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Review

# Resistance of human cytomegalovirus to ganciclovir/valganciclovir: A comprehensive review of putative resistance pathways ☆

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ABSTRACT

Human cytomegalovirus (HCMV) is a pathogen that can be life-threatening in immunocompromised individuals. Valganciclovir and its parent drug ganciclovir are currently the principle drugs used for the treatment or prevention of HCMV disease. The development of HCMV resistance to ganciclovir/valganciclovir has been documented in treated patients and is associated with the emergence of amino acid substitutions in the viral proteins pUL97, pUL54 or both. Generally, single amino acid substitutions associated with clinical resistance that alone do not confer decreased ganciclovir susceptibility in cell culture have been disregarded as causative or clinically significant. This review focuses on the analysis and mechanisms of antiviral drug resistance to HCMV. We also conducted a review of publicly available clinical and nonclinical data to construct a comprehensive list of pUL97 and pUL54 amino acid substitutions that are associated with a poor clinical response to the first line therapies ganciclovir and valganciclovir, or associated with reduced HCMV ganciclovir susceptibility in cell culture. Over 40 putative ganciclovir/valganciclovir resistance-associated substitutions were identified in this analysis. These include the commonly reported substitutions M460I/V and C592G in pUL97. There were additional substitutions that are not widely considered as ganciclovir/valganciclovir resistance-associated substitutions, including V466M in pUL97 and E315D in pUL54. Some of these ganciclovir/valganciclovir resistance-associated substitutions may confer cross-resistance to other HCMV therapies, such as cidofovir and foscarnet. Based on this review, we propose that there are more potential HCMV ganciclovir/valganciclovir resistance pathways than generally appreciated. The resulting comprehensive list of putative ganciclovir/valganciclovir resistance-associated substitutions provides a foundation for future investigations to characterize the role of specific substitutions or combinations of substitutions, which will enhance our understanding of HCMV mechanisms of ganciclovir/valganciclovir resistance and also provide insight regarding the potential for cross-resistance to other HCMV therapies.

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## 1. Introduction

As antiviral therapy has become widely used in various viral diseases, including disease associated with human cytomegalovirus (HCMV) infection, it has also become crucial to understand the mechanisms and clinical consequences of antiviral drug resistance, including the relevant virus, host and drug-related factors. Knowledge of the mechanisms of antiviral drug resistance has allowed for development of genotypic and phenotypic techniques for the timely diagnosis of resistance. Consequences of drug resistance for any viral infection may range from toxicity inherent in the use of antivirals designated as second-line in treatment guidelines to severe disease and even death from progressive viral infection when no effective alternative treatments are available. While the mechanisms and clinical impact of antiviral drug resistance have been well described for human immunodeficiency virus (HIV-1), hepatitis C virus (HCV), hepatitis B virus (HBV) and influenza virus, they have been less well characterized for HCMV even though drug-resistant HCMV is also a major clinical concern.

Cytomegaloviruses are members of the *Herpesviridae* family, and are capable of causing a variety of acute, latent and recurrent infections in humans and animals. HCMV, also designated as the human herpesvirus 5, is the prototype of the betaherpesvirus group (Roizman et al., 1981). The HCMV genome is about 230 kb and encodes more than 250 open reading frames. HCMV infection can be primary or secondary. Primary infection occurs in a HCMV seronegative susceptible host, whereas secondary infection represents reactivation of a latent infection or reinfection of a seropositive host.

As a general rule for all viral infections and antiviral drugs, antiviral drug pressure can select for the emergence of pre-existing drug resistant variants by conferring a survival advantage to those subpopulations that are relatively less susceptible to the antiviral agent. Alternatively, spontaneous changes in the viral genome generated during viral replication in the presence of drug selection can lead to the emergence of a drug resistant viral population. In either case, secondary consequences of resistance-associated nucleotide or amino acid substitutions can include alterations in viral pathogenicity, transmissibility, and genetic stability. Development of antiviral resistance can often increase in frequency and significance as antiviral agents are utilized more widely. Great strides have been made in the methods to characterize the mechanisms and development of antiviral drug resistance using biochemical assays or cell culture models, but there are still many limitations to these approaches. While these assays have become more sensitive, they often do not identify certain indirect mechanisms that may contribute towards clinically relevant drug resistance, such as compensatory viral fitness amino acid substitutions (Bloom et al., 2010), viral substitutions that only confer resistance when present in combination with other substitutions (Kobayashi et al., 2011; Sun et al., 2012), or viral- or host-specific (e.g., ribavirin, (Ibarra et al., 2011)) changes that impact drug metabolism. As an example of a potentially indirect mechanism of drug resistance for HCMV, the pUL54 K805Q substitution improved the fitness of a virus harboring the pUL54 T821I substitution associated with resistance to foscarnet (Martin et al., 2010a), although the clinical relevance of this particular observation remains unclear.

While many of the concepts and approaches to studying antiviral drug resistance generally apply to all viruses, HCMV brings along several unique challenges. In this article we review the current state of the field regarding the treatment of HCMV disease, specifically focusing on the study, mechanisms and clinical consequences of antiviral drug resistance. We also conducted a review of publicly available non-clinical and clinical data to construct a comprehensive list of amino acid substitutions that are associated with resistance to the most commonly used treatments for HCMV infection, ganciclovir and valganciclovir, and we discuss the potential for cross-resistance to other HCMV therapies. Finally, we highlight some of the major knowledge gaps in HCMV drug resistance to help stimulate further studies, which may ultimately help guide the development of new therapies and rational strategies that address the challenges of HCMV drug resistance.

## 2. HCMV infection and disease

The prevalence of HCMV infection is estimated to vary from about 45% in developed countries to near 100% in developing countries. Primary infection usually occurs during the first decades of life and humans are believed to be the only host of the virus. Transmission sources of HCMV include saliva, urine, semen, cervical and vaginal secretions, milk, stool, blood, cells, tissues, and organ transplants. Primary infection in immunocompetent adults is mainly asymptomatic or it may be associated with a self-limited mononucleosis-like syndrome and leads to a life-long latent infection. However, in patients with immature or compromised immune systems (i.e., congenitally infected newborns, patients with AIDS and transplant patients) HCMV infections are often symptomatic, causing a range of symptoms such as colitis and pneumonitis, and are associated with increased morbidity and mortality.

### 2.1. Congenital HCMV infection

In the United States, it is estimated that approximately 44,000 infants are born each year with congenital HCMV infection (Stagno and Britt, 2006). Approximately 10% of infected newborns are symptomatic at birth. Mortality in symptomatic infants is about 12% and approximately 90% of survivors experience significant morbidity from the infection, including mental retardation, sensorineural hearing loss, microcephaly, seizures, or other medical problems. No drugs are FDA-approved for antiviral treatment of congenital HCMV infection. However, a report by Kimberlin and his colleagues (Kimberlin et al., 2003) suggests that 6 weeks intravenous ganciclovir may have a role in treatment of neonates with symptomatic HCMV infection.

### 2.2. HCMV infection in AIDS patients

HCMV disease is common in patients with advanced HIV disease. Before the availability of highly active antiretroviral regimen (HAART), up to 45% of patients with AIDS acquired HCMV disease (Masur et al., 1996). HCMV retinitis is by far the most common manifestation of HCMV disease in patients with AIDS, accounting for 85% of the cases (Gallant et al., 1992). The introduction of HAART decreased the incidence of HCMV disease in patients with AIDS by more than 80% (Jabs et al., 2007). Currently FDA-approved drugs for the treatment of HCMV retinitis in patients with AIDS include intravenous ganciclovir, oral ganciclovir, ganciclovir intraocular implant, oral valganciclovir, intravenous foscarnet, and intravenous cidofovir. Oral valganciclovir is frequently used for initial treatment (Stewart, 2010).

### 2.3. HCMV infection in transplant recipients

HCMV is the most frequent opportunistic pathogen in transplant patients contributing significantly both to patient morbidity and mortality. The risk of developing HCMV disease after transplantation depends on a number of different factors, including the HCMV serologic status of both the donor and recipient, the type of transplant, and the level of immunosuppression. The clinical symptoms of HCMV infection range from asymptomatic HCMV viremia to tissue invasive HCMV disease. Because of the increased morbidity and mortality associated with HCMV infection in transplant patients, prevention of HCMV disease is a preferred strategy over waiting for established disease to occur and treating. Prophylactic therapy and pre-emptive therapy are the two major strategies used for prevention of HCMV disease (Boeckh and Ljungman, 2009; Tomblyn et al., 2009; Kotton et al., 2010; Razonable et al., 2013).

Although there have been no large studies comparing the two approaches, prophylaxis with oral valganciclovir has emerged as the most common strategy in solid organ transplant recipients due in part to the once daily dosing with this drug (Kotton, 2013). Valganciclovir has also emerged as the drug of choice for pre-emptive therapy and treatment of mild-to-moderate HCMV disease in solid organ transplant recipients. Intravenous ganciclovir, with or without administration of intravenous immunoglobulin, remains the standard for treatment of established severe HCMV disease (Kotton, 2013).

HCMV disease appears to be more severe in allogeneic stem cell recipients compared to the solid organ transplant recipients because of the more potent immunosuppressive therapy. Pneumonia is the most serious manifestation of HCMV disease, which is associated with a >50% mortality rate (Ljungman, 1995; Boeckh et al., 1996). Although prophylaxis is the preferred approach in preventing HCMV disease in solid organ transplant patients, pre-emptive therapy is more widely used in stem cell transplant patients because of the bone marrow toxicities of the current anti-HCMV drugs (Boeckh and Ljungman, 2009). Intravenous ganciclovir is the recommended drug of choice for pre-emptive therapy (Ljungman et al., 2011). Intravenous ganciclovir with or without administration of intravenous immunoglobulin is also the first-line treatment for established HCMV disease (Boeckh and Ljungman, 2009).

## 3. Antiviral agents for HCMV

There are limited therapeutic options for the treatment or prevention of HCMV disease in immunocompromised patients. Currently, four drugs have received FDA approval for the systemic treatment of HCMV disease: ganciclovir and its prodrug valganciclovir, foscarnet, and cidofovir. Another dug, fomivirsen, is approved for treatment of HCMV retinitis by intraocular injection.

### 3.1. Ganciclovir/valganciclovir

Ganciclovir (Cytovene®), in its intravenous formulation, was the first antiviral agent approved by FDA for the treatment of HCMV disease. It is an acyclic nucleoside analogue of 2′-deoxyguanosine, and must be phosphorylated to the triphosphate form to exert its antiviral activity by inhibiting viral DNA replication. It is not efficiently phosphorylated in most uninfected cells, thereby reducing toxicity, but is phosphorylated to ganciclovir monophosphate in HCMV-infected cells by the viral protein kinase (pUL97). Further phosphorylation to the triphosphate form occurs by cellular kinases.

Safety risks coupled with the inconvenience of administering the drug for a prolonged period of time led to the development of oral ganciclovir. Oral ganciclovir is approved for the prevention of HCMV disease in solid organ transplant recipients and in individuals with advanced HIV-1 disease at risk for developing HCMV disease. Oral ganciclovir is also approved for maintenance treatment only for HCMV retinitis in immunocompromised patients including patients with AIDS, in whom retinitis is stable following appropriate induction therapy. The poor bioavailability of the oral formulation raised concerns that the lower systemic exposure could be suboptimal and lead to emergence of drug resistance. In addition, the high pill burden for prophylaxis (four capsules three times daily) makes compliance challenging. These limitations lead to the development of valganciclovir (Valcyte®), a more orally bioavailable form of ganciclovir.

Valganciclovir is an L-valyl ester of ganciclovir. After oral administration, valganciclovir is rapidly absorbed and hydrolyzed by intestinal and hepatic esterases to ganciclovir. The absolute bioavailability of ganciclovir from oral valganciclovir tablets (~60%) is up to 10 times greater than the bioavailability of oral ganciclovir (6–9%) (Brown et al., 1999). Valganciclovir is approved for the treatment of HCMV retinitis in adults with AIDS, the prevention of HCMV disease in adult solid organ transplant (SOT) patients at high risk for HCMV disease (Paya et al., 2004), and the prevention of HCMV disease in pediatric kidney and heart transplant patients $\geqslant$4 months of age. Valganciclovir has not only replaced oral ganciclovir for the prevention of HCMV disease but has also become the recommended and most commonly used agent to treat solid organ transplant recipients with mild-to-moderate HCMV disease (Asberg et al., 2007).

### 3.2. Other agents used for HCMV therapy

Foscarnet (Foscavir®) was the second drug approved for the treatment of HCMV retinitis in AIDS patients. Foscarnet sodium is the trisodium salt of phosphormophonic acid, a pyrophosphonate analogue. Foscarnet inhibits the activity of the viral DNA polymerase by binding to the pyrophosphate binding site and blocking cleavage of pyrophosphate from the terminal nucleoside triphosphate added to the growing DNA chain. Foscarnet does not require phosphorylation by kinases and is therefore active against pUL97 kinase variants that confer resistance to ganciclovir/valganciclovir (mechanism of drug resistance is described in greater detail below). Foscarnet is considered a second-line therapy due to its relatively toxic safety profile. Its role in HCMV infections has been limited to patients who are failing ganciclovir therapy, presumably due to drug resistance, or those who cannot be treated with ganciclovir due to dose-limiting neutropenia (Razonable and Emery, 2004).

Cidofovir (Vistide®, CDV) is an acyclic monophosphate nucleotide analogue of 2′-deoxycytidine with cell culture antiviral

activity against various DNA viruses including herpesviruses and adenovirus (De Clercq and Holy, 2005). Viral kinases are not needed for the initial phosphorylation because a single phosphate group is present in its native form. Cidofovir inhibits DNA replication by viral DNA polymerase after phosphorylation to its active diphosphate form by cellular kinases. Cidofovir is approved for the treatment of HCMV retinits in AIDS patients. It is available only as an intravenous formulation because of its poor oral bioavailability, although the relatively long half-life of the active metabolite allows for an intermittent dosing schedule (Yang and Datema, 1991). The clinical use of cidofovir is limited because of adverse events such as nephrotoxicity and neutropenia. Additionally, cidofovir has been shown to be carcinogenic, teratogenic, and to cause hypospermia in animal studies (Vistide Package Insert, 1996). In clinical practice, cidofovir is generally used in patients with established HCMV disease who are failing treatment with ganciclovir/valganciclovir, foscarnet or both.

Fomivirsen (Vitravene®) is an antisense phosphorothioate oligonucleotide that blocks the replication of HCMV mRNA. It is 21 nucleotides long, comprised of a sequence that is complementary to the mRNA transcribed from the major immediate-early transcriptional unit of HCMV (Mulamba et al., 1998). Fomivirsen was approved by FDA in 1998 for treatment of HCMV retinitis, but is no longer marketed in the United States.

Acyclovir (Zovirax®, ACV) is a guanosine analogue with cell culture antiviral activity against various DNA viruses including herpesviruses (O'Brien and Campoli-Richards, 1989). It is converted into acyclo-guanosine monophosphate (acyclo-GMP) by viral thymidine kinase. Subsequently, the monophosphate form is further phosphorylated into the active triphosphate form, acyclo-guanosine triphosphate (acyclo-GTP), by cellular kinases. As a substrate, acyclo-GTP is incorporated into viral DNA, resulting in premature chain termination. Acyclovir is approved for the treatment of herpes simplex virus (HSV) and varicella zoster virus (VZV) infections. High doses of acyclovir and its prodrug valacyclovir have been used off label for HCMV prophylaxis. However, due to the high pill burden and the risk of neurologic adverse events, valganciclovir has become the preferred drug for prophylaxis.

Leflunomide (Arava®) is an immunomodulatory drug inhibiting mitochondrial enzyme dihydroorotate dehydrogenase which plays a key role in the de novo synthesis of the pyrimidine ribonucleotide uridine monophosphate (rUMP) (Fukushima et al., 2007). There are published reports of a beneficial effect of leflunomide in some cases (Levi et al., 2006; Mossad and Avery, 2007), but failures (in haemopoietic transplantation) in other cases (Battiwalla et al., 2007).

### 3.3. Investigational anti-HCMV agents

A number of other anti-HCMV agents are in development and in the future may provide alternatives to currently approved drugs. New agents with greater antiviral potency or novel mechanisms of action may be particularly important for the treatment of HCMV infections that are resistant to one or more of the currently available therapies.

Investigational anti-HCMV drugs currently include maribavir, CMX001, letermovir, cycloprapavir, and artesunate. Maribavir is a novel inhibitor of the pUL97 kinase and has been investigated for prophylaxis of HCMV. Unfortunately, the drug failed to demonstrate efficacy in two Phase III trials; one in allogeneic hematopoietic stem cell transplant recipients (Marty et al., 2011) and the other in liver transplant recipients at high risk for HCMV disease (Winston et al., 2012). CMX001 is a cidofovir analog with enhanced oral bioavailability and activity active against a broad range of herpes viruses including many drug-resistant HCMV isolates (Quenelle et al., 2010). Letermovir (AIC246) inhibits the replication of HCMV by a novel mechanism, presumably by targeting the pUL56 subunit of the viral terminase complex (Goldner et al., 2011; Lischka et al., 2010). Cyclopropavir (MBX-400) is a methylenecyclopropane guanosine nucleoside analog with a mechanism of action similar to that of ganciclovir/valgancyclovir (Gentry et al., 2010). Artesunate (ART) is a derivative of the Chinese herb Artemisia annua and has been used to treat malaria (Reyburn, 2010), but has also been reported to have anti-HCMV activity in nonclinical and clinical studies (Efferth et al., 2002; Efferth et al., 2008; Kaptein et al., 2006; Schnepf et al., 2011; Wolf et al., 2011; Shapira et al., 2008).

## 4. HCMV gene targets of antiviral drugs

### 4.1. HCMV replication components involved in current treatment

In general, HCMV gene expression is divided into three sequential kinetic classes: α (immediate early), β1 and β2 (delayed early), and γ1 and γ2 (late), based on the time of expression after virus infection (Grant et al., 1981; Stinski et al., 1991). Expression of β and γ gene products requires the synthesis of functional α gene products that act as transcriptional trans-activators. Delayed early β1 genes are transcribed between 4 and 8 h post-infection, while β2 genes are expressed approximately between 8 and 24 h post-infection. Most of the β2 gene products, such as viral DNA polymerase, are involved in viral DNA synthesis (Wright et al., 1964).

The HCMV DNA polymerase, pUL54, which is a β2 delayed early product encoded by the UL54 gene (also referred as the HCMV pol gene), is the central enzyme involved in viral DNA replication and the primary drug target for current therapies. It is a relatively large, 140 kD protein, consisting of 1242 amino acids based on the AD169 HCMV reference strain. Its range of activities includes viral DNA synthesis, as well as a 3′- to 5′-exonuclease proof-reading activity to increase replication fidelity (Kouzarides et al., 1987; Pari and Anders, 1993). Both DNA and amino acid sequence analyses have revealed that the UL54 gene has significant homology to the pol genes of other herpesviruses, such as herpes simplex virus type 1 (HSV-1) and Epstein–Barr virus (EBV) (Shepp et al., 1985). In addition, herpesvirus DNA polymerases share striking sequence similarities with eukaryotic DNA polymerases α and δ and many other DNA polymerases of viruses and bacteriophages in several discrete regions that define a family of α-like polymerases (Wong et al., 1988; Sullivan et al., 1993). This homology has been used to define seven conserved regions of the pUL54 protein, labeled I–VII in order of decreasing homology.

The UL97 gene, which is also a β2 delayed early gene and has conserved homologues in all herpes sub-families (Michel et al., 1998), encodes pUL97. This protein has homologies with protein kinases, and may impair HCMV growth (Michel et al., 1996; Prichard et al., 1999; Wolf et al., 2001; Gill et al., 2012). The pUL97 kinase activity plays a key role in the phosphorylation of the nucleoside ganciclovir, which is necessary for generation of the active triphosphate polymerase inhibitor (Littler et al., 1992; Sullivan et al., 1992). Experiments with recombinant vaccinia viruses expressing a range of mutant pUL97 proteins have indicated the C-terminal region of pUL97 (amino acids 292–707) binds and phosphorylates ganciclovir, while the central portion (amino acids 305–365) has a role in phosphorylating both ganciclovir and pUL97 itself (Michel et al., 1998). The N-terminal region of pUL97 (amino acids 1–199) contains a nuclear localization signal.

### 4.2. Mechanisms of resistance to HCMV drugs

Variants with reduced susceptibility to ganciclovir/valganciclovir occur naturally in circulating HCMV (Drew et al., 1993).

Furthermore, antiviral resistance develops in a stepwise fashion (Muller and Krausslich, 2009; Nijhuis et al., 2009). Prior to treatment, the entire virus population contains a small fraction of less drug susceptible variants due to error-prone replication by the viral DNA polymerase. When subsequent antiviral treatment does not suppress viral replication completely, for example due to suboptimal levels of antiviral drugs or extreme immunosuppression of the host, a selection process favoring these variants occurs. For example, ganciclovir-resistant pUL97 mutants may have some subtle loss of growth fitness, as supported by in vivo viral population dynamic studies (Emery et al., 1999). Since they can replicate in the presence of the antiviral drug, they are able to evolve and acquire additional amino acid substitutions that enhance replicative fitness and possibly increase resistance, ultimately resulting in a large population of highly resistant and replication competent viruses. This model is supported by the observation that major risk factors for HCMV drug resistance are the residual capacity of the host's immune system to control viral replication and the overall level and duration of viremia in the presence of drug (Drew, 2000). Clinicians should keep these selection mechanisms in mind when monitoring viral loads in treated patients. Furthermore, the timing of samples obtained for antiviral drug susceptibility testing combined with the sensitivity of applied assays has to be considered. For example, analyzing phenotypic susceptibility early during antiviral treatment may not predict eventual treatment failure due to an underlying drug resistant viral population that has yet to predominate in the viral population.

The development of resistance to ganciclovir has been documented during the treatment of HCMV retinitis in patients with AIDS. Before HAART was introduced, HCMV antiviral treatment was usually administered in the presence of overt disease, followed by lifelong maintenance therapy. The incidence of HCMV-related complications and death in patients with AIDS has declined due to the introduction of HAART (Palella et al., 1998; Kedhar and Jabs, 2007), but still remains a concern in patients with low $CD4^+$ T cell counts. Therefore, diagnosis and monitoring of active HCMV viremia and, in many cases, long-term antiviral therapy against HCMV are life-saving for patients at risk for severe HCMV disease. Unfortunately, the likelihood of drug resistance emergence in patients with AIDS appears to increase with duration of therapy. Jabs et al. (1998) reported that approximately 2–7% of patients with AIDS have ganciclovir resistant HCMV after 2–3 months of drug therapy, while a study by Boivin et al. (2001) demonstrated that up to 15–28% of patients have drug resistant virus after 9 or 18 months of therapy. Ganciclovir-resistant HCMV is a clinical problem in organ transplant recipients as well (Bhorade et al., 2002; Limaye, 2002; Boivin et al., 2009). Patients with drug-resistant HCMV strains often have virological failure and might have unfavorable clinical outcomes (Boivin et al., 2001).

Pathways leading to the development of resistance to ganciclovir have been reported from selection of resistant virus in cell culture passage experiments, as well as treatment studies of HCMV retinitis in patients with AIDS (Lurain and Chou, 2010; James and Prichard, 2011). Amino acid substitutions conferring resistance to ganciclovir have been detected in the pUL97 kinase (decreasing phosphorylation of ganciclovir) and in the pUL54 DNA polymerase (decreasing inhibition of the viral DNA polymerase by ganciclovir triphosphate), sometimes occurring in both within a single resistant variant. The passage of laboratory HCMV strain AD169 in cell culture in the presence of increasing concentrations of ganciclovir (from 1.5 to 3000 μM) resulted in the selection of amino acid substitutions in pUL97 (M460I and L595S) and in pUL54 (L545S, V812L, P829S and D879G) (Gilbert and Boivin, 2005). Resistance to ganciclovir has also been studied in other herpesviruses. Similar to what was found in HCMV, amino acid substitutions conferring resistance to ganciclovir have been detected in the kinase and DNA polymerase in HSV and HHV-6 (Freifeld and Ostrove, 1991; Nakano et al., 2009; Isegawa et al., 2009; Baldwin, 2011).

Resistance to foscarnet in the laboratory or clinical setting has been mapped to amino acid substitutions in pUL54. Cross-resistance has been observed between ganciclovir and foscarnet in several laboratory and clinical isolates with both genotypic and phenotypic resistance. Resistance to cidofovir has been difficult to select in the laboratory (Cihlar et al., 1998a), and has not been reported in clinical isolates, although this may be due to the shorter treatment duration and limited use of this agent. Nevertheless, several ganciclovir-resistant HCMV clinical isolates or laboratory-selected strains with specific pUL54 amino acid substitutions are clearly cross-resistant to cidofovir (Gilbert and Boivin, 2005).

## 5. Methodologies used for resistance analysis

### *5.1. Phenotypic methods used to characterize resistance to HCMV drugs*

Phenotypic testing of clinical isolates was the primary method for HCMV drug susceptibility testing before the establishment of genotypic testing. The gold standard for phenotypic characterization of HCMV drug susceptibility has historically been the plaque reduction assay (PRA), which measures viral spread in cell culture based on the quantity of newly formed viral plaques that emerge in the presence of different antiviral drug concentrations (Table 1). However, inter-assay and especially inter-laboratory standardization of the PRA has been hampered by technical challenges and often poor reproducibility of the assay (Landry et al., 2000). Consequently, efforts have been made to develop new assays that are more amenable to standardization.

Several other phenotypic or combined genotypic/phenotypic methods have been used to characterize HCMV drug susceptibility and resistance (Table 1). Reporter cell lines have been developed to measure HCMV spread in cell culture reflected by cell-expressed luciferase activity (Gilbert and Boivin, 2005), green fluorescent protein (GFP) (Ueno et al., 2006; Ueno and Ogawa-Goto, 2009), or enhanced green fluorescent protein (EGFP) (Luganini et al., 2008). Another approach uses quantitative real-time PCR to measure the declining number of HCMV genome copies in cell culture due to different concentrations of the antiviral agent (Schnepf et al., 2009). Marker transfer approaches have been developed to generate recombinant HCMV viruses and characterize the impact of specific genetic changes or entire gene cassettes on viral susceptibility and resistance to antiviral drugs. These recombinant viruses may express various reporter proteins in infected cells, such as secreted alkaline phosphatase (Chou et al., 2005), GFP (Marschall et al., 2000), enhanced yellow fluorescent protein (Dal et al., 2008) or EGFP (Chevillotte et al., 2009). Finally, a biochemical assay has been developed to measure the incorporation of digoxigenin-labeled nucleotides by recombinant pUL54 polymerase enzymes (Ducancelle et al., 2007). These relatively newer methods of phenotypic analysis are often easier to perform and better standardized than the PRA, and are important tools to identify and characterize resistance-associated amino acid substitutions. However, all assays, including the PRA, generally have not been in widespread use for routine clinical decision making as they lack clinically validated data defining cutoffs in $EC_{50}$ values that likely reflect clinical drug resistance.

An important limitation of phenotypic analysis of clinical isolates is the impact of mixed populations of wild-type and drug resistant virus and the potential bias of the assays for detecting wildtype virus phenotypic susceptibility. Detection of mixed viral populations has been reported in patients with HCMV viremia (Eckle et al., 2004; Jabs et al., 2006; Ross et al., 2011), and while

**Table 1**
Phenotypic assays used to characterize resistance to HCMV drugs.

| Phenotypic assay/ readout | Summary of methodology | Technical/practical challenges |
|---|---|---|
| Plaque reduction assay | a. Clinical or recombinant isolates grown in presence of varying drug concentrations.<br>b. Viral spread in culture quantified by plaque assay.<br>c. Drug effective concentrations (e.g., $EC_{50}$ values) calculated and compared for different isolates. | a. Inter-assay and inter-laboratory standardization very difficult.<br>b. Technically challenging and time consuming, and not amenable to automated or high throughput analysis.<br>c. Potential bias to wild-type phenotype when clinical isolates include a mixture of wild-type and drug resistant variants. |
| Reporter cell lines | a. Clinical or recombinant isolates grown in presence of varying drug concentrations.<br>b. Cell line used for infection carries a reporter gene (e.g., luciferase) under the control of HCMV promoter.<br>c. Viral spread in culture quantified by reporter assay (e.g., plate reader, flow cytometry).<br>d. Drug effective concentrations (e.g., $EC_{50}$ values) calculated and compared for different isolates. | a. No standardization for different reporter cell lines.<br>b. Although genotypic data may be informative, construction and phenotypic analysis of recombinant viruses not amenable to routine clinical use.<br>c. Potential bias to wild-type phenotype when clinical isolates include a mixture of wild-type and drug resistant variants. |
| Real-time PCR | a. Clinical or recombinant isolates grown in presence of varying drug concentrations.<br>b. Viral spread in culture quantified by measuring the declining number of genome copies.<br>c. Drug effective concentrations (e.g., $EC_{50}$ values) calculated and compared for different isolates. | a. Relatively expensive.<br>b. DNA readout does not distinguish between infectious and noninfectious virus particles.<br>c. Potential bias to wild-type phenotype when clinical isolates include a mixture of wild-type and drug resistant variants. |
| Marker transfer | a. Recombinant viruses are generated to encode specific genetic changes or entire gene cassettes.<br>b. Viral spread in culture quantified by reporter or other assay.<br>c. Drug effective concentrations (e.g., $EC_{50}$ values) calculated and compared for different isolates, or for parental and recombinant HCMV constructs. | a. Most direct method to understand impact of specific genetic changes on HCMV drug susceptibility, but mutant strains technically challenging to generate.<br>b. Although genotypic data may be informative, construction and phenotypic analysis of recombinant viruses not amenable to routine clinical use.<br>c. Results may be impacted by background sequence. |
| Polymerase Biochemical assays | a. HCMV UL54 DNA polymerase gene amplified by PCR.<br>b. Construct expression vectors harboring the HCMV DNA polymerase.<br>c. DNA polymerase activity assayed by measuring the incorporation of digoxigenin deoxyuridine triphosphate (DIG-dUTP) into the growing DNA chain. | a. Does not recapitulate entire viral life cycle.<br>b. Cannot characterize impact of other viral proteins (pUL97 kinase).Cannot account for impact on viral fitness.<br>c. Require cidofovir diphosphate/ganciclovir triphosphate (active forms) for conducting assay. |

*Abbreviation*: $EC_{50}$, 50% effective concentration.

HCMV phenotypic assays can sometimes detect minority drug resistant populations, the sensitivity of these assays likely varies depending on the specific viral variants. Drew et al. (2001) state that a phenotypic assay probably only exceeds the cut-off level for resistance when at least 50% of the viral population is resistant. Poor sensitivity of phenotypic assays for detecting mixed populations of drug resistant and drug susceptible virus has been noted for other viruses including influenza (Wetherall et al., 2003) and HCV (Verbinnen et al., 2012).

Marker transfer is generally the method of choice to provide a link between viral genetic changes and phenotypic drug susceptibility. In these experiments, amino acid substitutions suspected to confer resistance are introduced into a well-defined, drug-sensitive viral background, and the recombinant viruses are tested phenotypically. Early protocols for marker transfer were based on either co-transfection of HCMV DNA or transfection of HCMV infected cells with linear DNA containing the mutated sequence. After recombination of the DNA in the transfected cell, recombinant viruses bearing the desired amino acid substitution could be reconstituted and then plaque purified in order to avoid wild-type virus contamination (Lurain et al., 1992; Sullivan et al., 1992; Baldanti et al., 1996; Bourgeois et al., 1997; Cihlar et al., 1998a; Faizi et al., 1998; Chou et al., 2000; Mousavi-Jazi et al., 2001a, 2001b). These approaches do not always produce a clonal virus population due to residual wild-type virus contamination or the introduction of other substitutions during the viral amplification process, influencing the results of drug susceptibility testing.

Other methods use a drug-sensitive parental HCMV strain that bears unique restriction sites in the target gene. The parental DNA is digested with restriction enzymes and cotransfected with a plasmid encoding the amino acid substitution(s) of interest in the respective gene. Recombination between overlapping sequences in the plasmid and the digested parental DNA in cotransfected cells results in viable recombinant virus harboring the desired gene cassette or amino acid substitution(s) (Chou, 2008; Chou and Marousek, 2008; Chou et al., 2003, 2005; Drew, 2006; Iwasenko et al., 2009; Marfori et al., 2007; Scott et al., 2007; Springer et al., 2005; Weinberg et al., 2003). This method highly increases the selective pressure for the formation of recombinant virus compared to wild-type virus, but plaque purification is still necessary. Of note, the problem of wild-type virus contamination is circumvented using a biochemical assay, where only the mutated enzymes, and not viruses, are generated and tested for drug susceptibility (Ducancelle et al., 2005, 2007).

More recently, bacterial artificial chromosome (BAC) technology has been applied to generate clonal recombinant viruses for marker transfer experiments. Since all progeny viruses start from BAC DNA from one single bacterial colony, clonal virus populations are generally obtained (although the potential for other changes during viral amplication can still apply). Several protocols have been published for the generation of recombinant BACs for HCMV marker transfer purposes, based on a flp recombinase (Martin et al., 2006), RED recombinase (Chou, 2009), or RED-GAM recombinase system (Chevillotte et al., 2009). Only the latter two are markerless mutagenesis methods, meaning that they leave no trace of the recombination procedure in the BAC sequence (Tischer et al., 2006; Warming et al., 2005). BAC mutagenesis is efficient and relatively fast, and is now becoming the method of choice for the characterization of the many still uncharacterized resistance-associated substitutions.

Although marker transfer experiments offer a powerful approach to better understand the mechanisms of HCMV drug resistance, several limitations remain. One concern is that the genetic background may influence the ability of a particular substitution

to confer resistance. Furthermore, the number of needed marker transfers has increased as it is becoming clearer that combinations of substitutions play an important role in HCMV drug resistance. For example, certain combinations of resistance-associated substitutions in the UL97 and UL54 gene products have been shown to have a synergistic effect on drug resistance (Ducancelle et al., 2007; Mousavi-Jazi et al., 2001a, 2001b; Scott et al., 2007), while others have been shown to partially compensate for replicative fitness defects caused by individual substitutions (Ijichi et al., 2002; Martin et al., 2010a). However, these results have been conflicting. In the case of the pUL54 D605E substitution, there are also reports that show it does not have compensatory effects (Chou et al., 2005). Another important limitation of marker transfer-based assays, and phenotypic assays in general, is that the length of drug exposure is generally not representative of the drug exposure in treated patients. For these and other reasons noted above, substitutions associated with clinical drug resistance do not always confer obvious reductions in phenotypic susceptibility in cell culture or biochemical assays (Chou et al., 2002). Therefore, the lack of a phenotypic fold-shift by itself does not necessarily prove that a particular genetic variant has no impact on drug resistance. Despite the numerous challenges and lack of standardization of phenotypic assays, a >5-fold reduction in HCMV phenotypic susceptibility to GCV has been proposed as a basis for switching therapy (Kotton et al., 2010).

### 5.2. Genotypic methods used to characterize resistance to HCMV drugs

Genotypic assays take advantage of knowing specific resistance-associated substitutions in the drug target gene(s). Genotypic drug resistance testing is used extensively for guiding treatment decisions for other viruses, particularly HIV and hepatitis B virus, but are used relatively less frequently for HCMV. As an example for ganciclovir genotypic resistance testing, the UL97 and UL54 genes can be amplified by PCR, the PCR product analyzed by Sanger DNA sequencing, and amino acid coding substitutions already known to be associated with ganciclovir resistance can be identified and reported. Other HCMV genotypic methods that have been used include restriction fragment length polymorphism of PCR products (Novak et al., 2008; Pignatelli et al., 2003), and genotype-specific PCR assays (Mattick et al., 2004). Genotypic assays can be directly applied to clinical samples if there is sufficient HCMV DNA in, for example, plasma or cerebrospinal fluid, or can be used on cultured isolates. Several commercial laboratories can perform HCMV genotypic assays, which have a typical turnaround time of <3 days.

The main limitation with genotypic assays used in clinical practice is that they require prior knowledge of key drug resistance-associated substitutions to distinguish them from other sequence substitutions/polymorphisms believed to have no impact on drug activity. The known drug resistance-associated substitutions are typically those that have been demonstrated to confer reduced HCMV drug susceptibility in phenotypic assays, and substitutions that may contribute less directly towards drug resistance (as discussed above) are generally disregarded as being clinically relevant and often are not even reported. Another limitation of some current HCMV genotypic analysis methods is they are not capable of detecting and quantifying minority drug resistant viral populations. Next-generation sequencing (NGS) technologies (Gorzer et al., 2010) can enable detection of far smaller viral subpopulations, and have been explored in the context of HIV-1, HBV, and HCV (Margeridon-Thermet et al., 2009; Simen et al., 2009; Svarovskaia et al., 2012). While the NGS technology has not been fully developed for routine HCMV diagnostic purposes, in the future it may provide the ability to identify drug resistance at an earlier stage, prior to the predominance of drug resistant viral populations (Sahoo et al., 2013).

A major advantage with the use of genotypic assays is the potential to identify novel mechanisms of HCMV drug resistance. Viral genetic changes associated with treatment or prophylaxis failure can be identified, reported and further characterized as appropriate to determine their potential impact on HCMV drug susceptibility, including cross-resistance to other drugs. Continually monitoring HCMV genetic changes for potentially novel resistance mechanisms could lead to further refinement of genotypic and phenotypic drug resistance assays, which in turn could help optimize treatment decision making.

## 6. Analysis of ganciclovir/valganciclovir resistance pathways

Amino acid changes that confer reduced HCMV susceptibility to ganciclovir/valganciclovir are believed to map entirely to the pUL97 kinase and pUL54 DNA polymerase (Lurain and Chou, 2010; James and Prichard, 2011). There are numerous pathways for HCMV to become resistant to ganciclovir/valganciclovir and many different resistance-associated substitutions have been reported. Multiple resistance pathways are possible complicating the identification of resistance pathways from clinical samples. An additional complication is the lack of a baseline comparator for resistance analyses in isolates from patients experiencing failure in prophylaxis studies.

To better understand the full breadth of potential mechanisms of ganciclovir/valganciclovir resistance, we conducted a comprehensive review of the literature to identify and compile specific amino acid substitutions potentially associated with resistance to ganciclovir/valganciclovir. Although both clinical efficacy and phenotype data were considered, our analysis focused primarily on amino acid substitutions that have been clinically associated with resistance to ganciclovir/valganciclovir. We use the term "clinically associated" to refer to substitutions that have been observed in subjects who failed ganciclovir/valganciclovir treatment.

The list of resistance-associated amino acid substitutions were constructed based on the following criteria:

- single substitution with supporting phenotypic data ($\geqslant$2-fold reduction in susceptibility to ganciclovir),
- substitutions at positions identified in cell culture selection studies,
- substitutions reported at conserved positions from multiple independent studies in literature ($\geqslant$2 patients failing ganciclovir or valganciclovir treatment or prophylaxis),
- substitutions at conserved positions with other known resistance-associated substitutions,
- codon usage (e.g., multiple nucleotide changes within a codon required for a substitution),
- clear treatment-emergent amino acid substitution in $\geqslant$2 patients.

"Conserved" positions are defined as any position having 100% identity based on a BLAST search from GenBank using the AD169 pUL97 or pUL54 sequences. A potential limitation of this definition is that unlike other viruses like HIV-1 and influenza, there are not as many HCMV sequences (~200) deposited to GenBank, which could lead to underestimation of codon variability. For example, a few amino acid positions, such as A692, the collection of complete UL54 putative wildtype sequences from GenBank did not reveal any heterogeneity and therefore the percent identity is listed as 100% (i.e. all sequences were A692). Nonetheless, both A692S and A692V have been reported in the literature (Lurain and Chou, 2010). The sequences containing A692S and A692V may not have

been deposited to GenBank or may have been partial pUL54 sequences. Nevertheless, there are some clear examples of conserved amino acid motifs that are identical throughout the herpesvirus polymerases, such as 3′–5′ exonuclease domain (Exo I through III) and polymerization domain (I through VII) (Lurain et al., 1992; Ito and Braithwaite, 1991). There are also some conserved subdomains and residues present in all serine-threonine kinases, including the three glycines in the P-loop, the lysine amino acid position 355, and some other residues in the catalytic and activation loops (Hanks et al., 1988; Wilks et al., 1989; He et al., 1997; Marschall et al., 2001; Gershburg and Pagano, 2008).

### 6.1. pUL97 kinase

Using the resistance analysis criteria outlined above, there were 33 amino acid substitutions and 6 amino acid deletions, all at either conserved positions or at same positions at well-established resistance-associated substitutions, which were identified as being associated with resistance to valganciclovir or ganciclovir (Table 2). Most of these amino acid substitutions were reported in multiple studies in the literature, including the well established resistance substitutions M460V/I, H520Q, C592G, A594V, L595S, C603W. Amino acid substitutions L405P, M460I/V/T, V466G, C518Y, H520Q, C592G, A594E/G/T/V/P, L595F/S/W, E596G, K599T, C603W/R, C607Y, and all of the deletion variants were reported to confer at least a 2-fold reduction in susceptibility to ganciclovir in phenotypic assays.

Amino acid substitutions V466M, A591V, L595T, C603S, and C607F have been observed in patients failing treatment or prophylaxis, but have been shown to confer a <2-fold reduction in susceptibility to ganciclovir in phenotypic assays. Despite the lack of a clear resistance phenotype, each of these substitutions occurs at a conserved site where other substitutions have been shown to be associated with ganciclovir/valganciclovir resistance. Additionally, reductions in susceptibility of <2-fold have been reported to be clinically significant (Chou et al., 2002).

We are not aware of any published phenotypic data to characterize amino acid substitutions A591D, E596D, K599E/M, C603Y, and C607S. However, each substitution occurs at the same position as a confirmed resistance-associated amino acid substitution, and all have been observed in patients failing treatment or prophylaxis. Amino acid substitution G598S does not have a ganciclovir $EC_{50}$ value reported directly in comparison to a wild-type reference, although based on recombinant phenotyping it appears to confer significant ganciclovir resistance (Baldanti et al., 2002a, 2002b).

Other less common resistance-associated substitutions would be relatively difficult to generate at the molecular level. For example, A594E, L595T and L595W require either a transversion mutation or 2 nucleotide mutations to generate the specific amino acid substitution, which would not be enriched frequently by DNA replication error alone in the absence of drug selection.

**Table 2**
Ganciclovir resistance-associated amino acid substitutions in the pUL97 kinase.

| AA | Fold shift in susceptibility to GCV | Reference(s) |
|---|---|---|
| L405P | 2.5–2.7 | 1, 2 |
| M460I/V/T | I: 4<br>V: 5–10<br>T: 9.3 | 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 36 |
| V466G/M | G: 3.5–7<br>M: 1.3 | 2, 14, 15 |
| C518Y | 12 | 38 |
| H520Q | 5–10 | 16, 17, 18, 19 |
| del 590–593 | 3–10 | 20 |
| A591D/V | D: NA<br>V: 1.3 | 8, 18, 21, 22, 23 |
| C592G | 2–3 | 3, 12, 22, 24 |
| A594E/G/T/V/P | E: 3<br>G: 13.5<br>T: 2.7<br>V: 8.6<br>P: 2.9 | 2, 3, 5, 6, 8, 11, 12, 13, 18, 24, 25, 26. 36, 37 |
| L595F/S/W/T | F: no $EC_{50}$ ratio available, $EC_{50}$ = 31.9 uM<br>S: 8.5<br>W: 5.1<br>T: no $EC_{50}$ ratio available, $EC_{50}$ = 17.6 μM | 2, 3, 5, 9, 11, 12, 13, 18, 22, 36 |
| del 595 | 13 | 27 |
| del 595–603 | 8.4 | 28 |
| E596D/G | D: NA<br>G: 2.3 | 5, 18, 23 |
| K599E/M/T[a] | E: NA<br>M: NA<br>T: 5.9 | 23, 29 |
| del 597–600 | NA | 35 |
| del 600–601 | NA | 24 |
| del 601–603 | 15 | 7 |
| C603W/R/S/Y | W: 5–10<br>R: 3.6<br>S: 1.9<br>Y: NA | 2, 5, 12, 13, 15, 23, 30 |
| C607F/S/Y | F: 1.6<br>S: NA<br>Y: 12.5 | 14, 18, 31, 32, 33, 34, 35 |

1: Martin et al. (2006); 2: Chou (2010); 3: Chou et al. (2005); 4: Lurain et al. (1994); 5: Boutolleau et al. (2009); 6: Scott et al. (2004); 7: Marfori et al. (2007); 8: Wolf et al. (1995); 9: Gilbert and Boivin (2005); 10: Palmer et al. (2010); 11: Arslan et al. (2010); 12: Hantz et al. (2010); 13: Myhre et al. (2011); 14: Boivin et al. (2001); 15: Martin et al. (2010b); 16: Hanson et al. (1995); 17: Li et al. (2007); 18: Chou et al. (2002); 19: Schreiber et al. (2009); 20: Sullivan et al. (1992); 21: Erice (1999); 22: Smith et al. (1997); 23: Wolf et al. (1998); 24: Hu et al. (2002); 25: Bourgeois et al. (1997); 26: Kuo et al. (2003); 27: Baldanti et al. (1995); 28: Chou and Meichsner (2000); 29: Faizi et al. (1998); 30: Chou et al. (1997); 31: Baldanti et al. (1998); 32: Reddy et al. (2007); 33: Lurain et al. (2002); 34: Baldanti et al. (2002a,b); 35: Campanini et al. (2012); 36: Chou and Bowlin (2011); 37: Ijichi et al. (2002); 38: Zhang et al. (2013).

[a] Developed in cell culture studies only.

### 6.2. pUL54 DNA polymerase

There were 32 amino acid substitutions and 1 amino acid deletion that were identified as being associated with resistance to valganciclovir or ganciclovir (Table 3). All of these substitutions/deletions occur at conserved sites or at the same positions as well-established resistance-associated substitutions. Amino acid substitutions D301N, N408D/K/S, N410K, F412C/L/S, D413A/E, L501F/I, T503I, K513E/N/R, I521T, P522A/S, L545S/W, Q578H, D588N, E756K, V781I, V787L, L802M, A809V, T813S, T821I, A834P, G841A, A987G, and del 981–982 are reported to confer a $\geqslant$2-fold reduction in susceptibility to ganciclovir. Amino acid substitutions F412V, P488R, L516R, C539R, and V812L were identified only in cell culture selection studies and were reported to confer a $\geqslant$2-fold reduction in susceptibility to ganciclovir (Chou et al., 2003; Cihlar et al., 1998b; Gilbert and Boivin, 2005). Other single amino acid substitutions selected in cell culture have been reported to confer <2-fold reductions in ganciclovir susceptibility (F595I, L862F, V946L), or have an unclear phenotype (P829S) (Gilbert et al., 2011).

Amino acid substitutions E315D, P522L, Q578L, D588E, S695T, I726T/V, D879G, and A972V have been shown to confer a <2-fold reduction in susceptibility to ganciclovir in phenotypic assays. However, most of these substitutions occur at a conserved site. Substitutions P522L, Q578L, D588E, and A972V are located at the same position as confirmed resistance-associated amino acid

**Table 3**
Ganciclovir resistance-associated amino acid substitutions in the pUL54 DNA polymerase.

| AA | Fold Shift in Susceptibility to GCV | Cross-Resistance to CDV/FOS | Reference |
|---|---|---|---|
| D301N | 2.6 (viral fitness defect) | CDV: 3X, FOS: 0.5X | 1 |
| E315D | 0.8 | | 2, 3 |
| N408D/K/S | D: 4.9 | D: CDV: 5.6X, FOS: 1.3X | 4, 5, 6, 7, 8, 39 |
| | K: 5.3 | K: CDV: 5.4X, FOS: 1.6X | |
| | S: 3.1 | S: CDV: 7.5 | |
| N410K | 2.9 | CDV: 3X, FOS: 0.8X | 1 |
| F412C/L/S/V[a] | C: 3.6 | C: CDV: 10X, FOS: 1.2–2.5X | 5, 7, 9, 10, 11, 12, 35 |
| | L: 4.6 | L: CDV: 9.4X, FOS: 1.1X | |
| | S: 5.3 | S: CDV: 13X, FOS: 0.8X | |
| | V: 4.3 | V: CDV: 15.5, FOS: 1.1 | |
| D413A/E | A: 6.5 | A: CDV: 10.9X, FOS: 0.8X | 1, 8, 13, 14, 15 |
| | E 4.8 | E: CDV: 4.3X, FOS: 0.8X | |
| P488R[a] | 3.5 | CDV: 7.9 | 12 |
| L501F/I | F: no $EC_{50}$ ratio available, $EC_{50}$ = 80.9 uM | F: CDV: NA, FOS: no $EC_{50}$ ratio available, $EC_{50}$ = 234.1 uM | 5, 7, 11, 14, 16, 17, 18, 19 |
| | I: 6–20 | I: CDV: 9X, FOS: 1.4X | |
| T503I | 2.9 | CDV: 6.1X, FOS: 0.5X | 1, 5 |
| K513E/N/R | E: 5 | E: CDV: 9.1, FOS: 1.4 | 5, 7, 9, 20, 21, 22 |
| | N: 6 | N: CDV: 12.5, FOS: 1.1 | |
| | R: no $EC_{50}$ ratio available, $EC_{50}$ = 36.7 μM | R: no $EC_{50}$ ratio available, CDV $EC_{50}$ = 7.2 μM, FOS $EC_{50}$ = 200 μM | |
| L516R[a] | 2.1 | CDV: 5.1X, FOS: 0.8X | 1 |
| I521T | 3 | CDV: 3.9X, FOS: 0.9X | 23, 24 |
| P522A/L/S | A: 3 | A: CDV: 4.1X, FOS: 1.0X | 7, 14, 17, 23, 25, 26 |
| | L: 1.3 | L: CDV: 1.4X, FOS: 1.2X | |
| | S: 3 | S: CDV: 3.6, FOS: 1.1 | |
| C539R[a] | 3.2 | CDV: 13.3 | 12 |
| L545S/W | S: 3–7.4 | S: CDV: 9X, FOS: 1.2X | 7, 12, 27, 28, 29 |
| | W: 4.9 | W: CDV: 6.3, FOS: 1.3X | |
| Q578H/L | H: 3.3 | H: CDV: 2.3, FOS: 4.5 | 12, 38 |
| | L: 1.9 | L: CDV: 0.8, FOS: 3.0 | |
| D588E[b]/N | E: 1.3 | E: CDV: 1.1X, FOS: 2.3X | 5, 7, 30 |
| | N: 4 | N: CDV: 2.7X, FOS: 3.2X | |
| F595I[a] | 1.3 | CDV: 1.2X, FOS: 2.0 | 12, 36 |
| G629S | NA | NA | 2, 27 |
| S695T | 1.1 | CDV: 0.5X, FOS: 1.2 | 27, 37 |
| I726T/V | T: 1.9 | T: CDV: 1.8, FOS: 1.1 | 5, 38 |
| | V: 1.8 | V: CDV: 1.9, FOS: 1.2 | |
| E756K | K 3.5 | K:CDV: 2.2X, FOS: >8X | 1, 14 |
| V781I | 1–4 | CDV: 1.2X, FOS: 5.2X | 5, 7, 9 |
| V787L | 2–3 | CDV: 1X, FOS: 4.1X | 25, 31 |
| L802M | 4 | CDV: 1.8X, FOS: 10.8X | 5, 8, 9, 10 |
| A809V | 2.4 | CDV: 1.9X, FOS: 3.3X | 32, 33 |
| V812I[a] | 2.5 | CDV: 3.2X, FOS: 2.9X | 22, 29 |
| T813S | 2.5 | CDV: 2.7X, FOS: 4.9X | 32 |
| T821I | 4.5 | CDV: 1.9X, FOS: 21X | 5, 7 |
| P829S[a] | Unclear, too many other aa substitutions | | 29 |
| A834P | 6.1 | CDV: 3.0, FOS: 6.4 | 4, 8, 17 |
| G841A/S | A:3.2 | A: CDV: 2.6X, FOS: 4.3X | 14, 32, 38 |
| | S: NA | S: NA | |
| L862F[a] | 1.7 | CDV: 0.9X, FOS: 1.1X | 12 |
| D879G | 1.2 | CDV: 0.9X, FOS: 0.9X | 29, 36 |
| V946L[a] | 1.1 | CDV: 0.9X, FOS: 2.4 | 12 |
| A972V | 1 | CDV: 0.9X, FOS: 0.9X | 9, 18, 37 |
| del 981–982 | 8.3 | CDV: 2.8X, FOS: 3.6X | 1, 20 |
| A987G | 5.3 | CDV: 11.3X, FOS: 1.2X | 6, 7, 8, 20, 25, 34 |

1: Chou et al. (2003); 2: Boutolleau et al. (2009); 3: Chou et al. (2010); 4: Scott et al. (2007); 5: Smith et al. (1997); 6: Kuo et al. (2003); 7: Cihlar et al. (1998a); 8: Hantz et al. (2010); 9: Mousavi-Jazi et al. (2001a,b); 10: Chou et al. (1997); 11: Lurain et al. (1992); 12: Hakki and Chou (2011); 13: Marfori et al. (2007); 14: Erice et al. (1997); 15: Seo et al. (2001); 16: Ducancelle et al. (2004); 17: Reddy et al. (2007); 18: Scott et al. (2004); 19: Lurain et al. (2002); 20: Drew et al. (2006); 21: Smith et al. (1998); 22: Cihlar et al. (1998b); 23: Chou et al. (2008); 24: Eckle et al. (2000); 25: Jabs et al. (2001); 26: Foulongne et al. (2004); 27: Boivin et al. (2005); 28: Erice (1999); 29: Gilbert and Boivin (2005); 30: Springer et al. (2005); 31: Levi et al. (2006); 32: Chou et al. (2007a,b); 33: Chou et al. (1998); 34: Sullivan et al. (1993); 35: Chou (2011); 36: Gilbert et al. (2011); 37: Chevillotte et al. (2010); 38: (Valcyte Package Insert, 2013); 39: Hantz et al. (2013).

[a] Developed in cell culture studies only.

[b] Neither emerged in cell culture nor in vivo but subjected to recombinant phenotyping.

substitutions and have been observed in patients failing treatment or prophylaxis. Substitutions E315D and D879G have also been observed in multiple patients failing treatment or prophylaxis.

We are not aware of any published phenotypic data for amino acid substitutions G629S, and G841S. Substitution G629S has been observed in multiple patients failing prophylaxis. Substitution G841S occurs at the same position as the established resistance-associated substitution G841A.

Although we expect some of these single amino acid substitutions in Tables 2 and 3 to have little direct impact on HCMV susceptibility to ganciclovir, they may augment the effect of other substitutions in HCMV, either by directly enhancing resistance or indirectly by increasing fitness. For example, the HCMV DNA polymerase amino acid substitution K805Q improved the fitness of the T821I amino acid substitution associated with high levels of resistance to foscarnet (Martin et al., 2010a). It has also been demonstrated that combination of amino acid substitutions in pUL97

and pUL54 resulted in greater reduction in susceptibility to ganciclovir than in HCMV with either amino substitution alone (Chou et al., 2007a,b; Gilbert et al., 2011). In one recent study, a recombinant virus with pUL54 amino acid substitution D413A exhibited ganciclovir and cidofovir resistance but remained susceptible to foscarnet (Chou and Marousek, 2008). Moreover, after a limited number of passages in the absence of selective pressure, the plating efficiency of the unpurified D413A recombinant virus under 400 μM foscarnet was about 30-fold greater than that of the parental strain passaged under the same conditions. These results indicate that at least some amino acid substitutions listed in Table 3 could facilitate the subsequent development of other substitutions associated with drug resistance.

## 7. Cross-resistance between ganciclovir/valganciclovir and other drugs

Antiviral drugs with similar targets and mechanisms of action often have overlapping resistance pathways. Given that all approved HCMV drugs target the pUL54 polymerase to inhibit viral DNA replication, it is conceivable that some degree of cross-resistance will occur between all of the drugs. Cross-resistance between ganciclovir/valganciclovir and cidofovir or foscarnet has been reported for several amino acid substitutions in the DNA polymerase (Table 3). Amino acid substitutions D301N, N408D, N410K, F412C/V, D413A/E, L501I, T503I, L516R, I521T, P522A, L545S, D588N, E756K, V812L, T813S, G841A, A987G, and del 981–982 all confer a $\geqslant$2-fold reduction in susceptibility to cidofovir in phenotypic assays. Amino acid substitutions D588N, V781I, V787L, L802M, A809V, V812L, T813S, T821I, and G841A all confer a $\geqslant$2-fold reduction in susceptibility to foscarnet. There are multiple reports of treatment failure associated with some of these amino acid substitutions, indicating possible cross-resistance (Smith et al., 1997; Eckle et al., 2000; Scott et al., 2004). There are no controlled clinical trials that have been conducted to support the use of specific treatment options where HCMV resistance to ganciclovir/valganciclovir is suspected. Clinical management guidelines have been proposed based largely on expert opinion assessing published case reports and anecdotal experience (Boeckh and Ljungman, 2009; Kotton et al., 2010; Preiksaitis et al., 2005; Schreiber et al., 2009).

Two independent genes have been reported to be associated with maribavir drug resistance in cell culture. The first gene is UL97 (Biron et al., 2002; Chou et al., 2007a,b; Chou and Marousek, 2008) and the second is UL27 (Komazin et al., 2003; Chou et al., 2004). No cross-resistance has been detected between most of the viruses resistant to maribavir and those resistant to one or more of the current HCMV antiviral drugs (Drew et al., 2006). However, there was a recent report of amino acid substitutions F342S, V356G, V466G, and P521L in the pUL97 conferred reduced susceptibility to ganciclovir, maribavir and cyclopropavir (Chou et al., 2013). There are currently ongoing Phase 2 studies evaluating the activity of high doses of maribavir in subjects with HCMV refractory or resistant to ganciclovir/valganciclovir or foscarnet.

The frequency with which prior treatment with acyclovir elicits ganciclovir resistance remains unclear. In one study, subjects receiving prolonged high-dose acyclovir did not have evidence of ganciclovir resistance (Drew et al., 1995). However, there is a report of preexisting resistance before receiving any ganciclovir but after receiving acyclovir (Erice et al., 1989).

## 8. Conclusions and future directions

There are five drugs that have received FDA approval for the treatment of systemic or localized HCMV disease: ganciclovir and its prodrug valganciclovir, foscarnet, cidofovir, and fomivirsen. HCMV drug resistance studies have been limited by technical challenges and the lack of standardization of antiviral susceptibility assays. The recognition that specific amino acid substitutions in pUL97 and pUL54 are associated with different antiviral susceptibility patterns has prompted the development of molecular laboratory methods for the detection of drug resistant strains in clinical specimens.

Our review and analysis of the literature indicate there are potentially more HCMV ganciclovir/valganciclovir resistance pathways than generally appreciated. We compiled a listing of 85 resistance-associated amino acid substitutions and 7 deletions in pUL97 and pUL54. In clinical isolates, seven pUL97 substitutions, (M460V/I, H520Q, C592G, A594V, L595S, C603W) are the most frequently reported and validated ganciclovir resistance-associated substitutions. Although we identified several potentially novel amino acid substitutions that appear to be clinically associated with ganciclovir/valganciclovir resistance, and potentially associated with cross-resistance to other drugs, we anticipate that with further characterization not all substitutions will necessarily be proven causally related to ganciclovir/valganciclovir resistance. Questions that remain to be addressed include: What is the precise clinical impact of these resistance-associated substitutions and deletions alone or in combination? Does their impact vary in the context of prophylaxis versus treatment? Why do some substitutions that are clinically associated with resistance have no obvious effect on HCMV phenotypic resistance to ganciclovir, and are there other reasons (e.g., unrelated to drug resistance or replicative fitness) for the detection of these?

Because of these remaining questions the listings included in this review are not intended to guide treatment decisions at this time, as additional studies are clearly needed, particularly for potentially novel resistance substitutions and in the context of both available and investigational therapies. These listings are intended to generate hypotheses and guide future studies aimed at better understanding how to detect and minimize the impact of drug resistance in clinical practice. Examples of next steps include the continued phenotypic evaluation of clinical isolates, systematic phenotypic characterization of complex combinations of amino acid substitutions observed in clinical isolates, and evaluations of specific amino acid substitutions on HCMV replication capacity/fitness. Additionally, genotypic analyses of clinical isolates should continue to be conducted to substantiate uncommon but clinically relevant drug resistance pathways, and also to identify potentially novel resistance pathways for further characterization. As detection of antiviral resistance improves and new treatment options are introduced, additional strategies to combat the emergence of antiviral resistance can be developed and existing strategies refined.

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## References

Arslan, F., Tabak, F., Avsar, E., Midilli, K., Mert, A., Ozaras, R., Soysal, T., Ozturk, R., Ferhanoglu, B., 2010. Ganciclovir-resistant cytomegalovirus encephalitis in a hematopoietic stem cell transplant recipient. J. Neurovirol. 16, 174–178.

Asberg, A., Humar, A., Rollag, H., Jardine, A.G., Mouas, H., Pescovitz, M.D., Sgarabotto, D., Tuncer, M., Noronha, I.L., Hartmann, A., 2007. Oral valganciclovir is noninferior to intravenous ganciclovir for the treatment of cytomegalovirus disease in solid organ transplant recipients. Am. J. Transplant. 7, 2106–2113.

Baldanti, F., Michel, D., Simoncini, L., Heuschmid, M., Zimmermann, A., Minisini, R., Schaarschmidt, P., Schmid, T., Gerna, G., Mertens, T., 2002a. Mutations in the UL97 ORF of ganciclovir-resistant clinical cytomegalovirus isolates differentially affect GCV phosphorylation as determined in a recombinant vaccinia virus system. Antiviral Res. 54, 59–67.

Baldanti, F., Sarasini, A., Drach, J.C., Zemlicka, J., Gerna, G., 2002b. Z-isomers of 2-hydroxymethylcyclopropylidenemethyl adenine (synadenol) and guanine (synguanol) are active against ganciclovir- and foscarnet-resistant human cytomegalovirus UL97 mutants. Antiviral Res. 56, 273–278.

Baldanti, F., Silini, E., Sarasini, A., Talarico, C.L., Stanat, S.C., Biron, K.K., Furione, M., Bono, F., Palu, G., Gerna, G., 1995. A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. J. Virol. 69, 796–800.

Baldanti, F., Underwood, M.R., Stanat, S.C., Biron, K.K., Chou, S., Sarasini, A., Silini, E., Gerna, G., 1996. Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. J. Virol. 70, 1390–1395.

Baldanti, F., Underwood, M.R., Talarico, C.L., Simoncini, L., Sarasini, A., Biron, K.K., Gerna, G., 1998. The Cys607 Tyr change in the UL97 phosphotransferase confers ganciclovir resistance to two human cytomegalovirus strains recovered from two immunocompromised patients. Antimicrob. Agents Chemother. 42, 444–446.

Baldwin, K., 2011. Ganciclovir-resistant human herpesvirus-6 encephalitis in a liver transplant patient: a case report. J. Neurovirol. 17, 193–195.

Battiwalla, M., Paplham, P., Almyroudis, N.G., McCarthy, A., Abdelhalim, A., Elefante, A., Smith, P., Becker, J., McCarthy, P.L., Segal, B.H., 2007. Leflunomide failure to control recurrent cytomegalovirus infection in the setting of renal failure after allogeneic stem cell transplantation. Transpl. Infect. Dis. 9, 28–32.

Bhorade, S.M., Lurain, N.S., Jordan, A., Leischner, J., Villanueva, J., Durazo, R., Creech, S., Vigneswaran, W.T., Garrity, E.R., 2002. Emergence of ganciclovir-resistant cytomegalovirus in lung transplant recipients. J. Heart Lung Transplant. 21, 1274–1282.

Biron, K.K., Harvey, R.J., Chamberlain, S.C., Good, S.S., Smith III, A.A., Davis, M.G., Talarico, C.L., Miller, W.H., Ferris, R., Dornsife, R.E., Stanat, S.C., Drach, J.C., Townsend, L.B., Koszalka, G.W., 2002. Potent and selective inhibition of human cytomegalovirus replication by 1263W94, a benzimidazole L-riboside with a unique mode of action. Antimicrob. Agents Chemother. 46, 2365–2372.

Bloom, J.D., Gong, L.I., Baltimore, D., 2010. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. Science 328, 1272–1275.

Boeckh, M., Ljungman, P., 2009. How we treat cytomegalovirus in hematopoietic cell transplant recipients. Blood 113, 5711–5719.

Boeckh, M., Stevens-Ayers, T., Bowden, R.A., 1996. Cytomegalovirus pp65 antigenemia after autologous marrow and peripheral blood stem cell transplantation. J. Infect. Dis. 174, 907–912.

Boivin, G., Gilbert, C., Gaudreau, A., Greenfield, I., Sudlow, R., Roberts, N.A., 2001. Rate of emergence of cytomegalovirus (CMV) mutations in leukocytes of patients with acquired immunodeficiency syndrome who are receiving valganciclovir as induction and maintenance therapy for CMV retinitis. J. Infect. Dis. 184, 1598–1602.

Boivin, G., Goyette, N., Gilbert, C., Covington, E., 2005. Analysis of cytomegalovirus DNA polymerase (UL54) mutations in solid organ transplant patients receiving valganciclovir or ganciclovir prophylaxis. J. Med. Virol. 77, 425–429.

Boivin, G., Goyette, N., Rollag, H., Jardine, A.G., Pescovitz, M.D., Asberg, A., Ives, J., Hartmann, A., Humar, A., 2009. Cytomegalovirus resistance in solid organ transplant recipients treated with intravenous ganciclovir or oral valganciclovir. Antivir. Ther. 14, 697–704.

Bourgeois, C., Sixt, N., Bour, J.B., Pothier, P., 1997. Value of a ligase chain reaction assay for detection of ganciclovir resistance-related mutation 594 in UL97 gene of human cytomegalovirus. J. Virol. Methods 67, 167–175.

Boutolleau, D., Deback, C., Bressollette-Bodin, C., Varnous, S., Dhedin, N., Barrou, B., Vernant, J.P., Gandjbakhch, I., Imbert-Marcille, B.M., Agut, H., 2009. Resistance pattern of cytomegalovirus (CMV) after oral valganciclovir therapy in transplant recipients at high-risk for CMV infection. Antiviral Res. 81, 174–179.

Brown, F., Banken, L., Saywell, K., Arum, I., 1999. Pharmacokinetics of valganciclovir and ganciclovir following multiple oral dosages of valganciclovir in HIV- and CMV-seropositive volunteers. Clin. Pharmacokinet. 37, 167–176.

Campanini, G., Zavattoni, M., Cristina, E., Gazzolo, D., Stronati, M., Baldanti, F., 2012. Multiple ganciclovir-resistant strains in a newborn with symptomatic congenital human cytomegalovirus infection. J. Clin. Virol. 54, 86–88.

Chevillotte, M., Ersing, I., Mertens, T., von Einem, J., 2010. Differentiation between polymorphisms and resistance-associated mutations in human cytomegalovirus DNA polymerase. Antimicrob. Agents Chemother. 54, 5004–5011.

Chevillotte, M., Schubert, A., Mertens, T., von Einem, J., 2009. Fluorescence-based assay for phenotypic characterization of human cytomegalovirus polymerase mutations regarding drug susceptibility and viral replicative fitness. Antimicrob. Agents Chemother. 53, 3752–3761.

Chou, S., 2008. Cytomegalovirus UL97 mutations in the era of ganciclovir and maribavir. Rev. Med. Virol. 18, 233–246.

Chou, S., 2009. Diverse cytomegalovirus UL27 mutations adapt to loss of viral UL97 kinase activity under maribavir. Antimicrob. Agents Chemother. 53, 81–85.

Chou, S., 2010. Recombinant phenotyping of cytomegalovirus UL97 kinase sequence variants for ganciclovir resistance. Antimicrob. Agents Chemother. 54, 2371–2378.

Chou, S., 2011. Phenotypic diversity of cytomegalovirus DNA polymerase gene variants observed after antiviral therapy. J. Clin. Virol. 50, 287–291.

Chou, S., Bowlin, T.L., 2011. Cytomegalovirus UL97 mutations affecting cyclopropavir and ganciclovir susceptibility. Antimicrob. Agents Chemother. 55, 382–384.

Chou, S., Lurain, N.S., Thompson, K.D., Miner, R.C., Drew, W.L., 2003. Viral DNA polymerase mutations associated with drug resistance in human cytomegalovirus. J. Infect. Dis. 188, 32–39.

Chou, S., Marousek, G., Boivin, G., Goyette, N., Farhan, M., Ives, J.A., Elston, R., 2010. Recombinant phenotyping of cytomegalovirus sequence variants detected after 200 or 100 days of valganciclovir prophylaxis. Transplantation 90, 1409–1413.

Chou, S., Marousek, G., Guentzel, S., Follansbee, S.E., Poscher, M.E., Lalezari, J.P., Miner, R.C., Drew, W.L., 1997. Evolution of mutations conferring multidrug resistance during prophylaxis and therapy for cytomegalovirus disease. J. Infect. Dis. 176, 786–789.

Chou, S., Marousek, G., Li, S., Weinberg, A., 2008. Contrasting drug resistance phenotypes resulting from cytomegalovirus DNA polymerase mutations at the same exonuclease locus. J. Clin. Virol. 43, 107–109.

Chou, S., Marousek, G., Parenti, D.M., Gordon, S.M., LaVoy, A.G., Ross, J.G., Miner, R.C., Drew, W.L., 1998. Mutation in region III of the DNA polymerase gene conferring foscarnet resistance in cytomegalovirus isolates from 3 subjects receiving prolonged antiviral therapy. J. Infect. Dis. 178, 526–530.

Chou, S., Marousek, G.I., 2008. Accelerated evolution of maribavir resistance in a cytomegalovirus exonuclease domain II mutant. J. Virol. 82, 246–253.

Chou, S., Marousek, G.I., Senters, A.E., Davis, M.G., Biron, K.K., 2004. Mutations in the human cytomegalovirus UL27 gene that confer resistance to maribavir. J. Virol. 78, 7124–7130.

Chou, S., Marousek, G.I., Van Wechel, L.C., Li, S., Weinberg, A., 2007a. Growth and drug resistance phenotypes resulting from cytomegalovirus DNA polymerase region III mutations observed in clinical specimens. Antimicrob. Agents Chemother. 51, 4160–4162.

Chou, S., Meichsner, C.L., 2000. A nine-codon deletion mutation in the cytomegalovirus UL97 phosphotransferase gene confers resistance to ganciclovir. Antimicrob. Agents Chemother. 44, 183–185.

Chou, S., Miner, R.C., Drew, W.L., 2000. A deletion mutation in region V of the cytomegalovirus DNA polymerase sequence confers multidrug resistance. J. Infect. Dis. 182, 1765–1768.

Chou, S., Van Wechel, L.C., Lichy, H.M., Marousek, G.I., 2005. Phenotyping of cytomegalovirus drug resistance mutations by using recombinant viruses incorporating a reporter gene. Antimicrob. Agents Chemother. 49, 2710–2715.

Chou, S., Waldemer, R.H., Senters, A.E., Michels, K.S., Kemble, G.W., Miner, R.C., Drew, W.L., 2002. Cytomegalovirus UL97 phosphotransferase mutations that affect susceptibility to ganciclovir. J. Infect. Dis. 185, 162–169.

Chou, S., Wechel, L.C., Marousek, G.I., 2007b. Cytomegalovirus UL97 kinase mutations that confer maribavir resistance. J. Infect. Dis. 196, 91–94.

Chou, S., Ercolani, R.J., Marousek, G., Bowlin, T.L., 2013. Cytomegalovirus UL97 kinase catalytic domain mutations that confer multidrug resistance. Antimicrob. Agents Chemother. 57, 3375–3379.

Cihlar, T., Fuller, M.D., Cherrington, J.M., 1998a. Characterization of drug resistance-associated mutations in the human cytomegalovirus DNA polymerase gene by using recombinant mutant viruses generated from overlapping DNA fragments. J. Virol. 72, 5927–5936.

Cihlar, T., Fuller, M.D., Mulato, A.S., Cherrington, J.M., 1998b. A point mutation in the human cytomegalovirus DNA polymerase gene selected in vitro by cidofovir confers a slow replication phenotype in cell culture. Virology 248, 382–393.

Dal, P.F., Andrei, G., Daelemans, D., Winkler, M., Piette, J., de Clercq, E, Snoeck, R., 2008. Fluorescence-based antiviral assay for the evaluation of compounds against vaccinia virus, varicella zoster virus and human cytomegalovirus. J. Virol. Methods 151, 66–73.

De Clercq, E., Holy, A., 2005. Acyclic nucleoside phosphonates: a key class of antiviral drugs. Nat. Rev. Drug Discov. 4, 928–940.

Drew, W.L., 2000. Ganciclovir resistance: a matter of time and titre. Lancet 356, 609–610.

Drew, W.L., 2006. Is combination antiviral therapy for CMV superior to monotherapy? J. Clin. Virol. 35, 485–488.

Drew, W.L., Anderson, R., Lang, W., Miner, R.C., Davis, G., Lalezari, J., 1995. Failure of high-dose oral acyclovir to suppress CMV viruria or induce ganciclovir-resistant CMV in HIV antibody positive patients. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 8, 289–291.

Drew, W.L., Miner, R., Saleh, E., 1993. Antiviral susceptibility testing of cytomegalovirus: criteria for detecting resistance to antivirals. Clin Diagn. Virol. 1, 179–185.

Drew, W.L., Miner, R.C., Marousek, G.I., Chou, S., 2006. Maribavir sensitivity of cytomegalovirus isolates resistant to ganciclovir, cidofovir or foscarnet. J. Clin. Virol. 37, 124–127.

Drew, W.L., Paya, C.V., Emery, V., 2001. Cytomegalovirus (CMV) resistance to antivirals. Am. J. Transplant. 1, 307–312.

Ducancelle, A., Alain, S., Petit, F., Sanson Le Pors, M.J., Mazeron, M.C., 2007. Development and validation of a non-radioactive DNA polymerase assay for studying cytomegalovirus resistance to foscarnet. J. Virol. Methods 141, 212–215.

Ducancelle, A., Belloc, S., Alain, S., Scieux, C., Malphettes, M., Petit, F., Brouet, J.C., Sanson Le Pors, M.J., Mazeron, M.C., 2004. Comparison of sequential cytomegalovirus isolates in a patient with lymphoma and failing antiviral therapy. J. Clin. Virol. 29, 241–247.

Ducancelle, A., Gravisse, J., Alain, S., Fillet, A.M., Petit, F., Pors, M.J., Mazeron, M.C., 2005. Phenotypic characterisation of cytomegalovirus DNA polymerase: a method to study cytomegalovirus isolates resistant to foscarnet. J. Virol. Methods 125, 145–151.
Eckle, T., Jahn, G., Hamprecht, K., 2004. The influence of mixed HCMV UL97 wildtype and mutant strains on ganciclovir susceptibility in a cell associated plaque reduction assay. J. Clin. Virol. 30, 50–56.
Eckle, T., Prix, L., Jahn, G., Klingebiel, T., Handgretinger, R., Selle, B., Hamprecht, K., 2000. Drug-resistant human cytomegalovirus infection in children after allogeneic stem cell transplantation may have different clinical outcomes. Blood 96, 3286–3289.
Efferth, T., Marschall, M., Wang, X., Huong, S.M., Hauber, I., Olbrich, A., Kronschnabl, M., Stamminger, T., Huang, E.S., 2002. Antiviral activity of artesunate towards wild-type, recombinant, and ganciclovir-resistant human cytomegaloviruses. J. Mol. Med. (Berl.) 80, 233–242.
Efferth, T., Romero, M.R., Wolf, D.G., Stamminger, T., Marin, J.J., Marschall, M., 2008. The antiviral activities of artemisinin and artesunate. Clin. Infect. Dis. 47, 804–811.
Emery, V.C., Cope, A.V., Bowen, E.F., Gor, D., Griffiths, P.D., 1999. The dynamics of human cytomegalovirus replication in vivo. J. Exp. Med. 190, 177–182.
Erice, A., 1999. Resistance of human cytomegalovirus to antiviral drugs. Clin. Microbiol. Rev. 12, 286–297.
Erice, A., Chou, S., Biron, K.K., Stanat, S.C., Balfour Jr., H.H., Jordan, M.C., 1989. Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Engl. J. Med. 320, 289–293.
Erice, A., Gil-Roda, C., Perez, J.L., Balfour Jr., H.H., Sannerud, K.J., Hanson, M.N., Boivin, G., Chou, S., 1997. Antiviral susceptibilities and analysis of UL97 and DNA polymerase sequences of clinical cytomegalovirus isolates from immunocompromised patients. J. Infect. Dis. 175, 1087–1092.
Faizi, K.R., Mori, S., Eizuru, Y., Kumura, I.K., Minamishima, Y., 1998. Genetic analysis of a ganciclovir-resistant human cytomegalovirus mutant. Antiviral Res. 40, 95–103.
Foulongne, V., Turriere, C., Diafouka, F., Abraham, B., Lastere, S., Segondy, M., 2004. Ganciclovir resistance mutations in UL97 and UL54 genes of Human cytomegalovirus isolates resistant to ganciclovir. Acta Virol. 48, 51–55.
Freifeld, A.G., Ostrove, J.M., 1991. Resistance of viruses to antiviral drugs. Annu. Rev. Med. 42, 247–259.
Fukushima, R., Kanamori, S., Hirashiba, M., Hishikawa, A., Muranaka, R.I., Kaneto, M., Nakamura, K., Kato, I., 2007. Teratogenicity study of the dihydroorotate-dehydrogenase inhibitor and protein tyrosine kinase inhibitor Leflunomide in mice. Reprod. Toxicol. 24, 310–316.
Gallant, J.E., Moore, R.D., Richman, D.D., Keruly, J., Chaisson, R.E., 1992. Incidence and natural history of cytomegalovirus disease in patients with advanced human immunodeficiency virus disease treated with zidovudine. The Zidovudine Epidemiology Study Group. J. Infect. Dis. 166, 1223–1227.
Gentry, B.G., Kamil, J.P., Coen, D.M., Zemlicka, J., Drach, J.C., 2010. Stereoselective phosphorylation of cyclopropavir by pUL97 and competitive inhibition by maribavir. Antimicrob. Agents Chemother. 54, 3093–3098.
Gershburg, E., Pagano, J.S., 2008. Conserved herpesvirus protein kinases. Biochim. Biophys. Acta 1784, 203–212.
Gilbert, C., Azzi, A., Goyette, N., Lin, S.X., Boivin, G., 2011. Recombinant phenotyping of cytomegalovirus UL54 mutations that emerged during cell passages in the presence of either ganciclovir or foscarnet. Antimicrob. Agents Chemother. 55, 4019–4027.
Gilbert, C., Boivin, G., 2005. New reporter cell line to evaluate the sequential emergence of multiple human cytomegalovirus mutations during in vitro drug exposure. Antimicrob. Agents Chemother. 49, 4860–4866.
Gill, R.B., James, S.H., Prichard, M.N., 2012. Human cytomegalovirus UL97 kinase alters the accumulation of CDK1. J. Gen. Virol. 93, 1743–1755.
Goldner, T., Hewlett, G., Ettischer, N., Ruebsamen-Schaeff, H., Zimmermann, H., Lischka, P., 2011. The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase. J. Virol. 85, 10884–10893.
Gorzer, I., Guelly, C., Trajanoski, S., Puchhammer-Stockl, E., 2010. Deep sequencing reveals highly complex dynamics of human cytomegalovirus genotypes in transplant patients over time. J. Virol. 84, 7195–7203.
Grant, S., Edmond, E., Syme, J., 1981. A prospective study of cytomegalovirus infection in pregnancy. I. Laboratory evidence of congenital infection following maternal primary and reactivated infection. J. Infect. 3, 24–31.
Hakki, M., Chou, S., 2011. The biology of cytomegalovirus drug resistance. Curr. Opin. Infect. Dis. 24, 605–611.
Hanks, S.K., Quinn, A.M., Hunter, T., 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241, 42–52.
Hanson, M.N., Preheim, L.C., Chou, S., Talarico, C.L., Biron, K.K., Erice, A., 1995. Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob. Agents Chemother. 39, 1204–1205.
Hantz, S., Cotin, S., Borst, E., Couvreux, A., Salmier, A., Garrigue, I., Merville, P., Mengelle, C., Attal, M., Messerle, M., Alain, S., 2013. Novel DNA polymerase mutations conferring cytomegalovirus resistance: input of BAC-recombinant phenotyping and 3D model. Antiviral Res. 98, 130–134.
Hantz, S., Garnier-Geoffroy, F., Mazeron, M.C., Garrigue, I., Merville, P., Mengelle, C., Rostaing, L., Saint, M.F., Essig, M., Rerolle, J.P., Cotin, S., Germi, R., Pillet, S., Lebranchu, Y., Turlure, P., Alain, S., 2010. Drug-resistant cytomegalovirus in transplant recipients: a French cohort study. J. Antimicrob. Chemother. 65, 2628–2640.
He, Z., He, Y.S., Kim, Y., Chu, L., Ohmstede, C., Biron, K.K., Coen, D.M., 1997. The human cytomegalovirus UL97 protein is a protein kinase that autophosphorylates on serines and threonines. J. Virol. 71, 405–411.
Hu, H., Jabs, D.A., Forman, M.S., Martin, B.K., Dunn, J.P., Weinberg, D.V., Davis, J.L., 2002. Comparison of cytomegalovirus (CMV) UL97 gene sequences in the blood and vitreous of patients with acquired immunodeficiency syndrome and CMV retinitis. J. Infect. Dis. 185, 861–867.
Ibarra, K.D., Jain, M.K., Pfeiffer, J.K., 2011. Host-based ribavirin resistance influences hepatitis C virus replication and treatment response. J. Virol. 85, 7273–7283.
Ijichi, O., Michel, D., Mertens, T., Miyata, K., Eizuru, Y., 2002. GCV resistance due to the mutation A594P in the cytomegalovirus protein UL97 is partially reconstituted by a second mutation at D605E. Antiviral Res. 53, 135–142.
Isegawa, Y., Hara, J., Amo, K., Osugi, Y., Takemoto, M., Yamanishi, K., Fukunaga, R., Shibata, M., Ohshima, A., Horiguchi, Y., Sugimoto, N., 2009. Human herpesvirus 6 ganciclovir-resistant strain with amino acid substitutions associated with the death of an allogeneic stem cell transplant recipient. J. Clin. Virol. 44, 15–19.
Ito, J., Braithwaite, D.K., 1991. Compilation and alignment of DNA polymerase sequences. Nucleic Acids Res. 19, 4045–4057.
Iwasenko, J.M., Scott, G.M., Rawlinson, W.D., Keogh, A., Mitchell, D., Chou, S., 2009. Successful valganciclovir treatment of post-transplant cytomegalovirus infection in the presence of UL97 mutation N597D. J. Med. Virol. 81, 507–510.
Jabs, D.A., Enger, C., Dunn, J.P., Forman, M., 1998. Cytomegalovirus retinitis and viral resistance: ganciclovir resistance. CMV Retinitis and Viral Resistance Study Group. J. Infect. Dis. 177, 770–773.
Jabs, D.A., Martin, B.K., Forman, M.S., Dunn, J.P., Davis, J.L., Weinberg, D.V., Biron, K.K., Baldanti, F., 2001. Mutations conferring ganciclovir resistance in a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis. J. Infect. Dis. 183, 333–337.
Jabs, D.A., Martin, B.K., Ricks, M.O., Forman, M.S., 2006. Detection of ganciclovir resistance in patients with AIDS and cytomegalovirus retinitis: correlation of genotypic methods with viral phenotype and clinical outcome. J. Infect. Dis. 193, 1728–1737.
Jabs, D.A., van Natta, M.L., Holbrook, J.T., Kempen, J.H., Meinert, C.L., Davis, M.D., 2007. Longitudinal study of the ocular complications of AIDS: 2. Ocular examination results at enrollment. Ophthalmology 114, 787–793.
James, S.H., Prichard, M.N., 2011. The genetic basis of human cytomegalovirus resistance and current trends in antiviral resistance analysis. Infect. Disord. Drug Targets 11, 504–513.
Kaptein, S.J., Efferth, T., Leis, M., Rechter, S., Auerochs, S., Kalmer, M., Bruggeman, C.A., Vink, C., Stamminger, T., Marschall, M., 2006. The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo. Antiviral Res. 69, 60–69.
Kedhar, S.R., Jabs, D.A., 2007. Cytomegalovirus retinitis in the era of highly active antiretroviral therapy. Herpes 14, 66–71.
Kimberlin, D.W., Lin, C.Y., Sanchez, P.J., Demmler, G.J., Dankner, W., Shelton, M., Jacobs, R.F., Vaudry, W., Pass, R.F., Kiell, J.M., Soong, S.J., Whitley, R.J., 2003. Effect of ganciclovir therapy on hearing in symptomatic congenital cytomegalovirus disease involving the central nervous system: a randomized, controlled trial. J. Pediatr. 143, 16–25.
Kobayashi, M., Yoshinaga, T., Seki, T., Wakasa-Morimoto, C., Brown, K.W., Ferris, R., Foster, S.A., Hazen, R.J., Miki, S., Suyama-Kagitani, A., Kawauchi-Miki, S., Taishi, T., Kawasuji, T., Johns, B.A., Underwood, M.R., Garvey, E.P., Sato, A., Fujiwara, T., 2011. In Vitro antiretroviral properties of S/GSK1349572, a next-generation HIV integrase inhibitor. Antimicrob. Agents Chemother. 55, 813–821.
Komazin, G., Ptak, R.G., Emmer, B.T., Townsend, L.B., Drach, J.C., 2003. Resistance of human cytomegalovirus to the benzimidazole L-ribonucleoside maribavir maps to UL27. J. Virol. 77, 11499–11506.
Kotton, C.N., 2013. CMV: Prevention, Diagnosis and Therapy. Am. J. Transplant. 13 (Suppl. 3), 24–40.
Kotton, C.N., Kumar, D., Caliendo, A.M., Asberg, A., Chou, S., Snydman, D.R., Allen, U., Humar, A., 2010. International consensus guidelines on the management of cytomegalovirus in solid organ transplantation. Transplantation 89, 779–795.
Kouzarides, T., Bankier, A.T., Satchwell, S.C., Weston, K., Tomlinson, P., Barrell, B.G., 1987. Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J. Virol. 61, 125–133.
Kuo, I.C., Imai, Y., Shum, C., Martin, D.F., Kuppermann, B.D., Margolis, T.P., 2003. Genotypic analysis of cytomegalovirus retinitis poorly responsive to intravenous ganciclovir but responsive to the ganciclovir implant. Am. J. Ophthalmol. 135, 20–25.
Landry, M.L., Stanat, S., Biron, K., Brambilla, D., Britt, W., Jokela, J., Chou, S., Drew, W.L., Erice, A., Gilliam, B., Lurain, N., Manischewitz, J., Miner, R., Nokta, M., Reichelderfer, P., Spector, S., Weinberg, A., Yen-Lieberman, B., Crumpacker, C., 2000. A standardized plaque reduction assay for determination of drug susceptibilities of cytomegalovirus clinical isolates. Antimicrob. Agents Chemother. 44, 688–692.
Levi, M.E., Mandava, N., Chan, L.K., Weinberg, A., Olson, J.L., 2006. Treatment of multidrug-resistant cytomegalovirus retinitis with systemically administered leflunomide. Transpl. Infect. Dis. 8, 38–43.
Li, F., Kenyon, K.W., Kirby, K.A., Fishbein, D.P., Boeckh, M., Limaye, A.P., 2007. Incidence and clinical features of ganciclovir-resistant cytomegalovirus disease in heart transplant recipients. Clin. Infect. Dis. 45, 439–447.
Limaye, A.P., 2002. Ganciclovir-resistant cytomegalovirus in organ transplant recipients. Clin. Infect. Dis. 35, 866–872.
Lischka, P., Hewlett, G., Wunberg, T., Baumeister, J., Paulsen, D., Goldner, T., Ruebsamen-Schaeff, H., Zimmermann, H., 2010. In vitro and in vivo activities of

the novel anticytomegalovirus compound AIC246. Antimicrob. Agents Chemother. 54, 1290–1297.
Littler, E., Stuart, A.D., Chee, M.S., 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature 358, 160–162.
Ljungman, P., 1995. Cytomegalovirus pneumonia: presentation, diagnosis, and treatment. Semin. Respir. Infect. 10, 209–215.
Ljungman, P., Hakki, M., Boeckh, M., 2011. Cytomegalovirus in hematopoietic stem cell transplant recipients. Hematol. Oncol. Clin. North Am. 25, 151–169.
Luganini, A., Caposio, P., Landolfo, S., Gribaudo, G., 2008. Phosphorothioate-modified oligodeoxynucleotides inhibit human cytomegalovirus replication by blocking virus entry. Antimicrob. Agents Chemother. 52, 1111–1120.
Lurain, N.S., Bhorade, S.M., Pursell, K.J., Avery, R.K., Yeldandi, V.V., Isada, C.M., Robert, E.S., Kohn, D.J., Arens, M.Q., Garrity, E.R., Taege, A.J., Mullen, M.G., Todd, K.M., Bremer, J.W., Yen-Lieberman, B., 2002. Analysis and characterization of antiviral drug-resistant cytomegalovirus isolates from solid organ transplant recipients. J. Infect. Dis. 186, 760–768.
Lurain, N.S., Chou, S., 2010. Antiviral drug resistance of human cytomegalovirus. Clin. Microbiol. Rev. 23, 689–712.
Lurain, N.S., Spafford, L.E., Thompson, K.D., 1994. Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. J. Virol. 68, 4427–4431.
Lurain, N.S., Thompson, K.D., Holmes, E.W., Read, G.S., 1992. Point mutations in the DNA polymerase gene of human cytomegalovirus that result in resistance to antiviral agents. J. Virol. 66, 7146–7152.
Marfori, J.E., Exner, M.M., Marousek, G.I., Chou, S., Drew, W.L., 2007. Development of new cytomegalovirus UL97 and DNA polymerase mutations conferring drug resistance after valganciclovir therapy in allogeneic stem cell recipients. J. Clin. Virol. 38, 120–125.
Margeridon-Thermet, S., Shulman, N.S., Ahmed, A., Shahriar, R., Liu, T., Wang, C., Holmes, S.P., Babrzadeh, F., Gharizadeh, B., Hanczaruk, B., Simen, B.B., Egholm, M., Shafer, R.W., 2009. Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. J. Infect. Dis. 199, 1275–1285.
Marschall, M., Freitag, M., Weiler, S., Sorg, G., Stamminger, T., 2000. Recombinant green fluorescent protein-expressing human cytomegalovirus as a tool for screening antiviral agents. Antimicrob. Agents Chemother. 44, 1588–1597.
Marschall, M., Stein-Gerlach, M., Freitag, M., Kupfer, R., van Den, B.M., Stamminger, T., 2001. Inhibitors of human cytomegalovirus replication drastically reduce the activity of the viral protein kinase pUL97. J. Gen. Virol. 82, 1439–1450.
Martin, M., Azzi, A., Lin, S.X., Boivin, G., 2010a. Opposite effect of two cytomegalovirus DNA polymerase mutations on replicative capacity and polymerase activity. Antivir. Ther. 15, 579–586.
Martin, M., Gilbert, C., Covington, E., Boivin, G., 2006. Characterization of human cytomegalovirus (HCMV) UL97 mutations found in a valganciclovir/oral ganciclovir prophylactic trial by use of a bacterial artificial chromosome containing the HCMV genome. J. Infect. Dis. 194, 579–583.
Martin, M., Goyette, N., Ives, J., Boivin, G., 2010b. Incidence and characterization of cytomegalovirus resistance mutations among pediatric solid organ transplant patients who received valganciclovir prophylaxis. J. Clin. Virol. 47, 321–324.
Marty, F.M., Ljungman, P., Papanicolaou, G.A., Winston, D.J., Chemaly, R.F., Strasfeld, L., Young, J.A., Rodriguez, T., Maertens, J., Schmitt, M., Einsele, H., Ferrant, A., Lipton, J.H., Villano, S.A., Chen, H., Boeckh, M., 2011. Maribavir prophylaxis for prevention of cytomegalovirus disease in recipients of allogeneic stem-cell transplants: a phase 3, double-blind, placebo-controlled, randomised trial. Lancet Infect. Dis 11, 284–292.
Masur, H., Whitcup, S.M., Cartwright, C., Polis, M., Nussenblatt, R., 1996. Advances in the management of AIDS-related cytomegalovirus retinitis. Ann. Intern. Med. 125, 126–136.
Mattick, C., Dewin, D., Polley, S., Sevilla-Reyes, E., Pignatelli, S., Rawlinson, W., Wilkinson, G., Dal, M.P., Gompels, U.A., 2004. Linkage of human cytomegalovirus glycoprotein gO variant groups identified from worldwide clinical isolates with gN genotypes, implications for disease associations and evidence for N-terminal sites of positive selection. Virology 318, 582–597.
Michel, D., Pavic, I., Zimmermann, A., Haupt, E., Wunderlich, K., Heuschmid, M., Mertens, T., 1996. The UL97 gene product of human cytomegalovirus is an early-late protein with a nuclear localization but is not a nucleoside kinase. J. Virol. 70, 6340–6346.
Michel, D., Schaarschmidt, P., Wunderlich, K., Heuschmid, M., Simoncini, L., Muhlberger, D., Zimmermann, A., Pavic, I., Mertens, T., 1998. Functional regions of the human cytomegalovirus protein pUL97 involved in nuclear localization and phosphorylation of ganciclovir and pUL97 itself. J. Gen. Virol. 79 (Pt 9), 2105–2112.
Mossad, S.B., Avery, R.K., 2007. Second look at leflunomide "failure" to control cytomegalovirus infection in the setting of renal failure. Transpl. Infect. Dis. 9, 260–261.
Mousavi-Jazi, M., Hokeberg, I., Schloss, L., Zweygberg-Wirgart, B., Grillner, L., Linde, A., Brytting, M., 2001a. Sequence analysis of UL54 and UL97 genes and evaluation of antiviral susceptibility of human cytomegalovirus isolates obtained from kidney allograft recipients before and after treatment. Transpl. Infect. Dis. 3, 195–202.
Mousavi-Jazi, M., Schloss, L., Drew, W.L., Linde, A., Miner, R.C., Harmenberg, J., Wahren, B., Brytting, M., 2001b. Variations in the cytomegalovirus DNA polymerase and phosphotransferase genes in relation to foscarnet and ganciclovir sensitivity. J. Clin. Virol. 23, 1–15.
Mulamba, G.B., Hu, A., Azad, R.F., Anderson, K.P., Coen, D.M., 1998. Human cytomegalovirus mutant with sequence-dependent resistance to the phosphorothioate oligonucleotide fomivirsen (ISIS 2922). Antimicrob. Agents Chemother. 42, 971–973.
Muller, B., Krausslich, H.G., 2009. Antiviral strategies. Handb. Exp. Pharmacol., 1–24.
Myhre, H.A., Haug, D.D., Kristiansen, K.I., Rollag, H., Leivestad, T., Asberg, A., Hartmann, A., 2011. Incidence and outcomes of ganciclovir-resistant cytomegalovirus infections in 1244 kidney transplant recipients. Transplantation 92, 217–223.
Nakano, K., Nishinaka, K., Tanaka, T., Ohshima, A., Sugimoto, N., Isegawa, Y., 2009. Detection and identification of U69 gene mutations encoded by ganciclovir-resistant human herpesvirus 6 using denaturing high-performance liquid chromatography. J. Virol. Methods 161, 223–230.
Nijhuis, M., van Maarseveen, N.M., Boucher, C.A., 2009. Antiviral resistance and impact on viral replication capacity: evolution of viruses under antiviral pressure occurs in three phases. Handb. Exp. Pharmacol., 299–320.
Novak, Z., Ross, S.A., Patro, R.K., Pati, S.K., Kumbla, R.A., Brice, S., Boppana, S.B., 2008. Cytomegalovirus strain diversity in seropositive women. J. Clin. Microbiol. 46, 882–886.
O'Brien, J.J., Campoli-Richards, D.M., 1989. Acyclovir. An updated review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs 37, 233–309.
Palella Jr., F.J., Delaney, K.M., Moorman, A.C., Loveless, M.O., Fuhrer, J., Satten, G.A., Aschman, D.J., Holmberg, S.D., 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N. Engl. J. Med. 338, 853–860.
Palmer, S.M., Limaye, A.P., Banks, M., Gallup, D., Chapman, J., Lawrence, E.C., Dunitz, J., Milstone, A., Reynolds, J., Yung, G.L., Chan, K.M., Aris, R., Garrity, E., Valentine, V., McCall, J., Chow, S.C., Davis, R.D., Avery, R., 2010. Extended valganciclovir prophylaxis to prevent cytomegalovirus after lung transplantation: a randomized, controlled trial. Ann. Intern. Med. 152, 761–769.
Pari, G.S., Anders, D.G., 1993. Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. J. Virol. 67, 6979–6988.
Paya, C., Humar, A., Dominguez, E., Washburn, K., Blumberg, E., Alexander, B., Freeman, R., Heaton, N., Pescovitz, M.D., 2004. Efficacy and safety of valganciclovir vs. oral ganciclovir for prevention of cytomegalovirus disease in solid organ transplant recipients. Am. J. Transplant. 4, 611–620.
Pignatelli, S., Dal, M.P., Rossini, G., Chou, S., Gojobori, T., Hanada, K., Guo, J.J., Rawlinson, W., Britt, W., Mach, M., Landini, M.P., 2003. Human cytomegalovirus glycoprotein N (gpUL73-gN) genomic variants: identification of a novel subgroup, geographical distribution and evidence of positive selective pressure. J. Gen. Virol. 84, 647–655.
Preiksaitis, J.K., Brennan, D.C., Fishman, J., Allen, U., 2005. Canadian society of transplantation consensus workshop on cytomegalovirus management in solid organ transplantation final report. Am. J. Transplant. 5, 218–227.
Prichard, M.N., Gao, N., Jairath, S., Mulamba, G., Krosky, P., Coen, D.M., Parker, B.O., Pari, G.S., 1999. A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. J. Virol. 73, 5663–5670.
Quenelle, D.C., Lampert, B., Collins, D.J., Rice, T.L., Painter, G.R., Kern, E.R., 2010. Efficacy of CMX001 against herpes simplex virus infections in mice and correlations with drug distribution studies. J. Infect. Dis. 202, 1492–1499.
Razonable, R.R., Asberg, A., Rollag, H., Duncan, J., Boisvert, D., Yao, J.D., Caliendo, A.M., Humar, A., Do, T.D., 2013. Virologic suppression measured by a cytomegalovirus (CMV) DNA test calibrated to the world health organization international standard is predictive of CMV disease resolution in transplant recipients. Clin. Infect. Dis. 56, 1546–1553.
Razonable, R.R., Emery, V.C., 2004. Management of CMV infection and disease in transplant patients. 27–29 February 2004. Herpes 11, 77–86.
Reddy, A.J., Zaas, A.K., Hanson, K.E., Palmer, S.M., 2007. A single-center experience with ganciclovir-resistant cytomegalovirus in lung transplant recipients: treatment and outcome. J. Heart Lung Transplant. 26, 1286–1292.
Reyburn, H., 2010. New WHO guidelines for the treatment of malaria. BMJ 340, c2637.
Roizman, B., Carmichael, L.E., Deinhardt, F., de-The, G., Nahmias, A.J., Plowright, W., Rapp, F., Sheldrick, P., Takahashi, M., Wolf, K., 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology 16, 201–217.
Ross, S.A., Novak, Z., Pati, S., Patro, R.K., Blumenthal, J., Danthuluri, V.R., Ahmed, A., Michaels, M.G., Sanchez, P.J., Bernstein, D.I., Tolan, R.W., Palmer, A.L., Britt, W.J., Fowler, K.B., Boppana, S.B., 2011. Mixed infection and strain diversity in congenital cytomegalovirus infection. J. Infect. Dis. 204, 1003–1007.
Sahoo, M.K., Lefterova, M.I., Yamamoto, F., Waggoner, J.J., Chou, S., Holmes, S.P., Anderson, M.W., Pinsky, B.A., 2013. Detection of cytomegalovirus drug resistance mutations by next-generation sequencing. J. Clin. Microbiol. 51, 1605–1613.
Schnepf, N., Boiteau, N., Petit, F., Alain, S., Sanson-Le Pors, M.J., Mazeron, M.C., 2009. Rapid determination of antiviral drug susceptibility of human cytomegalovirus by real-time PCR. Antiviral Res. 81, 64–67.
Schnepf, N., Corvo, J., Pors, M.J., Mazeron, M.C., 2011. Antiviral activity of ganciclovir and artesunate towards human cytomegalovirus in astrocytoma cells. Antiviral Res. 89, 186–188.
Schreiber, A., Harter, G., Schubert, A., Bunjes, D., Mertens, T., Michel, D., 2009. Antiviral treatment of cytomegalovirus infection and resistant strains. Expert Opin. Pharmacother. 10, 191–209.

Scott, G.M., Isaacs, M.A., Zeng, F., Kesson, A.M., Rawlinson, W.D., 2004. Cytomegalovirus antiviral resistance associated with treatment induced UL97 (protein kinase) and UL54 (DNA polymerase) mutations. J. Med. Virol. 74, 85–93.

Scott, G.M., Weinberg, A., Rawlinson, W.D., Chou, S., 2007. Multidrug resistance conferred by novel DNA polymerase mutations in human cytomegalovirus isolates. Antimicrob. Agents Chemother. 51, 89–94.

Seo, S.K., Regan, A., Cihlar, T., Lin, D.C., Boulad, F., George, D., Prasad, V.K., Kiehn, T.E., Polsky, B., 2001. Cytomegalovirus ventriculoencephalitis in a bone marrow transplant recipient receiving antiviral maintenance: clinical and molecular evidence of drug resistance. Clin. Infect. Dis. 33, e105–e108.

Shapira, M.Y., Resnick, I.B., Chou, S., Neumann, A.U., Lurain, N.S., Stamminger, T., Caplan, O., Saleh, N., Efferth, T., Marschall, M., Wolf, D.G., 2008. Artesunate as a potent antiviral agent in a patient with late drug-resistant cytomegalovirus infection after hematopoietic stem cell transplantation. Clin. Infect. Dis. 46, 1455–1457.

Shepp, D.H., Dandliker, P.S., de Miranda, P., Burnette, T.C., Cederberg, D.M., Kirk, L.E., Meyers, J.D., 1985. Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl] guanine in the treatment of cytomegalovirus pneumonia. Ann. Intern. Med 103, 368–373.

Simen, B.B., Simons, J.F., Hullsiek, K.H., Novak, R.M., Macarthur, R.D., Baxter, J.D., Huang, C., Lubeski, C., Turenchalk, G.S., Braverman, M.S., Desany, B., Rothberg, J.M., Egholm, M., Kozal, M.J., 2009. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. J. Infect. Dis. 199, 693–701.

Smith, I.L., Cherrington, J.M., Jiles, R.E., Fuller, M.D., Freeman, W.R., Spector, S.A., 1997. High-level resistance of cytomegalovirus to ganciclovir is associated with alterations in both the UL97 and DNA polymerase genes. J. Infect. Dis. 176, 69–77.

Smith, I.L., Taskintuna, I., Rahhal, F.M., Powell, H.C., Ai, E., Mueller, A.J., Spector, S.A., Freeman, W.R., 1998. Clinical failure of CMV retinitis with intravitreal cidofovir is associated with antiviral resistance. Arch. Ophthalmol. 116, 178–185.

Springer, K.L., Chou, S., Li, S., Giller, R.H., Quinones, R., Shira, J.E., Weinberg, A., 2005. How evolution of mutations conferring drug resistance affects viral dynamics and clinical outcomes of cytomegalovirus-infected hematopoietic cell transplant recipients. J. Clin. Microbiol. 43, 208–213.

Stagno, S., Britt, R., 2006. Cytomegalovirus Infections. In: Remingtonm, J., Klein, J., Wilson, C., Baker, C. (Eds.), Infectious Diseases of the Fetus and Newborn Infant, sixth ed. Elsevier Saunders, Philadelphia, pp. 739–781.

Stewart, M.W., 2010. Optimal management of cytomegalovirus retinitis in patients with AIDS. Clin. Ophthalmol. 4, 285–299.

Stinski, M., Malone, C., Hermiston, T., Liu, B., 1991. Regulation of human cytomegalovirus transcription. In: Wagner, E. (Ed.), Herpesvirus Transcription and Its Control. CRC, pp. 246–260.

Sullivan, V., Biron, K.K., Talarico, C., Stanat, S.C., Davis, M., Pozzi, L.M., Coen, D.M., 1993. A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to ganciclovir and phosphonylmethoxyalkyl derivatives. Antimicrob. Agents Chemother. 37, 19–25.

Sullivan, V., Talarico, C.L., Stanat, S.C., Davis, M., Coen, D.M., Biron, K.K., 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature 359, 85.

Sun, J.H., O'Boyle Ii, D.R., Zhang, Y., Wang, C., Nower, P., Valera, L., Roberts, S., Nettles, R.E., Fridell, R.A., Gao, M., 2012. Impact of a baseline polymorphism on the emergence of resistance to the hepatitis C virus nonstructural protein 5A replication complex inhibitor, BMS-790052. Hepatology 55, 1692–1699.

Svarovskaia, E.S., Martin, R., McHutchison, J.G., Miller, M.D., Mo, H., 2012. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. J. Clin. Microbiol. 50, 3267–3274.

Tischer, B.K., von, Einem, J., Kaufer, B., Osterrieder, N., 2006. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40, 191–197.

Tomblyn, M., Chiller, T., Einsele, H., Gress, R., Sepkowitz, K., Storek, J., Wingard, J.R., Young, J.A., Boeckh, M.J., 2009. Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective. Biol. Blood Marrow Transplant. 15, 1143–1238.

Ueno, T., Eizuru, Y., Katano, H., Kurata, T., Sata, T., Irie, S., Ogawa-Goto, K., 2006. Novel real-time monitoring system for human cytomegalovirus-infected cells in vitro that uses a green fluorescent protein-PML-expressing cell line. Antimicrob. Agents Chemother. 50, 2806–2813.

Ueno, T., Ogawa-Goto, K., 2009. Use of a GFP-PML-expressing cell line as a biosensor for human cytomegalovirus infection. Methods Mol. Biol. 515, 33–44.

Valcyte (valgancyclovir hydrochloride tablets) Package Insert, 2013. Roche Laboratories Inc.

Verbinnen, T., Jacobs, T., Vijgen, L., Ceulemans, H., Neyts, J., Fanning, G., Lenz, O., 2012. Replication capacity of minority variants in viral populations can affect the assessment of resistance in HCV chimeric replicon phenotyping assays. J. Antimicrob. Chemother. 67, 2327–2337.

Vistide (cidofovir injection) Package Insert, 1996. Gilead Science Inc.

Warming, S., Costantino, N., Court, D.L., Jenkins, N.A., Copeland, N.G., 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. 33, e36.

Weinberg, A., Jabs, D.A., Chou, S., Martin, B.K., Lurain, N.S., Forman, M.S., Crumpacker, C., 2003. Mutations conferring foscarnet resistance in a cohort of patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis. J. Infect. Dis. 187, 777–784.

Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., McKimm-Breschkin, J.L., Zambon, M., Hayden, F.G., 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J. Clin. Microbiol. 41, 742–750.

Wilks, A.F., Kurban, R.R., Hovens, C.M., Ralph, S.J., 1989. The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. Gene 85, 67–74.

Winston, D.J., Saliba, F., Blumberg, E., Abouljoud, M., Garcia-Diaz, J.B., Goss, J.A., Clough, L., Avery, R., Limaye, A.P., Ericzon, B.G., Navasa, M., Troisi, R.I., Chen, H., Villano, S.A., Uknis, M.E., 2012. Efficacy and safety of maribavir dosed at 100 mg orally twice daily for the prevention of cytomegalovirus disease in liver transplant recipients: a randomized, double-blind, multicenter controlled trial. Am. J. Transplant. 12, 3021–3030.

Wolf, D.G., Courcelle, C.T., Prichard, M.N., Mocarski, E.S., 2001. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc. Natl. Acad. Sci. USA 98, 1895–1900.

Wolf, D.G., Shimoni, A., Resnick, I.B., Stamminger, T., Neumann, A.U., Chou, S., Efferth, T., Caplan, O., Rose, J., Nagler, A., Marschall, M., 2011. Human cytomegalovirus kinetics following institution of artesunate after hematopoietic stem cell transplantation. Antiviral Res. 90, 183–186.

Wolf, D.G., Smith, I.L., Lee, D.J., Freeman, W.R., Flores-Aguilar, M., Spector, S.A., 1995. Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. 95, 257–263.

Wolf, D.G., Yaniv, I., Honigman, A., Kassis, I., Schonfeld, T., Ashkenazi, S., 1998. Early emergence of ganciclovir-resistant human cytomegalovirus strains in children with primary combined immunodeficiency. J. Infect. Dis. 178, 535–538.

Wong, S.W., Wahl, A.F., Yuan, P.M., Arai, N., Pearson, B.E., Arai, K., Korn, D., Hunkapiller, M.W., Wang, T.S., 1988. Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. 7, 37–47.

Wright Jr., H.T., Goodheart, C.R., Lielausis, A., 1964. Human Cytomegalovirus. Morphology by negative staining. Virology 23, 419–424.

Yang, H., Datema, R., 1991. Prolonged and potent therapeutic and prophylactic effects of (S)-1-[(3-hydroxy-2-phosphonylmethoxy)propyl]cytosine against herpes simplex virus type 2 infections in mice. Antimicrob. Agents Chemother. 35, 1596–1600.

Zhang, Y., Zhao, Z., Sun, J., Cao, G., Zhao, F., Hu, J., Liu, L., Ji, Y., 2013. A new mutation in the human cytomegalovirus UL97 gene may confer ganciclovir resistance in Chinese kidney transplant recipients. Arch. Virol. 158, 247–250.