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The Effect of Cell Type on the Efficacy of CMV Antiviral Drugs

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THE EFFECT OF CELL-TYPE ON THE EFFICACY OF CMV ANTIVIRAL DRUGS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abstract

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By Benjamin Meza, MS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Until recently, all *in vitro* drug susceptibility assays of cytomegalovirus (CMV) were performed in clinically irrelevant fibroblast cells. This study sought to test if drug susceptibility was affected by cell type. MRC-5 embryonic lung fibroblasts and ARPE-19 retinal pigmented epithelial cells were infected with BADrUL131-Y4 epithelial/fibroblast tropic virus under serial concentrations of ganciclovir (GCV) or maribavir (MBV). Virus was quantified using plaque reduction, GFP fluorescence, and yield reduction. Both drugs performed less efficiently in ARPE-19 cells. A cell type effect was observed for both plaque reduction and yield reduction assays with implications for the treatment of CMV

retinitis as well as other manifestations of CMV Disease that involve non-fibroblast cell types.

Chapter 1: Virus and Host

Human cytomegalovirus (CMV) accounts for one of the most significant infectious causes of birth abnormalities in the developed world and is an important pathogen for immunocompromised individuals such as AIDS patients and allogeneic transplant recipients. In immunocompetent adults, the virus is poorly pathogenic. Primary infection may be asymptomatic or produce mild cold-like symptoms, progressing to a latent infection (108). Like others in the betaherpes subfamily, CMV can persist for the lifespan of its host, using sophistocated means of evading the host immune system, replicating, and laying dormant. As such, epidemiological studies report from 40-80% of the US adult population are seropositive (68). One study of US residents six years and older tested for anti-CMV IgG and found the population's overall seroprevalence between 1988-1994 to be 58.9%. Naturally, seroprevalence increased with age and correlated strongly with markers of socioeconomic status, such as household income, education level, race/ethnicity, and type of medical insurance (105). Concurrently, some research has found seroprevalence rates greater than 90% among homosexual men, poor-socioeconomic groups, and residents of developing countries (106).

The virus's victims fall into three major categories of immunologically compromised patients: infants in the case of congenital or perinatal infection, AIDS patients, and allogeneic transplant recipients. All three of these populations lack a fully

functioning immune system and thus are at risk of becoming newly infected or reactivating latent virus.

1.1 Congenital & Perinatal CMV

A child can acquire CMV *in utero*, during delivery, from post natal blood transfusion, or from infected secretions such as saliva or breast milk. These routes are commonly classified into two categories: "congenital" if virus is isolated within three weeks of age and "perinatal" if isolated between three weeks and 12 months (109). This distinction is important in predicting probable sequelae. Furthermore, the outcomes of congenital CMV infection depend on whether it results from a primary or non-primary maternal infection.

Worldwide, up to 2.3% of all live births will present with congenital CMV infection as detected by urine polymerase chain reaction (95). While all geographic regions and socioeconomic classes are at risk, there are higher rates of incidence among dense populations and poor demographics (28). In the United States, congenital CMV affects approximately 1% of all live births each year, making it the most common congenital infection and a major cause of morbidity and mortality among infants. While the majority of these children will suffer from no serious pathologies from the virus, approximately 10% will be symptomatic at birth and another 10-15% who are asymptomatic at birth will experience sensorineural hearing loss or developmental problems during childhood (68). Mortality among symptomatic neonates may be as high as 30% and more than 90% of survivors will show signs of hearing loss, mental retardation, delay in psychomotor

development, or optic atrophy (62). In the US healthcare system, physical examination of newborns includes identifying signs of TORCH infections. TORCH is an acronym for some of the most common perinatal infections: toxoplasmosis, other (i.e. syphilis, human parvovirus, listeria, Coxsackie virus, etc.), rubella, cytomegalovirus, and herpesviruses (i.e. herpes simplex, Epstein-Barr virus, Varicella-zoster virus, etc). Of these agents, which often cause malformations and cognitive retardation, CMV is the most common (26). Some of the malformations associated with CMV include microcephaly, cerebral calcifications, chorioretinitis and blindness, and hepatosplenomegaly (26).

Congenital cytomegalovirus is transmitted through the placenta to the fetus via maternal infected leukocytes. Replicating in placental fibroblasts, syncytiotrophoblasts and cytotrophoblast cells, the virus moves through the umbilical cord, ultimately to be taken up by the fetal circulation (109). These same infected cells can be shed into the amniotic fluid that is swallowed by the fetus. An infected oropharynx and colonization of the gastrointestinal and genitourinary tracts result (28).

Though transmission from mother to child can occur throughout the entire pregnancy and during or after delivery, route of transmission and clinical outcome depend on both the timing of infection and sero-status of the mother. Intrauterine infection occurs in approximately 50% of primary maternal infections. In contrast, non-primary maternal infections make up only 3.2% of all congenital CMV infections (109). A study by Pass et al. showed that children born to mothers who contracted a primary CMV infection during their first trimester had a higher frequency of central nervous system (CNS) sequelae than did those born to mothers who contracted the virus later in gestation. The sequelae were

also much more severe in the latter group, consisting of mental retardation, cerebral palsy, seizures, chorioretinitis, and, especially, sensorineural hearing loss (83).

Perinatal infections are more common than congenital infections but symptoms tend to be less severe. In these cases, the virus may be contracted during parturition from infected cervical tissue or after birth from infected breast milk, a blood transfusion, fomites, and contact with other children. Ninety percent of infants presenting viremia will be asymptomatic. The other 10% who are symptomatic may have encephalitis, gastroenterological disorders, hepatitis, pneumonitis, splenomegaly, or thrombocytopaenia (109).

The differences between the congenital and perinatal prognosis necessitate screening for the disease within three weeks of age. The standard of practice for these screens is a urinary CMV polymerase chain reaction (PCR), which detects viral DNA in the urine. PCR can also be performed on saliva and amniotic fluid but sensitivity is highest in the urine (28).

Treatment of perinatal and congenital CMV infections is controversial, due in part to the lack of treatment options and because of the severe side effects of available treatments. Ganciclovir (GCV) is the only antiviral drug that has undergone extensive clinical trials in infants and neonates. One study conducted by the National Institute of Allergy and Infectious Diseases collaborative Antiviral Study Group (CASG) looked at the effect of GCV treatment on symptomatic congenital CMV infection with central nervous system (CNS) involvement (116). Parenteral treatment was carried out every 12 hours over a period of six weeks and, as expected from such a long period, most of the infants

suffered some degree of thrombocytopenia and neutropenia attributed to GCV. Nevertheless, the study found that at a six month follow-up, five of 30 babies (16%) showed hearing improvement or stabilization. Subsequently, a Phase III randomized controlled study of parenteral GCV treatment in neonates with symptomatic congenital CMV infection involving the CNS also found promising results (53). Neonates were treated every 12 hours for six weeks. At a six month follow-up, 84% of the treated group showed an improvement in hearing or maintenance of normal hearing, as compared to only 59% of the placebo group. Furthermore, none of the GCV group showed deterioration, whereas 41% of the placebo group suffered from a deterioration in hearing. At 12 months, 68% of the placebo group showed signs of worsening hearing, compared to only 21% of the GCV treated group. Again, most of the neonates (63%) had significant neutropenia. In both of these studies, a transient drop in CMV shedding at mucosal sites was detected yet upon termination of therapy, excretion inevitably resumed.

Data on the efficacy of other therapies in congenitally or perinatally infected individuals is limited. CMV hyperimmunoglobulin (CMV-Ig), which is concentrated plasma antibodies from individuals who have high anti-CMV ELISA titers, is in use among adults and in pregnant mothers who contract a primay CMV infection in their first trimester. There are a number of studies which have suggested that while CMV-Ig cannot prevent infection, it can prevent serious CMV disease (78, 79). Cases where congenitally infected infants were treated with other antiviral drugs, such as Cidofovir (CDV) and Foscarnet (FOS), appear in the literature but pose even greater side effects than GCV. In this situation, an ethical dilemma arises. To use these drugs as prophylaxis would be

unethical due to their highly toxic nature and potential as teratogens. Yet CMV disease can be just as devastating. Children who are symptomatic at birth would probably benefit from drug treatment but will always be a subset of children who are not symptomatic at birth but who will still develop neurological disorders associated with CMV disease.

1.2 CMV Infection and Disease among Transplant Recipients

Immunocompromised individuals are also at high risk for CMV infection. In fact, the severity of the disease is correlated with the degree of immunosuppression. Bone marrow transplant recipients and AIDS patients with very low CD4+ T-cell counts have the most severe disease. Solid organ transplant recipients, patients under an immunosuppressive chemotherapy regime, and subjects with other immunodeficiencies also suffer from CMV disease, though clinical diseases are predominant in each group (61).

In their 1998 review of organ transplant infections, Fishman and Rubin delineate two factors that determine the risk of infection after organ transplantation: epidemiologic exposure to pathogens and the recipient's net state of immunosuppression (31). The highest risk of infection comes from CMV, which newly arises or reactivates from latency in up to 75% of solid organ transplant recipients (SOT) (32). Risk of infection is highest among seronegative recipients who receive tissue or organs from seropositive donors (D+/R-). These individuals have infection rates upwards of 20 times higher than R-/D- individuals as determined by the number of individuals with primary or reactivated disease within one year of transplantation. Rates of infection among R+/D- and R+/D+ transplants

fall somewhere within this interval. It should be noted that 5% of D-/R- patients will also become infected as a result of blood transfusions, improper serological testing, and other inaccuracies (32). An individual's level of immunosuppression is contingent on a number of interrelated factors, including, but not limited to, immunosuppressive therapy (dose, time course of different drugs), presence of other immunomodulatory parasites (e.g., CMV, Epstein-Barr virus, Hepatitis B and C, human immunodeficiency virus), and any corporal outcomes associated with the procedure itself (e.g., injured tissue) (31). Other risk factors include: donor's age, recurrent rejection, HLA mismatches, hypertension, hypercholesterolemia (107).

CMV infection is distinguished from CMV disease. Both indicate the presence of viremia but the former is asymptomatic while the latter presents symptoms. An example of CMV infection without disease might be an individual who underwent SOT two years earlier and who is still moderately immunosuppressed. If the individual's immune system is able to suppress viral replication but is unable to eliminate cells which harbor the virus, PCR will detect viral DNA in the blood absent clinical manifestations of disease (66). The effects of CMV infection and disease can be divided into those that are direct and those that are indirect. Direct disease may be subdivided into CMV syndrome, which resembles mononuclosis- or flu-like symptoms (often with neutropenia), and tissue invasive disease presented as pneumonitis, gastrointestinal disease, hepatitis or retinitis (32). The indirect effects of active CMV infection appear to be much more complex. As CMV is an immunosuppressive virus, infection has been implicated in increased risk of bacterial, fungal, and other viral infections such as human herpesvirus 6 and 7 (HHV-6 and HHV-7)

Epstein Barr virus (EBV), and hepatitis C. Furthermore, coinfection with Epstein-Barr virus, an oncogenic gammaherpes virus, is associated with higher risk of post-transplant lymphoproliferative disease in patients with CMV (66). CMV has also been implicated in acute allograft rejection, graft loss, and chronic allograft rejection. Accelerated transplant vascular sclerosis caused by an inflammatory response to the latently infected vasculature may be a major contributor to these outcomes (107). Alloreactive T-cells may be a contributing factor to endothelial damage (51).

Currently, prophylaxis, preemption, and treatment of CMV disease in SOT patients centers around a handful of therapies including: GCV, CMV-Ig, foscarnet, and cidofovir. Ganciclovir was the first antiviral drug found to be effective against CMV infection (24). As it became available, rates of CMV mortality dropped and today they are a rare event (32). Today the controversy is not what to use but how to use it. Prophylaxis with ganciclovir and related drugs (i.e., acyclovir, valciclovir) significantly reduce the risk of CMV infection and disease, mortality, and disease caused by other infectious agents (10, 40). In D+/R- kidney, heart and combination heart-lung transplants, ganciclovir prophylaxis has been shown to effectively suppress viremia during the initial months of infection and prevent the direct and indirect effects of disease. Patient mortality may also be decreased. In contrast, prophylaxis has not found much support among other transplant groups (e.g., D+/R+, D-/R+, D-/R-, liver transplant recipients) (81). For these patients, a preemptive approach (i.e., treating after serum or leucocyte dectection of CMV DNA or pp65 viral antigen but before symptoms appear) may be on par with prophylaxis (66). One of the prevailing side effects of many anti-CMV drugs is neutropenia (42). In the case of

ganciclovir and its derivatives, this may be due to inhibition of DNA polymerase in hematopoietic progenitor cells (104). To counter this effect colony stimulating factors have been used concomitantly with GCV, with relative success (7).

1.3 Coinfection with the Human Immunodeficiency Virus

Another major cause of immunosuppression and partner of CMV infection is human immunodeficiency virus (HIV). The most common manifestation of CMV disease in HIV seropositive individuals is retinitis, making up 85% of cases (106). In developing nations, this pathology is the number one cause of visual loss in HIV-infected patients in great measure because of the lack of highly active antiretroviral therapy (HAART) (52). HAART is a novel approach of combining at least three antiretroviral drugs of different classes in the treatment of HIV. Before the therapy's introduction in the United States around 1995, CMV was one of the most important opportunistic infections of HIV sufferers, afflicting up to 40% of individuals with advanced HIV disease (106). New approaches to HIV treatment in the developed world has lead to increased life-expectancy and quality of life, with the suppression of HIV viraemia and concurrent reduced incidence of CMV disease in this population. There are, however, subgroups of patients still at risk for CMV disease. In particular, those HIV-infected individuals who have CD4+ T-cell counts below the critical threshold of 100/mm^3 are at increased risk for many opportunistic infections such as CMV. As it requires up to eight months for HAART therapy to induce that degree of reconstitution of cellular immunity in most patients, a vulnerable population

arises (70). Additionally, a proportion of individuals are either non-adherent or do not respond to HAART therapy (e.g., develop resistance).

Prior to the advent to HAART, about 30% of people living with AIDS would develop retinitis (41). HAART has decreased this number by 75% (44). The retina is isolated from the rest of the body by several ocular tissues including the outer blood-retinal barrier, made up of the retinal pigment epithelium. These barriers block efficient drug delivery for vitreoretinal diseases such as CMV retinitis. In the past, GCV was injected into eyes to treat CMV-retinitis, however, this procedure is highly invasive and the intravitreal half-life of GCV is only about 18 hours (73). Repeated intravitreal injections often result in complications such as vitreous hemorrhage, endophthalmitis, and retinal detachment (121). To overcome these barriers to drug delivery, intraocular controlled (sustained)-release systems have been devised, using a number of different technologies, including: drug infused liposomes (84), biodegradable microspheres (72), and implants that are either non-biodegradeable (93) or biodegradable (38, 54). These devices can effectively prevent retinal disease from becoming worse, but fall short of clearing the virus.

CMV has also been implicated in the progression of HIV to AIDS. In one study of 290 patients who contracted HIV either through sexual intercourse, infected blood products, or IV drug use, the researchers found that progression to AIDS was two-fold more rapid among patients who became CMV seropositive. This increased risk of progressing to AIDS, generated by a primary CMV infection, even affected patients with relatively high CD4+ T-cell counts (>100/mm^3) (91).

Ironically, effective therapies have also given rise to what is termed as immune reconstitution inflammatory syndrome. In these cases, an unusually high incidence of vitritis (inflammation of the eye chamber) has been recorded and is generally ascribed to the reaction of the regenerating immune system to persisting viral antigens, in part from CMV (106).

1.4 Microbiology of CMV

Cytomegalovirus is a member of the herpesviridae family. The family is made up of eight herpes viruses known to infect humans. These viruses are then divided into three subfamilies: alpha, beta, and gamma. Herpesviruses were originally classified into these subfamilies according to their *in vitro* and *in vivo* biological properties, including: tissue tropism *in vivo*, host cell range *in vitro*, and characteristics of *in vitro* infection (e.g., duration) (77). These classifications were later bolstered with the discovery of distinct genomic similarities within subfamilies (77).

The alpha subfamily, characterized by latent neurotropism, includes human herpes virus 1 (HHV-1) and 2 (HHV-2), more commonly known as herpes simplex types 1 and 2 respectively. This subfamily also includes varicella zoster virus, the virus that causes chickenpox and shingles. In the gamma group of lymphotropic herpesviruses, one finds Epstein-Barr virus (EBV) and KSHV. Both are potentially oncogenic and are associated with posttransplant lymphoproliferative disease (PTLD) and Kaposi's sarcoma, respectively. Cytomegalovirus (also classified as HHV-5), along with HHV-6 and HHV-7 (which both can cause roseola infantum), are categorized as beta herpesviruses and as of

yet do not have a single cell type of latent infection. Worthy of note, CMV is believed be carried latently in cells of myeloid lineage (e.g., monocytes) as an episome (98).

In all herpesvirus genomes, there are a common collection of about 40 highly conserved genes (17, 50), comprising 25-70% of the genes of a particular virus (67, 77). A phylogenic analysis of herpesviruses undertaken by McGeoch et al. traces the coevolution of these viruses and their hosts. According to their analysis, the three subfamilies may have arisen up to 220 million years ago and began parallel speciation with their hosts probably around the time of the mammalian radiation, 80 million years ago (67).

The CMV virion structure is much like other herpesviruses. A loose lipid bilayer, studded with a large number of glycoproteins encircles the viral tegument, a proteinaceous layer which itself surrounds the viral capsid. The icosahedral nucleocapsid is approximately 100nm in diameter and encases the viral DNA. The entire virion is approximately 150-200nm in diameter (61).

CMV has a double-stranded linear DNA genome of 230 kbp, the largest of any herpesvirus. The genome contains a unique long (UL) and a unique short (US) region, each flanked by repeat regions. The UL and US regions are able to isomerize such that the genome can exist in four isomers: prototype, inverted L, inverted S, inverted L and S. Isomerization of the UL and US regions is contingent on the direct repeat sequences found at the genome termini and their inverted counterparts found at the UL-US junction (71). Of the handful of laboratory-adapted strains of CMV, the study of two strains, originally intended as live attenuated vaccine candidates: AD169 and Towne, tell us much about the virus' genome. Analysis of the AD169 genome has uncovered 225 open reading frames

(ORF) coding some 213 unique proteins, whose functions are mostly unknown (61, 80). Each reading frame is named according to its region and a number designating its relative position (e.g., UL130, UL131, US32, US33, etc.). Other laboratory-adapted strains, such as Towne and Toledo, contribute additional distinct ORFs.

Viral genes are grouped into three kinetic classes: immediate early (IE or α), early (E or β), and late (L or γ). These categories correspond to the temporal appearance of the gene's products. IE genes, for example, are defined as those genes whose expression does not require the expression of other viral genes. Genes in this first kinetic class are often responsible for activating the transcription of E genes. The second kinetic class, early genes, does not require DNA synthesis but also cannot be expressed without the products of IE genes. They are often involved in DNA synthesis. Late genes require DNA synthesis and typically encode structural genes necessary to construct new viral progeny.

Chapter 2: Antiviral Drugs and Treatment

2.1 Cell-Type Tropism

At the crux of this paper's argument is the issue of CMV cell tropism. Immunohistochemical staining of autopsied tissues demonstrates the presence of CMV in a variety of cell types including: endothelial cells, epithelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, monocytes/macrophages, and granulocytes. Ductal epithelial cells are most commonly infected but, in disseminated infections, high concentrations of CMV infected cells have been found in the lung, pancreas, kidneys, and liver (3, 85).

In contrast, the *in vitro* biological properties of the virus have been studied almost exclusively in clinically irrelevant fibroblasts. To a large extent, this inclination has been driven by the convenience of laboratory-adapted strains of the virus that provide controlled, reproducible results but preferentially infect fibroblasts. As mentioned before, there are a handful of commonly used lab-adapted strains of CMV. Among them are AD169 and Towne. Both strains were originally developed as potential live attenuated vaccine candidates, both were serially passaged in fibroblast cells to reach high titers *in vitro* (~107), and both suffered gross genetic mutations in the process. Upon isolation from the adenoids of a 7-year-old girl undergoing tonsillectomy-adenoidectomy, AD169 was passaged over 50 times in fibroblast cells (88, 92). Towne has a similar history.

Derived from the urine of a congenitally infected 2-month-old infant, the virus was subsequently passaged over 125 times, including three endpoint dilutions (86, 88). In both instances, the viruses underwent numerous point mutations and rearrangements (15, 88). As a result of laboratory-adaptation, these two viruses and others like them replicate to high titers in fibroblasts, but poorly in other cell types such as epithelial or endothelial cells. Clinical isolates, however, replicate just as well in endothelial and epithelial cell cultures as fibroblasts (61, 113).

Because CMV has such a large genome and most of its genes have yet to be functionally characterized, associating biological and genetic differences between strains can be very difficult (88). Yet stark differences in cell tropism have been traced back to genetic alterations, which arise from serial passage of virus strains in fibroblast cell cultures, and not just epigenetic phenomena (100). In 2004, Hahn et al. used knockout mutants and trans-complementation to identify the *UL131-128* locus as the primary determinant of endothelial cell tropism. The locus contains two spliced mRNAs and three ORFs: *UL128*, *UL130*, and *UL131*. A deleterious mutation in any one of these ORFs can annul endothelial tropism (37). Such deleterious mutations have been found in both AD169 and Towne, which should not be surprising as it has been shown that as few as three passages of a clinical isolate in fibroblast culture can abolish endothelial tropism (6). In AD169, a single nucleotide insertion in the *UL131* locus generates a frameshift and truncation of the mRNA that *UL131* encodes (27, 113). Town has a similar frameshift in the *UL130* locus (74).

In 2005, Dai Wang and Thomas Shenk used allelic exchange to replace AD169's UL131 locus and flanking sequences with corresponding sequence from TR, a clinical isolate. In so doing, they restored endothelial and epithelial tropism to the virus. They also found that while the repaired virus (named BADrUL131-Y4) produced larger cell-free and cell-associated virus progeny in epithelial and endothelial cells than its lab-adapted parent (i.e., AD169), the amount of cell-free and cell-associated virus progeny produced from fibroblasts had decreased up to 100-fold (113). Research has not yet exposed what the selective advantage for deleterious mutations in the UL128-131 locus might be when viral strains are serially passed in fibroblast culture. Other such mutations that impact endothelial cell tropism have been discovered in the translocation of viral nucleic acid to the nucleus (99, 101).

2.2 Antiviral Treatment

Today, there are four pharmaceuticals licensed for CMV treatment: ganciclovir (Cytovene®, Roche) and its prodrug valganciclovir (Valcyte®, Roche), foscarnet (Foscavir®, AstraZeneca), and cidofovir (Vistide®, Pharmacia) (94). Despite this, CMV antiviral treatment is suboptimal due to toxicity, poor oral bioavailability, and resistance, associated with all these drugs. While there are a number of candidate compounds in development, only GW 1263W94 (Maribavir® or MBV, ViroPharma) has progressed as far as phase III clinical trials.

Ganciclovir (GCV) was the first drug to be effective in patients with life-threatening CMV infections (24) and the first to be specifically licensed for CMV

treatment (94). Since the late 1980's, GCV has become the treatment of choice for patients who are immunocompromised (68). The compound is a nucleoside analog of guanosine and a homolog of acyclovir, another successful anti-herpesvirus antiviral. As a nucleoside analog, GCV acts as a competitive inhibitor of the viral DNA polymerase. Though it is not an absolute chain terminator as acyclovir is, GCV slows the rate of DNA elongation and thus inhibits viral replication. *In vivo*, GCV must be phosphorylated to its active triphosphate form. This only occurs in virus-infected cells because the initial phosphorylation step is performed by a viral kinase, pUL97. Once pUL97 protein kinase/phosphotransferase phosphorylates GCV to its monophosphate form, cellular kinases convert it to its active triphosphate form, which can then act to inhibit viral DNA polymerase (24). Valganciclovir is a 1-valyl ester prodrug of GCV which dramatically increases the oral bioavailability of GCV from 5.6% to 60.9% (48).

Due to their toxicity profiles, second line therapies are generally reserved except when GCV is not an effective option, they include: foscarnet and cidofovir (35). Foscarnet (trisodium phosphonoformate) is an inorganic pyrophosphate analog. The drug acts directly on the viral DNA polymerase by noncompetitively binding its pyrophosphate binding site. By binding this site, deoxynucleotide triphosphates (dNTPs) are unable to bind the polymerase. Unlike GCV, foscarnet does not need to be phosphorylated for antiviral activity (94). Cidofovir is another inhibitor of the CMV DNA polymerase. The compound is an acyclic phosphonate nucleotide analog and, as it is administered in its monophosphate form, it does not require initial phosphorylation by UL97 protein kinase to have antiviral activity. Nevertheless, the monophosphate form does rely on cellular kinases

in much the same way GCV does and ultimately acts on the CMV DNA polymerase in the same manner (61, 94).

All of these drugs are associated with considerable toxicities. In immunocompromised individuals, myelosuppression (e.g., granulocytopenia, anemia and thrombocytopenia) by GCV can be the dose-limiting factor. While myelosuppression is of lesser concern for foscarnet (and thus recommended for patients diagnosed with bone marrow failure) (94), other toxicities, such as nephrotoxicity, electrolyte disturbances, nausea, penile ulcerations, and seizures, make some physicians reticent to use foscarnet except when necessary (46). Of the three, cidofovir may be the most toxic with side effects such as: nephrotoxicity, neutropenia, ocular toxicity and metabolic acidosis. In animal studies, the drug is both carcinogenic and teratogenic (94, 120).

Of the anti-CMV drugs on the horizon, MBV (5,6-dicholoro-2-(isopropylamino)-1,β-L-ribofuranosyl-1-H-benzimidazole, GW 1263W94, Maribavir®) is the most promising and has come the closest of any to licensure (i.e., phase III clinical trials). MBV comes from a class of new anit-CMV drugs called halogenated benzimidazole ribonucleosides. They are characterized by a benzimidazole moiety halogenated with chlorine or bromine, which substitutes for the purine base of the ribonuceloside. The benzimidazole, which is the bicyclic fusion of benzene and imidazole, is typically halogenated with elements such as chlorine or bromine. The sugar moiety can be derived either from furanose (e.g., MBV) or pyranose and can either be a D- or L- isomer. D-isomers of the halogenated benzimidazoles have been investigated for their anti-"terminase" activity, preventing packaging of viral DNA into capsids. MBV, an L-

isomer, appears to act by blocking the pUL97 viral kinase, thus inhbiting capsid egress from the nucleus by a yet unknown mechanism.

The L-isomer halogenated benzimidazole ribonuceloside, MBV, is thought to act on the viral kinase encoded by UL97 and potentially on the gene product of UL27, a paralog of UL97. UL97 is an early/late tegument protein (69) known to autophosphorylate serine and threonine residues at its amino-terminus (39) as well as the essential DNA processivity subunit pUL44 (58), the RNA polymerase II carboxy-terminal domain (8), and nucleoside analogs such as GCV. Structurally, pUL97 shares many typical protein kinase motifs, however, overall the enzyme is relatively divergent from other herpesvirus kinases (18, 58). Prichard et al. have done extensive work on the UL97 kinase. They found that if they deleted a large portion of *UL97* from a recombinant virus, the replication of the virus was severely impaired and peak titers dropped as much as 100-fold (87). Researchers working with similar null mutants have cataloged a number of viral defects that could shed light on the function of pUL97, including: modestly reduced DNA synthesis, inefficient DNA packaging (119), impaired nuclear egress (57, 119), and inappropriate aggregation and sequestration of viral proteins into nuclear aggresomes (89). Recently, researchers have suggested that pUL97 acts as a cellular CDK ortholog and induces cell cycle progression through phosphorylation of the tumor-suppressor retinoblastoma protein (43).

MBV induces the phenotypic changes similar to pUL97 null mutants (43, 57, 58, 87, 89, 119). Mutant strains of CMV that are resistant to MBV have been isolated in cell culture and the majority of their mutations are in the UL97 locus. However, some less resistant mutants have been isolated with mutations mapped to the UL27 ORF (20, 55).

UL27 may be a paralog of UL97 as it shares 12% amino acid identity (90). The *in vivo* function of the UL27 protein is not known.

According to the ViroPharma Website and a Drugs in Research and Development article, MBV is currently undergoing phase III clinical trials as a CMV prophylaxis in transplant recipients. After being classified as an orphan drug, the Food and Drug Administration (FDA) granted the drug fast-track status, whereby it has completed both phase I and II clinical trials (1). Data from the phase II trials have not yet been published but the phase I data on safety and pharmacokinetics in healthy and HIV infected subjects are available. The latter demonstrate a favorable safety profile with good bioavailability and relatively low toxicity (i.e., the most frequent adverse events were taste disturbance in 80% of subjects and headache in 53%) (115). Additional pharmacological studies provide similar optimistic outlooks(56, 60) and *in vitro* studies further support the efficacy of the drug (11, 117). Importantly, CMV strains which develop resistance to GCV, CDV, and PFA appear to maintain sensitivity to MBV (11, 22).

2.3 Objectives

The tradition of culturing and studying CMV in fibroblasts has limited *in vitro* testing of drug susceptibility to a clinically irrelevant cell type. The discovery that the UL128-131 locus is essential for epithelial and endothelial entry has allowed us to repair well-scrutinized strains of laboratory adapted virus and study them in more relevant cell types. Certain clinical outcomes, such as the poor efficacy of front line drugs (e.g., GCV) in treating CMV retinitis, suggest that infection of certain tissues is more resilient against

our current repertoire of antiviral drugs. This study sought to test the *in vitro* activity of two prominent anti-CMV drugs under epithelial cell culture conditions which had not previously been explored. Our results demonstrate that there is a strong cell type-dependence to the efficacy of GCV and MBV when tested *in vitro*. Both drugs tested were more effective in fibroblast culture under conditions typically used to evaluate drugs. Diminished efficacy in epithelial culture exposes how our current phenotypic assay of drug susceptibility overlooks cell type differences that become critical to clinical treatment of CMV disease. These results strongly advocate the use of multiple cell types when assaying drug efficacy *in vitro* and shed light on possible reasons why these two drugs may be limited in their current means of application.

Chapter 3: Cell-Type Effect on Drug Susceptibility

3.1 Materials and Methods

Antiviral Compounds. Ganciclovir (GCV) was provided generously by Mark Prichard while GW1263W94 (Maribavir®, MBV) was the generous gift from John Drach. GCV was dissolved in 0.1 M sodium hydroxide. MBV was dissolved in anhydrous dimethyl sulfoxide (DMSO).

Cells, Viruses, and Cell Culture Conditions. MRC-5 human fetal lung fibroblasts (ATCC CCL-171) and human ARPE-19 retinal pigmented epithelial cells (ATCC CRL-2302) were propagated in high glucose Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL) (DMEM). An immortalized human embryonic lung fibroblast cell line was established by transduction of primary cells with a defective retrovirus that expresses human telomerase reverse transcriptase (13) and puromycin resistance. The retrovirus was generated from plasmid pLPChTERT (Clontech), and cells were infected as previously described (4). Transduced cells were selected by several passages in media containing 1 μg of puromycin (Sigma)/ml. Cells were maintained at 37°C and 5% CO^2 in Dulbecco's modified Eagle medium-H with Gluta-max supplemented with 10% fetal bovine serum and 1 μg of puromycin/ml.

CMV strain BADrUL131-Y4 was derived from a bacterial artificial chromosome (BAC) clone of the CMV strain AD169 genome that had been modified in *E. coli* by Wang and Shenk to (1) contain a green fluorescent protein (GFP) reporter cassette to permit efficient detection and quantitation of viral infection (112), and (2) to express a functional UL131 protein, which permits efficient entry and replication in either ARPE-19 or MRC-5 cells (113). BADrUL131-Y4 was reconstituted by transfection of BAC DNA into ARPE-19 cells as described (36) and amplified by passage exclusively in ARPE-19 cells. Tissue culture supernatants (BADrUL131-Y4) were clarified by centrifugation, adjusted to 0.1 M sucrose, aliquoted, and stored at –80°C. Viral titers of $1x10^5$ (BADrUL131-Y4) were determined by limiting dilution in 96-well plates (25) using MRC-5s. Stocks of BADrUL131-Y4 were capable of entering ARPE-19 and MRC-5 cells with equal efficiency.

Phenotypic Assay of Drug Susceptibility (Experiment 1). Two clear/flat-bottomed 96-well plates (Costar) each containing confluent MRC-5 or ARPE-19 cells were uniformly infected with BADrUL131-Y4 for an average of 10 pfu/well and incubated for one hour. Twelve three-fold dilutions of either GCV or MBV were prepared in triplicate in a 96-well format. These drug dilutions were then transferred to infected cells to produce final concentrations as follows: 320 μM to $5.4x10^{-3}$ μM GCV and a no-drug control; 100 μM to $1.7x10^{-3}$ μM MBV and a DMSO control of the same molarity as the highest MBV concentration.

Plaque Reduction Assay. On day eleven, representative photos were taken of plaque formation (indentified by GFP-expressing cells) using a Nikon 15 Diaphoto 300

microscope equipped with a 470-525 nm UV filter. At each drug dilution, photos were taken of plaques (more appropriately termed "foci" because the cells do not lyse) that characterized the general size and morphology of the replicates. The following day, the numbers of plaques formed in both the MRC-5 and ARPE-19 plates were counted. While the total numbers of plaques were counted in each well of the MRC-5 plate, the ARPE-19 plate had smaller more abundant plaques. This made it impractical to read the total number of plaques in each well. Instead, the densest area of plaque formation was found and the maximum number of plaques that could be fit within the microscope's viewer was read. Several of these ARPE-19 wells were also counted in totality, as to provide a reference to compare the cell types (Data presented on Plaque Formation Table).

GFP Fluorometry. GFP-fluorescence was measured using a PerkinElmer Victor^2 1420 Multilable Counter plate reader with manufacturer provided fluorescence presets of 485/535nm wavelength (excitation/emission) for 0.1 second/well.

Yield Reduction Assay. As BADrUL131-Y4 forms plaques more slowly in MRC-5 cells than in ARPE-19 cells, the yield reductions were conducted on the ARPE-19 plate on day 19 and on the MRC-5 plate three days later on day 22. Fifty microliters of culture medium were removed from each well and transferred to the bottom-edge row of a 96-well plate filled with 200 µL/well of fresh medium (five-fold initial dilution). This row of 12 aliquots was then diluted vertically seven times with five-fold serial dilutions. Two-hundred microliters of diluted supernatant were then transferred to a replicate plate containing confluent cells. This procedure was repeated for all three replicates of each drug group. Yield reductions of supernatants derived from an infected plate of a given cell

type were carried out on a clear-walled/flat bottomed 96-well plate of the same cell-type (e.g., viruses from ARPE-19 plates were titrated on ARPE-19 cells).

Titrations were read as soon as clear plaques had formed but before secondary spread was suspected. For the ARPE-19 plates, this period was nine days after infection; for the MRC-5 plates, it was 11 days after infection.

Comparison of Cell-Variant Entry. Each of five different cell-line variants was seeded into a 96-well plate. Variants included high passage (hp, passage greater than 30), low passage (lp, passage less than 20), and telomerase immortalized (hTERT, also hp) cell variants of MRC-5 and ARPE-19 cell lines. The following cell variants were seeded in duplicate eight-well columns: lp-ARPE-19, hp-ARPE-19, lp-MRC-5, hp-MRC-5, and MRC-5 hTERT. Once all the cell monolayers were confluent, BADrUL131-Y4 was titrated at 10-fold dilutions from 103pfu/mL to 10-3pfu/mL in each cell variant duplicate. GFP-expressing cells were detected using a Nikon 15 Diaphoto 300 microscope equipped with a 470-525 nm UV filter and viral plaques were counted in each titration at 24, 48, and 72 hpi.

Viral Growth Curves (Experiment 2). White-walled, clear/flat-bottomed 96-well plates (Costar) were seeded with MRC-5 hTERT cells and eight identical plates were seeded with hp-ARPE-19 cells. Once confluent, the plates were uniformly infected with BADrUL131-Y4 at an MOI of about 0.02 and incubated for 12 hours. Plates were kept in sealable plastic bags to prevent evaporation. In a 96-well format, ten three-fold dilutions of either GCV or MBV were prepared in sextuplet using DMEM supplemented with 10% FCS and PSG. Infected cell plates were aspirated to remove virus containing supernatant

and washed twice with 200 µL/well phosphate buffered saline (PBS). Drug solutions were then transferred to infected plates to produce final drug concentrations as follows: 320 µM to $4.9x10^{-2}$ µM GCV and a no-drug control; 100 µM to $1.5x10^{-2}$ µM MBV, and DMSO diluted appropriately to match the highest concentration of DMSO in MBV containing cultures. In this manner, 24 replicates of each drug were generated per cell type. Note: Titration of all culture supernatants, for the calculation of virus yield was performed on MRC-5 hTERT cells, unlike in experiment 1.

Cytotoxicity Assays. Two white-walled, clear/flat bottomed 96-well plates (Costar) were seeded with MRC-5 hTERT cells and two identical plates were seeded with hp-ARPE-19 cells. Each plate contained 12 replicates of the following treatment groups: each of the three highest drug concentrations (i.e., 320 µM, 107 µM, and 36 µM GCV or 100 µM, 33 µM, and 11 µM MBV), cell controls without drug, and drug controls without cells (i.e., 320 µM GCV or 100 µM MBV). Triplicates of each drug/cell-type combination (i.e., GCV on MRC-5 hTERT, GCV on hp-ARPE-19, MBV on MRC-5 hTERT, MBV on hp-ARPE-19) were assayed for cell viability using the promega CellTiter-Glo® kit (Promega). CellTiter-Glo® uses an engineered luciferase reagent to quantify ATP in cell culture. ATP concentration directly correlates with the number of viable cells in culture. Plates were equilibrated to room temperature for 30 minutes. Using an Eppendorf epMotion 5070 Liquid Handling Workstation, 100 µL media was removed from wells to be tested. One hundred microliters of CellTiter-Glo® reagent was added to each well and the plates were orbitally mixed for two minutes. The plates were then allowed to equilibrate for another 20 minutes before reading each well's luminescence using a PerkinElmer Victor^2 1420

Multilable Counter at manufacturer provided presets for 1.0 second/well readings. Mean percent luminescence was calculated by dividing the luminescence of each drug treated group by the luminescence of the no-drug control.

Statistics. Fifty percent inhibitory concentration (IC_{50}) values were determined for plaque reduction and GFP-fluorescence by plotting data against a log3 scale of drug concentration. A four-parameter sigmoid line of best fit was determined using the Solver function in Microsoft Excel. Each replicate's IC_{50} was obtained from the drug concentration at the inflection point (mid-point) of this line. Yield reduction 90% inhibitory concentration (IC_{90}) values were calculated by normalizing data with a log10 transformation and then plotting this transformed data against a log3 scale of drug concentration. The same four-parameter sigmoid line of best fit was determined using the solver function in Microsoft Excel. Each replicate's IC_{90} was obtained from the drug concentration that correlated with a log10 decrease in viral titer below the curve's upper asymptote. The three replicate IC_{50}s were then averaged and means ± one standard deviation were presented in the text. Comparisons were made using the Browne-Forsythe test of equal variance followed by the appropriate one-tailed t-test.

3.2 Results

The physiology of cells in the human body can be very diverse. Everything from membrane receptor expression to the concentrations of important signalling molecules can be profoundly cell-type dependent. The susceptibility of HCMV to GCV has only been tested in fibroblast cells, which are physiologically distinct from more clinically relevant

epithelial cells. The susceptibility of fibroblast and epithelial tropic BADrUL131-Y4 was determined against serial dilutions of GCV in cell culture.

The three methods of quantifying viral replication had differing degrees of sensitivity. The first method employed, plaque reduction, was generally effective, but raised the concern that identification of plaques in two morphologically distinct cell types was too subjective and could be easily biased. It was hoped that quantification of GFP fluorescence might be a more objective means of measuring viral spread and yet not be so laborious as yield reductions, but this method was found to lack sensitivity. Ultimately, yield reductions proved to have the best combination of objectivity and sensitivity. Both experiment 1 and experiment 2 have GCV and MBV components. The technical lessons learned from the former were applied to the latter. The results for GCV will be presented first (both experiment 1 and 2), followed by MBV (both experiment 1 and 2).

In the first experiment, representative photographs were taken to document the morphology and nature of infection in each cell type: epithelial cells (ARPE-19) and fibroblasts (MRC-5). Then all three methods (i.e., plaque reduction, GFP fluorescence, and yield reduction) were tested to the effect of each drug on viral quantification.

Representative micrographs of cell infection and morphology. As shown in Figure 1, representative micrographs were taken on day 11 to document the different cell morphologies and the qualitative observation that drug susceptibility was not just expressed as a reduction in viral plaques. The most striking observation was that treatment of epithelial cells with either drug or fibroblasts with MBV, did not prevent GFP expression in cells of primary infection. Only in fibroblasts treated with GCV did

fluorescing cells dim and disappear. At high concentrations of drug, the great majority of those cells, which were first to fluoresce green remained green throughout the experiment. Unlike no-drug controls or lower drug concentrations, these cells that were green under high GCV or MBV concentration did not spread from cell-to-cell laterally, nor did secondary plaques appear, which form by virus release into the supernatant and infection elsewhere in the cell monolayer. As the viewer scanned from high drug concentration (Figure 1, A and C) to low drug concentration (Figure 1, B and D), single cells transitioned to larger foci.

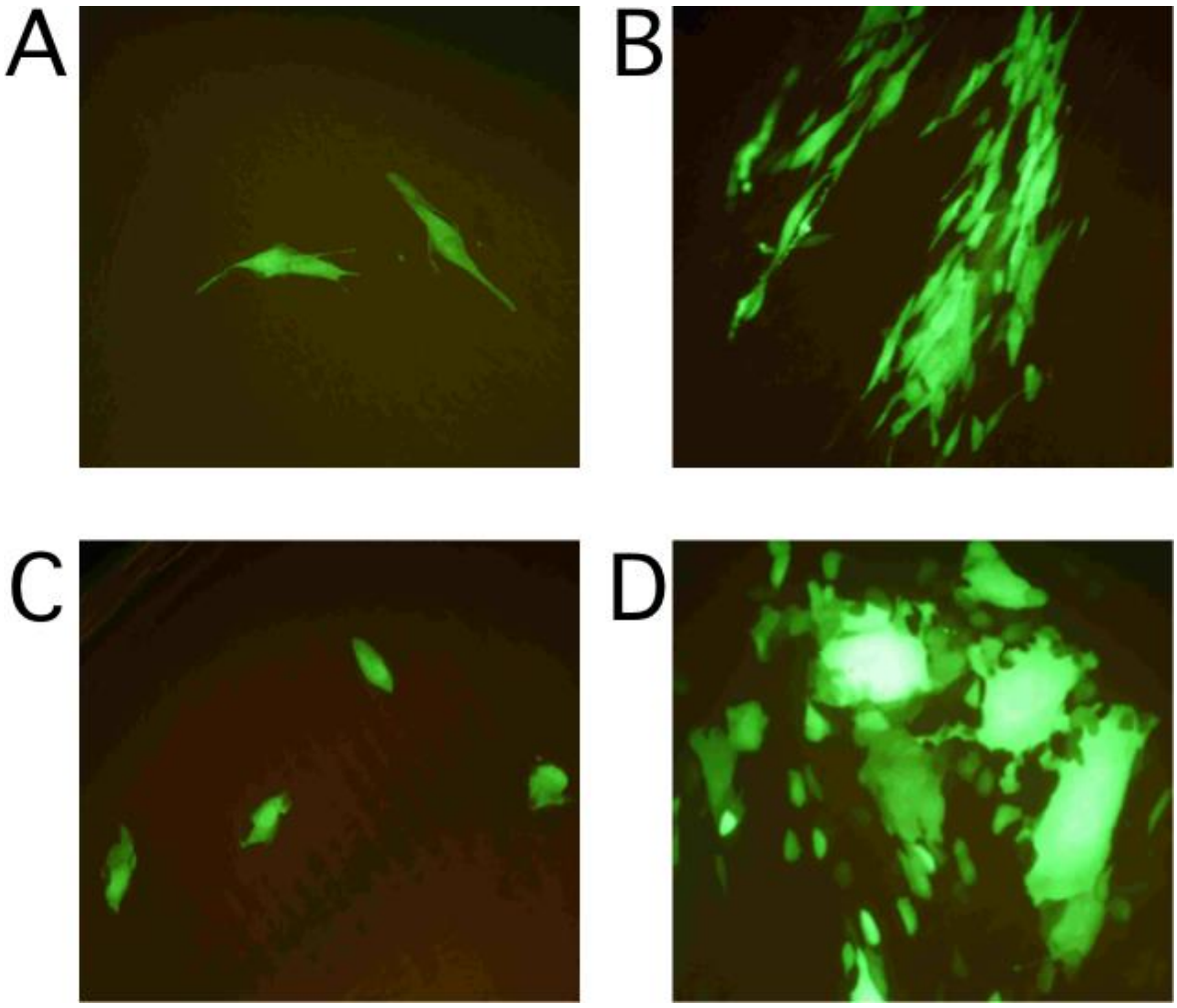

Figure 1. Representative micrographs of infected cell cultures treated with serial dilutions of ganciclovir (GCV). Photographs were taken 11 days post infection. (A) Infected fibroblasts with 12 μM GCV (higher concentrations were devoid of even singly infected cells). Infected cells are isolated and do not form plaques. (B) Infected fibroblasts at the lowest concentration of GCV (0.0054 μM) are tightly clumped and form neat, laterally expanding plaques that are randomly spaced across the monolayer. (C) Infected epithelial cells at the highest concentration of GCV (320 μM) are isolated and lateral spread indicative of plaque formation does not occur. (D) Infected epithelial cells at lowest concentration of GCV (0.0054 μM) form large syncitial plaques, spaced uniformly across the monolayer.

GCV is not efficient at reducing plaques in epithelial cells. For quantitative purposes of the plaque reduction assay, counting the number of viral foci, in contrast to individual green cells, became critical. Although the unique morphology of each cell type made this a dubious task, the fact that this particular strain of HCMV is highly fusogenic in epithelial cells but not in fibroblasts was particularly problematic (113, 114). Therefore, infected foci are easy to distinguish in fibroblasts but, in epithelial cells, a single large fluorescing cell strongly resembles a green focus of cells that have fused (Figure 1).

On day 12, the number of plaques at each drug concentration was counted. These data showed a drug-dependent change in green foci for both cell types. As shown in Figure 2, a curve fitting program was used to find a sigmoid curve of best fit and a concentration correlating with a 50% decrease in plaque formation (IC_{50}). Data analysis was performed by finding a line of best fit to each of the replicates, determining individual IC_{50} concentrations and then averaging the IC_{50} values to give the averages and corresponding standard deviations. The average fibroblast-IC_{50} was found to be 8 ± 3.7 μM GCV, while the average epithelial-IC_{50} was much higher at 100 ± 53 μM GCV. These data describe a 12-fold higher epithelial IC_{50} ($p = 0.0412$) and demonstrated that GCV is much less effective at inhibiting plaque formation in epithelial cells.

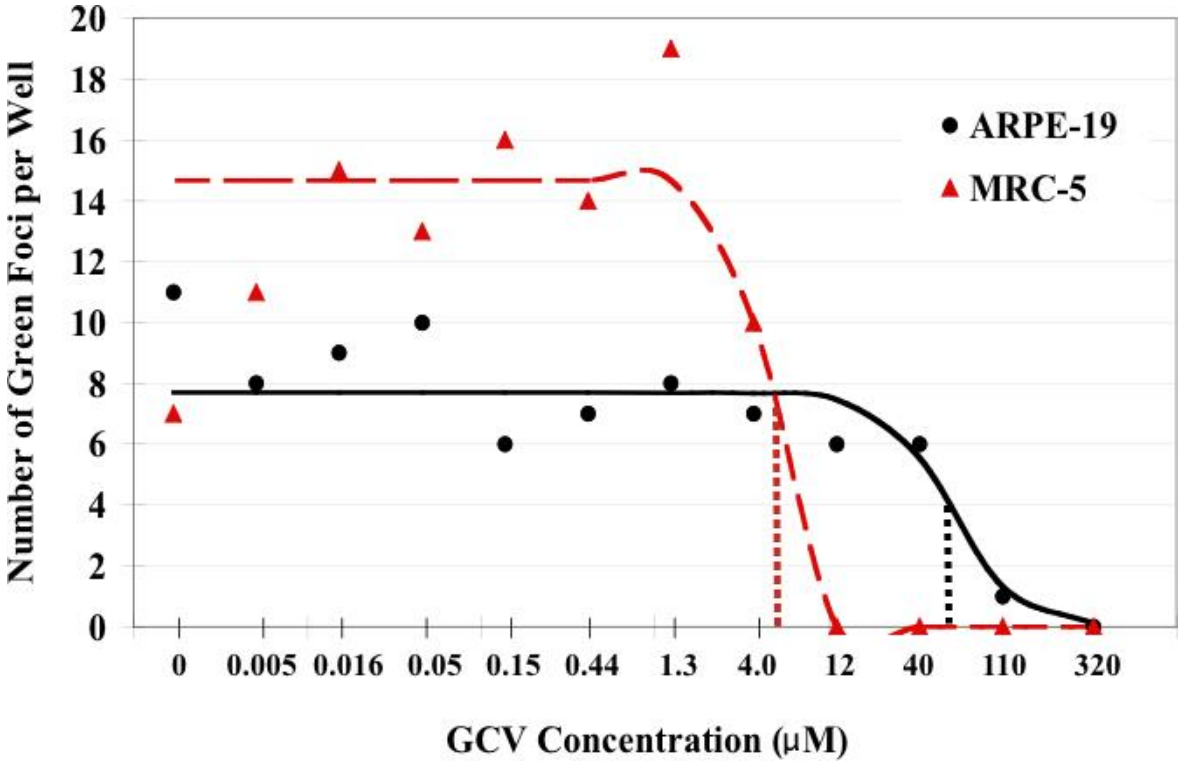

Figure 2. GCV inhibits plaque formation in fibroblasts more efficiently than in epithelial cells. The figure presents one representative replicate of plaque reduction data from each cell type fitted with a sigmoid curve. MRC-5 and ARPE-19 cell cultures were infected with BADrUL131-Y4 at an MOI of 0.01 (10 pfu/well), in the presence of serial dilutions of GCV at the concentrations indicated. On day 12, plaques were counted, graphed, and curve fitted. Fibroblast- and epithelial-IC_{50}s were calculated by finding the concentration at the inflection point of the line. Since there were many more plaques in the ARPE-19 monolayers, these data represent the total number of plaques viewed in the microscope's viewfinder at the most dense area of the well. Fibroblast data represent the total number of plaques per well.

Quantification of GFP fluorescence is inconclusive. As a more objective measure of viral spread over the cell monolayer, GFP fluorescence was measured on day 19. The data show a similar dose-dependency but neglected to capture the cell type effect that could be observed under the microscope. Average fibroblast- and epithelial-IC_{50}s were calculated in the same manner as for plaque reduction, however, these calculations were highly variable, even between replicates of the same cell type. A trend of low fibroblast-IC_{50}s and higher epithelial IC_{50}s emerged, but no statistically significant difference was detected between the cell types. One potential weakness of this experiment was the use of clear-walled plates which may have permitted interwell cross-talk.

GCV inhibition of yield in fibroblasts is more complete than in epithelial cells. Yield reduction, which evaluates the production of viral progeny by infected cells, may be the most pertinent test of antiviral efficacy because it evaluates the cumulative effect of the drug on the viral life cycle. To measure yield, cell-free supernatants were collected on day 19 (ARPE-19) or 22 (MRC-5) and titrated on the cell type from which they were harvested. Titrations were read 9 (ARPE-19) or 11 (MRC-5) days post infection. Titers were normalized using a log base 10 transformation and each replicate graphed against concentration. A sigmoid curve was fitted to the data. IC_{90} (concentration of drug which inhibits viral yield by 90%) values of each replicate were calculated by finding the concentration at which virus titer dropped one log unit from the sigmoid curve's upper asymptote. The average fibroblast-IC_{90} was 1.3 ± 0.06 μM GCV, while the average epithelial-IC_{90} was over four-fold greater at 5.5 ± 1.51 μM ($p = 0.0417$). Here, however, IC_{90} calculations incompletely illustrate the difference in drug efficacy. GCV completely

abolished new virus production in fibroblasts at a concentration of 4.0 μM (Figure 3). In contrast, the drug was incapable of completely eliminating production of new virus in epithelial cells even at 40 μM GCV (data from the highest drug concentration, 320 μM, was unusable because it succumbed to evaporation). This constituted as great as a 25-fold difference in the GCV concentration that could abolish the production of viral progeny and could account for the high incidence of CMV retinitis after GCV treatment.

Ensuring comparable entry between cell types. Another complication of the first experiment was differential entry between the cell types. Observation of early GFP expression suggested that the virus was not entering MRC-5 cells as quickly as ARPE-19 cells. There appeared to be more cells expressing green from primary infection in the ARPE-19 cells than the MRC-5 cells. Passage of primary cells in culture can change their physiology, so to see if a passage-dependent change in physiology was the reason for differential entry, BADrUL131-Y4 was titrated on a number of passage variants of MRC-5 or ARPE-19 cells. These included high passage, low passage, and, in the case of the MRC-5 cells, a high passage telomerase immortalized variant. High multiplicity of infection wells showed comparable entry in all five cell-line variants after 24 hours. Interestingly, after three days, the virus clearly showed higher (and more comparable) infectivity in the high passage variants. These results suggested the use of high passage ARPE-19 and MRC-5 hTERT cells for future study, so as to ensure comparable entry.

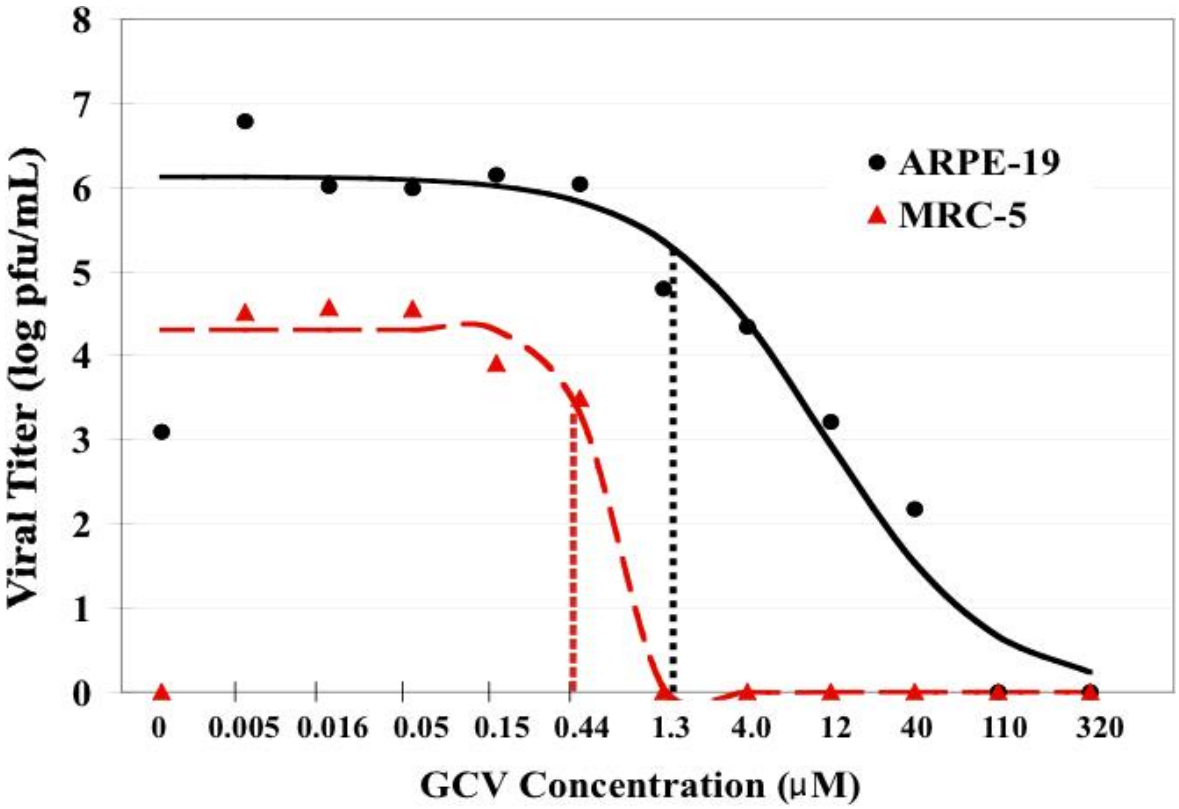

Figure 3. GCV inhibits viral yield in fibroblasts more efficiently than in epithelial cells. The figure presents one representative replicate of viral yield titers from each cell type fitted with a sigmoid curve. MRC-5 and ARPE-19 cell cultures were infected with BADrUL131-Y4 at an MOI of $1x10^5$ (20 pfu/well), in the presence of serial dilutions of GCV at the concentrations indicated. On day 19 (ARPE-19) and day 22 (MRC-5), culture supernatants were titrated. Titers were graphed, and curve fitted. Fibroblast- and epithelial-IC_{90}s were calculated by finding the concentration of drug that correlated with a log drop in viral yield from the upper asymptote.

Growth curves show reduced GCV efficacy in epithelial cells (Experiment 2).

Experiment two incorporated growth curves to monitor the effect of the drug over time. It followed a similar setup to the original design, but incorporated the aforementioned changes (e.g., white-walled plates, excluding edge wells from design, etc.). Growth curves showed how GCV impacted spread (quantified by GFP fluorescence) and virus yield over time (Figures 4 and 5, respectively). GFP growth curves provided little useful data as to the effect of cell type on GCV efficacy.

Viral yield growth curves portray highly divergent drug dynamics in each cell type. In both epithelial and fibroblast cultures, the highest drug concentrations suppressed viral titers to an undetectable level early on. In contrast, however, 12 μM GCV was highly effective in abolishing virus titers by nine days post infection (d.p.i) in fibroblast cells, whereas virus from the epithelial cells treated with the same dose saw resurgence at 11 d.p.i. At even lower concentrations (e.g., 4.0 μM), GCV appeared to be equally effective at suppressing early virus production but again, at day nine, became highly ineffectual in epithelial cells. At nine d.p.i., all of the epithelial virus titers began to rise at nearly identical rates, apparently unperturbed by the antiviral drug.

When virus titers began to diverge, the epithelial-IC_{90} began to rise substantially (see Table 1) and by 13 and 15 d.p.i., there was a significant difference between epithelial and fibroblast IC_{90}s ($p = 0.0014$ and $p = 0.0007$, respectively).

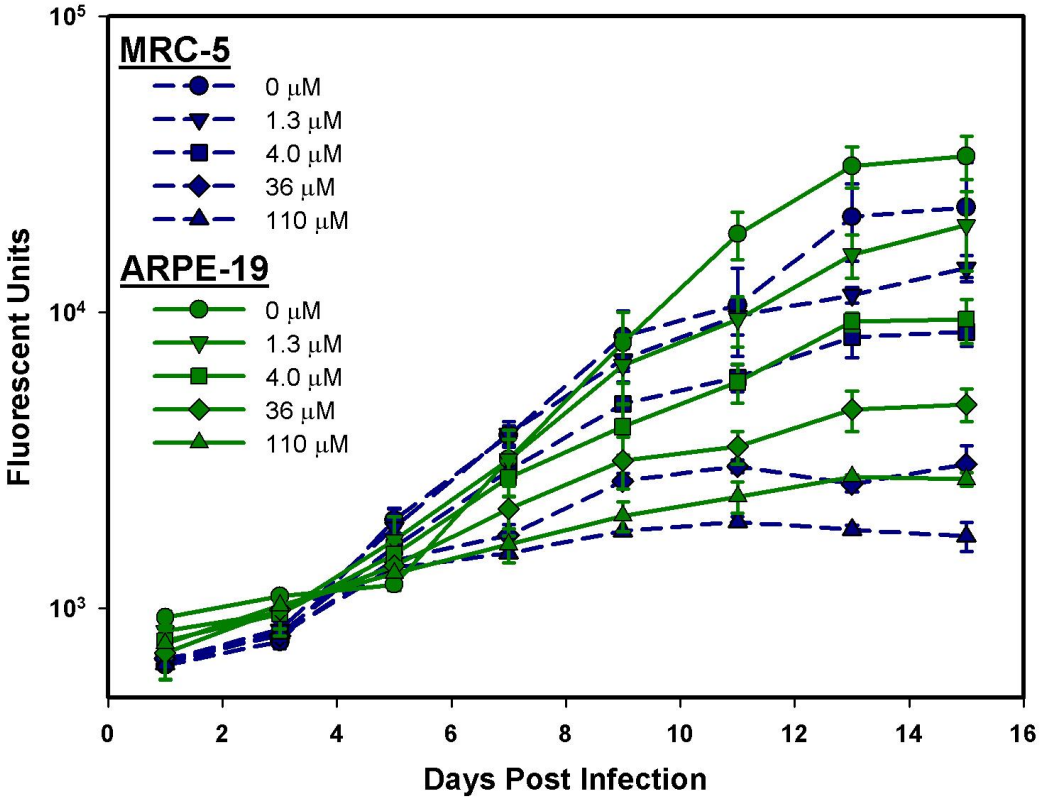

Figure 4. GFP fluorescence does not unmask differential efficacy of GCV in fibroblasts and epithelial cells. MRC-5 hTERT and ARPE-19 cells were infected with BADrUL131-Y4 at an MOI of 0.02 in the presence of indicated concentrations of GCV. GFP fluorescence was measured on the days indicated.

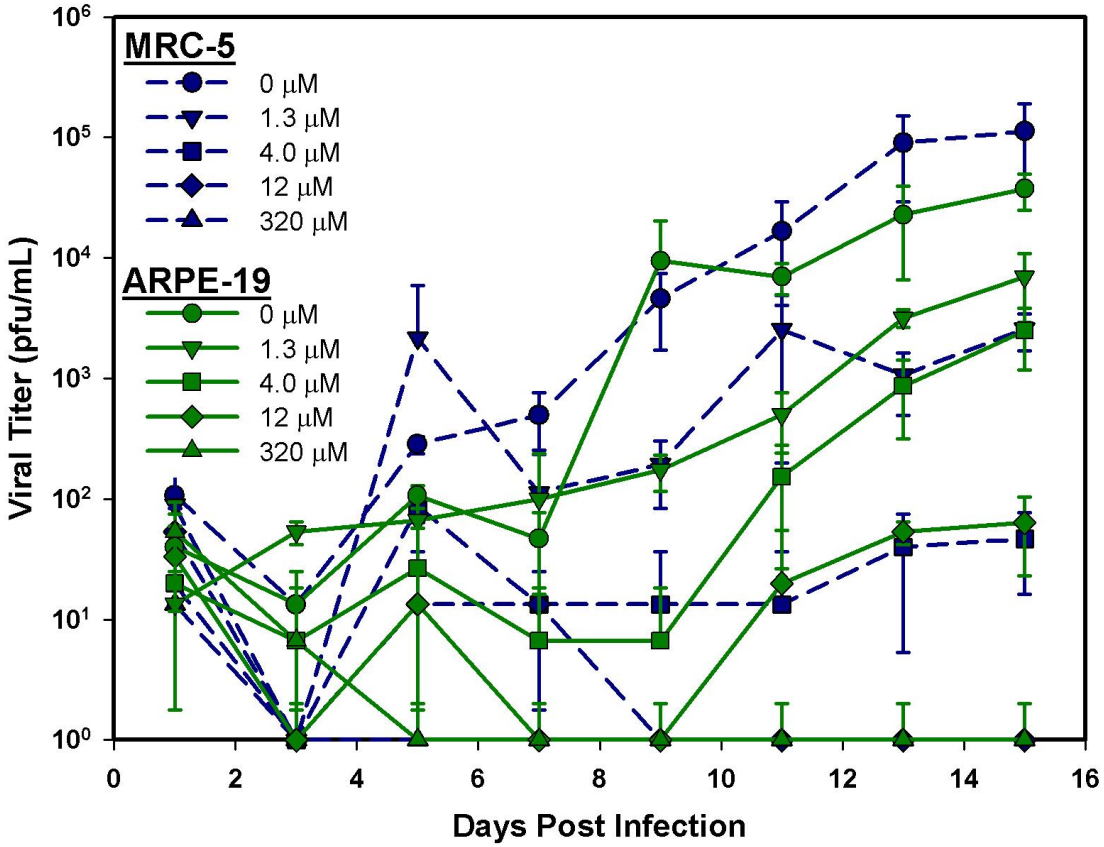

Figure 5. Yield reductions illustrate the stark contrast of GCV efficacy in fibroblast and epithelial cells. MRC-5 hTERT and ARPE-19 cells were infected with BADrUL131-Y4 at an MOI of 0.02 in the presence of indicated concentrations of GCV. Supernatants were removed and titered on MRC-5 hTERT cells on the days indicated.

Table 1. GCV IC_{90}s measured in MRC-5 and ARPE-19 cells.

	GCV IC_{90} [a]				
Day [b]	ARPE-19	± Σ	MRC-5	± Σ	E:F Ratio [c]
9	1.02	0.396	1.25	0.110	0.8
11	2.26	1.403	1.14	0.436	2.0
13	**3.08**	0.429	**0.83**	0.290	**3.7**
15	**4.20**	0.454	**0.84**	0.247	**5.0**

[a] Average of three replicate IC_{90}s.
[b] Day post infection.
[c] The ratio of epithelial (E) IC_{90} divided by the fibroblast (F) IC_{90}.
* Rows that are bolded describe a statistically significant difference between cell types ($\alpha = 0.05$).

MBV effectively inhibits plaque formation in fibroblast cells but poorly in epithelial cells. Experiment one provided strong evidence for a cell type effect on the efficacy of MBV in addition to GCV. This experiment tested GCV and MBV in parallel, using identical designs. Here, the data pertaining to MBV plaque reductions are presented. At the same time as GCV, MBV treated plates were photographed and plaques were counted to determine whether MBV's ability to inhibit plaque formation was at all affected by cell type. Plaque reduction uncovered a seven-fold lower average fibroblast-IC_{50} than epithelial-IC_{50}, as depicted in Figure 6. With a fibroblast-IC_{50} of 2.4 ± 1.49 μM and an epithelial IC_{50} of 17 ± 5.6 μM MBV ($p = 0.00094$), MBV was much better suited to inhibit plaque formation in fibroblast culture than epithelial cell culture.

Just as in the component of experiment one dedicated to GCV susceptibility testing, GFP fluorescence of MBV-treated cultures was inconclusive. Drug susceptibility curves were highly variable and showed no significant shift in IC_{50} between cell types.

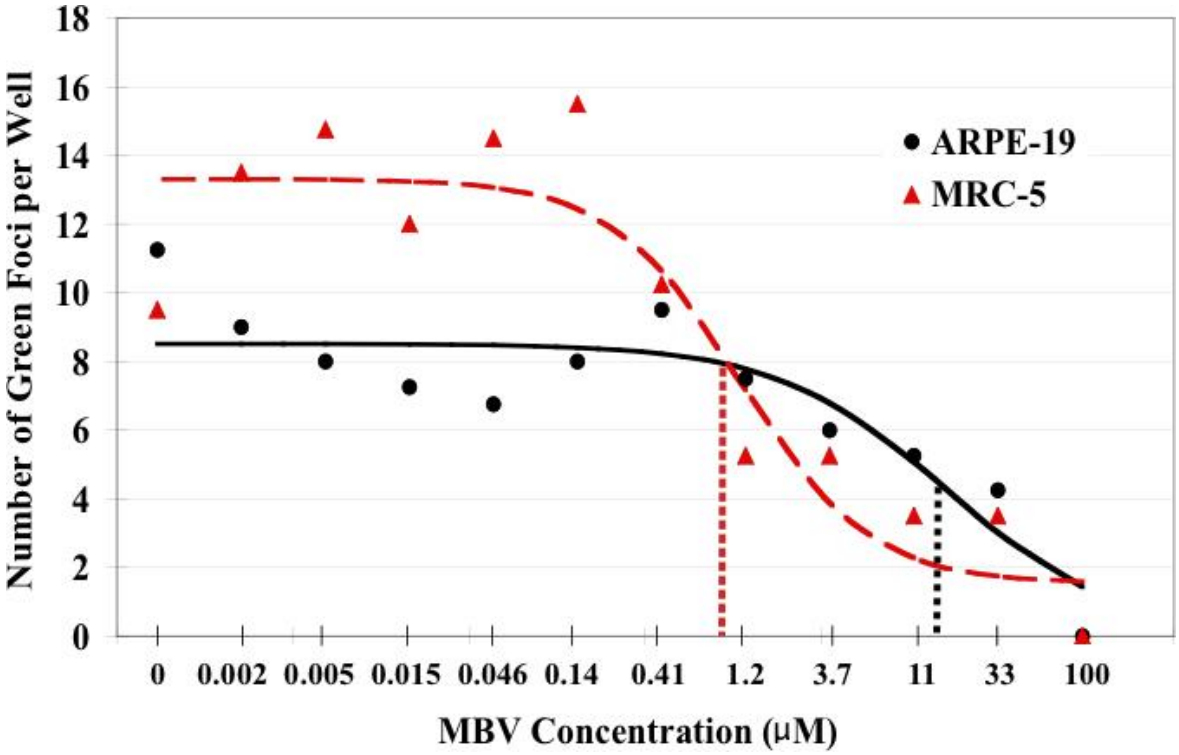

Figure 6. MBV inhibits plaque formation in fibroblasts more efficiently than in epithelial cells. The figure presents one representative replicate of plaque reduction data from each cell type fitted with a sigmoid curve. MRC-5 and ARPE-19 cell cultures were infected with BADrUL131-Y4 at an MOI of $1x10^5$ (10 pfu/well), in the presence of serial dilutions of GCV at the concentrations indicated. On day 12, plaques were counted, graphed, and curve fitted. Fibroblast- and epithelial-IC_{50}s were calculated by finding the concentration at the inflection point of the line. Since there were many more plaques in the ARPE-19 monolayers, these data represent the total number of plaques viewed in the microscope's viewfinder at the most dense area of the well. Fibroblast data represent the total number of plaques per well.

MBV does not block virus yield from epithelial cells. As in the initial GCV experiments, the most compelling evidence that drug efficacy is cell type-dependent was drawn from yield reduction data (Figure 7). The fibroblast-IC_{90} was found to be about two-fold lower than the epithelial-IC_{90}: 0.39 ± 0.002 μM versus 0.95 ± 0.271 μM, respectively ($p = 0.0354$). Yet the IC_{90} does not properly convey the extent of this cell-type disagreement. In this case, a qualitative analysis of the graphs is much more revealing. The data show how MBV was capable of completely extinguishing production of infectious virus in fibroblast cells at a concentration of 1.3 μM, but even at the highest concentration with reliable data (33 μM because 100 μM partially evaporated and data was lost), 25 times more concentrated, it was unable to do the same in epithelial cells. Referring to the plaque reductions above, we see that there were still green fluorescing cells of both cell types at the highest concentrations of drug. In fibroblast culture in 0.41 μM, these green foci were incapable of producing infectious virus, yet in epithelial culture at 26-fold higher MBV concentrations, they were releasing sufficient virus to achieve a titer of 10,000 infectious particles per mL.

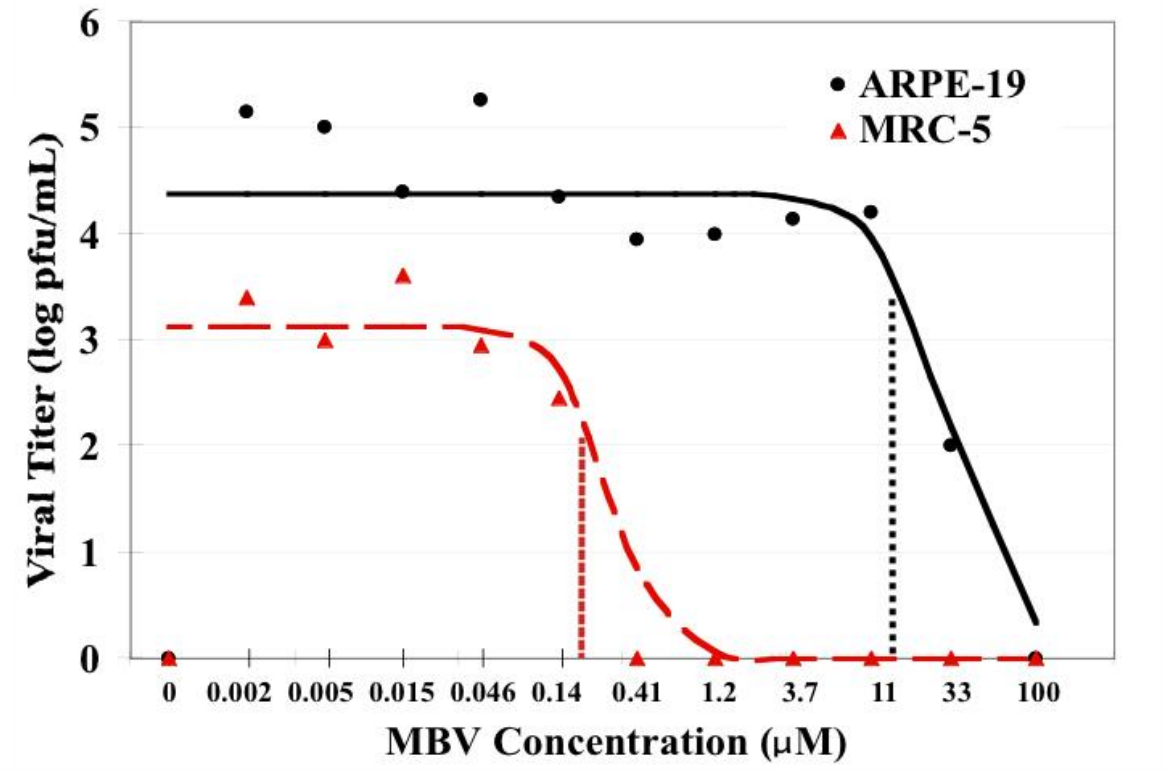

Figure 7. MBV efficiently inhibits viral yield in fibroblasts but is unable to do so at sub-cytotoxic doses in epithelial cells. The figure presents one representative replicate of viral yield titers from each cell type fitted with a sigmoid curve. MRC-5 and ARPE-19 cell cultures were infected with BADrUL131-Y4 at an MOI of $1x10^5$ (20 pfu/well), in the presence of serial dilutions of GCV at the concentrations indicated. On day 19 (ARPE-19) and day 22 (MRC-5), culture supernatants were titered. Titers were graphed, and curve fitted. Fibroblast- and epithelial-IC_{90}s were calculated by finding the concentration of drug that correlated with a log drop in viral yield from the upper asymptote.

Growth curves show much greater MBV suppression of viral yield in fibroblasts than epithelial cells (Experiment 2). Evaluating the efficacy of MBV in both cell-types with growth curves clarified the time dimension of the observed effect. This phase followed the same format as the GCV phase of experiment 2. Cell cultures were treated with serial dilutions of MBV and both GFP fluorescence and virus yield titrations were performed over a period of 15 days. Again, GFP fluorescence growth curves provided no statistically significant evidence of cell type dependence of MBV activity (see Figure 8).

As in the first experiment with MBV, yield reductions were highly illustrative of the reduced efficacy of MBV in epithelial as compared to fibroblast cells. As shown in figure 9, the viral no-drug control group in both cell types reached about the same peak titer at 15 days (10^5 pfu/mL), albeit at different rates. The most striking difference between the two cell types was observed at the highest drug concentrations. In fibroblasts, the highest MBV concentration (100μM) effectively eliminated extracellular infectious virus. Epithelial culture continued to produce sufficient viral progeny for an average extracellular titer of 10^2 pfu/mL. The other treatment groups paralleled this finding. At drug concentrations below 100 μM, infected epithelial cultures scarcely dropped one $\log_{10}$ below the no-drug control titers, while virus propagation in fibroblasts was severely curtailed above a concentration of 1.3 μM MBV (exemplary replicate from day 11 displayed in Figure 10).

Calculated IC_{90}s convey the cell-type divergence. This divergence could be observed after nine d.p.i (Table 2). From day nine to fifteen, every epithelial-IC_{90} was significantly higher than its fibroblast counterpart (highest $p = 0.0001$). Figure 9 shows

how viral titers from the epithelial culture were inhibited only at cytotoxic levels of the drug (100μM). A more nuanced look at the epithelial data reveals a biphasic curve that shows partial inhibition around the fibroblast-IC_{90} but then levels out (biphasic variation cannot be captured by a sigmoid curve).

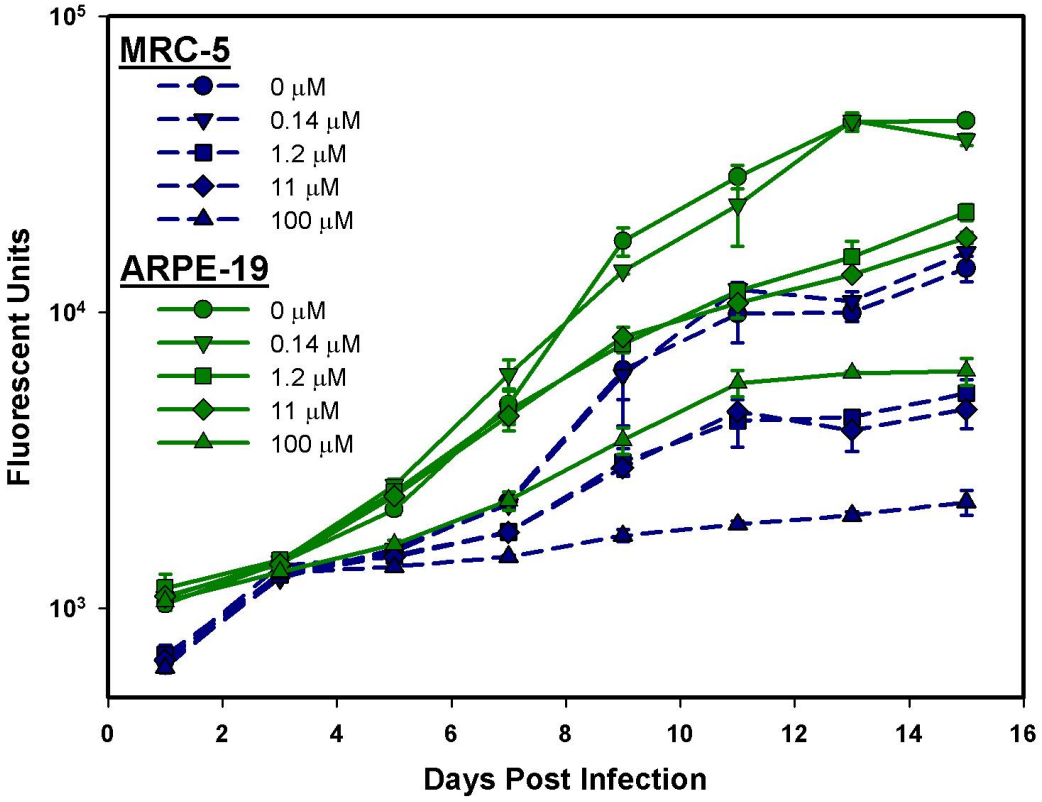

Figure 8. GFP fluorescence does not unmask differential efficacy of MBV in fibroblasts versus epithelial cells. MRC-5 hTERT and ARPE-19 cells were infected with BADrUL131-Y4 at an MOI of 0.02 in the presence of indicated concentrations of MBV. GFP fluorescence was measured on the days indicated.

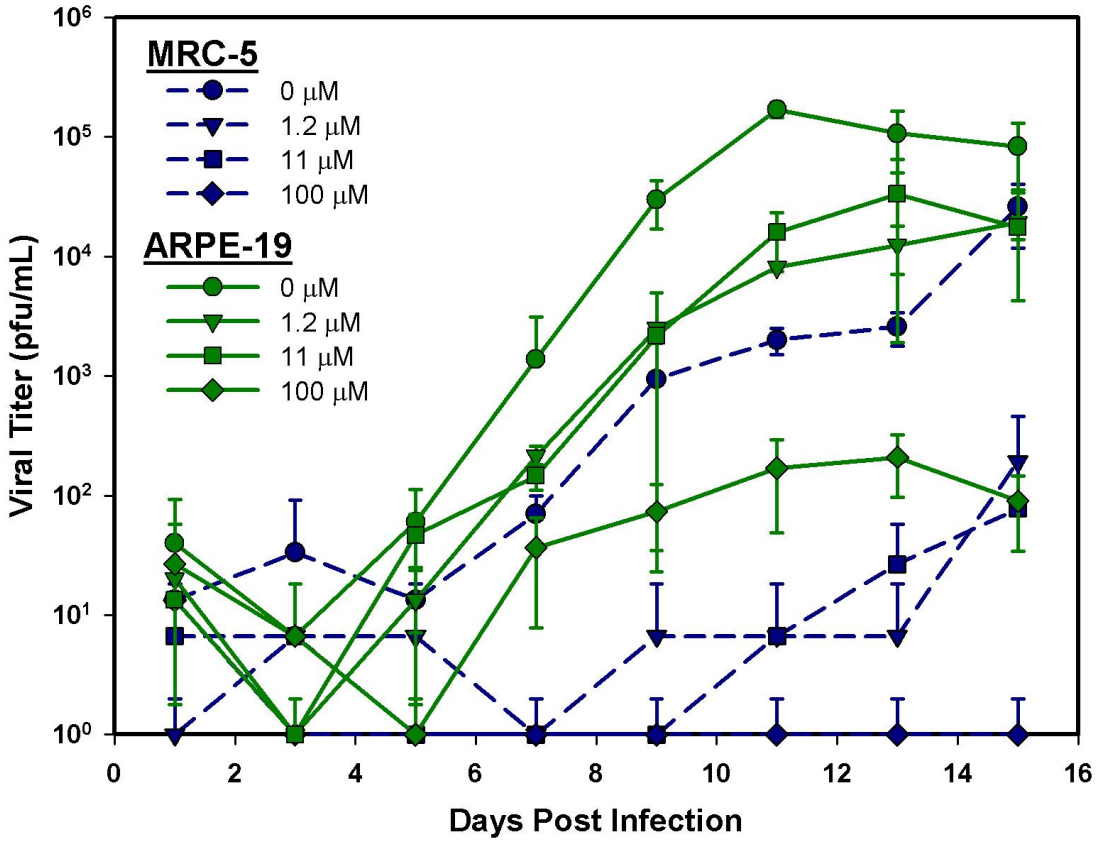

Figure 9. Yield reductions illustrate the stark contrast of MBV efficacy in fibroblast and epithelial cells. MRC-5 hTERT and ARPE-19 cells were infected with BADrUL131-Y4 at an MOI of 0.02 in the presence of indicated concentrations of GCV. Supernatants were removed and tittered on MRC-5 hTERT cells on the days indicated.

Table 2. MBV IC_{90}s measured in MRC-5 and ARPE-19 cells.

	MBV IC_{90} [a]				
Day [b]	ARPE-19	± Σ	MRC-5	± Σ	E:F Ratio [c]
9	**92.04**	2.774	**0.26**	0.142	**359.2**
11	**92.87**	2.011	**0.29**	0.148	**317.9**
13	**92.09**	1.588	**0.35**	0.187	**262.9**
15	**89.75**	1.764	**0.45**	0.453	**199.3**

[a] Average of three replicate IC_{90}s.

[b] Day post infection.

[c] The ratio of epithelial (E) IC_{90} divided by the fibroblast (F) IC_{90}.

* All rows show a statistically significant difference between cell types ($\alpha = 0.05$).

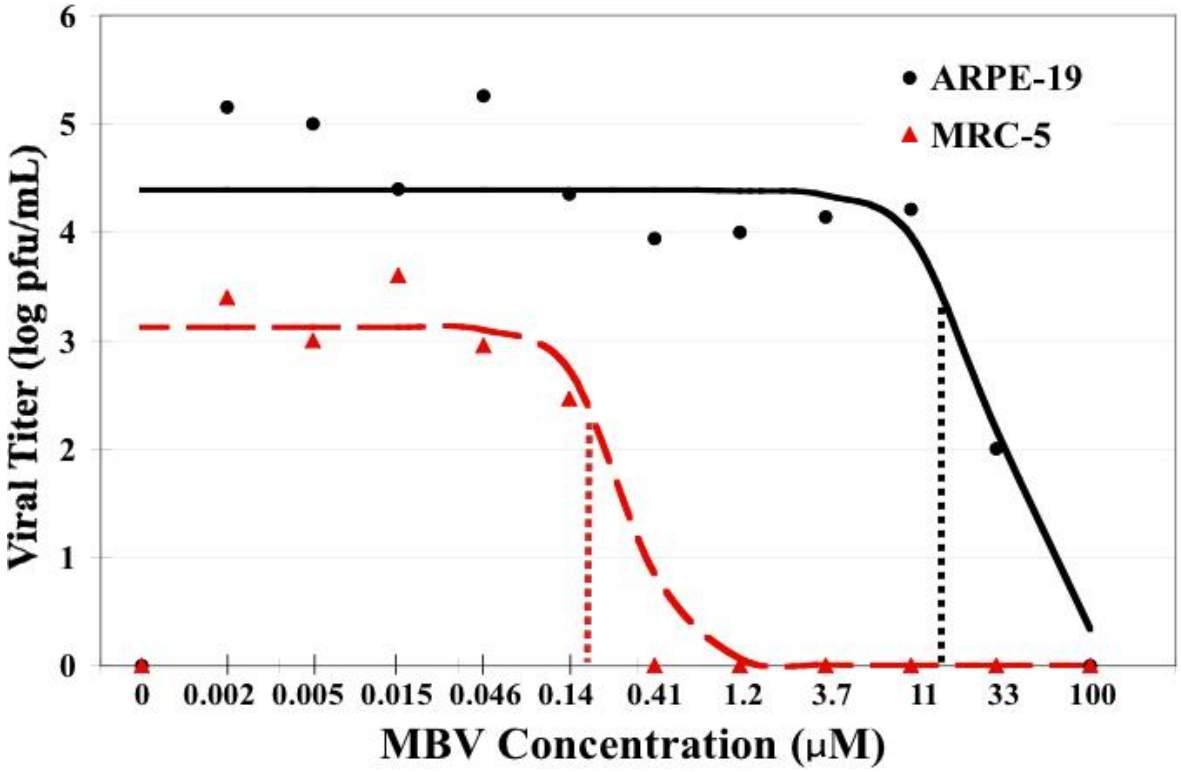

Figure 10. A cross-sectional view of viral yield curves shows that MBV efficiently inhibits viral yield in fibroblasts but is unable to do so at sub-cytotoxic doses in epithelial cells. The figure presents one representative replicate of day 11 viral titers from each cell type fitted with a sigmoid curve. MRC-5 and ARPE-19 cell cultures were infected with BADrUL131-Y4 at an MOI of $1x10^5$ (20 pfu/well), in the presence of serial dilutions of GCV at the concentrations indicated. Titers were graphed, and curve fitted. Fibroblast- and epithelial-IC_{90}s were calculated by finding the concentration of drug that correlated with a log drop in viral yield from the upper asymptote.

Both drugs show cytotoxicity at highest drug concentrations. To determine if a component of the drug inhibition seen at high concentrations of GCV or MBV was due to cytotoxic effects, a cell viability assay was performed. This assay reproduced the conditions of the second experiment at the highest three drug concentrations with mock infected cells. This was done in triplicate. The assay used to evaluate cell viability quantifies the amount of ATP within cultured cells using a luciferase based reagent. At nine, eleven, thirteen, and fifteen days post mock infection, cells were lysed, reagent added, and luminescence was quantified. Luminescence data were used to calculate a luminescence ratio. The luminescence produced by drug treated cells was divided by the luminescence of untreated cells and presented as a percentage of the no-drug control. An experimental group of the highest concentration of drug in cell-free media was also tested to assure that neither drug would have a direct effect on the assay reagent. Luminescence in this treatment group was never more than 2.5% of the no-drug control.

Raw luminescence decreased gradually in all the different treatment-cell groups over the six days on which it was recorded. GCV did not show a noteworthy degree of cytotoxicity in epithelial cells from days 9 to 15 (Figure 11). Fibroblasts, on the other hand, reacted enigmatically to GCV (Figure 11). Luminescence of cells treated with any of the three concentrations of GCV showed higher luminescence than their respective no-drug control, resulting in luminescence rising above 100%. The epithelial luminescence ratio dropped to its lowest point (90%) on day 15 while fibroblast culture remained above 100% throughout. The peak luminescence ratio was 224% in fibroblast culture treated

with 320 μM GCV and measured on day 15. The dose dependency of this effect suggests that it was a physiological change induced by the drug itself.

MBV showed similarly enigmatic results (Figure 12). Both cell types were adversely affected by 100 μM MBV on days 9 and 11, displaying a luminescence ratio of about 78%. Other drug concentrations had proportionally lesser effects on ATP levels in both cell-types. At later time points (i.e., days 13 and 15), the cells appeared to revcover. MBV appeared to improve ATP maintenance in both epithelial and fibroblast culture, although the effect was not as profound as in fibroblast culture treated with GCV. The luminescence ratio peaked at 147% of the no-drug control on day 15 in fibroblast culture treated with 33 μM MBV.

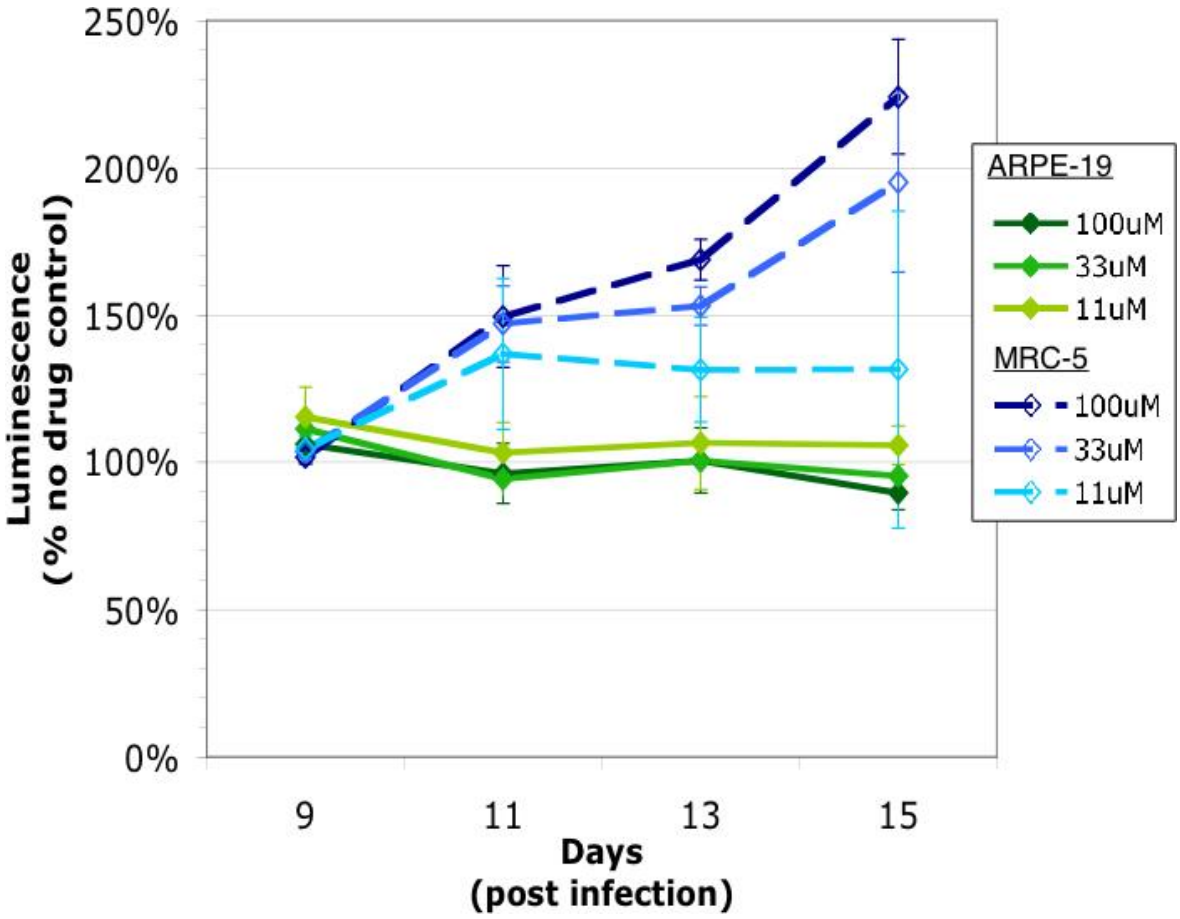

Figure 11. GCV's effect on cellular ATP levels. Epithelial (ARPE-19) and fibroblast (MRC-5) cultures were treated with the three highest concentrations of GCV or no drug and incubated for 9 to 15 days. At each time point, intracellular ATP in culture was quantified using a luciferase-based assay. Luminescence is presented as a percent of the no-drug control.

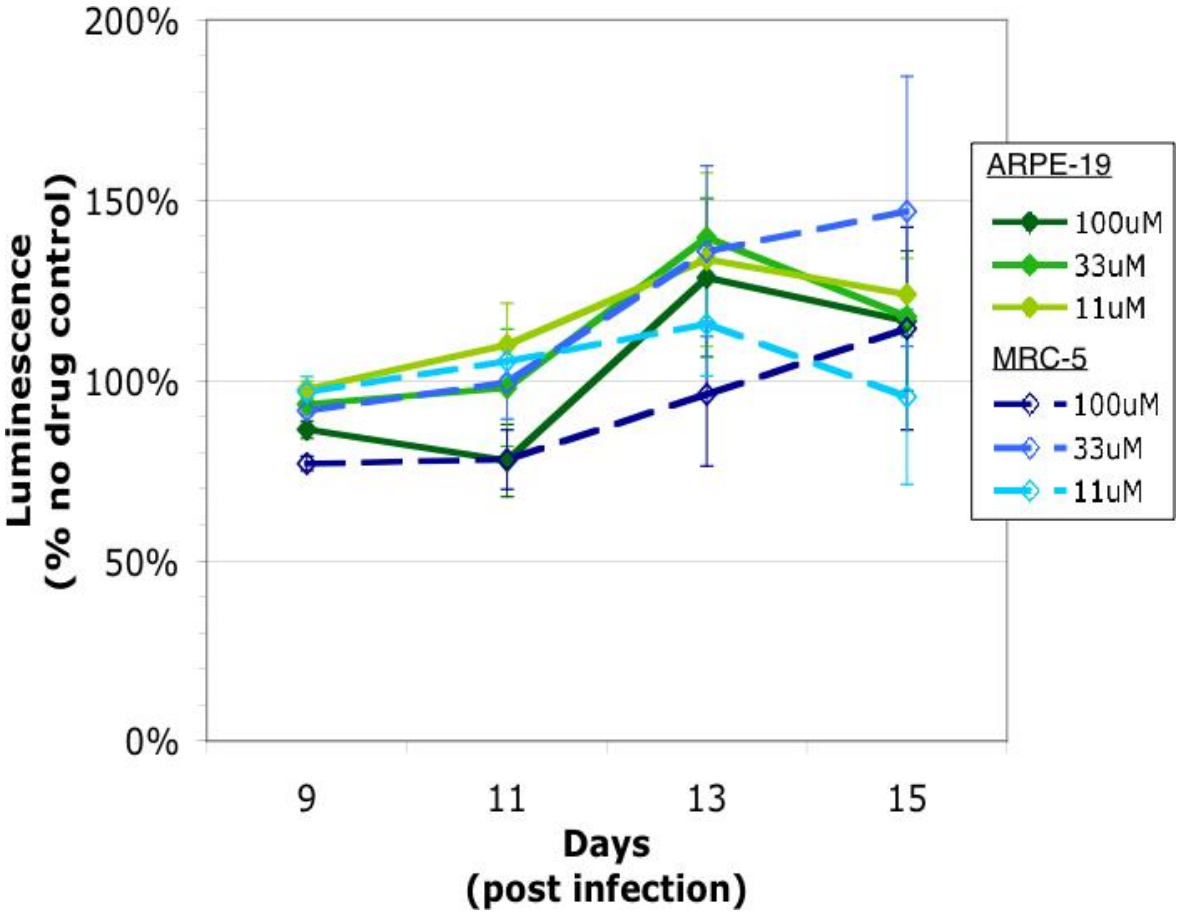

Figure 12. MBV's effect on cellular ATP levels. Epithelial (ARPE-19) and fibroblast (MRC-5) cultures were treated with the three highest concentrations of MBV or no drug and incubated for 9 to 15 days. At each time point, intracellular ATP in culture was quantified using a luciferase-based assay. Luminescence is presented as a percent of the no-drug control.

Discussion of Cell Type Specific Antiviral Drug Susceptibility

Historically, *in vitro* study of human cytomegalovirus (CMV) has been conducted in fibroblast cell culture. Yet viral transmission and the serious pathologies associated with CMV involve cell types other than fibroblasts *in vivo*. This unitary practice has lead to the adoption of a series of laboratory-adapted strains of CMV that, in contrast to clinical isolates, are no longer epithelial or endothelial tropic. The study of these laboratory-adapted viruses necessitates the use of fibroblast cell culture and therefore overlooks any phenotypic differences in viral infection generated by clinically relevant cell types. The discovery of a frameshift mutation in the *UL131* locus that blocks the endothelial and epithelial tropism of CMV strain AD169, and the repair of this mutation (BADrUL131-Y4) has enabled the study of this virus in non-fibroblast cell cultures. It has also enabled the phenotypic assessment of drug susceptibility in other cell types.

To our knowledge, no study has looked at the efficacy of either GCV or MBV in parallel cell cultures of both fibroblasts and epithelial cells. The cell types chosen for this study were based on precedent – MRC-5 fibroblasts are a common medium for the study of CMV infection *in vitro* – and clinical relevance – ARPE-19 retinal pigmented epithelial cells are associated with CMV retinitis. GCV, which is licensed for use by the Food and Drug Administration (FDA), is the treatment of choice for cytomegalovirus infection and disease. MBV is the most promising novel anti-CMV drug and the only anti-CMV drug in

phase III clinical trials. Each has distinct targets. GCV, a nucleotide analog of guanosine, targets the viral DNA polymerase and inhibits replication. MBV inhibits the viral gene product of UL97 which appears to be a kinase intricately involved in viral egress from the nucleus, as well as a number of other essential viral pathways.

Our results indicate that there is a cell type dependency to the antiviral activities of both GCV and MBV *in vitro*. The mechanism of this cell type dependency has yet to be discovered, but the implications of diminished efficacy of these drugs in epithelial cells are broad.

4.1 Technical Obstacles

Preliminary studies can require a great deal of trial and error to refine a method that optimizes sensitivity, reproducibility, and simplicity. A phenotypic drug susceptibility assay for the two cell types was attempted multiple times, each time with greater refinement. Some of the shortcomings of initial experiments have been described. For example, in the first experiment, edge wells of each plate contained critical control treatment groups and replicates. The long time course of the experiment combined with no precaution to minimize evaporation adversely impacted data collection in these critical wells, as virus grew poorly and cells appeared stressed. To avert this issue in later experiments, we used sealable bags to minimize evaporation and avoided edge wells entirely, taking all experimental data exclusively from interior wells. Another example was the use of clear-walled plates in early experiments. Clear-walled plates allow fluorescence leakage into neighboring wells. In instances where the two cell types showed equal total

fluorescence, leakage would have affected cell type IC_{50} calculation uniformally. Unfortunately, epithelial fluorescence typically surpassed fibroblast fluorescence, resulting in greater leakage across wells of epithelial plates and an upward shift of those fluorescence curves toward higher drug concentrations.

Overall, GFP proved to be a valuable method for quantifying primary infection over a short time course, as was the case in our neutralizing studies. In contrast, where the degree of viral spread was sought, GFP fluorescence did not seem to correlate as closely with secondary plaque formation. This could be for a number of reasons. GFP has a relatively long half life in mammalian cells (e.g., ~26 hours in mouse La-9 cells) (23) as compared to other reporter genes such as luciferase. Protein half-lives are contingent on rates of expression and degradation which could vary between cell types. If the kinetics of expression-degradation were such that the former outweighed the latter, GFP concentrations would increase overtime and those cells which were primarily infected would contribute to a much greater percentage of the total fluorescence. In this scenario, viral spread would be poorly quantified by GFP fluorescence. In contrast, reporter fluorescence would correlate well with primary infection and stand as a very good measurement tool. The latter scenario describes the use of GFP to quantify the relative amount of virus entering cells. An analogy might be a pair of water hoses laying side by side and gradually being turned on. If the two water flows are begun at different times and the delay between them is great enough, the first will be producing so much water that the observer may not even realize that the second is on. This dynamic is further complicated by the use of multiple cell types which may have distinct expression-degradation kinetics.

The GFP marker cassette of BADrUL131-Y4 is mediated by the simian virus 40 (SV40) early promoter, which may even have different expression kinetics than CMV viral genes such as IE-1 (113).

4.2 Possible Mechanisms of GCV Cell type-Dependent Susceptibility

Despite green fluorescence having significant drawbacks for the evaluation of drug susceptibility, both plaque reduction and yield reduction analysis found a statistically significant difference in GCV susceptibility. The average epithelial-IC_{50} by plaque reduction was 12-fold higher than its fibroblast correlate. Similarly, yield reductions exposed a five-fold higher epithelial-IC_{90} than fibroblast-IC_{90}. Cytotoxicity experiments failed to reveal underlying evidence of substantial cytotoxic effects, even at the highest GCV concentrations. There are a number of different possible explanations for this discrepancy.

The epithelial cells used here (ARPE-19) make up the outer component of the blood-retinal barrier (BRB) of the eye. Retinal pigmented epithelial cells regulate a number of physiological processes such as maintaining fluid and ion balance, transport of nutrients and waste products between the retina and the choroid vascular system, and preserving ocular homeostasis. The epithelium contains a number of transporters and channels which facilitate this function. Many of these transporters may be involved in the efflux of small molecules and pharmacological agents (65). A number of groups have looked at the intravitreal kinetics of GCV and proposed that elimination of GCV from the vitreous chamber probably involves some sort of active transport. Retention and

infiltration of GCV into the retina is mediated first by uptake and second by efflux across the BRB. A study investigating the activity of nucleobase and nucleoside transporters over a five minute period, demonstrated that GCV uptake into human ARPE-19 cells is primarily governed by passive diffusion (64).

On the other hand, there is evidence to suggest that mechanisms exist to expel GCV from ARPE-19 cells via active transport. Multidrug resistance-associated proteins (MRP) are members of the ATP-binding cassette (ABC) superfamily of membrane transporters. This particular family of transporters has been extensively studied as organic anion drug efflux pumps that are often upregulated in cancer cells (9). These pumps are naturally found in a plethora of different cell types throughout the body. MRP4 (also known as ABCC4) is a particular MRP that has been shown to efflux GCV and markedly increase GCV resistance in herpes simplex virus thymidine kinase (HSV-TK) expressing cells that over express the transporter (5). Recall that HSV-TK acts in the same way that CMV UL97 does: activating GCV by monophosphorylation. Cells transfected with HSV-TK activate GCV without CMV infection. Furthermore, MRP4 and related MRPs have been detected by quantitative PCR in both flask and filter cultures of ARPE-19 cells, though to a higher degree in the filter culture where cells differentiate and become polarized (110).

Although this same research has not been conducted in fibroblast cells, it is reasonable to hypothesize that such cells, which do not perform the same protective function as retinal epithelial cells, could express lower levels of MRPs and thus have a muted mechanism of drug efflux. This could certainly explain the greater susceptibility of these cells in culture. One might test this hypothesis by using antagonists of the major

MRPs in both cell types and monitor the degree of synergism they show with GCV. Knockout experiments could pinpoint more precisely the particular cellular genes involved in drug efflux.

Another hypothesis might be that guanosine triphosphate concentrations might differ between cell types. Nucleotide pools can vary appreciably depending on rates of synthesis, degradation and even cell cycle (16, 103). A higher ratio of endogenous GTP to GCV-triphosphate (GCV-TP) would competitively inhibit GCV incorporation into the viral genome by the viral polymerase, leading to weaker antiviral activity. In addition to differing levels of endogenous nucleotides, the metabolism of GCV could be different in the two cell types. The intracellular half-life of (GCV-TP) in MRC-5 cells has been estimated at roughly six (12) to 34 (102) hours depending on viral MOI. The data presented here shows that the highest drug concentrations suppressed viral titers from both cell types to a negligible level early in infection. In contrast, 12 μM GCV was highly effective in abolishing virus titers by nine days post infection (d.p.i) in fibroblast cells, while virus in the parallel epithelial treatment group saw resurgence at the same time point. If GCV were more quickly metabolized in epithelial cells, the drug might initially suppress viral titers but not have the longevity that it shows in fibroblasts. Differential expression of the protein kinases responsible for di- and tri-phosphorylation of GCV could be an explanation for differential metabolism. To determine if endogenous nucleotide levels were an influencing factor, nucleotide pools in the two cell types could be analyzed using chromatographic methods. GCV-TP levels could be monitored using radioactively tagged [H^3]GCV-TP.

4.3 Implications for a Cell type Difference in GCV efficacy.

Many methods of potentiating GCV activity are already being developed. The physiological isolation of the retina is an impediment for treatment of a myriad of vitreoretinal diseases. Proper delivery of pharmaceutical agents is paramount but extraordinarily difficult because the blood retinal barrier (BRB) separates the vitreous and retina from systemic circulation. A number of technologies have been developed to overcome this barrier. Early in the AIDS epidemic, doctors attempted to inject GCV directly into the vitreous of AIDS patients suffering from CMV retinitis (111). This was not an attractive treatment, however, because of the highly invasive nature of intraocular injections and because of the short half-life of GCV in the vitreous (7 hours) (63) necessitated multiple injections. As a result, a host of intravitreal polymeric drug delivery technologies are in development that prolong drug release and stabilize vitreal concentrations. A review of these devices sheds light on their diversity and innovation (121).

More recently, biochemists have attempted to exploit cellular amino acid transporters for better GCV delivery. The GCV prodrug valganciclovir was engineered to take advantage of one of these broad specificity transporters known as $ABC^{0,+}$ (34). By coupling an anionic amino acid to GCV in such a way that it will be cleaved later in metabolism, researchers could trick the transporter into pumping prodrug across the plasma membrane. This raises intracellular concentrations and improves drug efficacy. $ABC^{0,+}$ is found in a number of tissues, including the lung, colon, and eye. If the main

obstacle to GCV activity in ARPE-19 cells is related to drug transport, such a prodrug might add a productive compensatory mechanism.

Another method for potentiating GCV activity would be to exploit its competitive relationship with guanosine triphosphate (GTP). By lowering endogenous deoxynucleotide concentrations, one could increase the probability of GCV-TP incorporation by the viral polymerase. One means of lowering nucleotide pools is to inhibit the cellular ribonucleotide reductase responsible for reducing ribonucleotides to deoxyribonucleotides. A number of different ribonucleotide reductase inhibitors have been tested for the treatment of viral infections and cancer, including hydroxyurea (HU) and mycophenolic acid (MPA). HU has been used in conjunction with anti-HIV nucleotide analogs, in particular, didanosine (ddI) and adefovir, with great success (82). HU also shows synergistic activity when combined with GCV in the treatment of other herpesviruses and drug resistant viruses but has not been studied in treating CMV (76, 97). MPA has been shown to have a similar synergistic potential when combined with GCV (75).

In the clinic, cell type research is critically important. Before HAART, AIDS patients in particular had a very high incidence of CMV retinitis. GCV was transiently effective against the pathology but after a few months of treatment it would reappear. In 1996, a large multicenter retreatment trial was conducted that gave the median time for CMV retinitis relapse as: 1.3 months for foscarnet treatment, 2.0 months for GCV treatment, and 4.3 months for a GCV/foscarnet combination therapy ($p < 0.001$) (2). Other indicators of morbidity and mortality were equally bleak. Fortunately, HAART became

widely available that same year, bolstering patient cellular immunity and diminishing the urgency felt to find better treatments for CMV retinitis. The results of our studies shed light on why GCV may have been ineffectual in the eye, while effectively restricting CMV disease in other tissues. Further study is necessary to optimize drug delivery and explore possible synergistic combinations of GCV and other antiviral drugs.

4.4 Possible Mechanisms of MBV Cell type Dependent Susceptibility

To an even greater degree than GCV, MBV showed considerable cell type-dependent susceptibility. A plaque reduction assay exposed a seven-fold higher epithelial-IC_{50} than fibroblast-IC_{50} at a low MOI. Virus yield growth curves demonstrated an even larger gap between epithelial-IC_{90} and fibroblast-IC_{90}, as great as 200-350-fold. Except at cytotoxic levels, MBV was essentially ineffective at blocking the generation of infectious viral progeny in epithelial cells. Both cell types showed small amounts of cytotoxicity at the highest drug concentration, but this could not account for the dramatic difference in supernatant viral titers. Due to the still experimental status of MBV and the opacity of its action, instructive hypotheses are tenuous at best.

MBV inhibits the viral protein kinase encoded by *UL97*. pUL97 has been proposed as a cyclin-dependent kinase ortholog that is immune from normal cell cycle regulation (43). As a result, pUL97 promotes cell cycle progression. The specific activities of the UL97 kinase are still mysterious. It appears to phosphorylate retinoblastoma protein (Rb), deactivating it (43). In normal cells, the Rb family of proteins binds E2F transcription factors, thus blocking transcriptional activation of genes that induce cell cycle progression.

pUL97 inhibition of this pathway coupled with modification of other cellular proteins by viral enzymes such as pp71 (49), leads to unfettered cell cycle progression through G1/S and G2/M checkpoints. The cumulative effect of CMV infection does not appear to shorten the cell cycle but rather induces early transition into S phase, prolonged S and G2 phases, and ultimately metaphase arrest (45). Extrapolating simply from observations of a phenotypic difference in cell growth, one might hypothesize that faster replicating ARPE-19 cells might have less stringent cell cycle checkpoints.

A similar cell type-specific dynamic was elucidated from human lung bronchial epithelial cells (BEC) in comparison to human lung fibroblasts (HLF). The study showed that BECs were less susceptible to DNA-damage induced cell cycle arrest (33). Upon insult of ionizing radiation and DNA-damage, HLFs became terminally trapped in G1 arrest. In contrast, BECs continued proliferating after transient G1 and G2 delays. Additionally, radiation induced greater p53 and p21Cip1 increases in HLFs, while BECs showed higher prexposure basal levels of p53. One might point out the fact that the lung bronchial epithelium, while morphologically similar, is physiologically distinct from the retinal pigmented epithelium. The lung bronchial epithelium is constantly bombarded by DNA damaging reagents from noxious air pollution to endogenously produced defensive agents (e.g., superoxide radicals). This is not the case for the retinal pigmented epithelium. Nevertheless, cell type-specific cell cycle variations exist elsewhere. The literature is plush with cell cycle research conducted in fibroblasts, yet there are examples that infer cell cycle pathways from the study of other cell types. A study conducted in 2006 found that the p16INK4a (p16) tumor suppressor gene plays fundamentally different roles in p53

regulation depending on the cell type (122). In human mammary epithelial cells, p16 was found to inversely modulate p53 levels via an Rb-dependent mechanism. In contrast, neither human mammary fibroblasts nor human foreskin fibroblasts displayed this interdependence. Therefore, the potential for cell type-specific variations of cell cycle pathways exist.

The cell type effect observed in the MBV experiments above could be attributed to any number of cell cycle contributors. Recently, Chou et al recorded that MBV activity was dependent on culture conditions (21). The group points out that widely differing plaque reduction IC_{50}s have been described in the literature without clarification (11, 117). In their study, human foreskin fibroblasts exhibited a nearly 100-fold higher IC_{50} than embryonic lung fibroblasts grown under the same conditions. They further demonstrated that this gap could be virtually eliminated by adding a cellular kinase inhibitor (i.e., roscovitine, rapamycin, or LY294002) acting in a synergistic fashion with MBV. Roscovitine is an oligo-specific cyclin-dependent kinase inhibitor; rapamycin is an inhibitor of mTOR kinase, which is a regulator of protein translation; and LY294002 is an inhibitor of phosphatidylinositol-3-kinase. All have activity against CMV infection (14, 47, 59) but there is no data, as of yet, that determines if these kinase inhibitors have an effect directly on UL97 kinase function. These data posit the possibility that the cell type difference may be due to differential expression of a cellular kinase that can substitute for UL97 in MBV-treated ARPE-19 cells. Further study of the MBV-cellular kinase relationship is needed. Any such study should evaluate both the synergistic effects of combining different cellular kinase inhibitors with MBV and the effects, if any, of these

kinase inhibitors directly on the UL97 kinase. This relationship will help tease out any cellular kinases which may act as UL97 kinase surrogates.

4.5 Implications for a Cell type Difference in MBV efficacy.

Should our evidence prove to have significant clinical value, it might also support more robust *in vitro* testing of drug susceptibility that incorporates multiple clinically relevant cell types. Phenotypic assays of anti-CMV susceptibility should be performed in endothelial and epithelial cells, which are central in CMV pathology. This is not to say that data culled from fibroblasts is useless. In fact, clinical studies demonstrating a significant anti-CMV activity of MBV reinforce this point. Fibroblasts may function well as a neutral cell type control. In retrospect, MBV may have been abandoned and its mechanism of action overlooked had it been tested in ARPE-19 cells exclusively. The use of multiple, clinically relevant cell types to test the efficacy of antiviral drugs would be optimum.

Despite the fact that MBV clinical trials have shown promising results, their design is such that it would not have exposed the drug's ostensive Achilles' heel, proposed by our study. Recently, data from a multicenter, randomized, double-blind, placebo-controlled, dose ranging study of MBV in seropositive allogeneic stem-cell transplant recipients show that MBV can have a moderate effect on preventing CMV infection (monitored by CMV pp65 antigenemia and plasma DNA) compared with placebo (118). This study did not evaluate the ability of the drug to treat CMV disease, but rather discontinued participation at onset of CMV infection or disease. In addition, MBV displayed no evidence of

myelosuppression, as existing first line anti-CMV therapies do (e.g., GCV). A similar phase III clinical trial, which seeks to evaluate whether MBV prophylaxis leads to significant reduction of CMV disease, is ongoing (118). However, this study was conducted in transplant recipients and since CMV in transplant recipients manifests infrequently in the eye (29) (these patients develop CMV disease more frequently in the liver, gastrointestinal track, kidney, and lung) (31), the trial's design was not likely to have uncovered poor performance against retinal disease (which involves the retinal pigmented epithelium). A phase I clinical trial was performed on HIV-infected men in 1996 to evaluate the drug's safety profile, but all of the subjects were stable and asymptomatic (60). As of yet, MBV has not been assessed as a treatment for CMV retinitis. Even so, if approved, physicians may elect to prescribe MBV to treat CMV retinitis. The data presented herein may encourage the FDA to press for further testing or advise physicians to use caution when considering the use of MBV for treating CMV retinitis.

The combined data from both these drugs begs another question: can they be used together in combination therapy? In HIV therapy, the advent of the multidrug cocktail has revolutionized treatment. Many propose that a similar approach is needed for CMV. Two factors prevent this. First, all front line CMV antivirals have significant toxicity. GCV and its prodrug are strong myelosuppressants and potent renal toxicity limits the use of both cidofovir (CDV) and foscarnet (FOS). Second, all of these drugs have a common viral target, the viral DNA polymerase. The success of HAART has been in its ability to combine drugs with different targets to minimize the development of viral resistance. For

these reasons, MBV is a highly anticipated drug and must be assayed with GCV, the treatment of choice, to determine if there are drug interactions. A theoretical basis for antagonism exists between these drugs. GCV's activity depends on its phosphorylation by the UL97 protein kinase, while MBV's activity rests in its ability to inhibit this kinase. The literature is currently divided on the subject of GCV-MBV antagonism. In 2002, an article was published that found considerable synergy for combinations of MBV+CDV and MBV+FOS, but MBV+GCV was only additive (not antagonistic) (30). A year later, another study from the GlaxoSmithKline headquarters in North Carolina found the same additive interaction (at this time, GlaxoSmithKline owned the rights to MBV) (96). In 2006, however, a group in Portland, Oregon, which has worked extensively with MBV, found that MBV increased the GCV IC_{50} by 13-fold, a powerful antagonistic effect (19). The same group had previously determined the effect of culture conditions on MBV activity (21), which may influence the outcome of these assays. Today, the issue of GCV-MBV antagonism remains unresolved, but data such as these caution the use of GCV+MBV combination therapy in treating CMV of epithelial origin. Without understanding the mechanism behind the cell type discrepancy uncovered by our study, it is difficult to predict that MBV will not antagonize GCV because it has no activity in retinal pigmented epithelial cells or rather that MBV has no activity against CMV in these cells and combination therapy could potentially be even worse than GCV alone.

While these and other questions remained unresolved by this preliminary study of cell type dependence. Our results warrant further *in vitro* testing and more critical clinical trials to identify possible treatment concerns.

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