Current Topics in Virology
Vol. 11, 2013

Review

Antiviral drug resistance of human cytomegalovirus

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ABSTRACT

Human cytomegalovirus (CMV) is a common cause for human viral infection and is mostly asymptomatic in healthy individuals. However, CMV is the leading cause of congenital infection and is a common opportunistic pathogen in immunocompromised individuals. The currently approved antivirals have limited efficacy due to the development of resistance and severe toxicities. It is thus imperative to develop new therapeutics that are highly efficacious and without significant toxicities. The purpose of this review is to provide genotypic maps of drug resistance mutations, give an update of the progress of CMV antiviral drug resistance research, and showcase promising drug candidates.

KEYWORDS: cytomegalovirus, antiviral drug, drug resistance

INTRODUCTION

Human cytomegalovirus (CMV), also known as human herpesvirus-5 (HHV-5) is the cause of one of the most prevalent chronic viral infections in the world. It is estimated that approximately 60 percent of the United States population has been infected with the virus [1]. Most of these infections are largely asymptomatic; however, CMV can be a major cause of morbidity and mortality for congenitally infected infants and immunocompromised individuals in the setting of transplants and HIV-1 infection [2-4]. There are four drugs currently approved to treat CMV infection: ganciclovir (GCV), valganciclovir (vGCV), cidofovir (CDV) and foscarnet (FOS). Their clinical utility is limited, however, due to the development of resistance and severe toxicities [5]. Minimizing the development of resistance has proven difficult because all current antivirals have the same target, and CDV and FOS have severe toxicity issues. Thus, most patients are treated with GCV or vGCV until resistance develops. There are, however, several drug candidates with new viral targets, as well as drugs with potentially less toxicity in development. Here, we review CMV antiviral resistance, recent progress in drug development and provide genotypic maps of drug resistance mutations for the currently marketed antivirals, as well as those known to us in development.

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Drug targets and drug resistance

CMV drug resistance to currently marketed antivirals arises from mutations in two viral proteins: the DNA polymerase (UL54) and the viral kinase (UL97). UL54 is a polymerase consisting of 1242 amino acids that has polymerase and 3'-5' exonuclease activity, and it has numerous conserved functional regions that are shared amongst all family B DNA polymerases [6, 7]. It has seven conserved regions

that correspond with polymerase activity (I-VII) and three conserved regions that correspond with exonuclease activity (Exo I–Exo III). It also has a region that is conserved amongst nearly all mammalian and yeast delta DNA polymerases, termed deltaC. Since this region is so highly conserved it is theorized that it plays a role in active site formation.

Mutations in UL54 can confer resistance to all of the currently available antivirals (Fig. 1). The majority of mutations that confer resistance to the nucleotide analogs, GCV and CDV, are found in the Exo II and Exo III domains. Structural modeling suggests that the residues that cause the strongest resistance to GCV and CDV cause UL54 to be more rigid and locked in the closed "editing" mode, increasing the exonuclease activity of the polymerase [8]. For this reason, it is hypothesized that one mechanism of resistance for these antivirals is increased exonuclease activity favoring the removal of GCV and CDV from the DNA strand. However, some evidence has shown that CDV likely cannot be excised from the growing DNA strand by UL54, but this issue is still under debate [9]. On the other hand, a few mutations in the exonuclease domains confer FOS resistance, but most FOS resistance associated mutations are congregated in the polymerase domains II, III and VI. Further, most of the FOS resistance associated mutations in domains VI and III show low-level cross-resistance with CDV and GCV. The mechanism of resistance of UL54 mutations remains unclear perhaps due to insufficient structural information.

The viral kinase, UL97, is necessary for efficient viral replication. It phosphorylates serine and threonine residues of various cellular and viral proteins [10]. Loss of UL97 function significantly retards viral replication kinetics by inhibiting viral encapsidation and nuclear egress of viral particles from infected cells [11]. Concerning antiviral activity, UL97 is also necessary for the conversion of GCV and vGCV to their active form. Antiviral anabolism is illustrated in Fig. 2. Specifically, GCV and vGCV require three phosphorylations to be activated (Fig. 2). The first of these three phosphorylations is performed by UL97, while the other two are done by cellular kinases [12]. The other marketed antivirals are phosphorylated solely by cellular kinases or do not require phosphorylation (Fig. 2) [13]. Thus, mutations in the viral kinase that decrease binding affinity between UL97 and the drug can confer resistance to GCV and vGCV, but not CDV or FOS [14]. There are seven canonical mutations in UL97 that are found in over 80% of GCV resistant CMV isolates: M460V/L, H520Q, C592G, A594V, L595S and C603W [15] (Fig. 3).

Marketed CMV antivirals

Of the four drugs currently approved by the FDA to treat CMV infection, GCV and vGCV are the current first line therapy for CMV infection because of their relatively favorable toxicity profiles in comparison to FOS and CDV [16]. GCV is a deoxyguanosine triphosphate mimetic that acts by two mechanisms: (i) competitive inhibition of the incorporation of dGTP into the growing DNA strand during DNA synthesis and (ii) termination of DNA elongation upon incorporation into the DNA strand [17-19]. One downside of GCV is that it has very poor oral bioavailability [20]. On the other hand, vGCV, the valine esther of GCV, has excellent oral bioavailability [21].

The antivirals CDV and FOS are often second-line drugs due to clinical side effects, the most prevalent being acute renal toxicity [22, 23]. In addition, both drugs have poor oral bioavailability and are administered intravenously [24, 25]. CDV is a mimic of deoxycytosine triphosphate nucleotide, and acts by three distinct mechanisms: (i) competitive inhibition of the incorporation of deoxycytosine into the growing DNA strand during DNA elongation, (ii) decrease the polymerase activity upon incorporation into the DNA and (iii) termination of DNA elongation [26]. FOS is a pyrophosphate analog that binds near the pyrophosphate-binding site of UL54. The bound drug blocks the cleavage of the pyrophosphate moiety from the deoxynucleotide triphosphate. This halts DNA elongation [27]. Unlike GCV, FOS does not require phosphorylation and CDV is only phosphorylated by cellular kinases. Hence, mutations in UL97 do not confer resistance to CDV or FOS [13].

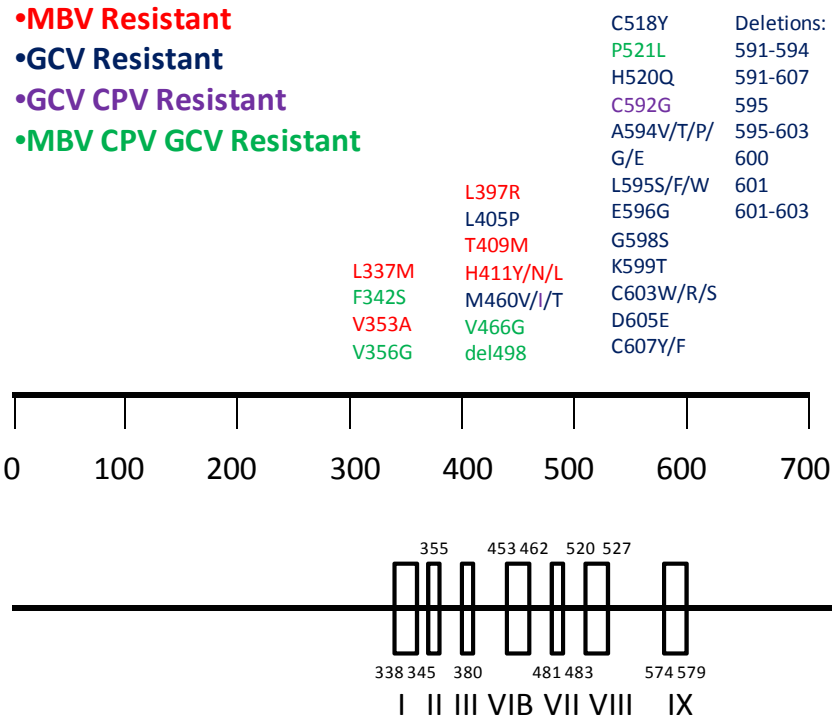

Fig. 1. Map of cytomegalovirus DNA polymerase UL54 functional domains, resistance mutations, and associated phenotypes. del: deletion; MBV: Maribavir; GCV: Ganciclovir; CPV: Cyclopropavir.

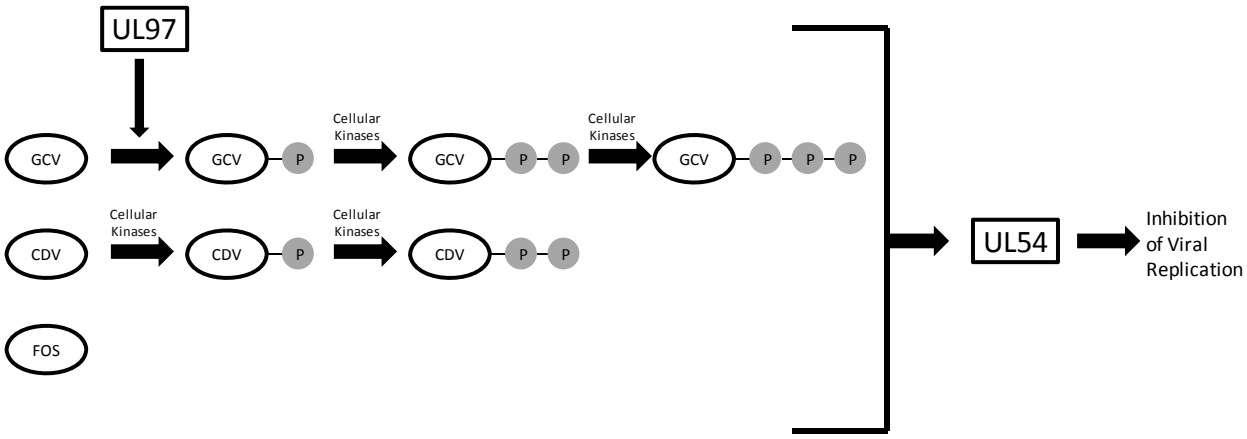

Fig. 2. Drug Anabolism. GCV: Ganciclovir; CDV: Cidofovir; FOS: Fosamprenavir. Each gray circle denotes a phosphate group. CMV proteins DNA polymerase (UL54) and the viral kinase (UL97) are squared. CDV and GCV require two and three phosphorylations, respectively, to be activated meanwhile FOS does not require any phosphorylation.

Potential new anti-cmv drug candidates

Treatment of CMV disease remains a serious problem for clinicians because of limited number of antivirals and toxicity. This is especially pertinent in patients with severe immune deficiency who require prolonged anti-CMV therapy because drug resistance develops in nearly 20 percent of patients within 12 months of starting GCV monotherapy [28].

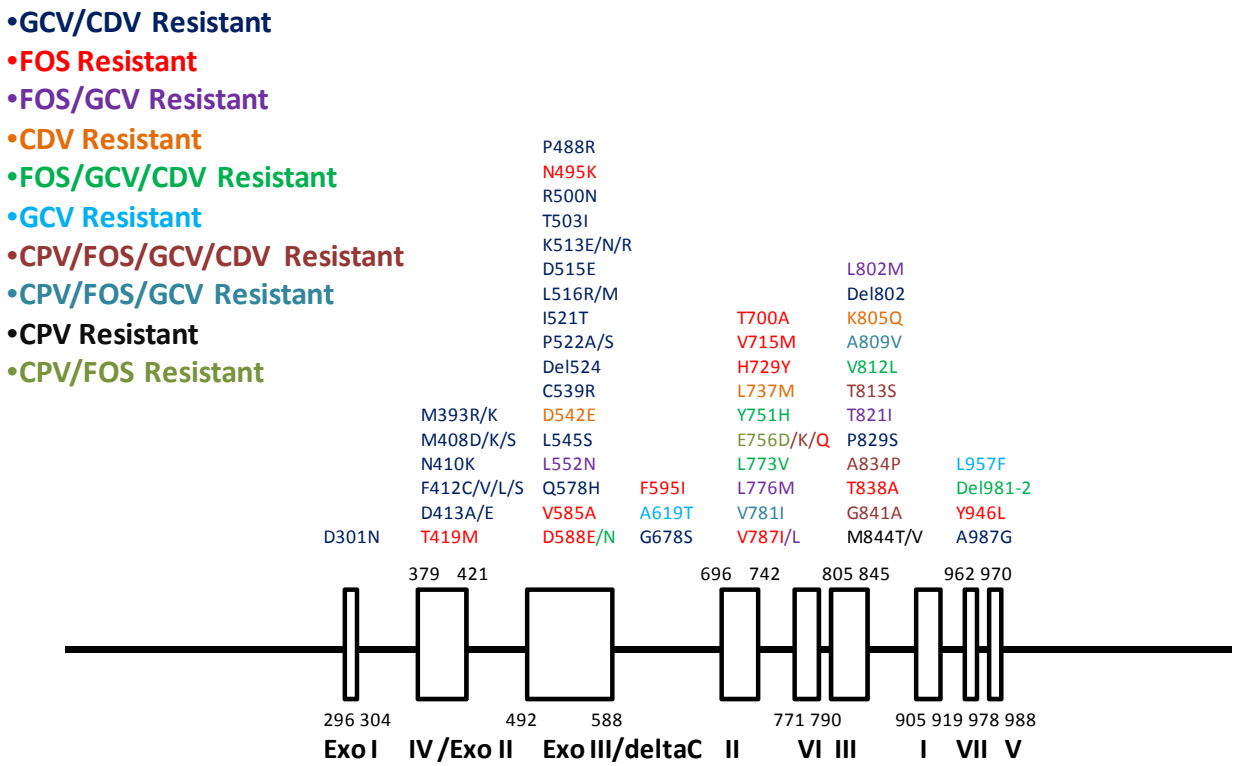

Fig. 3. Map of cytomegalovirus DNA polymerase UL97 functional domains, resistance mutations, and associated phenotypes. del: deletion; GCV: Ganciclovir; CPV: Cyclopropavir; FOS: Fosamprenavir; CDV: Cidofovir; Exo: exonuclease.

Combined therapy with CDV and/or FOS limits the resistance rate to less than five percent, but the trade off is the toxicity of CDV and FOS [29]. Further complicating this issue is the development of multi-drug resistant CMV [30]. Thus, there is a strong need to identify new targets for anti-CMV therapy and to develop novel antiviral compounds and strategies.

Nucleoside and nucleotide analogs

The DNA polymerase remains a viable target for newly developed antivirals. Nucleoside and nucleotide analogs work by competitively inhibiting UL54's active site and terminating elongation of a growing DNA strand upon incorporation. Newer drugs in this class are designed to have greater potency and less toxicity than GCV and CDV.

CMX001

CMX001 is a lipid conjugate of CDV that eliminates some of the major limitations of its parent compound. Unlike CDV, CMX001 can be administered orally and likely does not cause nephrotoxicity, even at high doses; however, diarrhea seems to be its limiting toxicity [31]. Further, CMX001 was found to be over a hundred times more potent *in vitro* than CDV [32]. In a recent phase II clinical trial of CMX001 in the setting of hematopoetic stem cell transplant patients, CMX001 had a statistically significant benefit *versus* placebo in preventing CMV viremia and CMV disease 13 weeks after the transplant (NCT00942305) [33]. Since, CMX001 is cleaved to CDV within the cell, it is likely to have the same resistance profile as CDV [34]; however, one recent study found a novel resistance mutation (D542E) that conferred a higher level of resistance to CMX001 than to CDV suggesting that the resistance profiles of these two drugs may somewhat differ [35]. The mechanisms of these differences in resistance remain unclear. Therefore, more phenotypic and genotypic characterizations are needed to determine the similarity of the resistance profile of CMX001 to CDV.

Cyclopropavir

Cyclopropavir (CPV) is a methylenecyclopropane nucleoside analog with *in vitro* and *in vivo* anti-CMV activity [36, 37] and low cytotoxicity [37] that has recently completed phase I clinical trials (NCT01433835). Similar to GCV, CPV must undergo initial phosphorylation by UL97 kinase [38], while the remaining phosphorylation steps are performed by cellular kinases. Unlike currently marketed CMV antivirals, CPV inhibits both UL54 and UL97 function [39, 40]. Therefore, resistance associated mutations can occur in both UL54 and UL97 (Fig. 1 and Fig. 3), which could be important in cross-resistance with other CMV drugs [40]. Specifically, seven canonical UL97 mutations are found in >80% of GCV-resistant clinical isolates (M460V/I, H520Q, C592G, A594V, L595S, C603W, and M460I). They commonly appear because of evolutionary pressure in drug selection experiments with CPV and confer 12 and 20 fold increased resistance [14]. The UL54 mutations that confer resistance to CPV map to the polymerase domains III and VI, in contrast to the other nucleoside analogs whose resistance primarily maps to the Exo II and Exo III domains. In addition, resistance has been found in viral isolates containing mutations in UL27, although the mechanism of this resistance is unknown [38].

UL97 inhibitors

UL97 kinase phosphorylates proteins that are responsible for viral encapsidation and nuclear egress of viral particles from infected cells [10]. UL97 inhibitors work by competitively inhibiting the function of the UL97 kinase thereby decreasing the viral replication kinetics.

Maribavir

Maribavir (MBV) is an L-riboside benzimidazole compound that inhibits the UL97 kinase [11]. Phase I trials indicated good oral bioavailability, did not indicate dose limiting toxicities, and the phase II trials demonstrated that prophylaxis with MBV reduced the rate of CMV infection in bone marrow transplant patients [41, 42]. As such, it has been given fast track status by the FDA. Interestingly, however, in a phase III trial investigating CMV prophylaxis study of stem-cell transplant patients, MBV was not effective [43]. It has been suggested that the dose of MBV in the study might have been too low [44]. Further, a recent study showed that MBV was effective against CMV infection with resistance associated mutations to GCV [45]. In this small study, six out of twelve patients with multi-drug resistant CMV isolates showed a reduction in CMV viral load. Resistance to MBV is associated with mutations in UL97 (Fig. 3) [46, 47]. Genotypic analysis indicates that these mutations are proximal to the ATP binding site [48], which supports the idea that MBV is a competitive inhibitor of UL97's ATP binding site [49]. Mutations in UL97 that confer resistance to MBV often do not confer resistance to GCV, which may make MBV a good candidate for combination therapy [50].

Viral packaging and processing inhibitors

The viral terminase complex directs the cleavage and packaging of CMV genomes [51]. The terminase complex consists of UL56, UL89, and UL104. Viral packaging and processing inhibitors work by inhibiting the function of one or more of these proteins.

Letermovir

Letermovir (AIC246) is a CMV inhibitor that targets UL56 [52]. It has an *in vitro* potency 400 times greater than GCV [53]. In clinical trials, AIC246 has been used to successfully treat CMV that is resistant to all currently marketed antivirals [54]. AIC246 has completed phase IIb clinical studies (NCT01063829) and seems to be well tolerated. Single resistance mutations in UL56 confer resistance to Letermovir [55]. The resistance profile of Letermovir does not overlap with other known terminase inhibitors, GW275175X and BAY 38-4766 [54].

Benzimidazole ribosides: GW275175X

Halogenated benzimidazole ribonucleosides were the first compounds identified that inhibited UL89 of the viral terminase complex with 1H-β-ribofuranosyl-2-bromo-5,6-dichlorobenzimidazole (BDCRB) being the best potential drug candidate [56]. BDCRB inhibits CMV replication *in vitro* [57] and *in vivo* [58]. However, development was stopped when it was demonstrated that BDCRB is cleaved *in vivo* to produce less active and more

cytotoxic aglycones [59]. A similar drug is GW275175X, which is a more stable analog of BDCRB that also inhibits UL89 [60]. Single mutations in UL89 confer resistance to the drug [60]. Its efficacy has been demonstrated *in vitro* [61] and *in vivo* [58] and seems to be the best candidate of this class for further development. However, to our knowledge no clinical studies are in progress.

Bay 38-4766

BAY 38-4766 is another viral terminase complex inhibitor that has been reported to have a broad spectrum of anti-CMV activity. Specifically, it has been documented to inhibit the replication of 36 CMV clinical isolates including 11 GCV resistant strains *in vitro* [62]. The drug has also exhibited antiviral *in vivo* activity in animal models [63]. Mutations that confer resistance to BAY 38-4766 map to both UL89 and UL56 [64]. This compound remains in the early phases of development and to our knowledge has yet to begin clinical trial. Table 1 summarizes the main drug targets and mechanisms of action.

Boron cluster modulated anti-CMV drugs

Similar to CMX001 and vGCV, modification of currently available antiviral agents can be a good strategy in increasing bioavailability, decreasing toxicity and improving overall efficacy. An intriguing new drug formulation technology has recently been applied to anti-CMV antivirals with promising preliminary results. Olejniczak *et al.* have modified the currently available antivirals CDV, GCV, and vGCV with lipophilic boron clusters in an effort to increase oral bioavailability and decrease toxicity [65]. For GCV and CDV there was no *in vitro* toxicity observed in five separate cell lines with concentrations up to 1000 μM and toxicity was only observed in vGCV above 300 μM, while maintaining the antiviral activity of the parent compound. Further *in vivo* experiments need to be preformed to assess the utility of this new technology.

Table 1. Drug targets and mechanisms of action.

Drug	Target	Mechanism of Action	Resistance Proteins
GCV	UL54	(i) Competitively inhibits incorporation of GTP (ii) Terminates DNA elongation	UL54 UL97
CDV	UL54	(i) Competitively inhibits incorporations dCTP (ii) Decrease polymerase activity upon incorporation (ii) Terminates DNA elongation	UL54
FOS	UL54	(i) Competitively inhibits pyrophosphate binding site	UL54
CMX001	UL54	Same as CDV	UL54
CPV	UL54 UL97	(i) Competitively inhibits UL54 (ii) Competitively inhibits UL97	UL54 UL97 UL27
MBV	UL97	(i) Competitively inhibits UL97	UL97 UL27
LTV	UL56	(i) Inhibits UL56	UL56
GW275175X	UL89	(i) Inhibits UL89	UL89
Bay 38-4766	UL56 UL89	(i) Inhibits UL56 (ii) Inhibits UL89	UL56 UL89

GCV: Ganciclovir; CDV: Cidofovir; FOS: Fosamprenavir; CMX001: Lipid Conjugate of CDV; CPV: Cyclopropavir; MBV: Maribavir; LTV: Letermovir; GW275175X: Benzimidazole Ribosides; Bay 38-4766: Viral Terminase Complex Inhibitor.

Risk factors for antiviral drug resistance

The major risk factors for development of drug resistant CMV are the duration of drug exposure, CMV load, and presence and level of immunodeficiency. During HIV infection, low CD4 count, higher HIV load and longer GCV duration have been associated with increased CMV antiviral resistance [28, 66, 67]. For transplant patients, many factors contribute to the development of resistances, including the type of transplant, whether solid organ transplant or a human stem cell transplant, the organ being transplanted, and the CMV serostatus of the recipient and the donor [16, 68, 69]. The most influential risk factor is the serostatus of the donor and recipient of the transplant, and the most likely scenario for the development of resistance is when a seronegative patient receives a CMV-seropositive organ (Donor+/Recipient-) [69]. While most of these observations have occurred in the setting of GCV or vGCV, it is likely that similar risk factors will be associated with resistance to other anti-CMV compounds described above.

Identifying drug resistance

Antiviral drug resistance should be suspected when there is no decrease in CMV levels (i.e. viral load) during prolonged antiviral use; typically considered 6 or more weeks [16]. While this may indicate antiviral resistance, it is not conclusive and laboratory tests are needed to confirm resistance. Phenotypic assays measure drug susceptibilities of viral isolates by growing the virus in the presence of various concentrations of an antiviral drug in order to determine the concentration of drug needed to inhibit 50% of the CMV population (IC_{50} value). For routine clinical purposes, these methods are often too time consuming, expensive and labor intensive. For example, determining the IC_{50} of the desired strain can take upwards of four to six weeks; therefore, phenotypic assays are usually relegated to research settings [47].

Genotypic assays can detect the presence of genetic sequence polymorphisms previously associated with antiviral resistance and have mostly replaced phenotypic assays in clinical practice. Population based sequencing is the most common genotypic technique as it is the cheapest and quickest to perform. One limitation is that it can only detect a mutation that is present in >20% of the sample [70]. Next generation sequencing is a much more sensitive method allowing for the detection of emerging resistance by detecting mutations that are in only 1% of the sample population [71]. This new method is often too expensive to employ regularly and is used primarily for research purposes.

Several open-access tools are available to screen for known resistance mutations. A very useful open access web tool for classifying UL54 and UL97 genotypic resistance was recently released that contains all of the relevant mutations to antiviral drug resistance that can be accessed from: http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/CMV/app/index.php?plugin=form&item=ul54 [72]. With the tool, one can input a partial or complete sequence of either UL54 or UL97 and it will determine if the sequence contains any polymorphisms and if those polymorphisms have been known to confer resistance. The group maintaining the database scans through the literature weekly to keep their tool up to date. Together, phenotypic and genotypic assays can be used to define the level of drug resistance conferred by viral genetic mutations. Further, structural modeling can be used to tease out potential mechanisms of the observed resistance.

CONCLUSION

CMV disease remains a serious concern for immunocompromised patients and treatment regimens are currently limited by the drug toxicity and lack of variability in drug targets. However, new drugs with reduced toxicity and unique targets of action appear to be on the horizon. These agents will likely open new treatment paradigms. For example, CMX001 could provide a better tolerated oral regimen for chronic CMV suppression, while combination therapy, like CMX001, CPV, and LTV, could thwart the development of resistance and become the new standard of care for active infection. With these new regimens we must remain diligent to identify, characterize and catalogue new CMV drug resistance patterns to maximize their clinical effectiveness.

ACKNOWLEDGMENTS

This work was partially supported by the CTRI NIH training grant TL1 TR000098-04 and partially supported by the Department of Veterans Affairs and grants from: the National Institutes of Health AI100665, AI080353, DA034978, AI36214, AI7462, AI69432, AI47745; the James B. Pendleton Charitable Trust.

CONFLICT OF INTEREST STATEMENT

FMG, HV, PJ, CI and DMS, AC: No conflict.

ABBREVIATIONS

CMV: Cytomegalovirus; HHV-5: Human herpesvirus-5; GCV: Ganciclovir; CDV: Cidofovir; FOS: Fosamprenavir; CMX001: Lipid Conjugate of CDV; CPV: Cyclopropavir; MBV: Maribavir; LTV: Letermovir; BDCRV: 1H-β-ribofuranosyl-2-bromo-5,6-dichlorobenzimidazole; IC_{50}: Drug Concentration that inihibits 50% of viral replication

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