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INVESTIGATING SYNERGY BETWEEN RIBONUCLEOTIDE REDUCTASE INHIBITORS
AND CMV ANTIVIRALS

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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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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Virginia Commonwealth University, August 2012

Major Director: Michael McVoy, Ph.D.
Professor, Department of Microbiology and Immunology

Cytomegalovirus (CMV) infections remain a significant problem in congenitally infected infants and immunocompromised individuals. Modest antiviral activities of currently approved drugs coupled with dose-limiting toxicities restrict effectiveness and promote development of resistance. The potential for ribonucleotide reductase (RR) inhibitors hydroxyurea (HU), Didox, and Trimidox to synergize, through reduction of nucleotide pools, with the deoxynucleotide analog Ganciclovir (GCV) was examined. A yield reduction assay that utilizes luciferase expressed by a recombinant virus as a surrogate measure of viral infectious units was developed and used to determine effective dose ranges for each drug. RR inhibitors exhibited intrinsic anti-CMV activities on their own with IC₅₀ values well below toxic levels. Moreover, RR inhibitors significantly synergized with GCV. These findings provide a rationale for exploration of RR inhibitors and deoxynucleotide analogs in anti-CMV combination therapy.

INTRODUCTION

Impact on human health-

Cytomegalovirus or CMV is a member of the *Herpesviridae* family of viruses. It is a common virus that infects people of all ages. CMV has a ubiquitous distribution, with between 40 and 90% of all adults worldwide carrying the virus. It infects between 50% and 80% of persons in the United States by 40 years of age. The vast majority of people infected with CMV do not show any symptoms. Primary CMV infections in immunocompetent individuals are mild or asymptomatic, or produce fever-like and mononucleosis-like symptoms. After primary infection the virus remains in a latent state for the entire lifetime of the host. However, CMV is highly pathogenic in immunosuppressed patients, especially AIDS and transplant patients. Moreover, congenital CMV infections are among the leading causes of birth abnormalities in developed countries ⁽¹⁾.

CMV infection and disease among transplant recipients-

CMV is a major pathogen of immunocompromised solid organ and bone marrow transplant patients. 80-90% of all post-transplant patients are infected by the virus, while the incidence of CMV disease is 30-40% ⁽²⁾. Severe disease is most common when the recipient is seronegative and receives organs or blood products from seropositive donors. However, CMV disease can also occur following reactivation from latency in seropositive transplant recipients. CMV disease has a number of clinical manifestations, including asymptomatic viremia, CMV syndrome (viremia with symptoms including fever and malaise) and tissue-invasive disease (*e.g.*, colitis,

pneumonitis, hepatitis, retinitis, and disease in other sites) ⁽³⁾. In BMT recipients, CMV-induced interstitial pneumonia is a frequent and endangering manifestation of CMV disease. The risk of CMV also varies with the type of organ transplant received: lung, small intestine, and pancreas transplant recipients are at higher risk than kidney and liver transplant recipients ⁽⁴⁾. These differences may be due to the net state of immunosuppression or other endogenous factors specific to the transplanted organ. The level of immunosuppression may depend on a number of factors, such as type and amount of immunosuppressive therapy or the presence of bacterial, fungal or other viral infections. Bone marrow transplantation (BMT) as a therapy of hematological malignancies is associated with a transient immunodeficiency. Accordingly, during the period of immunocompromise, transmission of donor-type CMV with the transplant as well as recurrence of CMV from latency established within the recipient`s organs both entail a risk for destructive virus replication in tissues resulting in multi-organ CMV disease ⁽⁵⁾.

CMV in HIV-infected patients-

Before the advent of highly active antiretroviral therapy (HAART), CMV was the most frequent opportunistic pathogen in patients with AIDS. It has been reported that CMV affects about 45% of AIDS patients at some point of time during the course of the disease. The common effects of CMV infection in this population include retinitis, gastrointestinal infection and neurologic disease. CMV retinitis accounts for 85% of all CMV disease in patients with AIDS. If untreated, CMV retinitis can progress to total retinal destruction and blindness ⁽⁶⁾. With the development of HAART, CMV infection in AIDS patients has become less common. Treatment with HAART suppresses HIV replication, resulting in a drop in HIV load and in immune recovery, resulting in a rise in CD4 T-cell counts. Thus, the incidence of opportunistic infections, such as CMV retinitis, declines. However, the long-term consequences of CMV infection in

AIDS patients still remain undefined. CMV retinitis and GI infection are still a problem for patients with delayed diagnosis of HIV and those who are intolerant or unresponsive to HAART⁽⁷⁾. It requires about eight months for HAART to induce reconstitution of immunity, thus a significant population still arises with a risk of CMV infection⁽⁸⁾.

Congenital and Perinatal CMV-

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⁽⁹⁾. In many cases, babies are either infected *in utero* or during delivery by transmission from mothers who suffer from primary infection or reactivation. CMV can also be transmitted to babies by post natal blood transfusions or infected saliva and breast milk. In industrialized countries up to 8% of CMV seronegative mothers contract primary CMV infection during pregnancy, of which roughly 50% will transmit to the fetus⁽¹⁰⁾. In contrast, the transmission rate when infection occurs prior to pregnancy is 0.5%-2%⁽¹¹⁾. Although the majority of infants are asymptomatic at birth, between 22-38% of infected fetuses are born with symptoms, which may include pneumonia, gastrointestinal, retinal, or neurological disease. CMV is now the most common viral cause of mental retardation, hearing deficit, and vision impairment of children in developed countries. Hearing loss occurs in approximately half of the infants with symptomatic and about 15% of infants with asymptomatic congenital CMV infection. Children who are asymptomatic at birth may also have mental retardation⁽¹²⁾. Preterm infants are at greater risk of symptomatic CMV infection than term infants. Breast milk is the main source of perinatal CMV infections⁽¹³⁾.

Molecular Biology of CMV-

The *Herpesviridae* are a large family of DNA viruses that cause diseases in animals, including humans. The word *herpein* (which means 'to creep') refers to the latent recurring infection which is typical to this group of viruses ⁽¹⁴⁾. The family *Herpesviridae* is divided into three subfamilies- alpha, beta and gamma. The alphaherpesviruses primarily target mucoc epithelial cells and latently infect neurons. This subfamily includes herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and varicella zoster virus (VZV), the virus that causes chickenpox and shingles. The gamma group of lymphotropic herpesviruses includes Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Both are potentially oncogenic and are associated with African Burkitt's lymphoma and Kaposi's sarcoma, respectively. CMV (also called as HHV-5), along with HHV-6 and HHV-7 (which can cause roseola infantum), are categorized as betaherpesviruses. This subfamily primarily targets monocytes, lymphocytes, T-cells and epithelial cells. CMV is believed to be carried latently in cells of myeloid lineage (*e.g.*, monocytes) ⁽¹⁵⁾.

Herpesviruses have large double-stranded linear DNA genomes (120- to 235-kb, encoding 70-200 genes) within an enveloped icosohedral capsid. CMV has the largest genome among the herpesviruses, 235-kb in length and encoding >200 genes. The genome has a unique long (UL) and a unique short (US) region, bounded by inverted repeats ⁽¹⁶⁾. Outside the capsid is the tegument, a protein-filled region that appears amorphous in electron micrographs. On the outside of the particle is the envelope, a lipid bilayer that contains numerous glycoproteins, such as gB, gH, gL, gM, gN, and gO, which are involved in attachment and entry of the virus into host cells ⁽¹⁷⁾.

The genes of CMV are expressed in three distinct phases, designated as immediate early, early and late. Expression of immediate early genes does not require the expression of other viral genes. Expression of early genes is dependent on the synthesis of immediate early gene products. Early transcripts have been mapped to all regions of the CMV genome and are often involved in DNA synthesis. Once the early genes have been synthesized, virus replication can occur and late gene synthesis ensues. The majority of late genes encode virus structural proteins (*e.g.* the capsid and tegument proteins and the envelope glycoproteins) ⁽¹⁸⁾.

Although the overall picture of CMV DNA synthesis appears typical of the herpesviruses, some novel features are emerging. Six herpesvirus-group-common genes encode proteins that likely constitute the replication fork machinery, including a two-subunit DNA polymerase, a helicase-primase complex and a single-stranded DNA-binding protein ⁽¹⁹⁾. Many herpesviruses encode two subunits (R1 and R2) that form a Ribonucleotide Reductase (RR), an enzyme that converts NTPs to dNTPs, which is important for efficient viral DNA synthesis. Unlike other herpesviruses, the β -herpesviruses, including CMV, lack the gene for the R2 subunit and their R1 subunit lacks residues important for catalytic activity. Thus, the RR expressed by CMV is non-functional ⁽²⁰⁾. Since CMV lacks a functional RR, it is predicted that CMV replication may be impaired by drugs that antagonize host RR.

CMV infects a variety of cell types including: endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, mesenchymal cells, hepatocytes, monocytes/macrophages, and granulocytes ⁽²¹⁾. However, the *in vitro* biological properties of CMV have been studied mostly in fibroblasts. This can be attributed to the fact that the two commonly used laboratory-adapted strains of CMV, AD169 and Towne, were initially serially passaged in fibroblast cells to reach high titers *in vitro* and both suffered gross genetic mutations in the process ⁽²²⁾. 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 about as well in endothelial and epithelial cell cultures as in fibroblasts. Thus, it may be important to study drug susceptibility of CMV in other clinically relevant cell types.

CMV Antivirals

Presently there are three drugs licensed for the treatment of systemic CMV infections: Ganciclovir (and its prodrug Valganciclovir), Foscarnet and Cidofovir. These drugs are beneficial in the treatment of CMV disease in adults. However, they have limitations of modest efficacy, dose-limiting toxicities, oral bioavailability and risk of development of resistance. None of these drugs is currently approved for use in treating congenital CMV infections.

Ganciclovir-

Ganciclovir (GCV) is widely used in the treatment and prophylaxis of several viruses belonging to the *herpesvirus* family, including CMV, HSV, VZV and EBV. It is the first drug found to be effective in treating established CMV infections. It continues to be the first-line treatment for CMV infection in AIDS and organ transplant patients ⁽²³⁾. GCV is a deoxyguanosine analog. It is converted to its mono-phosphate form by the CMV-encoded protein kinase pUL97, and subsequently, to its di- and tri- phosphate form by host cell kinases. GCV tri-phosphate incorporates into viral DNA and inhibits viral DNA polymerase by competitively inhibiting the incorporation of dGTP into the elongating viral DNA. GCV is not an absolute chain terminator but it slows down the synthesis of viral DNA and thus, slows down CMV replication ⁽²⁴⁾. Mutations in either pUL97 or the viral DNA polymerase (UL54) can confer resistance to GCV ⁽²⁵⁾.

In AIDS patients, CMV retinitis is a very common and sight-threatening infection. Intravenous GCV has been approved by the FDA for treating CMV retinitis in AIDS patients. Relapse of retinitis is controlled with ValGCV a (prodrug of GCV with a significantly higher oral bioavailability). Often treatment options include intraocular sustained-release GCV implants and intravitreal injections of GCV ⁽²⁶⁾. GCV is used in combination with CMV immune globulin to treat CMV pneumonia associated with bone-marrow transplant ⁽²⁷⁾. GCV is also used to treat CMV pneumonia and CMV retinitis in renal transplant patients ⁽²⁸⁾.

GCV is associated with severe toxicity issues. It most commonly causes hematological toxicity (neutropenia, anemia, and thrombocytopenia). Preclinical studies show that GCV can cause long-term reproductive toxicity leading to aspermatogenesis. In animal studies, GCV has been found to be a potential carcinogen and teratogen ⁽²⁹⁾.

Thus, despite of being the gold standard for management of CMV disease, GCV cannot be used in all patient populations because of the problems of toxicity and drug resistance. One clinical trial demonstrated that postnatal GCV therapy in symptomatic congenital CMV infection of the central nervous system prevented hearing deterioration. GCV is currently used to treat congenital CMV infections post-partum but is not the ideal treatment. GCV is not used to treat congenital CMV infections *in utero* because it is difficult to monitor neutropenia in the developing fetus ⁽³⁰⁾ and due to the risk of gonadal toxicity, teratogenicity, and carcinogenicity on long-term use ⁽³¹⁾.

Foscarnet-

Foscarnet (FOS) is considered as a second-line therapy for the treatment of CMV infections. FOS is a pyrophosphate analog. It directly and reversibly inhibits the viral DNA polymerase by blocking the pyrophosphate binding site on the enzyme and blocking cleavage of pyrophosphate

from the terminal nucleoside triphosphate added to the growing DNA chain. Unlike GCV, FOS does not need to be phosphorylated for antiviral activity. Mutations in the viral DNA polymerase, UL54, can confer resistance to FOS. Cross-resistance has been observed between GCV and FOS due to mutations in the *UL54* pol gene. Therapy with FOS has been shown to be effective in the treatment of CMV infections, especially CMV retinitis, in individuals infected with HIV. It is a preferred drug for patients who fail GCV therapy due to viral resistance when the mutations are in the *UL97* gene. It is also used in patients who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia or other toxic effects. However, FOS is associated with renal toxicity and electrolyte imbalance. This can lead to cardiac or neurological disorders and in certain cases, even death. FOS is not currently approved for use in treating pre- or post-partum congenital CMV infections ^{(32) (33)}.

Cidofovir-

Cidofovir (CDV) is another antiviral used in the treatment of CMV infections. CDV is an acyclic nucleoside phosphonate. It is converted to its mono- and then diphosphate form (triphosphate equivalent) by host cell kinases. The active CDV diphosphate incorporates into viral DNA, competitively inhibiting addition of dCTP. Thus, CDV inhibits viral DNA polymerase, resulting in chain termination. Unlike GCV, CDV is not phosphorylated by pUL97 kinase. Mutations in DNA polymerase UL54 can confer resistance to CDV. Cross-resistance has been observed between GCV and CDV due to mutations in the *UL54* gene. CDV is used to treat CMV retinitis in AIDS patients. It is a useful drug for patients resistant to GCV therapy due to mutations in the *UL97* gene. However, the major limitation of CDV is nephrotoxicity. CDV has found to be a carcinogen and teratogen in preclinical studies and is not approved for use during pregnancy ^{(32) (33)}.

Acyclovir-

Acyclovir (ACV) is another antiviral agent that is attractive to use because it is considerably less toxic as compared to GCV, FOS and CDV. ACV is a synthetic deoxyguanosine analog. Its mechanism of action is similar to that of GCV. It is converted to its mono-phosphate form by pUL97 and subsequently to its di- and tri- phosphate form by host cell kinases. The tri-phosphate form (ACV-TP) is the active form of the drug. It incorporates into viral DNA, inhibiting further addition of nucleotides to this strand because it lacks the 3` OH. Thus, ACV inhibits viral DNA polymerase, resulting in chain termination. ACV has approximately 100-times greater affinity for viral than cellular polymerase. Specificity of ACV-TP for viral polymerases, and that ACV gets mono- to tri- phosphorylated in infected cells because only infected cells express UL97, explains the low toxicity of this drug as compared to other currently used CMV antivirals ⁽³³⁾.

Among the herpesvirus family, ACV is widely used in the treatment and prophylaxis of HSV 1 and 2 as well as VZV infections. It is the safest drug in use for the treatment of genital herpes during pregnancy. A registry of over 1000 pregnant women who were treated with ACV during early pregnancy identified no increases in birth defects. Physicians who use ACV in pregnant patients for treatment of primary or recurrent HSV infections found that it not only treats the mother's condition but also reduces the likelihood of transmission to the neonate without unduly compromising fetal safety ⁽³⁴⁾.

ACV has limited efficacy against EBV and CMV ⁽³⁵⁾. Use of ACV in the treatment of CMV disease in transplant patients has been studied. In a randomized, placebo-controlled trial conducted to study the use of ACV for the prevention of CMV disease in recipients of renal allografts from cadavers, it was found that oral administration of ACV before transplantation reduces the rate of CMV infection and disease without affecting the survival rate of either grafts

or patients ⁽³⁶⁾. In another study, prophylaxis with intravenous ACV was found to significantly reduce the risk of CMV infection and disease in seropositive patients after allogeneic bone marrow transplantation ⁽³⁷⁾.

ACV is associated with relatively less toxicity issues as compared to the other CMV antivirals-GCV, FOS and CDV. However, ACV is a less efficient substrate for pUL97 than GCV, which in part explains the lower in vitro potency of ACV as compared to GCV in CMV-infected cells. Another reason for low potency of ACV can be attributed to its short half-life. ACV-TP has a four- to five-fold shorter half-life as compared to GCV-TP in infected cells, resulting in the lower intracellular levels of the active ACV-TP ⁽³³⁾. Thus, ACV is not much used to treat established CMV infections due to its low potency as compared to GCV.

Objectives-

The modest antiviral activity of currently approved drugs like GCV, FOS and CDV coupled with dose-limiting toxicities, limits their effectiveness and often results in the development of resistance. Unfortunately, drugs with low toxicity such as ACV do not have sufficient activity against CMV to be clinically useful. Development of new antiviral therapies that have improved efficacy as well as reduced toxicity to allow extended courses of therapy and adequate safety for treating congenital CMV infections in utero and postnatally is required.

We therefore propose to evaluate the potential of “combination therapy” to improve the efficacy of current therapy and reduce problems of drug resistance. Through synergy it may be possible to improve the potency of the current CMV antivirals by co-administration with drugs that reduce intracellular nucleotide pools (Figure 1). One such class of drugs is the RR inhibitors,

which reduce intracellular nucleotide pools by inhibiting the conversion of ribonucleotides to deoxyribonucleotides. We are considering three RR inhibitors for study- Hydroxyurea (HU), Didox and Trimidox.

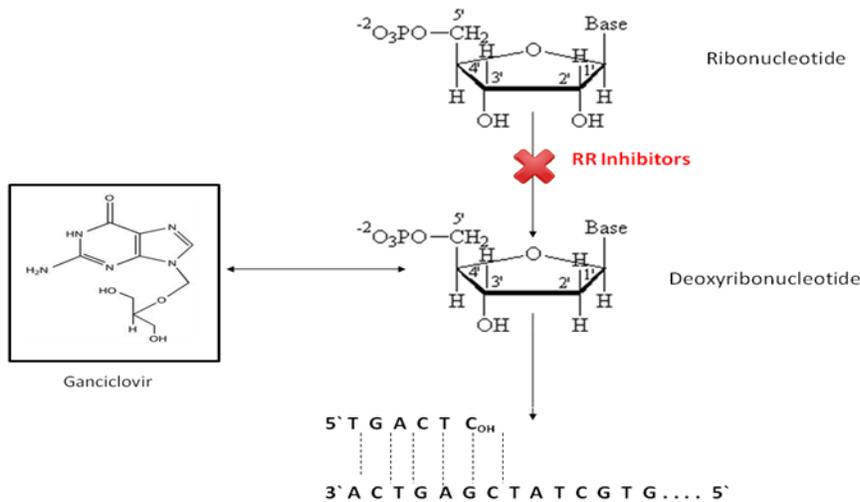


Figure 1- Mechanism of action of GCV in combination with RR inhibitors on CMV replication

Ribonucleotide Reductase Inhibitors-

RR is a crucial enzyme for nucleotide anabolism. It converts ribonucleotides to deoxyribonucleotides, the only metabolic pathway affording *de novo* biosynthesis of deoxyribonucleotides. The active site of RR consists of the active dithiol groups from the R1 subunit as well as the diferric iron center and the tyrosyl radical from the R2 subunit. RR inhibitors are a class of drugs that inhibit the conversion of ribonucleotides to deoxyribonucleotides and thus reduce the intracellular deoxyribonucleotide pools. Since RR plays a key role in DNA synthesis and cell growth control, RR inhibitors have been widely used in clinical cancer chemotherapy and anti-viral therapy (Table 1).

Hydroxyurea (HU)-

Hydroxyurea was first synthesized in 1869 by Dresler and Stein ⁽³⁸⁾. It is a drug that targets intracellular RR. It quenches the tyrosyl free radical at the active site of the R2 subunit, inactivating the enzyme. It acts specifically on the S-phase of the cell cycle, limiting *de novo* DNA synthesis. The anti-tumor activity of HU is attributed to its RR inhibitory effect. HU is employed as a first-line treatment of myeloproliferative disorders, such as polycythemia vera, thrombocythemia and primary myelofibrosis. HU also plays a role in combination therapy for management of malignant melanoma, head and neck cancers, and brain tumors ⁽³⁹⁾. There are a few reports of potential synergy between HU and radiation or alkylating agents for cancer treatment ⁽⁴⁰⁾.

HU is also implicated in the treatment of sickle cell anemia. It is the only approved medication in the United States for the treatment of sickle cell anemia and is widely used in children despite an indication limited to adults ⁽⁴¹⁾. HU increases the level of fetal hemoglobin, leading to a reduction in the incidence of vaso-occlusive crises in sickle cell patients. The exact mechanism of action of HU in this disease is not yet clear, but studies have shown that HU increases nitric oxide levels, causing soluble guanylyl cyclase activation, with a resultant rise in cyclic GMP, and the activation of gammaglobin synthesis necessary for fetal hemoglobin ⁽⁴²⁾.

HU has been used in the treatment of cancer, sickle cell anemia, and viral diseases for about four decades. Some limitations of its use include gastrointestinal disturbances, bone marrow depression, oral ulcers and skin rashes. Cutaneous toxicity is rare. Discontinuation of HU usually leads to slow resolution of the ulcers over several months ⁽⁴³⁾.

Didox and Trimidox-

Didox and Trimidox are RR inhibitors developed by the Richmond-based biopharmaceutical company Molecules For Health, Inc. Both drugs are dihydroxy derivatives of benzohydroxamic acid. Didox forms iron complexes, interferes with iron metabolism and scavenges tyrosyl free radicals. Thus, Didox exerts its RR inhibitory effect by destabilizing the R2 subunit of RR through its iron chelating properties ⁽⁴⁴⁾. Trimidox demonstrates a similar mechanism of action.

Didox and Trimidox are considered to be promising targets for cancer chemotherapy. In multiple myeloma cells, Didox was found to induce apoptosis and inhibit DNA repair. This study also showed that Didox, unlike other RR inhibitors that mainly target the pyrimidine metabolism pathway, targets both purine and pyrimidine metabolism pathways in multiple myeloma ⁽⁴⁵⁾. In the treatment of malignant glioma, Didox was found to synergize the activity of BCNU, a standard agent in the therapy of glioblastoma multiforme ⁽⁴⁶⁾. Didox is in Phase II trials for the treatment of advanced breast cancer ⁽⁴⁷⁾. Similarly, several studies have confirmed the effectiveness of Trimidox in combination cancer chemotherapy. *In vitro* and *in vivo* experiments have shown that Trimidox potentiates the anti-tumor activity of Cisplatin and Cyclophosphamide in leukemia-bearing mice ⁽⁴⁸⁾. Trimidox was also found to synergize the activity of Temozolomide, an alkylating agent, in malignant brain tumor cells ⁽⁴⁹⁾.

Antiviral activity of RR inhibitors-

The effect of HU on replication of Human Immunodeficiency Virus type 1 (HIV-1) has been investigated. HU inhibits HIV-1 replication in a dose-dependent manner. HU also potentiates the anti-HIV activity of several 2',3'-dideoxynucleoside analogs, in particular, didanosine (ddI), a reverse transcriptase inhibitor, through its activity of RR inhibition ⁽⁵⁰⁾. HU has established benefit as a component of drug cocktails for the treatment of HIV-1

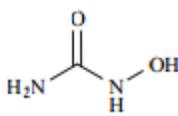
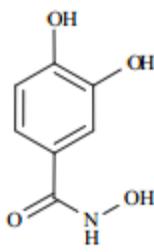
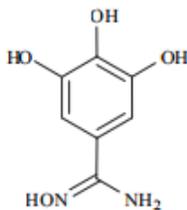
infection. However, the toxicity issues associated with HU limit its use only as salvage therapy to patients with late-stage AIDS. In a particular study the anti-retroviral effect of HU was compared to that of Didox and Trimidox in combination with didanosine. Didox and Trimidox appeared to be more effective and less myelosuppressive than HU when administered with ddI⁽⁵¹⁾. Thus, Didox and Trimidox might replace HU in combination anti-HIV therapy.

The combined effect of HU and some anti-HSV drugs like ACV, GCV, Penciclovir, Lobucavir and Brivudin has also been studied. It was found that HU by itself has little effect on HSV but it synergizes the antiviral activity of these anti-HSV drugs. The potentiating effect of HU can be attributed to depletion of intracellular dNTPs, thus favoring the nucleoside triphosphate analogs in their competition with the cellular dNTPs at the viral DNA polymerase⁽⁵²⁾.

In vitro studies have also shown that HU inhibits murine CMV (MCMV) replication and DNA synthesis⁽⁵³⁾. Recently, the effect of Didox on the pathogenicity of and host responses to MCMV infection was investigated. *In vitro* experiments suggested that Didox had moderate antiviral activity against MCMV. *In vivo* Didox administration did not decrease viral load in livers and spleen of MCMV-infected mice, and unexpectedly *in vivo* prophylactic Didox treatment had adverse effects of increased inflammatory cytokine transcription in liver and suppressed CD8⁺ T-cells⁽⁵⁴⁾.

Although Didox lacked beneficial efficacy when used alone against MCMV, it has not been evaluated for activity against human CMV or in combination with anti-CMV drugs. Previous data strongly suggest that RR inhibitors potentiate the activity of anti-HIV and anti-HSV drugs. We therefore sought to evaluate the potential for synergy between RR inhibitors and nucleoside analog inhibitors of CMV.

Table 1- Ribonucleotide Reductase Inhibitors

Drug	Mechanism of action	General Uses	Use in antiviral therapy	Toxicity
<p>HU</p> 	<p>-inhibits RR -increases fetal hemoglobin</p>	<p>-treatment of cancer -treatment of sickle cell anemia</p>	<p>-potentiates anti-HIV activity of ddI and other dideoxynucleoside analogs -synergizes HSV antivirals (GCV, ACV, etc.)</p>	<p>-gastrointestinal disturbances -bone marrow depression -oral ulcers -skin rashes</p>
<p>Didox</p> 	<p>- inhibits RR through iron chelation</p>	<p>-treatment of multiple myeloma -in phase II trials for treatment of breast cancer</p>	<p>- advantageous over HU when administered with ddI in HIV therapy - moderate antiviral activity against MCMV but lacked efficacy <i>in vivo</i></p>	<p>-myelosuppressive - increases inflammatory cytokines - suppresses CD8⁺ T-cells</p>
<p>Trimidox</p> 	<p>- inhibits RR through iron chelation</p>	<p>- potentiates the anti-tumor activity of Cisplatin and Cyclophosphamide -synergizes the activity of Temozolomide</p>	<p>advantageous over HU when administered with ddI in HIV therapy</p>	<p>- myelosuppressive</p>

MATERIALS AND METHODS

Cells-

Fetal lung fibroblast MRC-5 cells (ATCC CCL-171) and retinal pigment epithelium ARPE-19 cells (ATCC CRL-2302) were obtained from ATCC. MRC-5 cells were propagated in modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/L penicillin, 10 mg/L streptomycin (Gibco-BRL) (MEM). ARPE-19 cells 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). All cell cultures were maintained at 37°C in a 5% CO₂ atmosphere.

Viruses-

CMV strain BADrUL131-Y4 was derived from a BAC clone of the CMV strain AD169 genome that had been modified in *E.coli* by Wang and Shenk to contain a GFP reporter cassette for efficient detection and quantification of viral infection ⁽⁵⁵⁾. Subsequently the *UL131* mutation was repaired to express a functional UL131 protein that allows efficient entry and replication in both MRC-5 and ARPE-19 cells ⁽⁵⁶⁾. Strain RC2626 is a Towne strain CMV. A luciferase expression cassette was introduced into the *US2-US6* region to create recombinant virus RC2626 ⁽⁵⁷⁾. Due to mutation in *UL130*, RC2626 can replicate efficiently in fibroblasts but not in epithelial cells. Viral titers were determined by limiting-dilution in 96-well plates using MRC-5s. Stocks were stored at -80°C.

Drugs-

GCV and ACV were purchased from InvivoGen and Sigma respectively. HU was obtained from Sigma. Didox and Trimidox were a gift from Howard Elford (Molecules for Health Inc., Richmond, VA). All drugs were solubilized in water to produce stock solutions.

GFP-based assay for susceptibility of CMV to individual drugs-

Clear-walled, clear/flat-bottomed 96-well plates containing confluent monolayers of MRC-5 or ARPE-19 cells were infected with BADrUL131-Y4 (MOI=0.015) and incubated for 1 h. Twelve two-fold serial dilutions of the drugs (GCV, ACV, HU, Didox and Trimidox) were prepared in 200 μ l MEM in triplicate in a 96-well format. These drug dilutions were transferred to infected cells to produce final drug concentrations as follows- 0.06 to 62.5 μ M GCV, 0.4 to 467.5 μ M ACV, 9.7 to 10000 μ M HU, 0.97 to 1000 μ M Didox and Trimidox. Triplicates of no-drug controls and no-virus controls were included on each plate. After incubation for 14 days, fluorescence from the cultures was measured as relative fluorescence units (RFU) using Biotek Synergy HT Multimode Microplate Reader. Each experiment was performed three times for each drug. For comparability between experiments, data from each experiment were normalized by converting RFU to “percent maximum RFU” for that experiment. Best-fit four-parameter curves were fitted to the data (fluorescence measured at different drug concentrations) using Prism Software (Graph Pad, Inc.). Fifty-percent inhibitory concentrations (IC_{50}) (drug concentrations at which fluorescence was reduced by half) were determined as the inflection points of the four-parameter curves.

Luciferase reporter gene assay for susceptibility of CMV to individual drugs-

Clear -walled, clear/flat-bottomed 96-well plates containing confluent monolayers of MRC-5 fibroblast cells were infected with RC2626 (MOI=0.03) and incubated for 1 h. Twelve two-fold serial dilutions of the drugs (GCV, ACV, HU, Didox and Trimidox) were prepared in 200 μ l MEM in triplicate in a 96-well format. These drug dilutions were transferred to infected cells to produce final drug concentrations as follows- 0.06 to 62.5 μ M GCV, 0.4 to 467.5 μ M ACV, 9.7 to 10000 μ M HU, 0.97 to 1000 μ M Didox and Trimidox. Triplicates of no-drug controls and no-virus controls were included on each plate. After incubation for 5 days, 50 μ l supernatants were transferred to a black-walled, clear/flat-bottomed 96-well plate containing confluent MRC-5 monolayers. After 24, 100 μ l Steady-Glo luciferase assay reagent (Promega) was added and the luciferase activity was measured in relative light units (RLU) using Biotek Synergy HT Multimode Microplate Reader. For comparability between experiments, means of data from three independent experiments were normalized by converting RLU to “percent maximum RLU” for each experiment. Best-fit four-parameter curves were fitted to the data. IC₅₀ were determined as above. Each experiment was performed at least three times for each drug.

Analysis of the effect of drug combinations on CMV replication-

Drug-drug combination analyses for GCV and RR inhibitors (HU, Didox or Trimidox) were performed using the luciferase reporter gene assay described above. The experimental design utilized a checkerboard dilution matrix consisting of all possible combinations of two-fold serial dilutions of the two drugs and the drugs used individually. The RR inhibitor (HU/Didox/Trimidox) was serially diluted along the ordinate, while GCV was diluted along the abscissa. The concentration of GCV ranged from 0 to 8 μ M and that of the RR inhibitors ranged

from 0 to 600 μM for HU, 0 to 250 μM for Didox, and 0 to 150 μM for Trimidox. The drug combinations were analyzed for synergy/antagonism using MacSynergy II software ⁽⁵⁸⁾.

Toxicity Assay-

Black-walled, clear/flat-bottomed 96-well plates containing confluent monolayers of MRC-5 fibroblast cells were incubated with twelve two-fold serial dilutions of the drugs (GCV, ACV, HU, Didox and Trimidox) or with drug combinations (GCV + HU/Didox/Trimidox in a checkerboard format as above) in 200 μl MEM in triplicate. Triplicates of no-drug controls and no-cell controls were included on each plate. After incubation for 5 days, 100 μl of supernatants were removed and 100 μl CellTiter-Glo assay reagent (Promega) was added to each well. Luminescence was measured as above. Each experiment was performed in duplicate for individual drugs and in triplicate for each drug combination. Best-fit four-parameter curves were fitted to the data (luciferase activities measured at different drug concentrations) using Prism Software and used to calculate 50% toxic dose (TD_{50}) values (amount of drug required to reduce luciferase activity by 50%).

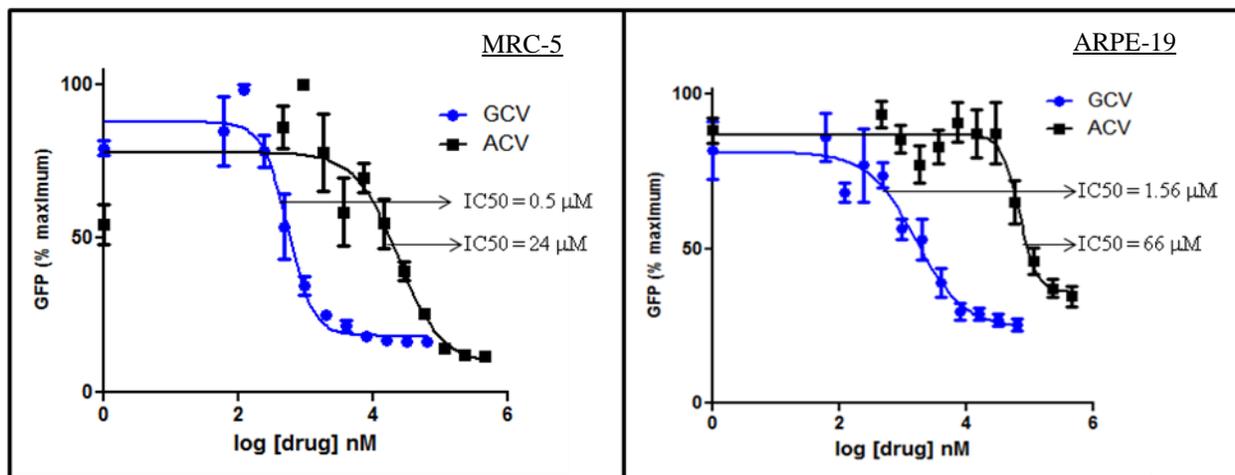
RESULTS

GFP-based assay for susceptibility of CMV to individual drugs-

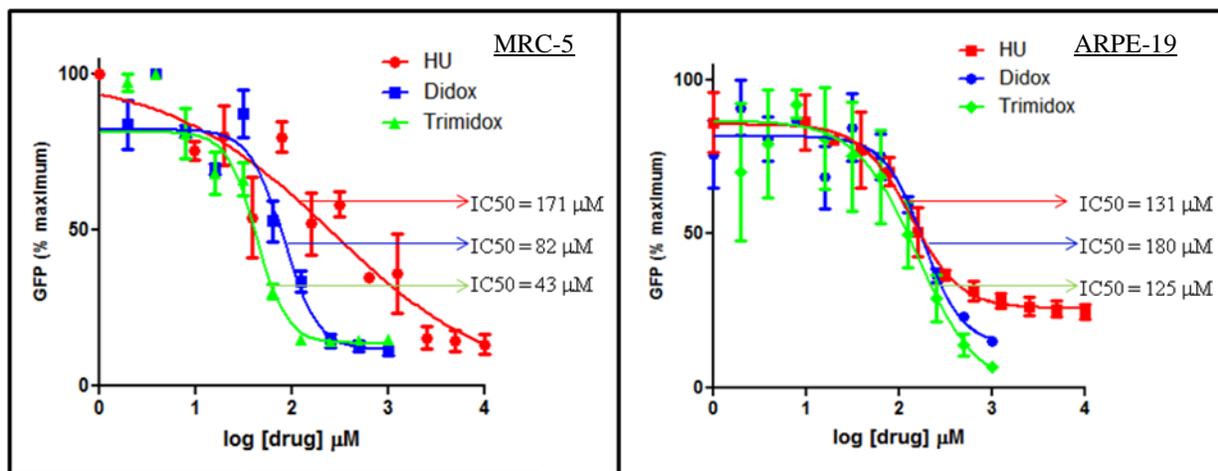
The GFP-based assay was used to determine susceptibilities of CMV to GCV, ACV, HU, Didox, and Trimidox in MRC-5 and ARPE-19 cells. Confluent monolayers of MRC-5 or ARPE-19 cells were infected with GFP-tagged CMV BADrUL131-Y4 (MOI=0.015) and incubated with twelve two-fold serial dilutions of the drugs (GCV, ACV, HU, Didox and Trimidox) prepared in triplicate in a 96-well format. After incubation for 14 days, GFP signal from the cultures was determined as a measure of viral spread (surrogate for viral plaque formation). Best-fit four-parameter curves were fitted to the data using Prism Software and used to calculate IC₅₀ values for each drug.

Figure 2 shows dose response curves from triplicate data for each drug. From these curves the IC₅₀s for each drug were determined. The results are summarized in Table 2. IC₅₀s for antivirals GCV and ACV in MRC-5 cells were calculated as 0.5 μM and 24 μM, respectively, and in ARPE-19 cells as 1.56 μM and 66 μM, respectively. These results suggest that GCV has more efficacy than ACV against CMV, as can be seen by its much lower IC₅₀ than ACV. The IC₅₀s for RR inhibitors HU, Didox and Trimidox against CMV in MRC-5 cells were found to be 171 μM, 82 μM, and 43 μM respectively. This suggests that in MRC-5 the potency of these RR inhibitors against CMV are in the order Trimidox > Didox > HU. However, in ARPE-19 cells, the IC₅₀s of these drugs were not found to be much different. The IC₅₀s for HU, Didox and Trimidox in ARPE-19 cells were found to be 131 μM, 180 μM, and 125 μM respectively.

These studies were conducted in two different cell types because of concerns that antiviral activities could vary significantly between cell types. This was a particular concern for RR inhibitors because they target host reductase, which could be expressed to a greater or lesser extent in different cell types. However, we observed only a two- to three- fold difference in the IC_{50} s of the drugs in MRC-5 vs. ARPE-19 cells. Based on these results, subsequent synergy studies were conducted in only one cell type (MRC-5 cells).



(a)



(b)

Fig 2- CMV inhibition by antivirals and RR inhibitors in two different cell types (fibroblasts and epithelial cells). Confluent MRC-5 or ARPE-19 cultures in 96-well plates were infected with BADrUL131-Y4 (MOI = 0.015) and incubated in the presence of different concentrations of the indicated drugs for 14 days. Fluorescence in the cultures was determined as a measure of virus replication and spread in each well. Best-fit four parameter curves were fitted to the data and used to calculate IC_{50} values for each drug. Each data point represents the means of three independent experiments.

Table 2- IC₅₀s measured by GFP-based assay in two cell types.

Drug	IC ₅₀ (μM) MRC-5 cells	IC ₅₀ (μM) ARPE-19 cells
GCV	0.5	1.56
ACV	24	66
HU	171	131
Didox	82	180
Trimidox	43	127

Developing the Luciferase Reporter Gene Assay-

Yield reduction reflects the ability of antiviral agents to inhibit production of infectious virus. It is a powerful technique for evaluating the efficacy of potential antiviral compounds. It is a stringent measure of the process of virus replication, and hence drug activity. The capacity of drugs to reduce virus titer by several orders of magnitude *in vitro* correlates well with *in vivo* drug efficacy. Yield reduction permits the determination of antiviral activity over a wide dynamic range of 0-10⁶ pfu/ml as compared to the GFP-based assay which showed a dynamic range of at most 2 logs. Thus, yield reduction may be more suitable than the GFP-based assay for detecting moderate synergistic effects of drug combinations.

However, the major disadvantage of yield reduction assay is that it is time consuming and laborious to titrate infectious virus in triplicate 96-well plates, as needed for synergy studies. In an attempt to reduce the assay time and to produce quantitative results comparable to infectious virus yield, we developed a luciferase-based assay to quantitate viral yield.

Growth Characteristics of RC2626

The first aim was to determine whether luminescence can be utilized as a surrogate marker for viral yield so as to use luciferase activity as a read out in viral yield reduction assay. Black-walled, clear/flat-bottomed 96-well plates containing confluent monolayers of MRC-5 fibroblast cells were infected with 3-fold serial dilutions of a stock of known titer of RC2626. The inoculums ranged from $0.1-10^4$ pfu/well. The cultures were incubated and luciferase activity in the cells was measured every 24 h post infection (p.i.) for seven days using the Steady-Glo Luciferase Assay System.

Figure 3a shows curves obtained by plotting luciferase activity as a function of time after infection with four different MOIs of RC2626 (0.03, 0.1, 0.3, 1). Peak levels were reached about five days p.i but were similar on day three and later regardless of MOI. Luciferase activity was only MOI-dependent at 24 and 48 h.p.i. Figure 3b shows the relationship between infectious units added per well and luciferase activity measured 24 and 48 h later. A linear relationship was observed between infectious units of virus and luciferase activity. However, because the 24 h data gave a more linear relationship over a broader dynamic range (10^1 to 10^4 pfu/well), subsequent assays using luciferase activity as a surrogate for infectious units in culture supernatants (*i.e.*, virus yield) were conducted 24 h.p.i.

These results suggest that the luciferase reporter assay can be used as a marker for viral yield within this range. Moreover, this method allows measurement over a very wide dynamic range of >3 logs. It is time and labor saving as compared to measuring infectious yield by virus titration. This assay can enable accurate quantification of small changes in the luciferase activity (virus yield) with the addition of varying amounts of anti-CMV agents.

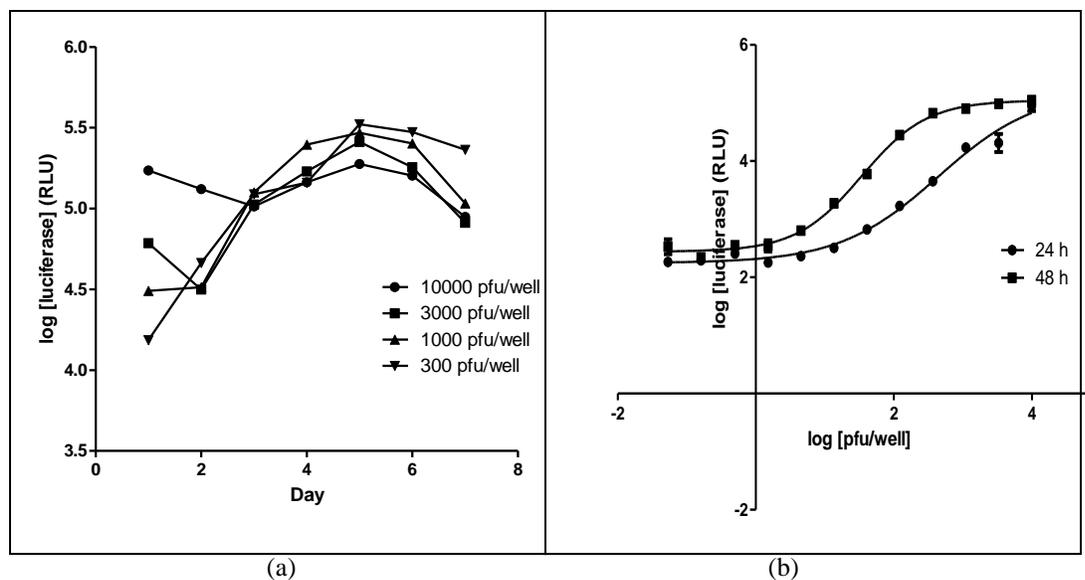


Fig 3- (a) Luciferase expression after infection with RC2626. Cells were infected with RC2626 at a range of MOIs and luciferase expression was determined in cell-lysates at the indicated times after infection. For simplicity only four MOIs are shown. (b) Luciferase data collected 24 and 48 h.p.i are plotted vs. the full set of MOIs used (converted to pfu/well).

Optimizing the luciferase reporter gene assay

The assay was further optimized to work within its linear range (10^1 - 10^4 pfu/well or 10^2 - 10^5 RLU as determined in figure 3) such that changes in the viral titer are reflected by changes in luciferase activity. To optimize the protocol an experiment was designed to determine susceptibility of RC2626 to HU. Fibroblast cultures in a 96-well plate were infected with RC2626 (MOI=0.03) and incubated in the presence of different concentrations of HU for five days. On the fifth day three different volumes of the culture supernatants (10 μ l, 50 μ l, and 100 μ l) were transferred to fresh MRC-5 cells and the resulting luciferase activities were measured 24 hours p.i. (Figure 4). Based on results in Figure 3, to be in the linear response range the peak luciferase activity should be below 10^5 RLU. Thus, the 10 and 50 μ l supernatant transfers were below this threshold, while 100 μ l transfer was potentially out of the linear range. This conclusion was supported by the IC_{50} values calculated from each data set.

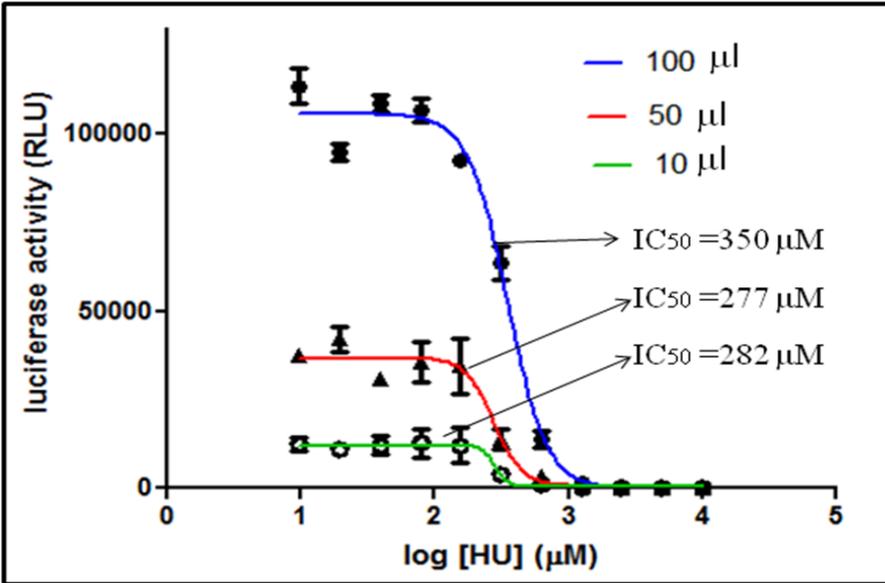


Fig 4- Dose response curves for HU inhibition of viral yield in fibroblast cells. Confluent MRC-5 cultures in 96-well plates were infected with RC2626 (MOI 0.03) and incubated in the presence of different concentrations HU for five days. Three different volumes (10 μ l, 50 μ l, and 100 μ l) of the day five culture supernatants were transferred to a fresh plate of confluent MRC-5 cells and incubated for 24 h. IC_{50} s were calculated from the luciferase activities in the cells measured 24 hours p.i.

That the IC_{50} for HU shifted from 350 μ M for data obtained by transfer of 100 μ l supernatant to 277 μ M and 282 μ M for 50 μ l and 10 μ l transfers, respectively, suggests that the 100 μ l transfer may have exceeded the upper limit of the linear range, thus requiring higher HU concentrations to reduce virus titer into the linear range and artificially elevating the apparent IC_{50} . That no difference was observed between IC_{50} s from 50 and 10 μ l transfers suggests that these data sets were within the linear range and provided accurate measurement of the IC_{50} . Thus, subsequent experiments used 50 μ l supernatant transfers for measurement of viral yield as this provided a wider dynamic range of signal than 10 μ l and yet remained within the linear range.

Luciferase reporter gene assay for susceptibility of CMV to individual drugs-

The luciferase reporter assay described above was used to determine susceptibilities of RC2626 to GCV, ACV, HU, Didox, and Trimidox. Each IC_{50} determination was conducted using triplicate cultures and for each drug IC_{50} values were determined from three independent experiments. Table 3 shows the results of each independent IC_{50} determination, their means and standard deviations. Figure 5 shows the dose response curves for combined data from three independent experiments for each drug. To improve comparability between experiments, RLU were normalized as “percent maximum RLU” for each experiment before being averaged between experiments. IC_{50} s for antivirals GCV and ACV were calculated as 0.6 μ M and 24 μ M respectively. This confirms that ACV has limited efficacy against CMV as compared to GCV. The IC_{50} s for RR inhibitors HU, Didox and Trimidox against CMV were found to be 221 μ M, 103 μ M, and 36 μ M, respectively. This suggests that Trimidox is more potent than Didox which in turn is more potent than HU.

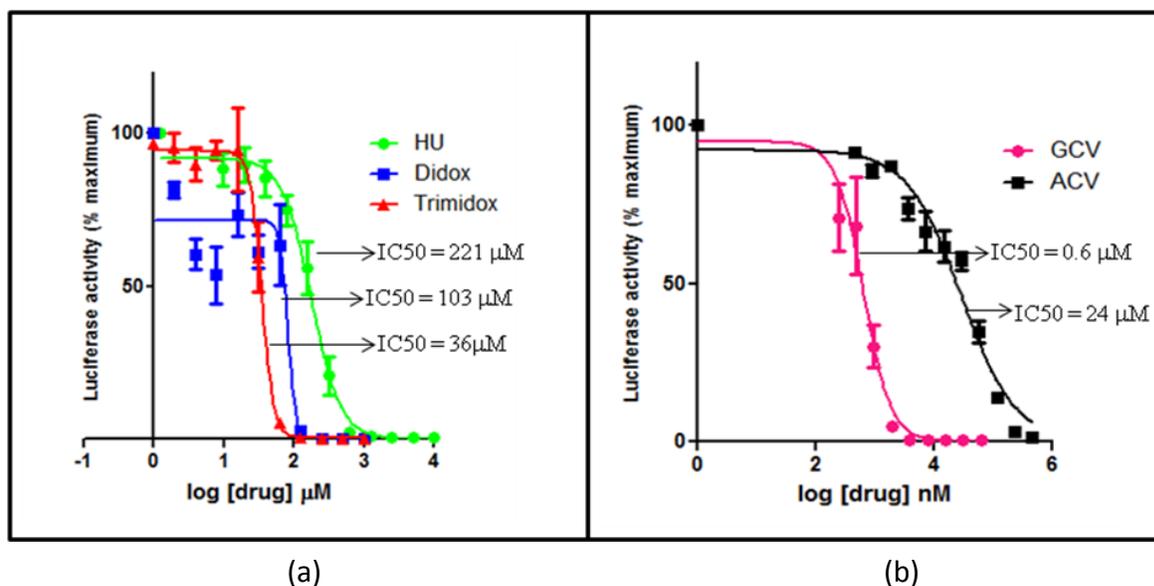


Fig 5- CMV inhibition by antivirals and RR inhibitors. Confluent MRC-5 cultures in 96-well plates were infected with RC2626 (MOI = 0.03) and incubated in the presence of different concentrations of the indicated drugs for five days. 50 μ l of day-five culture supernatants were transferred to fresh confluent MRC-5 cultures. After 24 h luciferase activities were determined. Data from three independent experiments were normalized by converting RLU to “percent maximum RLU” for each experiment then averaged. Best-fit four-parameter curves were fitted to the data and used to calculate IC_{50} values for each drug.

Table 3- Yield Reduction IC_{50} values of individual drugs determined by luciferase reporter assay

Drug	IC_{50} (μ M)*	Mean IC_{50} (μ M)	Standard Deviation	Toxicity IC_{50} (μ M)
GCV	0.6 0.634 0.716	0.6	0.06	ND
ACV	25.99 20.59 25.92	24	3.1	ND
HU	277 210.7 176	221	52	14500
Didox	120 107 82	103	19.31	256
Trimidox	34.73 38.13 36.28	36	1.718	166

* results of three independent experiments, ND-not determined

Analysis of the effect of drug combinations on CMV replication-

Synergy is the interaction of two or more agents or forces so that their combined effect is greater than the sum of their individual effects. Therapeutic synergy is when the inhibitory effect of two drugs is greater than the sum of their individual inhibitory effects. The eagerness for using drug combinations to achieve synergy is tempered by concerns that the therapeutic synergy of a combination may be accompanied by synergistic toxicity.

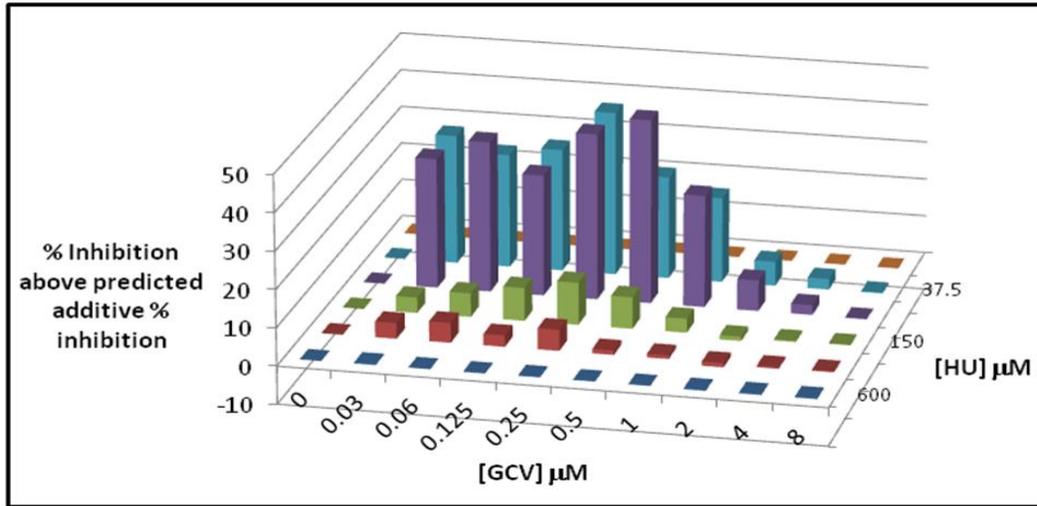
Confluent MRC-5 cultures in 96-well plates were infected with RC2626 (MOI = 0.03). The experimental design utilized a checkerboard dilution matrix of all permutations of two-fold serial dilutions of each of the drugs, including the drugs used individually. The RR inhibitor (HU/Didox/Trimidox) was serially diluted along the ordinate, while GCV was diluted along the abscissa. 50 μ l of day-five culture supernatants were transferred to fresh confluent MRC-5 cultures. After 24 h luciferase activities were determined as a measure of infectious virus in the transferred supernatants. Each experiment was performed three times.

Synergy between GCV and RR inhibitors was evaluated using MacSynergy II software ⁽⁵⁸⁾. MacSynergy II uses luciferase values to calculate % inhibitions for the two drugs when used alone. A theoretical additive value for each drug combination is calculated based on the values generated by the drugs alone. The theoretical additive values are subtracted from the observed experimental values generated by each drug combination to give a value of synergy (positive value) or antagonism (negative value). These synergy and/or antagonism values are plotted on a three-dimensional graph with their corresponding drug combinations. Areas of the graph below zero indicate antagonism, whereas areas above zero indicate synergy. If the interactions are additive, the surface appears as a horizontal plane at 0% above the calculated additive surface in the resulting difference plots.

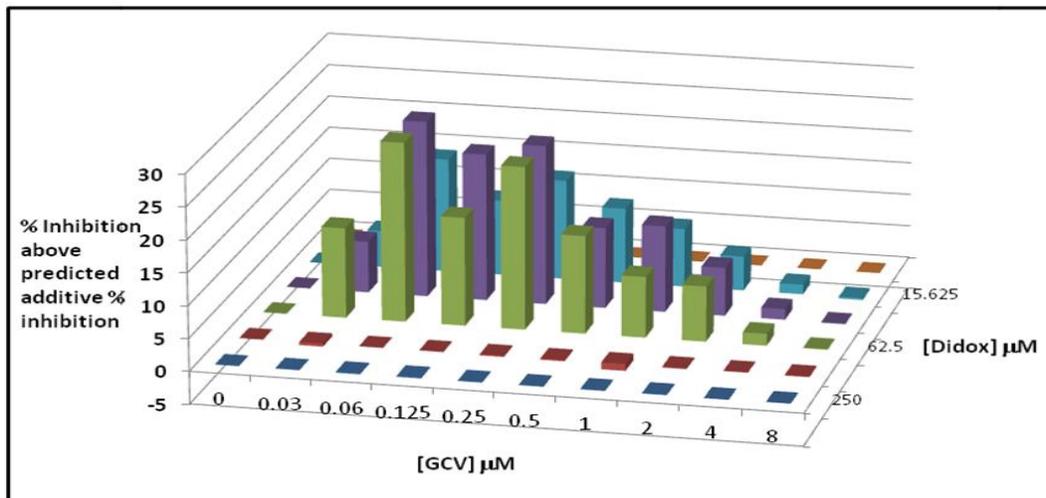
Figure 6a shows synergistic inhibition of CMV replication by a combination of GCV and HU. Sixty different concentration combinations of GCV+HU were analyzed for their activity against CMV. The combination of 75 μM HU + 0.5 μM GCV exhibited maximum synergy wherein the observed inhibitory effect of the combination was found to be ~50% greater than the predicted additive inhibition of the two drugs. Considerable synergy (30-40 %) was observed at very low doses of GCV (0.03, 0.06, 0.125, 0.25 μM) in combination with low doses of HU (37.5 and 75 μM). This suggests that addition of small amounts of HU (concentrations less than the IC_{50} of HU, which is 221 μM) can increase the effectiveness of GCV against CMV.

Similarly, combination of GCV and Didox also exhibited synergy (Figure 6b). Between 5-30% synergy was observed at concentrations of GCV ranging from 0.03-2 μM and concentrations of Didox ranging from 15-60 μM . These concentrations of Didox which are synergistic with GCV are less than the IC_{50} of Didox (103 μM).

Likewise, combinations of GCV and Trimidox were also tested for their ability to inhibit CMV replication using the luciferase reporter gene assay and the results were analyzed using MacSynergy II. Concentrations of Trimidox ranged from 0-150 μM . About 20% synergy was observed at concentrations of GCV ranging from 0.03-0.5 μM and concentrations of Trimidox ranging from 9-36 μM . Combination of GCV with Trimidox appears to be less synergistic as compared to HU and Didox (Figure not shown).



(a)



(b)

Fig 6- Synergistic inhibition of CMV replication by GCV + HU/Didox. A checkerboard assay of GCV and HU/Didox combinations was evaluated for CMV yield reduction using the luciferase reporter gene assay. Synergistic effects (Z axis) for all drug combinations were calculated as % inhibition (observed) - % inhibition (predicted additive).

Toxicity Assay-

Toxicities for each RR inhibitor (HU, Didox, Trimidox) and drug combinations (GCV + HU/Didox/Trimidox) were determined using the CellTiter-Glo Assay (Promega). The CellTiter-Glo reagent consists of a stable form of luciferase derived from the gene from firefly *Photuris pennsylvanica*. The reagent causes cell lysis and results in generation of a luminescent signal on reaction between luciferin and ATP released from the cells. The luminescence signal is proportional to the amount of ATP present in the cells and that in turn is proportional to the number of viable cells present in the culture.

MRC-5 fibroblast cells were incubated with twelve two-fold serial dilutions of the drugs (GCV, ACV, HU, Didox and Trimidox) in a 96-well format. After incubation for 5 days, 100 μ l supernatants were discarded and 100 μ l CellTiter-Glo assay reagent was added to each well and luminescence was measured. Each experiment was performed in duplicate. Best-fit four-parameter curves were fitted to the data (luciferase activities measured at different drug concentrations) using Prism Software.

Figure 7 shows TD₅₀ values for each drug calculated from toxicity assays performed in duplicate. From these curves, the TD₅₀s for RR inhibitors HU, Didox and Trimidox were calculated as 14500 μ M, 256 μ M and 166 μ M, respectively (Table 3). This suggests that HU might be considerably less toxic as compared to Didox and Trimidox. These results also suggest that the anti-CMV activities of these drugs are not due to non-specific toxicities since the CMV inhibitory IC₅₀s of these drugs are much lower than their TD₅₀s. Also, HU, Didox and Trimidox exhibit some toxicity at 600 μ M, 250 μ M and 150 μ M, respectively, but each are non-toxic at concentrations which show synergy with GCV.

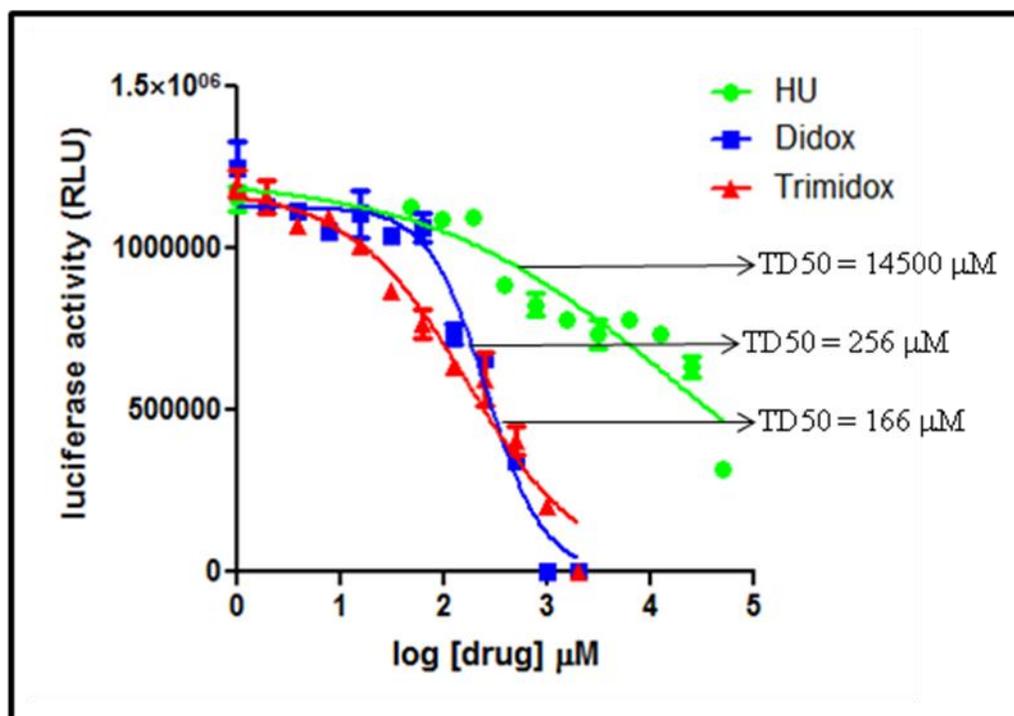
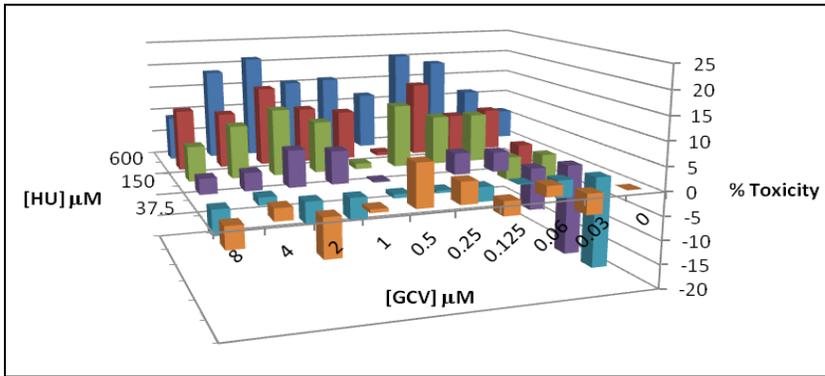
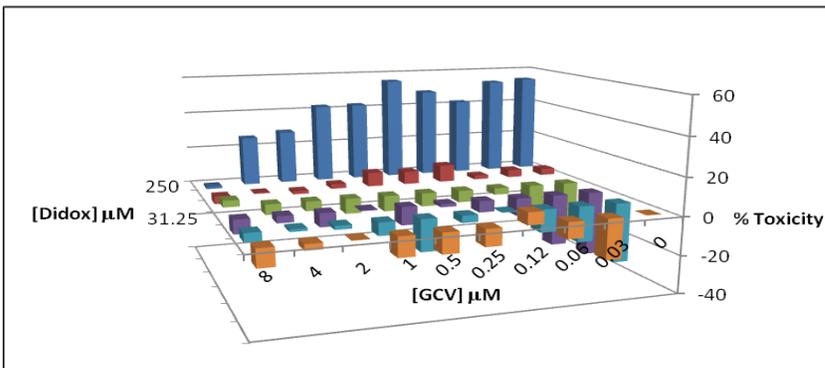


Fig 7- Toxicity of RR inhibitors. Confluent MRC-5 cultures in 96-well plates were incubated in the presence of different concentrations of the indicated drugs for five days. Cell viability was measured using CellTiter-Glo reagent. Best-fit four-parameter curves were fitted to the data and used to calculate TD₅₀ values for each drug.

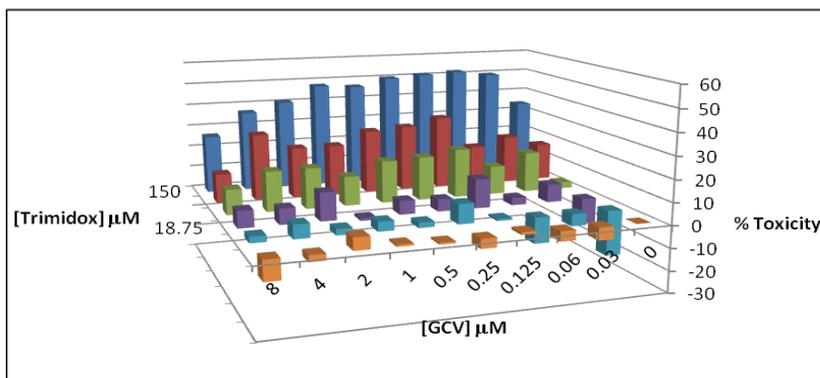
The three drug combinations (GCV + HU/Didox/Trimidox) were also evaluated for synergistic toxicity. The results are shown in Figure 8. Our results suggest that GCV is non-toxic at the concentrations used in these experiments. Combinations of GCV + HU exhibit very low toxicity (< 10%) at concentrations which are synergistic against CMV replication (*i.e.*, 37.5 and 75 μM HU). Toxicities of 10-21 % were observed for drug combinations using more than 75 μM HU (Figure 8a). GCV + Didox exhibited toxicity (60%) only at high doses of Didox (250 μM). No toxicity was observed at Didox concentrations which exhibited synergy (15-60 μM) (Figure 8b). Similarly, combinations of GCV + Trimidox were less toxic at Trimidox concentrations which exhibited synergy (9-36 μM) (Figure 8c). Significant toxicity was observed for combinations involving greater than 36 μM Trimidox.



(a)



(b)



(c)

Fig 8- Toxicity of drug combinations. A checkerboard assay of GCV and HU/Didox/Trimidox combinations was evaluated for toxicity. Total toxicity (Z axis) for all drug combinations was calculated as % cell growth inhibition of no-drug controls.

DISCUSSION

Plaque reduction assay is one of the basic approaches used to assay antivirals. Plaque reduction assays use a constant number of viral particles and vary the concentration of test substance. Antiviral activity is assessed as the reduction in the number of viral plaques in a cell monolayer. This assay most commonly involves infection with virus followed by counting of viral plaques (foci) as a measure of viral replication. Traditionally plaques are identified by cytopathic effect (CPE), but CPE can be difficult to read in some cell types, as is the case for ARPE-19 cells. Methods to identify infected cells, such as antibody staining or the use of GFP-tagged viruses can facilitate plaque counting under these circumstances. However, the major disadvantage of this assay is that it is difficult to count as many as 50-100 plaques per well with consistent accuracy and counting plaques is labor intensive. Determination of plaques in cell types that result in distinct plaque morphologies can also be subjective and biased. Thus, we hoped that quantification of GFP fluorescence might be a more objective and comparable means of measuring viral spread in different cell types.

Yield reduction assay is another basic approach used to assay antivirals. It reflects the ability of antiviral agents to inhibit production of infectious virus rather than formation of a plaque. It is a powerful technique for evaluating the efficacy of potential antiviral compounds. It is a more stringent measure of the process of virus replication, and hence drug activity, as compared to the plaque reduction assay. The capacity of drugs to reduce virus titer by several orders of magnitude *in vitro* correlates well with *in vivo* drug efficacy. Compared to plaque reduction, yield reduction

permits the determination of antiviral activity over a much wider dynamic range (for CMV, 0-10⁶ pfu/ml). This allows the measurement of more profound drug effects that by plaque assay might be too subtle to detect. However, titration of the virus to determine yield is very labor intensive and may not be practical for synergy studies. In an attempt to reduce the assay time and to produce quantitative results comparable to measuring infectious units by titration, we developed a luciferase-based assay to quantitate viral yield.

In vitro drug susceptibility studies of CMV have almost exclusively been conducted in fibroblast cell culture. However, CMV infects a variety of other cell types including: endothelial cells, epithelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, monocytes/macrophages, and granulocytes. Thus, it might be necessary to study drug susceptibility of CMV in several clinically relevant cell types. Indeed, recent studies from our group found that the investigational anti-CMV drug Maribavir is active in fibroblasts but has no anti-CMV activity in ARPE-19 epithelial cells (Meza and McVoy, unpublished data). In our GFP-based assay, the cell types chosen were based on standard – MRC-5 fibroblasts (a regular means for the study of CMV infection *in vitro*) and clinical relevance – ARPE-19 retinal pigment epithelium cells (relevant *in vivo* in CMV retinitis). GFP-expressing CMV strain BADrUL131-Y4 was used in this assay because of its ability to efficiently enter and replicate in both MRC-5 and ARPE-19 cells. Susceptibility of BADrUL131-Y4 to antivirals (GCV, ACV) and RR inhibitors (HU, Didox, Trimidox) was tested using the GFP-based spread assay in the two different cell types- fibroblast and epithelial cells. For the luciferase reporter gene assay (yield assay), luciferase-expressing CMV strain RC2626 was used. Due to a mutation in *UL130*, RC2626 can replicate efficiently in fibroblasts but not in epithelial cells. Thus, susceptibility of RC2626 to each drug was tested using this assay in MRC-5 cells only.

Our results from the GFP-based assay (fibroblasts and epithelial cells) as well as luciferase assay indicate that GCV has much lower IC_{50} s than ACV (0.6 vs. 24 μ M in fibroblasts and 1.5 vs. 66 μ M in epithelial cells). This confirms earlier reports that GCV is more potent than ACV against CMV (literature reported IC_{50} s for GCV = 0.5-8 μ M and ACV = 64-273 μ M). Although in general IC_{50} s were up to two-fold lower in fibroblasts vs. epithelial cells, that these differences were neither large nor statistically significant led us to conclude that for the compounds tested, cell type (fibroblast vs. epithelial cells) does not have a significant impact on antiviral activity.

In vitro studies have also shown that HU inhibits murine CMV (MCMV) ⁽⁵³⁾. HU has also been reported to inhibit human CMV *in vitro* and although an IC_{50} was not determined, cytopathic effect endpoint titration numbers reported suggest an IC_{50} of about 100 μ M ⁽⁵⁹⁾. *In vitro* experiments found that Didox has moderate antiviral activity against MCMV (IC_{50} =10-25 μ M). However, these RR inhibitors have not yet been extensively studied for their activity against human CMV. Our results suggest that HU, Didox, and Trimidox have anti-CMV activity when used alone. When tested in MRC-5 cells using the GFP-based assay and luciferase assay, the potency of RR inhibitors against CMV was found to be in the order Trimidox > Didox > HU. We might have expected a cell type-dependence for RR inhibitors since the expression levels of RR can vary with cell type. However, our results indicate that the anti-CMV activities of HU, Didox, and Trimidox *in vitro* were only two- to three-fold different in MRC-5 vs. ARPE-19 cells (Table 1). Since each of the drugs was found to have similar efficacy in both cell types, performing drug susceptibility assays and synergy studies in MRC-5 cells only is justified. However, the use of multiple, clinically relevant cell types to test the efficacy of antiviral drugs would be optimum to reinforce our observations.

In a study conducted to evaluate the potential of HU to cause adverse effects on reproduction and development in humans, the peak plasma achievable non-toxic concentrations of HU were found to be 20-50 mg/L (264-660 μM)⁽⁶⁰⁾. Since the plasma achievable concentration of HU is greater than our measured IC_{50} ($\sim 200 \mu\text{M}$), HU might have potential use as an anti-CMV drug *in vivo*. This also suggests that doses used for combination therapy, which are likely to target levels of HU below its IC_{50} should be achievable and non-toxic. Thus, the effects of HU on CMV replication *in vivo* need to be investigated. Recently, the effect of Didox on the pathogenicity of and host responses to MCMV infection was investigated. *In vitro* experiments suggested that Didox had moderate antiviral activity against MCMV ($\text{IC}_{50}=10-25 \mu\text{M}$). However, *in vivo* Didox administration did not decrease viral load in livers and spleen of MCMV-infected mice, and paradoxically *in vivo* prophylactic Didox treatment had adverse effects of increased inflammatory cytokine transcription in liver and suppressed CD8^+ T-cells. Although Didox was not found to be effective on its own against MCMV, its activity needs to be further investigated as part of anti-CMV drug combinations.

Combinations of GCV + RR inhibitors were tested for their efficacy against CMV using the luciferase reporter assay. Considerable synergy (30-40 %) was observed at very low doses of GCV (0.03, 0.06, 0.125, 0.25 μM) in combination with low doses of HU (37.5 and 75 μM). This indicates that addition of HU at concentrations considerably below its IC_{50} (221 μM) increases the effectiveness of GCV against CMV. Combinations of GCV + Didox/Trimidox were also found to be synergistic in inhibition of CMV replication.

Thus, using a combination of GCV + RR inhibitors to treat CMV infections could allow a decrease in the GCV dose and hence alleviate problems such as dose-limiting toxicity and development of drug-resistance. The synergy plots will assist in determining the drug

combinations that exhibit optimal synergy *in vitro* so that such combinations can be further tested in animal models.

Toxicities for RR inhibitors (HU, Didox, Trimidox) and drug combinations (GCV + HU/Didox/Trimidox) indicate that HU might be considerably less toxic as compared to Didox and Trimidox (Table 3). The results further suggest that the anti-CMV activity of these drugs are not due to non-specific toxicities since the CMV inhibitory IC₅₀s of these drugs are much lower than their TD₅₀s. Moreover, while HU, Didox and Trimidox exhibit some toxicity at high concentrations, each is non-toxic at concentrations which show synergy with GCV. The three drug combinations (GCV + HU/Didox/Trimidox) were also evaluated for synergistic toxicity. Our results suggest that GCV is non-toxic at the concentrations used in these experiments. Combinations of GCV + HU exhibit very low toxicity at concentrations which are synergistic against CMV replication. GCV + Didox exhibited toxicity (up to 60%) only at high doses of Didox (250 µM) but again, GCV did not impact toxicity- it was mostly due to Didox alone. No toxicity was observed at Didox concentrations which exhibited synergy. Similarly, combinations of GCV + Trimidox were less toxic at Trimidox concentrations which exhibited synergy. Of the three drug combinations, GCV + HU exhibited the lowest toxicity. This observation is in accordance with our individual drug toxicity results which indicate that the TD₅₀ for HU is much greater than Didox and Trimidox.

Since GCV + HU exhibits maximum synergy with minimal toxicity, it might be the best candidate for further animal studies. Our results suggest that a combination of 0.25 µM GCV + 37.5 µM exhibits optimal synergy and therefore should be considered for *in vivo* studies. A major drawback of animal studies is that CMVs are extremely species-specific and do not infect species other than their natural hosts. Nevertheless animal studies are essential for pre-clinical

drug development. A considerable amount of CMV immunology has been deduced from mouse models. However, MCMV is not very sensitive to GCV and thus might not be a very useful model. Guinea pig CMV (GPCMV) is the only small animal model for congenital infections. Susceptibility of GPCMV to GCV has been improved by inserting the HCMV UL97 gene into the GPCMV genome in place of the homolog, GP97 (IC50 of GCV= 174 μ M in wild-type GPCV vs. 15 μ M in GPCV::*UL97*)⁽⁶¹⁾. This might be a good animal model for testing GCV + HU. Rhesus CMV (RhCMV) is found to infect the fetus with similar sequelae to human CMV but transmission during pregnancy has not been demonstrated. Disadvantages of this model include increased cost of the animals and limited availability of seronegative animals. Thus, the rhesus model could be utilized but only on a limited scale.

Our data suggests that RR inhibitors may be useful in combination with GCV. HU may be a preferred candidate due to strong synergy with GCV, low toxicity, and clinical history. As a potential candidate for use for treatment of CMV infections in humans, HU has the advantage of being a licensed drug for sickle cell anemia and cancer treatment. Therefore, HU could be used with GCV in compassionate use situations. However, large scale clinical trial comparing GCV to GCV + HU would be needed to determine clinical benefit of combination therapy.

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