction of the viral tegument phosphoproteins is essential for the maturation of the infectious virion which contains the viral DNA, nucleocapsid, tegument proteins, and envelope studded with viral glycoproteins (figure 6). Assuming that this cascade of events is non-random, it could be blocked at various points along the pathway inhibiting the production of mature infectious virus particles.

In the current study we have tested the hypothesis that the antiviral activity of A77 1726 is a result of its inhibition of phosphorylation of pp150, leading to an inhibition of the assembly of the mature infectious virion. Utilizing a CMV gene array and immunoblot analysis we have determined that A77 1726 does not inhibit the synthesis of viral transcripts or viral proteins. Rather, as demonstrated by $^{32}$P-orthophosphate labeling experiments, the phosphorylation of the late structural proteins pp150 is inhibited by this agent. Inhibition of phosphorylation of pp150 was shown to be associated with attenuation of protein/protein interactions between pp150/pp28 and pp150/pp65 when these interactions were analyzed via co-immunoprecipitation assays. Immunohistochemistry showed an alteration in the intracellular localization of pp150 in A77 1726-treated cells. We conclude that A77 1726, by inhibiting the phosphorylation of pp150, disrupts the cascade of events that lead to the assembly of a complete infectious HCMV viral particle.
Figure 6. A schematic illustrating the putative late stages of CMV assembly.
1. The viral DNA is inserted into the newly formed nucleocapsid.
2. The nucleocapsid-DNA complex transverses the inner leaflet of the nuclear membrane aided by late viral structural proteins.
3. The nucleocapsid-DNA complex now exits the nucleus through the outer leaflet of the nuclear membrane once again aided by late viral structural proteins.
4. The nucleocapsid-DNA complex acquires a light tethering of tegument proteins in the cytoplasm.
5. Late structural proteins (tegument proteins and glycoproteins) are translated within the ER and transported to the golgi complex.
6. The late structural proteins are post-translationally modified in the golgi. The glycoproteins contained in the cytoplasmic vesicle interact with tegument proteins in the cytoplasm.
7. This complex of glycoproteins, tegument proteins, and cell membrane (from vesicle) interacts with the “immature” virion to form a complete infectious virion.
3. 3. Materials and Methods

3. 3. 1. Cells and Viruses.

MRC5 human fibroblast cells (American Type Culture Collection, Rockville, MD) were propagated in Eagle’s Minimal Essential Medium (MEM, Sigma Chemical Co.) supplemented with 10% fetal bovine serum (U.S. Bio-Technologies Inc., Parker Ford, PA), 1% essential amino acids, 2% nonessential amino acids, and 0.5% vitamins (Sigma Chemical Co.). Human umbilical vein endothelial cells (HUVEC) were isolated from cord vessels as previously described\textsuperscript{106,107} and propagated in endothelial growth medium (ECGM), consisting of M-199 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (U.S. Bio-Technologies), 50µg/ml bovine brain extract\textsuperscript{108}, 12 U/ml sodium heparin (Sigma chemical Co., St. Louis, MO), and 20mM HEPES buffer. All growth surfaces for HUVEC were pretreated with human fibronectin (Upstate Biotechnology, Inc., Lake Placid, NY), 25 µg/ml. CMV strain VHL/E, isolated from duodenal biopsy material of a bone marrow transplant recipient\textsuperscript{109} was propagated in HUVEC to preserve its natural endothelial cytopathogenicity. CMV strain Towne, acquired from ATCC, was propagated in MRC5 cells.

3. 3. 2. Analysis of CMV gene expression.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with HCMV strain Towne at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of
100uM A77 1726 for various times post infection. Viral mRNA was isolated from total RNA collected 8 and 48 hours post infection using a mRNA isolation kit (Qiagen). 250ng of mRNA was subjected to reverse transcription by using oligo(dT) and 3’ gene specific primers in the presence of [32P]dCTP. RNA was degraded; unincorporated nucleotides were removed; and the labeled cDNA was then hybridized to the membrane-bound array containing 206 HCMV open reading frames (ORFs) along with actin as a control.110

3. 3. 3. Analysis of Protein expression.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with HCMV strain Towne at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (4-96 hrs). Proteins were isolated from untreated or A77 1726-treated cells by incubating in a lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM phenylmethylsulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.) for 30min at 4°C. An equal volume of loading buffer containing 2% beta-mercaptoethanol was added to each lysate prior to boiling. Insoluble material was removed by centrifugation. Proteins were separated on a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Osmonics, Inc., Westborough, MA). The membranes were blocked overnight at 4°C in a blocking solution consisting of 5% non-fat dried milk and 0.2%
Tween-20 in PBS on an orbital shaker. Membranes were probed with antibodies specific for viral proteins, UL123 encoded IE-1, UL44, and UL99 (pp28) (generously provided by Dr. William Britt, University of Alabama Birmingham), overnight at 4°C in blocking solution on an orbital shaker. Membranes were rinsed repeatedly with Tris-buffered saline containing 0.1% Tween-20. Anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as a secondary antibody. Proteins were visualized using ECL western blot detection reagents (Amersham) and Hyperfilm ECL (Amersham).

3. 3. 4. $[^{32}P]$-orthophosphate labeling of viral proteins.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with HCMV strain Towne at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (48-96 hrs). Four hours pre-harvest cells were incubated in phosphate free medium (Gibco) for two hours. Two hours pre-harvest cells were incubated in phosphate free medium supplemented with $[^{32}P]$-orthophosphate to give a final concentration of 50µCi/ml of medium (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for two hours. Cells were treated with trypsin (Worthington Biochemical Corp., Freehold, NJ), collected in phosphate-free medium; and pelleted by centrifugation (1200rpm, 10 minutes). Proteins were isolated using a lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM phenylmethylsulfonyl fluoride
(PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.). Protein lysates were pre-cleared with irrelevant isotype-matched control antibodies, IgG1, IgG2a, and IgG2b (Dako Corporation, Carpinteria, Ca) and protein G sepharose beads (Amersham). Lysates were rocked for 3 hours and protein complexes pelleted by centrifugation (13,000rpm, 10 minutes). Antibodies specific for UL32 (pp150) (generously provided by Dr. William J. Britt, University of Alabama Birmingham, Birmingham, Al) were combined with protein G sepharose beads in order to immuno-precipitate pp150. The proteins were separated on a 7.5% SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane and the membrane was exposed to Kodak X-AR biomax film (Kodak Industrie, Cedex France) for various times to produce an autoradiograph. Pp150 bands were then visualized on the membrane using an anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.) as the secondary antibody, ECL detection reagents (Amersham), Hyperfilm ECL (Amersham) as described above.

3.3.5 Immunohistochemical staining.
HUVEC were pretreated for 30 minutes with fresh ECGM alone or ECGM supplemented with 200uM A77 1726, then inoculated with HCMV strain VHL/E at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 200uM A77 1726 for various times post infection (48-120 hrs), acetone fixed, and
immunoperoxidase stained as described previously. Viral proteins were stained with primary antibodies specific to pp150, or and irrelevant isotype-matched antibodies as specificity controls.

3.3.6. Co-immunoprecipitation analysis.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100μM A77 1726, then inoculated with HCMV strain TOWNE at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 100μM A77 1726 for various times post infection. Proteins were isolated using a lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanadate, 0.05M NaF, 10mM phenylmethylsulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.). Proteins were immuno-precipitated as described above using antibodies specific for viral proteins pp28, pp65, pUL69, and pp150. The immuno-precipitated proteins were separated on four identically loaded 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Each membrane was blocked overnight in blocking reagent (see above). Each of the four membranes was then probed with one of the antibodies listed above. Proteins were then visualized using an anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.) as the secondary antibody, ECL detection reagents (Amersham), Hyperfilm ECL (Amersham) as described above.
3. 4. Results

3. 4. 1. A77 does not inhibit the transcription of immediate early (IE), early (E), or late (L) viral genes.

We have hypothesized that the antiviral mechanism of A77 1726 is due to an inhibition of phosphorylation of one or more of the viral tegument phosphoproteins, not due to an inhibition of viral gene transcription or protein translation. To determine if A77 1726 had an inhibitory effect on viral transcription, human fibroblast (MRC-5) cells incubated in the presence or absence of 100μM A77 1726 for 8 and 48 hours was reverse transcribed in the presence of $^{32}$P-radiolabeled nucleotides. In order to quantify the amount of viral mRNA transcribed in the presence and absence of A77 1726, these radiolabeled products were hybridized to a cytomegalovirus gene array to compare the quantities of all viral transcripts produced in HCMV infected cells incubated in the presence or absence of A77 1726.

HCMV gene array data, demonstrated that A77 1726 does not inhibit transcription of any viral genes (figure 7). Table 1 lists the amounts of each transcript, IE-1, UL44, UL32, and b-actin, based on the phospho-intensity of the spot that correlates with each transcript normalized to the phospho-intensity of b-actin, a control cellular transcript.
Figure 7. A77 1726 does not inhibit immediate early, early, or late viral RNA transcription. Viral mRNA was isolated from CMV-infected MRC-5 fibroblasts incubated in the presence (B) or absence (A) of 100 µM A77 1726 for 48 hours post inoculation. The mRNA was reverse transcribed in the presence of [32P]dCTP. Quantities of 32P-labeled cDNA copied from mRNA that was prepared from the same number of CMV-infected cells in the presence or absence of A77 1726 was used as a probe on a CMV gene array containing all 206 open reading frames. The arrays contain actin as a cellular transcript control.
A significant difference was deemed to be greater than or less than a three fold
difference, which would correlate to >3.000 or <0.3333 when comparing CMV
transcripts in untreated cells to those in A77 1726 treated cells. When comparing the
normalized values of each transcript from CMV infected control cells with
normalized values of each transcript from A77 1726 treated CMV infected cell we
found no significant difference between transcript levels.

Table 1. A77 1726 does not inhibit immediate early, early, or late
viral RNA transcription. Phospho-intensity of gene array spots
correlating to the open reading frames for immediate early 2, UL44
(early), UL32 (pp150, late), and actin (cellular control). Values are
normalized to the control spot, actin. Column one: gene name. Column
two: intensity in the absence of 100 µM A77 1726. Column three:
intensity in the presence of 100 µM A77 1726. Column four: ratio of
intensities between RNA isolated in the absence of A77 1726 and RNA
isolated in the presence of A77 1726.

<table>
<thead>
<tr>
<th>Gene</th>
<th>CMV Volume Normalized to actin</th>
<th>CMV + A77 Volume Normalized to actin</th>
<th>CMV:CMV +A77 Volume Normalized to actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE-2</td>
<td>1.2114</td>
<td>1.1307</td>
<td>1.0714</td>
</tr>
<tr>
<td>UL44</td>
<td>1.4246</td>
<td>2.294</td>
<td>0.621</td>
</tr>
<tr>
<td>UL32</td>
<td>1.2225</td>
<td>1.4141</td>
<td>0.8645</td>
</tr>
<tr>
<td>actin</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
3. 4. 2. A77 does not inhibit the translation of immediate early (IE), early (E), or late (L) proteins.

We have shown that A77 1726 inhibits the assembly of CMV virions and subsequent production of infectious virus. Silva et.al. have shown that a HCMV mutant virus lacking UL99 (pp28) does not acquire an envelope. In order to determine if A77 1726 inhibits viral protein production or alters the maintenance of viral protein levels, immunoblotting analysis was used to quantitate IE, E, and L viral proteins collected from HCMV infected cells incubated in the presence or absence of 100uM A77 1726 for various times post infection.

Total protein lysates were collected 0, 8, 24, 48, 72, and 96 hours post infection from fibroblast cells infected with Towne HCMV incubated in the presence or absence of 100uM A77 1726. Figure 8 shows results from immunoblot experiments in which proteins were detected with antibodies specific for (a) IE-1, (b) UL44, (c) UL32, and (d) GAPDH. When comparing protein samples taken from untreated HCMV infected control fibroblasts to A77 1726 treated HCMV infected fibroblasts our results show that A77 1726 does not inhibit the production or maintenance of immediate early proteins (Fig. 8A). IE-2 protein was used as a representative for all IE proteins. Similar results were found when comparing the relative amount of UL44 (representing early proteins) found in the presence of A77 1726 versus the amount found in the absence of A77 1726 (Fig. 8B). When comparing protein samples of untreated HCMV infected fibroblasts to A77 1726 treated HCMV infected fibroblasts, UL99 (Fig. 8C) protein levels, representative of
late proteins, was not affected by the addition of A77 1726. The same was true for multiple late proteins including pp71, pp65, ppUL69, UL65, minor capsid protein, and the major capsid protein (data not shown).

3. 4. 3. A77 1726 alters the phosphorylation of late viral structural protein pp150.

The effect of A77 1726 on viral replication is not due to an inhibition in the synthesis of viral mRNA, DNA, or proteins. We have hypothesized that A77 1726 inhibits the phosphorylation of one or more viral tegument proteins, many of which are phosphoproteins. \(^{32}\)P-orthophosphosphate labeling of pUL32 (pp150) in the presence or absence of A77 1726 showed a distinct difference in the phosphorylation of the late structural tegument phosphoprotein. Prior to harvesting HCMV infected cells incubated in the presence or absence of A77 1726, MRC5 cells were starved of phosphate for two hours, then incubated in culture media supplemented with \(^{32}\)P-orthophosphosphate for two hours. Once harvested, the cells were lysed in order to collect protein, and pp150 was immunoprecipitated with an antibody specific for pp150. Immunoprecipitated pp150 from samples collected 48, 72, and 96 hours post infection was subjected to immunoblotting and autoradiography (see material and methods).
Figure 8. A77 1726 does not inhibit immediate early, early, or late protein synthesis. Cell lysates, collected at various times post inoculation, from CMV-infected MRC-5 fibroblasts incubated in the presence or absence of 100 μM A77 1726 were analyzed by immunoblotting with antibodies against: (A) Immediate Early 1 protein, (B) UL44 protein, (C) pp28 protein. GAPDH protein was used as a loading control.
Figure 9 (western blot) shows that the relative amount of pp150 is not affected by A77 1726. The increase in pp150 accumulation from 48 to 96 hours post infection is not affected by A77 1726. When analyzing the autoradiograph (Fig 9 bottom panel) a decrease in phospho-intensity can be seen in all lanes corresponding to the presence of A77 1726, demonstrating an inhibition of phosphorylation of pp150 by A77 1726. $^{32}$P-orthophosphate labeling of pp65 also showed an attenuation of phosphorylation of pp65 (data not shown) in the presence of A77 1726, but no inhibitory effect was seen in the phosphorylation of pp28. While it has been documented that A77 1726 inhibits protein kinase activity, these studies are the first to show an inhibition of phosphorylation of HCMV viral proteins.

3. 4. 4. A77 1726 alters the intracellular localization of pp150.

Previous work has shown that pp150, pp65, and pp28 localized specifically within the cytoplasm of HCMV infected cells.$^{43,66}$ We utilized dual immunohistochemical staining to determine the intracellular localization of pp150 and other structural proteins in infected human umbilical vein endothelial cells (HUVEC). Acetone fixed HCMV infected HUVEC and MRC5s (data not shown) were incubated in the presence or absence of A77 1726 for various times post infection, then immunohistochemically stained with antibodies specific for each
protein. As shown in figure 10 (left panels) pp150 is expressed at 48 hours post infection and it migrates to specific locations within the cytoplasm by 96 hours post infection (figure 10, left panels). However in the presence of A77 1726 this localization pattern is altered. In both untreated and A77 1726-treated cells, pp150 visible 48 hours (figure 10, upper panels). At 72 hours (figure 10, middle panels) pp150 is diffusely spread throughout the cytoplasm in both populations. By 96 hours post infection (figure 10, bottom left) pp150 has localized to specific foci within the cytoplasm of untreated cells. However, in A77 1726-treated cells at 96 hours post
infection pp150 continues to be found diffusely spread throughout the cytoplasm. This diffuse staining pattern persists at 120 hours post infection (data not shown). In the presence of A77 1726 we were never able to visualize pp150 in the specific locations we found it to localize to in HCMV infected control cells late in infection.

3. 4. 5. A77 1726 inhibits the interactions between pp150 and other viral tegument proteins.

A77 1726 inhibits the production of infectious HCMV particles. We have shown that the phosphorylation of pp150 is inhibited in the presence of A77 1726 and that this inhibition is associated with an alteration of the intracellular localization of pp150 (figure 10) and other viral tegument proteins (See Chapter IV). We speculate that viral tegument proteins interact with each other late in viral replication to allow for the formation of a viral tegument and subsequent envelopment. To test this hypothesis we have immuno-precipitated viral tegument proteins pp150, pp28, and pp65 and used immuno-blotting to determine if other viral tegument protein co-immunoprecipitate with pp150, pp65, or pp28.
Figure 10. A77 1726 alters the intracellular localization of pp150. Immunohistochemical stain of CMV VHL/E-infected human umbilical vein endothelial cells (HUVEC) incubated in the presence or absence of 200 µM A77 1726 (noted by axes) and fixed in acetone at 48 hours post inoculation, 72 hours post inoculation, or 96 hours post inoculation. Cells were stained with primary antibodies specific for pp150 (blue stain) and major capsid protein (red stain). Bound antibody complexes were localized with biotinylated horse anti-mouse antibody, and visualized with avidin-conjugated alkaline phosphatase + Fast Blue (blue) and horseradish peroxidase + amino-ethylcarbazole (red). Cells were counterstained with hematoxylin.
Viral tegument proteins were immunoprecipitated with either anti-pp150, anti-pp65, or anti-pp28 antibodies from protein lysates collected 72 hours post infection from infected cells incubated in the presence or the absence of A77 1726. These immunoprecipitated samples were subjected to electrophoresis through triplicate 7.5% SDS poly-acrylamide gels with samples loaded in the same orientation and then transferred to nitrocellulose membranes. Each of the three membranes was then probed with one of the three antibodies listed above. The proteins were then visualized with a horseradish peroxidase conjugated secondary antibody.

Figure 11 shows that the interaction between pp28 and pp150 is attenuated in the presence of A77 1726. Immunoprecipitation of pp65 and pp150 shows apparent interactions of these tegument proteins with pp28 (fig. 11A). Probing a correlate membrane with pp150 confirms the interaction between pp150 and pp28 in the absence of A77 1726. In the presence of A77 1726 the interaction between pp150 and pp28 is attenuated. The interactions between pp65 and pp150 and between pp65 and pp28 are also attenuated in the presence of A77 1726 (figure 11B). To our knowledge this is the first demonstration of such viral protein-protein interactions. These interactions support previous findings that have shown pp28, pp65, and pp150 localizing to a compartment that overlaps an endoplasmic reticulum gogi-intermediate compartment.\textsuperscript{43,66}
3.5. Discussion

We have previously shown that A77 1726, the active metabolite of Leflunomide, inhibits the production of infectious HCMV virus particles in vitro and in vivo. Electron microscopy data showed that CMV infected cells incubated in the presence of A77 1726 produced “immature” virus particles that lacked both a complete tegument and envelope.\textsuperscript{20} In the current study we have tested the hypothesis that the inhibition of virus production is due to an inhibition of the phosphorylation of CMV tegument phosphoproteins. \textsuperscript{32}P-orthophosphosphate labeling

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{A77 1726 alters the interaction of late structural proteins. CMV infected MRC5 cells were incubated in the presence or absence of 100\mu M A77 1726 for 72 hours post inoculation. pp28, pp65, and pp150 were immuno-precipitated from separate protein lysates and subjected to electrophoresis in three identical SDS-PAGE gels. The proteins were then transferred to nitrocellulose membranes and detected by immuno-blot with antibodies against (A) pp150 and (B) pp28.}
\end{figure}
experiments illustrated the inhibition of the phosphorylation of pp150, a HCMV tegument phosphoprotein, in the presence of the experimental immunosuppressive agent Leflunomide. In the presence of A77 1726, the intracellular protein level of pp150 is not affected but the intracellular localization of pp150 is altered. Co-immunoprecipitation experiments show that the interaction between pp150 and pp28 is also attenuated in the presence of A77 1726.

Tegument phosphoprotein pp150 has been shown to bind to the nucleocapsid through its amino one-third end. It is hypothesized that this may occur when the complete nucleocapsid exits the nucleus of an infected cell. Recent work has suggested a site for viral assembly, within HCMV infected cells, in a pp150 containing juxtanuclear structure. Viral tegument phosphoprotein pp28 localizes to a cytoplasmic compartment that overlaps the endoplasmic reticulum-golgi-intermediate compartment (ERGIC), which may be the site of assembly. Tegument protein pp28 has also been shown to be essential for the late stages of viral replication, specifically the envelopment of mature infectious virus particles. We have shown that A77 1726 inhibits the phosphorylation of pp150 at 48, 72 and 96 hours post infection, the time when pp150 is localized to the ERGIC. The inhibition of phosphorylation of pp150 is associated with an alteration in the intracellular localization of pp150.

Pp150 localizes to discrete foci in HCMV infected human umbilical vein endothelial cells (HUVEC) at 96 hours post infection, and these specific regions may be where pp150 is interacting with pp28 and pp65 allowing the assembly of mature infectious virus particles to proceed. In the presence of A77 1726 pp150 never localizes to these

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specific locations, instead it continues to be diffusely spread throughout the cytoplasm of infected cells. The localization of pp150 may be determined by its phosphorylation state; therefore an alteration in the phosphorylation state of pp150 could lead to a dysregulation in the localization of pp150 and disruption of the cascade of events necessary for maturation of CMV particles.

The localization of pp150 may also be important for its ability to interact with pp28 and pp65. We have shown that pp150 interacts with pp28 and pp65 at 72 hours post infection in HCMV infected cells. In the presence of A77 1726 this interaction is attenuated. It is possible that pp150 is the bridge between the nucleocapsid and the rest of the tegument proteins. Nucleocapsids, with pp150 bound to the capsid proteins, may migrate to specific sites of assembly allowing other proteins to begin forming the tegument; this cascade of events most likely ends when the immature virion localizes to structures containing pp28 and pp65 near the ERGIC allowing for the final envelopment of the tegument to occur. The interaction of pp150 and pp28 or pp65 may be necessary in order for the tegument proteins bound to the nucleocapsid to become enveloped. An alternative to this speculation may be that the phosphorylation of pp150 may determine the interaction of pp150 with the nucleocapsid, thus an inhibition of pp150 phosphorylation would not allow pp150 to bind to the nucleocapsid and inhibit further tegumentation. If this alternative inhibition occurs then the altered intracellular localization of pp150 may be due to a lack of interaction with the nucleocapsid. In either case, the interaction of pp150 with both pp28 and pp65 is attenuated in the presence of A77 1726, and it has been shown
that each of these three tegument phosphoproteins localize to specific sites within the cytoplasm of HCMV infected cells that may be potential sites of assembly. Transmission electron microscopy data has shown an inhibition of tegumentation of HCMV virus particles in the presence of A77,\textsuperscript{20} which we have now demonstrated is associated with an inhibition of the phosphorylation of pp150.

Current therapeutic options for treatment of HCMV, with the exception of CytoGam\textsuperscript{®}, inhibit viral replication at the stage of DNA synthesis. Such treatment options have given rise to multiple drug resistant viral variants. It is necessary to find alternative approaches to treat immunosuppressed patients that suffer from possible life threatening effects of CMV infection. Leflunomide is an experimental immunosuppressive agent that has shown antiviral effects both \textit{in vitro} and \textit{in vivo}. A77 1726, the active metabolite of Leflunomide, has two known mechanisms of action, inhibition of protein kinase activity and inhibition of pyrimidine synthesis. Previous work has shown that the inhibition of HCMV virus production is not due to pyrimidine depletion since the addition of exogenous uridine could not restore viral activity. We have shown in this study that the inhibition of viral replication is associated with an inhibition of phosphorylation of pp150, a HCMV tegument phosphoprotein. The inhibition of viral activity due to the inhibition kinase activity is a novel finding that may lead to future therapeutic agents that utilize this novel antiviral activity. Equally important is that the phosphorylation of structural proteins can be a crucial step in the formation of infectious virus particles.
CHAPTER 4

INHIBITION OF HCMV PPUL69 PHOSPHORYLATION BY THE EXPERIMENTAL IMMUNOSUPPRESSIVE AGENT LEFLUNOMIDE: A NOVEL ANTI-VIRAL STRATEGY.

4. 1. Abstract

Despite progress in antiviral chemotherapy, cytomegalovirus (CMV) continues to complicate the clinical course of many allograft recipients. Leflunomide is an experimental immunosuppressive agent currently in Phase I clinical trials. Its active metabolite, A77 1726, inhibits protein kinase activity and pyrimidine synthesis. We have previously demonstrated that this agent exhibits antiviral activity against CMV in vitro and in vivo. A77 does not inhibit viral DNA synthesis; rather it appears to disrupt virion assembly by inhibiting nucleocapsid tegumentation. In the current study we have tested the hypothesis that the antiviral activity of A77 1726 is a result of its inhibition of phosphorylation of one or more viral structural proteins. We have previously verified that the antiviral mechanism is not related to viral gene transcription, since mRNA isolated from A77 treated (or untreated) CMV-infected fibroblasts hybridized to a CMV gene array containing all 208 open reading frames showing no significant difference in levels of any viral transcripts between A77-treated and untreated infected cells (See Chapter III). Western blot analysis of protein
extracts prepared from A77-treated (or untreated) CMV-infected fibroblasts at intervals spanning 4-96 hours post-inoculation showed no quantitative effect on production of immediate early, early, or late viral proteins. \(^{32}\)P-orthophosphate labeling experiments demonstrate a reduction in the phosphorylation of ppUL69. In addition, immunohistochemical staining showed discrete changes in localization of ppUL69 in A77-treated cells. Results of these studies suggest that Leflunomide may inhibit complete infectious virion assembly by altering the phosphorylation states of one or more viral structural proteins, and suggest a novel class of molecular targets for further antiviral drug development.

4. 2. Introduction

Despite progress in antiviral chemotherapy, cytomegalovirus (CMV) continues to complicate the clinical course of many allograft recipients. HIV infection and pharmacological immunosuppresion may contribute to the reactivation of latent CMV or allow for primary infection in these patient populations. Interstitial pneumonia, diffuse gastrointestinal mucosal ulceration, hepatitis, retinitis, and destructive inflammatory lesions in a variety of locations, are serious and often life-threatening complications that can arise from CMV infection or reactivation.\(^{54}\) Current approved therapeutic options for treatment of CMV disease include ganciclovir, valganciclovir (oral ganciclovir), phosphoformate (foscarnet), cidofovir, and anti-CMV hyper immune globulin (CytoGam®). With the exception of CytoGam®, all of these agents act in a similar manner by inhibiting viral DNA synthesis, either through the inhibition of chain elongation\(^{102,84}\) or by direct binding of
the viral DNA polymerase; hence viral variants are emerging which exhibit multi-drug resistance. Leflunomide [N-(4-trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486] is an experimental immunosuppressive agent with demonstrated effectiveness against acute and chronic rejection, and is currently in Phase I clinical trials in human transplant recipients. A77 1726, [N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotoamide], the active metabolite of Leflunomide, has two known mechanisms of action, inhibition of protein kinase activity and inhibition of pyrimidine synthesis. We have previously demonstrated that this agent inhibits production of multiple isolates of CMV in vitro, including multi-drug resistant variants, and reduces viral load by 4-6 orders of magnitude in an animal model. This antiviral activity is not accountable to A77-mediated pyrimidine depletion since addition of exogenous uridine, which restores intracellular pyrimidine levels to normal, does not reconstitute viral activity. Unlike currently prescribed anti-CMV drugs, A77 does not inhibit viral DNA synthesis. Rather, as demonstrated by transmission electron microscopy, it appears to act at a late stage of assembly by inhibiting formation of the virion tegument, the major components of which are viral phosphoproteins.

Our data (see chapter III) has shown that the novel antiviral effects of A77 1726 is associated with an inhibition of the phosphorylation of the viral phosphoprotein pp150, a major constituent of the viral tegument. ppUL69 another protein found in the HCMV virion is also affected by A77 1726. The viral protein encoded by the UL69 open reading frame (ORF), expressed as an early-late gene
product, has been shown to stimulate genes expressed under the control of a HCMV early promoter in co-transfection assays. Antiserum generated to detect UL69 protein has been used to show that ppUL69 localizes to intranuclear inclusions, characteristic of HCMV infection. A follow up study by Winkler showed that there are three isoforms of ppUL69 found within the cytoplasm of HCMV infected cells, but only one of these isoforms, a phosphorylated form, has been found in the virus particle. This study demonstrated that ppUL69 was not found in a membrane fraction of virions treated with detergents, and since ppUL69 is found in dense bodies it has been concluded that the ppUL69 isoform found in the HCMV virion is a part of the tegument. Hayashi et al. showed that a mutant virus lacking ppUL69 did not replicate with wild type kinetics. TNsubUL69-UL69, a HCMV mutant virus that lacks both the UL69 gene and protein, was not able to induce a block in the cell cycle, which is seen in a wild type infection. TNsubUL69+UL69, a HCMV mutant virus that lacks the UL69 gene but contains the protein product encoded by UL69 in the virus particle, was able to induce a block in the cell cycle upon infection. We report in this study that A77 1726 inhibits the phosphorylation of ppUL69. Associated with a reduction in phosphorylation of ppUL69 is an alteration in the cytoplasmic localization of ppUL69 in the presence of A77 1726. A77 1726 inhibits the tegumentation of HCMV virions and may do so by inhibiting the phosphorylation of late structural proteins such as pp150 and ppUL69.
4. 3. Materials and Methods

4. 3. 1. Cells and Viruses.

MRC5 human fibroblast cells (American Type Culture Collection, Rockville, MD) were propagated in Eagle’s Minimal Essential Medium (MEM, Sigma Chemical Co.) supplemented with 10% fetal bovine serum (U.S. Bio-Technologies Inc., Parker Ford, PA), 1% essential amino acids, 2% nonessential amino acids, and 0.5% vitamins (Sigma Chemical Co.). Human umbilical vein endothelial cells (HUVEC) were isolated from cord vessels as previously described\textsuperscript{106,107} and propagated in endothelial growth medium (ECGM), consisting of M-199 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (U.S. Bio-Technologies), 50\(\mu\)g/ml bovine brain extract,\textsuperscript{108} 12 U/ml sodium heparin (Sigma chemical Co., St. Louis, MO), and 20mM HEPES buffer. All growth surfaces for HUVEC were pretreated with human fibronectin (Upstate Biotechnology, Inc., Lake Placid, NY), 25 \(\mu\)g/ml. CMV strain VHL/E, isolated from duodenal biopsy material of a bone marrow transplant recipient\textsuperscript{109} was propagated in HUVEC to preserve its natural endothelial cytopathogenicity. CMV strain Towne, acquired from ATCC, was propagated in MRC5 cells.

4. 3. 2. Analysis of UL69 gene expression.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100\(\mu\)M A77 1726, then inoculated with HCMV strain Towne at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of
100uM A77 1726 for various times post infection (8 and 48 hrs). Viral mRNA was isolated from total RNA collected 8 and 48 hours post infection using a mRNA isolation kit (Qiagen). 250ng of mRNA was subjected to reverse transcription by using oligo(dT) and 3’ gene specific primers in the presence of [32P]dCTP. RNA was degraded; unincorporated nucleotides were removed; and the labeled cDNA was then hybridized to the membrane-bound array containing 206 HCMV open reading frames (ORFs). 110

4. 3. 3. Analysis of UL69 protein expression.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with HCMV strain Towne at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (0-96 hrs). Proteins were isolated from untreated or A77 1726-treated cells by incubating in a lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM phenylmethylsulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.) for 30min at 4°C. An equal volume of loading buffer containing 2% beta-mercaptoethanol was added to each lysate prior to boiling. Insoluable material was removed by centrifugation. Proteins were separated on a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Osmonics, Inc., Westborough, MA). The membranes were blocked overnight at 4°C in a blocking solution consisting of 5% non-fat dried milk and 0.2%
Tween-20 in PBS on an orbital shaker. Membranes were probed with an antibody specific for viral protein ppUL69 (a generous gift from Dr. William Britt, University of Alabama Birmingham), overnight at 4°C in blocking solution on an orbital shaker. Membranes were rinsed repeatedly with Tris-buffered saline containing 0.1% Tween-20. Anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as a secondary antibody. ppUL69 was visualized using ECL western blot detection reagents (Amersham) and Hyperfilm ECL (Amersham).

4. 3. 4. Analysis of ppUL69 phosphorylation.

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with HCMV strain Town at a M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (48 and 72 hrs). Four hours pre-harvest cells were incubated in phosphate free medium (Gibco) for two hours. Two hours pre-harvest cells were incubated in phosphate free medium supplemented with [³²P]-orthophosphate to give a final concentration of 50µCi/ml of medium (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for two hours. Cells were treated with trypsin (Worthington Biochemical Corp., Freehold, NJ), collected in phosphate-free medium; and pelleted by centrifugation (1200rpm, 10 minutes). Proteins were isolated using a lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM
phenylmethylsulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and
leupeptin (Sigma Chemical Co.). Protein lysates were pre-cleared with irrelevant
isotype-matched control antibodies, IgG1, IgG2a, and IgG2b (Dako Corporation,
Carpinteria, Ca) and protein G sepharose beads (Amersham). Lysates were rocked
for 3 hours and protein complexes were pelleted by centrifugation (13,000rpm, 10
minutes). An antibody specific for ppUL69 (generously provided by Dr. William J.
Britt, University of Alabama Birmingham, Birmingham, Al) was combined with
protein G sepharose beads in order to immuno-precipitate ppUL69. The protein was
separated on a 7.5% SDS-polyacrylamide gel. Proteins were then transferred to a
nitrocellulose membrane and the membrane was exposed to Kodak X-AR biomax
film (Kodak Industrie, Cedex France) for various times to produce an autoradiograph.
ppUL69 bands were visualized on the membrane using an anti-mouse
immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz
Biotechnology Inc.) as the secondary antibody, ECL detection reagents (Amersham),
Hyperfilm ECL (Amersham) as described above.

4.3.5. Immunohistochemical staining.

HUVEC were pretreated for 30 minutes with fresh ECGM alone or ECGM
supplemented with 200uM A77 1726, then inoculated with HCMV strain VHL/E at a
M.O.I. of 1 PFU/cell. Cells were incubated in the presence or absence of 200uM A77
1726 for various times post infection (48 and 72 hrs), acetone fixed, and
immunoperoxidase stained as described previously. Viral proteins were stained with a primary antibody specific to ppUL69 and irrelevant isotype-matched antibodies as specificity controls.

4. 4. Results

4. 4. 1. A77 1726 does not inhibit the transcription or translation of immediate early, early, or late genes.

A HCMV gene array was utilized to demonstrate that the inhibitory effects of A77 1726 on viral replication was not due to an inhibition of viral gene transcription. Viral mRNA isolated from HCMV infected human fibroblast (MRC-5) cells, incubated in the presence or absence of 100uM A77 1726 for 8 and 48 hours, was reverse transcribed in the presence or radiolabeled nucleotides. The radiolabeled cDNA’s were then used to probe a membrane containing nucleotide sequences correlating to 206 open reading frames (ORFs) of the HCMV genome.

Figure 12 shows gene array data from 48 hours post infection. Circled on the array are the spots corresponding to the UL69 ORF (figure 12, black circles) and actin (figure 12, white circles). We have previously demonstrated that A77 1726 does not inhibit viral gene transcription. UL69, highlighted in figure 12, is transcribed in the presence of A77 1726. A three fold increase or decrease in intensity was considered significant, and table 2 illustrates that A77 1726 does not significantly decrease the intensity of UL69.
Figure 12. A77 1726 does not inhibit immediate early, early, or late viral RNA transcription. Viral mRNA was isolated from CMV-infected MRC-5 fibroblasts incubated in the presence (B) or absence (A) of 100 µM A77 1726 for 48 hours post inoculation. The mRNA was reverse transcribed in the presence of [32P]dCTP. Quantities of 32P-labeled cDNA copied from mRNA that was prepared from the same number of CMV-infected cells in the presence or absence of A77 1726 was used as a probe on a CMV gene array containing all 206 open reading frames. The spot corresponding to UL69 ORF is highlighted by the black circles. The arrays contain actin (white circle) as cellular transcript controls.
To be sure that there was no loss in protein, which could constitute an inhibition of viral replication, we used immunoblot analysis to determine the effect of A77 1726 on protein production and maintenance. MRC-5 cells were infected with HCMV and incubated in the presence or absence of 100ul A77 1726 for 0, 8, 24, 48, 72, and 96 hours post infection in order to visualize the onset of protein production and subsequent protein levels throughout infection. Proteins were collected, subjected to electrophoresis, and transferred to nitrocellulose membranes. We have previously shown that A77 1726 has no inhibitor effect on the production of immediate early, early, or late viral proteins (see Chapter III). We visualized ppUL69 using an antibody specific to it to determine if A77 1726 inhibited the production or altered the stability of this protein. Figure 13 represents a western blot that illustrates

<table>
<thead>
<tr>
<th>Gene</th>
<th>CMV Volume Normalized to actin</th>
<th>CMV + A77 Volume Normalized to actin</th>
<th>CMV:CMV +A77 Normalized to actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE-2</td>
<td>1.2114</td>
<td>1.1307</td>
<td>1.0714</td>
</tr>
<tr>
<td>UL44</td>
<td>1.4246</td>
<td>2.294</td>
<td>0.621</td>
</tr>
<tr>
<td>UL69</td>
<td>1.8848</td>
<td>2.1228</td>
<td>0.8879</td>
</tr>
<tr>
<td>UL99</td>
<td>3.1757</td>
<td>2.5127</td>
<td>1.2638</td>
</tr>
<tr>
<td>actin</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. A77 1726 does not inhibit immediate early, early, or late viral RNA transcription. Phospho-intensity of gene array spots correlating to the open reading frames for immediate early 2, UL44 (early), UL69, UL99 (pp28) (late), and actin (control). These values are normalized to the control spot, actin. Row one: gene name. Row two: intensity in the absence of 100 µM A77 1726. Row three: intensity in the presence of 100 µM A77 1726. Row four: a comparative ratio of intensities between RNA isolated in the absence of A77 1726 and RNA isolated in the presence of A77 1726.

To be sure that there was no loss in protein, which could constitute an inhibition of viral replication, we used immunoblot analysis to determine the effect of A77 1726 on protein production and maintenance. MRC-5 cells were infected with HCMV and incubated in the presence or absence of 100ul A77 1726 for 0, 8, 24, 48, 72, and 96 hours post infection in order to visualize the onset of protein production and subsequent protein levels throughout infection. Proteins were collected, subjected to electrophoresis, and transferred to nitrocellulose membranes. We have previously shown that A77 1726 has no inhibitor effect on the production of immediate early, early, or late viral proteins (see Chapter III). We visualized ppUL69 using an antibody specific to it to determine if A77 1726 inhibited the production or altered the stability of this protein. Figure 13 represents a western blot that illustrates
ppUL69 production (Fig. 13). As hypothesized, based on previous findings (Chapter III), A77 1726 did not inhibit the production or the maintenance of ppUL69. GAPDH was used as a loading control to show equal protein amounts were loaded.

4.4.2. A77 1726 inhibits the phosphorylation of ppUL69.

We have hypothesized that A77 1726 inhibits the phosphorylation of one or more viral tegument proteins, many of which are phosphoproteins. $^{32}$P-orthophosphate labeling of ppUL69 in the presence or absence of A77 1726 showed a distinct difference in the phosphorylation of the early-late tegument phosphoprotein. Prior to harvesting HCMV infected cells incubated in the presence or absence of A77 1726, MRC5 cells were starved of phosphate for two hours, then incubated in culture media supplemented with $^{32}$P-orthophosphate for two hours. Once harvested, the cells were lysed in order to collect protein, and ppUL69 was immunoprecipitated. Immunoprecipitated ppUL69 from samples collected 48 and 72 hours post infection was subjected to immunoblot and an autoradiograph was made as explained in the methods section. Using the immunoblot and autoradiograph we were able to determine that A77 1726 inhibits the phosphorylation of ppUL69 late in viral replication.
Figure 13. A77 1726 does not inhibit pUL69 protein synthesis. Cell lysates, collected at various times post inoculation, from CMV-infected MRC-5 fibroblasts incubated in the presence or absence of 100 μM A77 1726 were analyzed by immunoblotting with an antibody against pUL69. GAPDH protein was used as a loading control.

Figure 14 shows that the relative amount of ppUL69 is not affected by A77 1726. When analyzing the autoradiograph (figure 14, bottom panel), a decrease in phospho-intensity can be seen in all lanes correlating to the presence of A77 1726. No distinct difference in ppUL69 protein levels (figure 14, western blot) in conjunction with a lack in phospho-intensity (figure 14, autoradiograph) in the A77 1726 treated samples illustrates the inhibition of phosphorylation of ppUL69 by A77 1726. $^{32}$P-orthophosphate labeling of pp65 and pp150 also showed an abrogation of phosphorylation of each protein (data not shown) in the presence of A77 1726. It has been documented that A77 1726 inhibits protein kinase activity, these results are the first to show that the inhibition of kinase activity actually affects HCMV viral proteins.
To confirm these results we took advantage of a new technique available from Molecular Bio-Probes that allowed us to stain phosphoproteins in a SDS polyacrylimide gel. Then using the SYPRO total protein stain we were able to determine if A77 1726 effected the phosphorylation of ppUL69 by comparing the amount of protein present to the amount of phosphorylation. We immunoprecipitated ppUL69 from protein samples collected from CMV infected cells incubated in the presence and absence of 100ul A77 1726 for 48 and 72 hours post infection. Each sample was then loaded onto two 7.5% SDS polyacrylimide gels and subjected to electrophoresis. One gel was stained with SYPRO total protein stain (figure 15, lower panel) and the other was fixed, stained with Pro-Q phosphostain, and de-stained per protocol (figure 15, upper panel).

To determine if the technique worked as published, we treated a portion of total protein samples from HCMV infected control cells with lambda phosphatase to dephosphorylate phosphoproteins. The sample treated with lambda phosphatase showed no difference in total protein levels (figure 15, lower panel lanes 1 and 2), but the Pro-Q gel showed a marked decrease in intensity for all proteins present in the lambda treated lanes (figure 15, upper panel lanes 1 and 2), proving the technique worked properly. When analyzing ppUL69 in the SYPRO stained gel (figure 15, lower panel) we noticed that there seemed to be two forms of ppUL69 collected from the cytoplasm of HCMV infected cells incubated in the presence or absence of A77 1726.
Figure 14. A77 1726 reduces the phosphorylation of ppUL69. CMV infected MRC5 cells incubated in the presence or absence of 100µM A77 1726 for various times post inoculation were starved of phosphate for two hours. The phosphate free media was then supplemented with 32P-orthophosphate. After a two hour incubation the cells were harvested and protein lysates were collected. UL69 was immuno-precipitated with a monoclonal antibody specific for UL69. The immuno-precipitates were subjected to electrophoresis and transferred to a nitrocellulose membrane. To determine protein phosphorylation levels, autoradiographs were made from membranes (upper panel). To relate phosphorylation levels to protein abundance, the membranes were then analyzed by immunoblot with an antibody specific for UL69 (lower panel).
There was only one ppUL69 band present in the Pro-Q stained gel (figure 15, upper panel), suggesting there is only one isoform of ppUL69 that is phosphorylated. In the presence of A77 1726 the phosphorylated band of ppUL69 is less intense than the bands correlating with ppUL69 collected from HCMV infected control cells. Pro-Q/SYPRO staining in conjunction with $^{32}$P-orthophosphate labeling of ppUL69 shows that A77 1726 inhibits the phosphorylation of ppUL69.

**Figure 15. A77 1726 alters the phosphorylation of pp150.** Cell lysates were collected 72 and 96 hours post inoculation from CMV-infected MRC-5 fibroblasts incubated in the presence or absence of 100 µM A77 1726. UL69 was immunoprecipitated from the cell lysates and electrophoresed. The gel was then stained with PRO-Q Phosphoprotein stain to visualize phosphorylated proteins (upper panel). A correlate gel was stained with SYPRO Ruby Red stain to visualize total protein (lower panel). Total cell lysates from 96 hour post inoculation treated or not treated with λ phosphatase were used as technical controls.
4.4.3 A77 1726 alters the intracellular localization of ppUL69.

HCMV ppUL69 has been shown to localize to intranuclear inclusions when using an antiserum generated to detect ppUL69, and thus has been labeled a protein that localizes exclusively to the nucleus. Yet ppUL69 is found within the tegument of a HCMV virus particle, therefore we believe that ppUL69 could be found in the cytoplasm of HCMV infected cells. Immunohistochemical staining of ppUL69, with a monoclonal antibody specific to ppUL69, demonstrates that ppUL69 is found in the cytoplasm of HCMV infected cells late in viral replication (figure 16, bottom left panel). We did visualize ppUL69 in the nucleus of infected cells at 48 hours post infection (figure 16, top left panel) and earlier (data not shown), but late in viral replication ppUL69 was found predominately in the cytoplasm of HCMV infected cells. ppUL69, stained red in figure 16, seems to migrate from the nucleus to the cytoplasm by 72 hours post infection, in the absence of A77 1726 (figure 16, left panels), which suggests that ppUL69 may be in the process of being incorporated in the tegument of virus particle. Contrary to this pattern of migration, we see ppUL69 being retained in the nucleus when HCMV infected cells are incubated in the presence of A77 1726 for longer than 48 hours post infection (figure 16, right panels).

4.5. Discussion

We have previously shown that A77 1726 inhibits the production of mature infectious HCMV particles and we hypothesize that A77 1726 interferes with the phosphorylation of one or more HCMV tegument phosphoproteins resulting in a
Figure 16. **A77 1726 alters the intracellular localization of UL69.**
Immunohistochemical stain of CMV VHL/E-infected HUVEC incubated in the presence (right panels) and absence (left panels) of 200 µM A77 1726 and fixed in acetone at 48 hours post inoculation (top panels) or 72 hours post inoculation (bottom panels). Cells were stained with a primary antibody specific for UL69, followed by incubation with a secondary antibody (biotinylated horse anti-mouse). Bound antibody complexes were visualized after incubation with avidin D/peroxidase by development in amino-ethylcarbazole (red stain, black arrows). Cells were counterstained with hematoxylin (blue stain).
block of virus assembly. In this study we have shown that the phosphorylation of ppUL69, a phosphoprotein found in the tegument of HCMV virions, is inhibited at late stages in viral replication. Although transcript and protein levels are not affected when infected cells are treated with A77 1726, the intracellular localization of ppUL69 changes when the phosphorylation state of ppUL69 is altered. Winkler et al. demonstrated that there are three isoforms of ppUL69 found in HCMV infected cells, but only one of these isoforms, the phosphorylated form, was found in isolated virions. The only isoform found in the HCMV virion being the phosphorylated form suggests that the phosphorylation of ppUL69 is necessary in order for ppUL69 to be incorporated into the HCMV virion. If this is truly the case, then an inhibition in the phosphorylation of ppUL69 would not allow ppUL69 to be assimilated into a HCMV virion. Could this block impede the maturation of the HCMV virion? Hayashi et al. used a HCMV virus that lacked UL69 to shown that it could replicate, but not to wild type kinetics. ppUL69 may not be essential for HCMV to replicate, but must play a factor in the efficiency of replication.

ppUL69 has been shown to transactivate gene expression, and play a role in blocking cell cycle progression. Multiple investigators have described ppUL69 as a nuclear protein. Our own immunohistochemical staining demonstrated that ppUL69 does localize to the nucleus early in HCMV infection. Extended studies show that ppUL69 can be found in the cytoplasm of infected cells, which we feel would make sense since the UL69 protein is incorporated into the virion. Recent work regarding the HCMV assembly process has shed light onto where tegumentation and
envelopment of the HCMV particle takes place. Sanchez et al. have shown that HCMV tegument proteins localize to specific locations, possible sites of assembly, in the cytoplasm of HCMV infected cells.\textsuperscript{43} When ppUL69 is incorporated into the virion is debatable. It is possible that ppUL69 could become a part of the tegument in the nucleus as the nucleocapsid transverses the nuclear membrane, but it is equally possible that ppUL69 assimilates into the tegument in the cytoplasm, much like what is hypothesized for pp28.\textsuperscript{66}

Since we find ppUL69 in the cytoplasm of HCMV infected cells at 72 hours post infection in the absence of A77 1726, but retained in the nucleus of HCMV infected cells in the presence of A77 1726 at the same time point, we hypothesize that ppUL69 is incorporated into the HCMV tegument in the cytoplasm. Keeping in mind that A77 1726 blocks the phosphorylation of ppUL69 at 72 hours post infection, the same time we see an alteration in the intracellular localization of ppUL69, we feel this evidence shows some necessity of phosphorylation for the incorporation of ppUL69 into the tegument of HCMV. We would be naive to believe that by abrogating the phosphorylation of only ppUL69, A77 1726 inhibits the tegumentation of HCMV particles. We have also shown that A77 1726 inhibits the phosphorylation of pp150. This block in phosphorylation, much like with ppUL69, correlates with an alteration of the intracellular localization of pp150 (see chapter III). Considering a vast majority of tegument proteins are phosphoproteins, A77 1726 could potentially have an effect on multiple proteins. We
have observed a significant difference in the phosphorylation of ppUL69, pp150, and other tegument proteins, but A77 1726 only seems to affect the intracellular localization of ppUL69 and pp150.

It is still unknown how exactly the tegument forms around the nucleocapsid of HCMV virion, but recent work seems to suggest that the tegumentation of the virion is not a completely random event. ppUL69 leaves the nucleus late in viral replication and is incorporated into the tegument of HCMV particles. Could ppUL69 play a role in chaperoning other proteins found in the nucleus (pp71 and pp65) into the cytoplasm in order to be incorporated into the maturing virus particle? Our work has shown that A77 1726 disrupts the migration of ppUL69 from the nucleus into the cytoplasm. This disruption could be blocking an order of events that leads to the tegumentation of HCMV nucleocapsids and formation of full infectious virions. Our previous studies showed that when HCMV infected cells were treated with A77 1726, only naked nucleocapsids, which lacked a tegument and envelope, could be visualized by transmission electron microscopy. If an order of events must occur in order for the nucleocapsid to acquire a tegument, A77 1726 is disrupting these events and therefore inhibiting the production of infectious HCMV particles.
CHAPTER 5

PRELIMINARY STUDIES DEMONSTRATING THE IN VITRO INHIBITION OF POLYOMAVIRUS STRAIN BK (BKV) BY THE EXPERIMENTAL IMMUNOSUPPRESSIVE AGENT LEFLUNOMIDE.

5. 1. Overview

The following chapter contains an introduction reviewing pertinent literature relative to BKV. The materials and methods used in this section will precede the experimental results. A discussion that will summarize the experimental findings and suggest future work will follow the results.

5. 2. Classification and Nomenclature

Viruses classified in the family Polyomaviridae were once organized in a subfamily of Papoviridae. Considered small DNA tumor viruses, the Polyomaviridae have been extensively studied since they were discovered by Gross in 1953. The majority of work done with polyomaviruses have been done with simian virus 40 (SV40), but two human strains of polyomavirus, JC virus (JCV) and BK virus (BKV), have been studied since they were isolated from two different patients. Much of the work done to understand the replication cycle and virion structure comes from work done with SV40, so assume the following review is representative for all polyomaviruses, including BKV. All material specific for BKV will be clearly stated.
5. 3. Polyomavirus structure

5. 3. 1. Virion Structure

The polyomavirus capsid proteins, VP1, VP2, and VP3, form a T=7 icosahedral capsid. There are 360 copies of VP1, the major capsid protein, and one copy of VP2 or VP3 at each pentamer (~72 pentamers). Exposure to the outside of the virion is achieved only by the VP1 proteins, which bind to carbohydrates on the cell surface. Contained in the capsid of BKV is a singular linear copy of the viral genomic DNA. Infectious virus particles, virions complexed with cellular histones H2A, H2B, H3, and H4 in the form of chromatin, empty capsids, virions lacking viral DNA, and capsids containing cellular DNA, represent the three types of virus particles that can be isolated from preparations of polyomavirus infected cells. Figure 17 is a transmission electron micrograph illustrating viral particles found in infected human fibroblasts, MRC-5 cells (figure 17).

5. 3. 2. Polyomavirus genome and protein functions

The polyomavirus genome contains 5153 nucleotide pairs that can be divided into two distinct regions, an early and late region. The early region encodes the proteins necessary for viral DNA synthesis, the viral T antigens. The late region encodes the proteins that make up the capsid, VP1, VP2, and VP3. Found in between
these regions is an origin of replication, where DNA synthesis initiates, and distinct regions where transcription proceeds in opposite directions.\textsuperscript{117} Though the exact sequences of the T antigens and the viral proteins, VP1, VP2, and VP3, may vary between the polyomaviruses, the functions of each are conserved between the differing viruses.

\textbf{Figure 17.} Electron micrograph of BKV particles found BKV infected MRC-5 cells.
5. 4. Polyomavirus gene products

5. 4. 1. Early proteins

Early proteins translated from the BKV genome include the small and large T antigens. The small t antigen has been shown to be dispensable for the growth of SV40 in cultured cells, but other work suggests that the small t antigen plays a role, along with the large T antigen, in cell cycle progression.

The large T antigen, which has been extensively studied in context of SV40, is a multifunctional protein. The large T antigen is post-translationally modified in many ways, one of which is phosphorylation. The phosphorylation of the large T antigen occurs at two clusters within the peptide sequence. It is possible that the phosphorylation of the large T antigen may influence the localization of the protein since the phosphorylation sites are near two nuclear localization sites. The phosphorylation state of the large T antigen does regulate the ability of the large T antigen to assist in the replication of viral DNA. A hypophosphorylated form of the large T antigen is more efficient at binding to DNA and proceeding with DNA replication in vitro.

The large T antigen, a DNA binding protein, can bind to multiple sites in the viral origin of DNA replication. Depending on where the large T antigen binds determines the functional properties of the T antigen. Binding at one site regulates the transcription of early mRNAs. In the presence of physiologic levels of ATP the large T antigen prefers to bind at another site that will allow for the synthesis of viral DNA. Phosphorylation has been shown to be a factor in the DNA replication.
synthesis regulated by the large T antigen, allowing T antigen hexamers to bind at the origin of replication and begin bidirectional unwinding of the viral DNA.\textsuperscript{126} Transfected DNA containing a SV40 origin of replication can be unwound by the large T antigen, confirming the T antigen helicase activity, which aides in viral DNA synthesis.\textsuperscript{127}

5.4.2. Late proteins

16S and 19S are the two sets of late mRNAs produced during a polyomavirus infection. From the 16S mRNAs VP1 is translated, and VP2 and VP3 are translated from the 19S mRNAs. The coding sequence for VP3 is contained within the VP2 coding region, but VP3 can be made in the absence of the initiation codon for VP2.\textsuperscript{128} The viral proteins, VP1, VP2, and VP3, are all synthesized in the cytoplasm of infected cells and then translocate to the nucleus where the capsid is formed. Mutational analysis has demonstrated the importance of the N-terminal sequence of VP1 and the C-terminal sequences of VP2 and VP3 in the nuclear localization of the viral proteins.\textsuperscript{129} VP1 may help the translocation of VP2 and VP3 and vice versa.\textsuperscript{130,131} The exact mechanism of encapsidation has yet to be elucidated, phosphorylation may be an important mediator in this process, but more work needs to be done to completely understand the relevant events in this process.
5. 5. Polyomavirus replication cycle

The replication cycle of polyomaviruses can be broken into early and late stages. The early stages of replication begin with attachment at the cell membrane and continue to the beginning of DNA replication. The late stages of polyomavirus replication continue through DNA replication and end with the egress of newly formed viral particles. Work with SV40 demonstrated the importance of MHC class I molecules and adsorption of viral particles. One particular study showed that antibodies directed to MHC class I molecules could inhibit efficient attachment and entry of the SV40 viral particles into monkey kidney cells.\textsuperscript{132} After the viral particles enter the cytoplasm it is unclear how entry into the nucleus happens. Electron microscopy has shown viral particles in cytoplasmic vesicles\textsuperscript{133} that can be seen fusing with the nuclear membrane suggesting a transportation of particles from the cell membrane to the nucleus through cellular vesicles.\textsuperscript{134}

Once in the nucleus, the viral particle is uncoated and the genome is used as a template for the transcription of early mRNAs. As discussed above, the early proteins translated from the early mRNAs play a vital role in the regulation of their transcription and the initiation of viral DNA replication (V. 2. 3. 1. above). Viral DNA is replicated with the help of the large T antigen, an origin of replication, and other cellular machinery used to synthesize cellular DNA.\textsuperscript{135} Once viral DNA has begun to replicate the late viral transcripts begin to be transcribed. VP1, VP2, and VP3 translocate to the nucleus and encapsidation ensues, how this occurs is still unknown. Electron microscopy has shown viral progeny in the cytoplasm of infected
cells and it is suggested that particles transported in a cellular vesicle may be released as the vesicular membrane fuses with the cell membrane allowing the viral particle to be released.\textsuperscript{13}

5. 6. BKV nephropathy

Eighty percent (80\%) of the adult population demonstrate serological evidence of past polyomavirus exposure. Much like HCMV (as discussed in previous chapters) initial infection proceeds asymptotically, but BKV is retained in the kidney as a latent entity.\textsuperscript{136,137} Pharmacological immunosuppression prescribed to ensure graft preservation often leads to reactivation of BKV in renal allograft recipients. BKV nephropathy can be diagnosed by histological analysis of biopsies taken from transplant recipients. In these biopsies viral alterations can be seen in the tubular epithelium, this alteration is distinctly different than cellular inflammation resulting from rejection.\textsuperscript{138} Some studies have documented rates as high as 70\% graft loss in renal transplant populations suffering from BKV nephropathy.\textsuperscript{139,140} Recent work has suggested more favorable outcomes due to advancements in detection and diagnosis and possibly improvements in management of disease.\textsuperscript{141} BKV associated nephropathy still has clinicians “scratching their heads” trying to preserve renal grafts in concordance with fighting infection.
5. 7. BKV antiviral strategies

To date no Food and Drug Administration (FDA) approved therapies exist for the treatment of BKV nephropathy. One promising agent, cidofovir, approved for treatment of HCMV, has shown anecdotal evidence of suppressing BKV infection in renal transplant patients.\textsuperscript{142} In vitro testing has also demonstrated an inhibition of SV40 and mouse polyomavirus by cidofovir.\textsuperscript{143} Unfortunately cidofovir is nephrotoxic and patients must be closely monitored to assure damage to the kidneys is limited.\textsuperscript{144} Leflunomide, an experimental immunosuppressive agent, has been shown to be a potential treatment for BKV nephropathy. Clinical data showed a decrease in BKV titers in concordance with stable or improving renal function.\textsuperscript{18} In the following study we have tested the hypothesis that A77 1726, the active metabolite of Leflunomide, inhibits the production of BKV \textit{in vitro} without inhibiting viral DNA synthesis or protein translation.

5. 8. Materials and methods

5. 8. 1 Cells and Viruses

MRC5 human fibroblast cells (American Type Culture Collection, Rockville, MD) were propagated in Eagle’s Minimal Essential Medium (MEM, Sigma Chemical Co.) supplemented with 10\% fetal bovine serum (U.S. Bio-Technologies Inc., Parker Ford, PA), 1\% essential amino acids, 2\% nonessential amino acids, and 0.5\% vitamins (Sigma Chemical Co.). Polyomavirus strain BK (BKV), acquired from ATCC, was propagated in MRC5 cells.
5.8.2. Assay of infectious virus production

To determine the affect of A77 1726 on BKV production, 24-well plates consisting of a monolayer of MRC-5 cells were incubated in the presence of various concentrations of A77 1726 (0, 12.5, 25, 50, 75, and 100µM), in the presence or absence of 200µM exogenous uridine, for 30 minutes then washed twice with phosphate buffered saline (PBS). Monolayers were inoculated with BKV at a M.O.I of 0.0004 PFU/cell by centrifugal enhancement (300 x g, 30 min). After a 30 minute incubation at 37°C the monolayers were washed twice with PBS and fresh medium, supplemented with various concentrations of A77 1726 (as above), in the presence or absence of 200µM exogenous uridine, was added to the monolayers. After a 10-14 day incubation, with MEM changes at 48 hour intervals, cells were washed twice in PBS, harvested by brief trypsin digestion (Worthington Biochemical Corp., Freehold, NJ). Following sonication, the homogenate was inoculated in serial dilution onto confluent monolayers of MRC-5 cells, in six well plates, by centrifugal enhancement (as above). After a 30 minute incubation at 37°C, the monolayers were washed twice with PBS, and fresh medium supplemented with 3% agarose was added. After a 10-14 day incubation (adding fresh medium, supplemented with 3% agarose, after 7 days), the monolayers were formalin fixed and stained with crystal violet stain. Foci of infection (plaques) were counted with a dissecting scope and data was plotted as means of triplicate wells ± SD as PFU/ml.
5.8.3. Analysis of Protein expression

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with BKV at a M.O.I. of 0.0004 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (48-96 hrs.). Proteins were isolated by incubating cells in lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM phenylmethyisulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.) for 30min at 4°C. Protein lysates were pre-cleared with irrelevant isotype-matched control antibody, IgG2a (Dako Corporation, Carpinteria, Ca) and protein G sepharose beads (Amersham). Lysates were rocked for 3 hours and protein complexes were pelleted by centrifugation (13,000rpm, 10 minutes). A monoclonal antibody specific for BKV large T antigen (Chemicon International, Ternecula, CA) was combined with protein G sepharose beads in order to immuno-precipitate the large T antigen. An equal volume of loading buffer containing 2% beta-mercaptoethanol was added to each immunoprecipitate prior to boiling. Insoluble material and protein G beads were removed by centrifugation. Proteins were separated on a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Osmonics, Inc., Westborough, MA). The membranes were blocked overnight at 4°C in a blocking solution consisting of 5% non-fat dried milk and 0.2% Tween-20 in PBS on an orbital shaker. The membrane was probed with a monoclonal antibody specific for BKV large T antigen (Chemicon International,
Ternecula, CA), overnight at 4°C in blocking solution on an orbital shaker.

Membranes were rinsed repeatedly with Tris-buffered saline containing 0.1% Tween-20. Anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as a secondary antibody. Large T antigen bands were visualized using ECL western blot detection reagents (Amersham) and Hyperfilm ECL (Amersham).

5. 8. 4. Dot blot analysis of viral DNA

MRC-5 monolayers in a 75cm² culture flask were inoculated with BKV at a M.O.I. of 0.0004 PFU/cell and incubated in the presence or absence of 100µM A77 1726, in the presence or absence of 200µM exogenous uridine. 48 or 96 hours post inoculation cells were harvested by brief trypsin digestion, DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) per protocol for the isolation of Genomic DNA from tissue culture cells. After equalization of concentrations, extracted DNA was serially diluted in 96-well microtiter plates and transferred to a nylon membranes as described elsewhere (Waldman Transplantation 1999). The cDNA probe was generated from PCR amplification of viral genomic DNA with primers: BKV-1A: 5’ CTA GCA CTT TTG GGG GAC CTA G 3’ and BKV-1B: 5’ GGA AGA ATC CAT GGA GCT CAT G 3’.145 The cDNA probe was both labeled and visualized with the ECL Direct Nucleic Acid Labeling and Detection Systems kit (Amersham Biosciences, Piscataway, NJ).
5. 8. 5. Analysis of BKV early protein phosphorylation

MRC5 fibroblasts were pretreated for 30 minutes with fresh medium alone or medium supplemented with 100uM A77 1726, then inoculated with BKV at a M.O.I. of 0.0004 PFU/cell. Cells were incubated in the presence or absence of 100uM A77 1726 for various times post infection (8-96 hrs). Proteins were isolated by incubating cells in lysis buffer consisting of 0.05M Tris HCl, 0.15M NaCl, 1% Triton-X100, 10mM Na Orthovanidate, 0.05M NaF, 10mM phenylmethylsulfonyl fluoride (PMSF), and 0.05mg/ml of aprotinin, pepstatin A and leupeptin (Sigma Chemical Co.) for 30min at 4°C. Protein lysates were pre-cleared with irrelevant isotype-matched control antibody, IgG2a (Dako Corporation, Carpinteria, Ca) and protein G sepharose beads (Amersham). Lysates were rocked for 3 hours and protein complexes were pelleted by centrifugation (13,000rpm, 10 minutes). A monoclonal antibody specific for BKV large T antigen (Chemicon International, Ternecula, CA) was combined with protein G sepharose beads in order to immuno-precipitate the large T antigen. The immunoprecipitates were separated on a two identically loaded 7.5% SDS-polyacrylamide gels. Each gel was stained with either Pro-Q Phosphoprotein stain (Molecular Probes Inc., Eugene, OR) or Sypro Ruby Red total protein stain (Molecular Probes Inc., Eugene, OR) and visualized following supplied protocol.
5. 9. Results

5. 9. 1. Infection of human fibroblasts with polyomavirus strain BK (BKV)

Previous reports suggest that BKV replicates most efficiently in kidney epithelial cells of primate origin and to a lesser extent human fibroblasts. In order to determine if BKV would replicate in the \textit{in vitro} model system performed in our lab, we inoculated human fibroblasts (MRC-5) as described above (see V.8.2.). We visualized distinct cytopathic foci within the infected monolayer as compared to uninfected control cells. Rounding of cells occurs early in infection, and as the infection proceeds the fibroblasts begin to take on a distinct morphology by seven days post inoculation that is unlike the long narrow shaped uninfected fibroblasts (figure 18). Using this documented cytopathic determinant we assayed for viral production using standard plaque assay techniques. Infected monolayers from six well culture plates yielded between $1.28 \times 10^3$ and $3.58 \times 10^4$ plaque forming units (PFU)/ml for each of two BKV sub-strains propagated in our lab, F5 and F6 respectively. Propagating higher titers of BKV in human fibroblast cells could not be accomplished with our model system (BKV viral titers compared to HCMV are very low). Roughly 20-50 foci of infection could be found within a cell monolayer of human fibroblasts in one well of a 6 well culture plate.
Figure 18. MRC-5 cells infected with BKV show definitive cytopathic morphology. MRC-5 human fibroblasts were infected with BKV at an M.O.I. of 0.0004 PFU/cell. Mock infected MRC-5 control cells seven days post inoculation (A) compared to BKV infected MRC-5 cells seven days post inoculation (B) illustrates a foci of infection as seen under phase contrast (20x) microscopy.
Figure 19 is a series of phase contrast micrographs representative of cell monolayers infected with BKV incubated in the presence or absence of 100µM A77 1726. Individual infected cells are seen in A77 1726 treated and untreated cultures at 48 hours post infection (figure 19, A and C), but cytopathology cannot be seen spreading to adjacent cells in the A77 1726 treated cultures late in infection (seven days), represented by a lack of infected foci in the monolayer (figure 19, B and D). From these results we conclude that A77 1726 inhibits the production of BKV in vitro.

5.9.2. A77 1726 inhibits the production of infectious BKV

We have developed an in vitro model system of BKV infection in human fibroblasts (MRC-5 cells), and plaque assay data demonstrates an inhibition of BKV production in vitro. Primary inoculation of MRC-5 cells was allowed to progress in the presence or absence of various concentrations of A77 1726, in the presence or absence of 200µM exogenous uridine, 10-14 days, until roughly 20-50 foci of infection could be observed through phase contrast microscopy. Secondary inocula were collected by harvesting and sonication of the infected cell monolayer and virus yield was quantified by plaque assay.
Figure 20 shows that A77 1726 inhibits the production of infectious BKV in a dose dependant manner. At concentrations as low as 12.5 µM A77 1726 there is almost complete inhibition of BKV production. Toxicity tests have shown that MRC-5 cells are still viable at concentrations of greater than 100 µM A77 1726. Uninfected cells can still be observed in A77 1726 treated cultures and these cells show no sign of stress due to toxicity. The addition of exogenous uridine can counter act the inhibition of pyrimidine synthesis, one documented mechanism of A77 1726. If the inhibition

Figure 20. A77 1726 restricts dissemination of BKV. Phase contrast micrographs (20x) taken from BKV infected MRC-5 cells incubated in the presence of 100 µM A77 1726 for 48 hr (C) or 168 hr (D) post inoculation or the absence of A77 1726 for 48 hr (A) or 168 hr (B) post inoculation. White arrows represent BKV infected cells. Black arrow represents foci of infection.
of BKV was due to a depletion of pyrimidine levels, incubating the A77 1726 treated samples in the presence of 200µM exogenous uridine should restore virus yield to wild type levels. Data represented in figure 20 demonstrate that the addition of exogenous uridine could not restore viral replication. These results suggest that the antiviral mechanism of A77 1726 against BKV is independent of the ability of Leflunomide to inhibit pyrimidine biosynthesis.

5.9.3. A77 1726 does not inhibit viral DNA synthesis

A77 1726 has two known mechanisms of action, inhibition of protein kinase activity and inhibition of pyrimidine synthesis. To further test if the inhibition of BKV was due to a depletion in pyrimidines and to determine the impact of A77 1726 upon viral DNA synthesis, we inoculated confluent monolayers of MRC-5 cells with BKV and incubated the infected cells in the presence or absence of 100µM A77 1726 ± 200µM exogenous uridine, for 48 or 96 hours post inoculation. We isolated viral DNA and utilized dot blot analysis to determine if A77 1726 had an effect on viral DNA synthesis.

Figure 21 presents data generated by dot blot analysis. No decrease in quantity of viral DNA isolated from A77 1726-treated infected cells is detectable compared with untreated infected control cells. This finding alone argues against pyrimidine depletion as an antiviral mechanism, and is further substantiated by lack of detectable effect of exogenous uridine upon viral DNA synthesis.
Figure 20. **A77 1726 inhibits production of infectious BKV in a dose-dependent manner.** Plaque assay of polyomavirus strain BKV in MRC-5 cells in the presence of various concentrations of A77 1726. Data points represent mean plaque forming units per ml of inoculate.
5. 9. 4. Leflunomide does not inhibit the translation of the large T antigen

The large T antigen is a protein vital for the synthesis of BKV DNA. The T antigen binds to viral DNA, acts as a helicase, and associates with ATP to facilitate DNA synthesis.\textsuperscript{125,127} To determine if A77 1726 had an effect on the transcription and subsequent translation of the large T antigen we determined the relative amount of T antigen present in BKV infected cells through western blot analysis. MRC-5 cells were inoculated with BKV and incubated in the presence or absence of 100\mu M A77 1726 for 48, 72, and 96 hours post inoculation. The large T antigen was immunoprecipitated from protein lysates collected from infected monolayers and visualized by western blot.

Figure 22 shows that A77 1726 has no inhibitory effect on the synthesis and accumulation of BKV large T antigen. When comparing the lanes corresponding to A77 1726 treated BKV infected cells to lanes corresponding to infected control cells it is apparent that the relative intensities of the bands are comparable. Although A77 1726 treated BKV infected cells seem to have slightly more large T antigen, there is clearly no inhibition of large T antigen translation in A77 1726-treated cells.
5.9.5. A77 1726 does not affect the phosphorylation of BKV large T antigen

Chapters III and IV demonstrate an inhibition of the phosphorylation of HCMV structural proteins by A77 1726. When we determined that A77 1726 inhibits the production of BKV *in vitro*, we hypothesized that there may be a similar mechanism of inhibition occurring with respect to BKV replication. In order to test
the affect of A77 1726 on large T antigen phosphorylation we utilized new techniques available from Molecular probes designed to quantify the amount of phosphorylation of phosphoproteins. Pro-Q phosphoprotein stain and Sypro Ruby Red stain, when used in conjunction, can quantify the amount of phosphorylation of specific proteins. We incubated BKV infected MRC-5 cells for various times post inoculation in the presence or absence of 100µM A77 1726. After immunoprecipitating BKV large T antigen, we stained the SDS-PAGE gel with Sypro Ruby Red total protein stain to visualize the total protein present in each sample. In a correlate gel we stained the immunoprecipitated protein with Pro-Q phosphoprotein stain to visualize the phosphoproteins in the gel.

According to Molecular probes, the intensity of bands in the Pro-Q stained gel directly correlate to the amount of phosphorylation of the specific proteins stained (ie. the more intense the band, the more phosphorylated amino acids on that protein). It is then possible to compare the total protein intensities, those bands represented in the Sypro Ruby Red stained gel, to the intensity of the bands stained in the Pro-Q gel to determine the amount of phosphorylation. The amount of phosphorylation can then be compared between different samples. We utilized this approach to compare the phosphorylation of the large T antigen in the presence of A77 1726 to the phosphorylation of the large T antigen in the absence of A77 1726.
The upper portion of figure 23 represents large T antigen stained with Pro-Q phosphoprotein stain. Sypro Ruby Red stained large T antigen is represented in the lower set of bands. It is evident from this figure that A77 1726 does not inhibit the phosphorylation of BKV large T antigen. The intensity of bands representing the large T antigen in the Pro-Q stained gel show no relative difference between samples collected from A77 1726 treated infected cells when compared to samples collected from untreated infected control cells. Sypro Ruby Red stained gels confirm the results demonstrated by western blot analysis demonstrating that A77 1726 has no
inhibitory affect on the translation of BKV large T antigen. BKV large T antigen can be visualized in all lanes representing various times post infection and the intensity of bands representing large T antigen are not lost when infected cells are treated with A77 1726.

5. 10. Discussion

We have previously shown that A77 1726, the active metabolite of Leflunomide, can inhibit the production of infectious HCMV virions. Poduval et al. has demonstrated that Leflunomide could be a potential therapeutic agent against BKV in renal transplant patients. In this study we have demonstrated that A77 1726 can inhibit the production of BKV infectious particles in vitro. The exact mechanism of BKV inhibition has not been established, but our results illustrate that A77 1726 does not inhibit the production of BKV large T antigen or BKV viral DNA. A77 1726 has been shown to inhibit the phosphorylation of some cellular proteins, but Pro-Q phosphoprotein staining demonstrates that A77 1726 does not inhibit the phosphorylation of BKV large T antigen, a process necessary for efficient virus production.
We were able to develop an in vitro model system to test the effect of A77 1726 on BKV viral replication. Distinct cytopathology could be seen as early as 48 hours post infection and foci of infection were obvious by 72 hours post inoculation. Using this system we were able to test the effect of A77 1726, in a dose dependent manner, on BKV replication. Viral replication was inhibited at near minimal A77

Figure 23. A77 1726 has no affect on the phosphorylation of BKV large T antigen. Cell lysates were collected at various times post inoculation from BKV-infected MRC-5 fibroblasts incubated in the presence or absence of 100 µM A77 1726. BKV large T antigen was immunoprecipitated from the cell lysates and electrophoresed. The gel was then stained with PRO-Q Phosphoprotein stain to visualize phosphorylated proteins. A correlate gel was stained with SYPRO Ruby Red stain to visualize total protein.
molar concentrations and foci of infection could not be visualized in monolayers of BKV infected fibroblasts treated with A77 1726. The immunosuppressive mechanism of A77 1726 has been partially attributed to the inhibition of pyrimidine biosynthesis, and the addition of exogenous uridine can restore pyrimidine to normal levels. We have incorporated the addition of exogenous uridine into our studies to determine if the antiviral activity of A77 1726 is due to pyrimidine depletion. The addition of uridine was not able to reconstitute viral replication in plaque assays, ruling out this mechanism.

Viral DNA synthesis is a necessity to produce infectious viral particles. Cidofovir, one therapeutic agent showing anecdotal inhibition of BKV, inhibits DNA chain elongation and subsequent production of BKV. Southern (dot) blot analysis showed that A77 1726 had no inhibitory affect on the synthesis of BKV viral DNA, thus ruling it out as a possible mechanism of action again BKV. Western blot analysis demonstrated A77 1726 had no inhibitory affect on the translation of large T antigen, a protein essential for virus production. Small T antigen and the structural proteins VP1, VP2, and VP3 could not be visualized due to a lack in appropriate antibodies specific for each protein. We are confident that the inhibition of BKV virus production is not due to an inhibition of viral DNA synthesis or large T antigen production. The inability to test the other viral protein levels limits our ability to determine if A77 1726 inhibits the production (or maintenance) of BKV viral proteins.
The inhibition of phosphorylation of multiple HCMV structural proteins is associated with an inhibition of HCMV production. BKV large T antigen phosphorylation is not affected by A77 1726. We were not able to test the phosphorylation states of the VP1, VP2, and VP3 due to a lack of antibodies specific for these proteins, but doing so could greatly enhance our understanding of the antiviral mechanism of A77 1726 with respect to BKV. To our knowledge no work has been done to show definitive evidence of how the BKV structural protein encapsidate the viral genome. It may be possible that a phosphorylation event coordinates the association of the BKV structural proteins. If A77 1726 inhibits this phosphorylation event then the viral structural proteins may not localize to areas of encapsidation or associate with other structural proteins. The development of antibodies specific for BKV structural proteins VP1, VP2, and VP3 is essential for determining how A77 1726 inhibits BKV replication.

Our work thus far has demonstrated that A77 1726 inhibits the production of BKV in vitro. Other work has suggested that Leflunomide is promising as a therapy to treat BKV infected renal transplant recipients. Immunohistochemical staining would give insight into the affect of A77 1726 on the intracellular localization of BKV proteins in the presence or absence of A77 1726. Leflunomide may inhibit the ability of viral proteins to localize to specific sites of replication or assembly, thus inhibiting the production of infectious virus particles. In vitro phosphate labeling analysis would confirm if the large T antigen or other BKV proteins were phosphorylated in the presence of A77 1726. If the phosphorylation of one or more
BKV proteins was demonstrated then a multitude of other assays (2D gel analysis, *in vitro* kinase assays, and/or co-immunoprecipitation assays) could be done to determine the mechanism of action of A77 1726 and shed light onto BKV processes that are not well understood.
6. 1. Conclusions from experimental results

In the current study we have tested two hypotheses both of which have one common factor, Leflunomide. Based on previous studies we have hypothesized that the antiviral activity of A77 1726, the active metabolite of Leflunomide, is a result of its inhibition of phosphorylation of one or more HCMV structural proteins, leading to an inhibition of the assembly of the mature infectious virion. CMV gene array and western blot analysis demonstrated that A77 1726 does not inhibit viral gene expression or protein production. Further analysis illustrated an inhibition of phosphorylation of two viral structural proteins, pp150 and ppUL69. The intracellular localization of both pp150 and ppUL69 were altered, and pp150 no longer co-immunoprecipitated with pp28 and pp65 when infected cells were incubated in the presence of A77 1726. From these results we have made four conclusions:

1) A77 1726 (Leflunomide) inhibits assembly of complete infectious virions, but does not do so through inhibition of viral gene transcription or viral protein synthesis.
2) A77 1726 inhibits phosphorylation of viral structural phosphoproteins (ppUL69 and pp150) found in the tegument of the complete infectious virion.

3) A77 1726 disrupts the interaction of pp150 with pp28.

4) A77 1726 alters the intracellular localization of ppUL69 and pp150.

The second hypothesis we tested was that A77 1726, the active metabolite of Leflunomide, will inhibit the production of BKV \textit{in vitro} without inhibiting viral DNA or protein synthesis. By developing an \textit{in vitro} model system we were able to test the effects of A77 1726 on BKV production. Plaque assay data demonstrated that A77 1726, at a minimal molar concentration, could almost completely inhibit the production of infectious BKV, and the addition of exogenous uridine could not recapitulate virus growth. We utilized southern (dot) blot analysis to illustrate that A77 1726 has no effect on BKV DNA synthesis, and western blot analysis showed no inhibition of BKV large T antigen by A77 1726. A new technique available through
Molecular Probes was used to determine that A77 1726 had no inhibitory affect on the phosphorylation of BKV large T antigen. From these experimental results we have made three conclusions:

1) **A77 1726 inhibits the production of BKV in vitro**

2) **A77 1726 does not inhibit the production of BKV via inhibiting early protein production or viral DNA synthesis**

3) **A77 1726 does not inhibit the production of BKV through the inhibition of the phosphorylation of BKV large T antigen.**

Although A77 1726 inhibits both HCMV and BKV in vitro, these conclusions suggest that A77 1726 may inhibit these viruses through slightly different mechanisms. Until further studies are completed with BKV we cannot be assured that this is truly the case. HCMV is a complex virus with morphology unlike BKV. BKV virions consist of a viral DNA genome and a nucleocapsid, whereas the HCMV genome-containing-nucleocapsid is surrounded by a protein matrix, the tegument, and an outer glycoprotein containing envelope. BKV gene products number five, where HCMV gene products exceed 200. HCMV encodes over 20 phosphoproteins found in the tegument, and limited work done with BKV has not determined whether or not the T antigens are the only encoded phosphoproteins. We have extended previous
findings by determining some of the HCMV proteins affected by A77 1726, and we have shown that A77 1726 can inhibit the production of BKV \textit{in vitro}. Many more studies need to be done to determine the exact process(es) affected by A77 1726 with respect to these two viruses.

We have ruled out pyrimidine depletion as the antiviral mechanism of A77 1726 (II. 9. Inhibition of HCMV by Leflunomide \textit{in vitro}, V. 9. 2. and V. 9. 3.) by adding exogenous uridine to infected cells treated with A77 1726. The only other known mechanism attributed to A77 1726 is an inhibition of protein phosphorylation. An association between the inhibition of HCMV production and the inhibition of phosphorylation of multiple tegument proteins is evident in this study. The phosphoproteins affected by A77 1726, pp150 and ppUL69, show altered intracellular localization in the presence of A77 1726. Sanchez et al. have shown that pp150 localizes to specific regions in the cytoplasm of HCMV infected cells.\textsuperscript{43,66} If the localization of pp150 is a determining factor in the assembly of infectious HCMV particles, the alteration of this localization by A77 1726 could lead to a block in the assembly process. The protein encoded by UL69 has three isoforms and only one of these forms, the phosphorylated form, is incorporated into the virion.\textsuperscript{39} The fact that only one of these forms is present in the virion suggests that the phosphorylation of ppUL69, and possibly other tegument proteins, is a determining factor for incorporation.
HCMV assembly has not been studied in detail. Events occurring after nucleocapsid assembly until recently were only hypothesized. The current study is the first work, to our knowledge, demonstrating this novel antiviral mechanism. Currently prescribed agents to control HCMV have helped to promote the isolation of multi-drug resistant viruses. These mutant viruses are limiting the effectiveness of current approved HCMV antiviral agents, thus new treatment options must be tested, understood, and developed in order to treat patients suffering from the debilitating complications arising from HCMV infection.

The current preliminary data demonstrating the efficacy of A77 1726 against BKV \textit{in vitro} may lead to future studies defining the anti-BKV mechanism. Although these studies are preliminary, we can be confident that pyrimidine biosynthesis inhibition is not the key factor in BKV inhibition since the addition of exogenous uridine could not counteract the inhibition of BKV replication by A77 1726. A77 1726 may inhibit the phosphorylation of the viral structural proteins, but until we have antibodies specific to the structural proteins we cannot be sure. BKV has a simple assembly process compared to HCMV, but this process has not been documented in great detail. Future studies defining a mechanism of A77 1726 as an anti-BKV agent could lead to further understanding of the BKV replication cycle.
6.2. Future experiments and considerations

The current study has lead to new hypotheses that must be tested in order to completely define the antiviral mechanism(s) of A77 1726. Leflunomide inhibits the phosphorylation of multiple tegument proteins leading to an alteration of intracellular localization and inhibition of some protein/protein interactions. We now hypothesize that A77 1726 inhibits the production of infectious HCMV particles by inhibiting the phosphorylation of pp150 and ppUL69. In order to test this hypothesis, experiments must be designed to examine the downstream affects of inhibiting the phosphorylation of these two proteins. Mutagenesis studies could determine the viral protein phosphorylation sites essential for proper intracellular localization of the tegument proteins and subsequent assembly of a complete infectious virion. To determine which kinases are inhibited by A77 1726, in vitro kinase assays could be utilized. It is very important to design studies that will test this hypothesis in the context of a viral infection. Transfection systems utilizing over-expressed mutant viral proteins may be simple, but may not represent what occurs during an infection.

Obtaining monoclonal antibodies specific for BKV viral structural proteins is crucial in determining the anti-BKV mechanism of A77 1726. With only five functional proteins encoded by BKV, determining which viral proteins may be affected by A77 1726 should be relatively simple with the proper reagents. Pro-Q phosphostaining and in vitro phosphate labeling studies could be extended to test all five viral proteins. Imaging of the BKV particle through electron microscopy did not allow for the comparison of the morphology of the particles in the presence or
absence of A77 1726 (data not shown). BKV utilizes cellular factors to replicate and it may be possible that no viral proteins are directly affected by A77 1726. Cellular chaperone proteins or cellular proteins involved in the assembly process of BKV could be the factors negatively affected by A77 1726 leading to a block in the production of BKV particles. If cellular factors are altered in the presence of A77 1726 then determining the anti-BKV mechanism of A77 1726 becomes even more difficult.

6.3. Summary

Leflunomide is a novel immunosuppressive agent that has been shown to have antiviral effects against HCMV and BKV in vitro. HCMV and BKV are completely different entities that replicate using different cellular and viral factors. A77 1726, the active metabolite of Leflunomide, has two activities, the inhibition of pyrimidine biosynthesis and the inhibition of protein kinase activity. The addition of exogenous uridine was not able to recapitulate HCMV or BKV replication when added to infected cells treated with A77 1726, suggesting that the antiviral affect of A77 1726 can be contributed to an inhibition in kinase activity of either viral or cellular kinases. The phosphorylation of two HCMV proteins, pp150 and ppUL69, is inhibited by A77 1726, leading to an altered intracellular location of each protein. A protein/protein interaction between pp150 and other HCMV tegument proteins is disrupted in the presence of A77 1726 possibly leading to an inhibition in the assembly of the complete infectious HCMV virions. Preliminary studies with BKV have illustrated
an inhibitory affect of A77 1726 on BKV replication. The inhibition of BKV is not attributed to a block in viral DNA synthesis or early protein synthesis. Large T antigen phosphorylation is not altered in the presence of A77 1726, but we were unable to test the BKV viral structural proteins VP1, VP2, or VP3 due to a lack in appropriate antibodies. Understanding the mechanism of A77 1726 is going to lead to future understanding of HCMV and BKV replication and the development of novel antiviral therapeutics that target alternate processes in the replication cycles of both HCMV and BKV.
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