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The biology of cytomegalovirus drug resistance

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Abstract

Purpose of review—To assess recently published data on cytomegalovirus (CMV) antiviral drug resistance

Recent findings—Resistance is typically encountered after prolonged ganciclovir treatment for post-transplant primary CMV infection, and is diagnosed by the detection of characteristic mutations in the viral UL97 kinase and UL54 DNA polymerase genes in clinical specimens. One of seven canonical UL97 mutations is detected in most cases of ganciclovir resistance, but many viral sequence variants of unknown relevance are being reported after drug exposure *in vitro* and *in vivo*. Rapid technical advances in recombinant phenotyping have shown that many of these variants confer no detectable drug resistance, while some unusual resistance mutations are newly confirmed. All currently marketed CMV antiviral drugs, including foscarnet and cidofovir, target the viral DNA polymerase, and cross-resistance may result from some UL54 mutations. To decrease cross-resistance and toxicity, there is an ongoing effort to develop anti-CMV drugs with different resistance pathways and alternative targets, such as the UL97 kinase or UL56-UL89 terminase enzymes.

Summary—An increasing volume of information correlating CMV genotypes and drug susceptibility phenotypes is becoming available. This will improve the interpretation of sequence-based assays currently used for clinical diagnosis, and guide the development of new antiviral drugs.

Keywords

Cytomegalovirus; antiviral therapy; drug resistance

Introduction

Antiviral therapy for human cytomegalovirus (CMV) infection has long been available and is routinely used in immunocompromised hosts [1], especially transplant recipients where detailed management guidelines have been developed for this important opportunistic viral pathogen [2]. Currently marketed anti-CMV drugs, namely ganciclovir (GCV), its oral prodrug valganciclovir (vGCV), foscarnet (FOS), and cidofovir (CDV), all target the viral DNA polymerase. Issues of drug toxicity and antiviral resistance [3] require ongoing efforts to define molecular mechanisms, optimize diagnostic testing, and develop novel therapeutics

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[4]. This article is intended as an overview and update on developments in CMV drug resistance since a recent comprehensive review [3].

Genetic mechanisms of CMV drug resistance

Viral UL97 kinase and UL54 DNA polymerase gene mutations are well-documented mechanisms of resistance to current antivirals [3]. The UL97 kinase normally phosphorylates serine and threonine residues of various cellular and viral proteins, and is required for efficient CMV replication [5]. This kinase also effects the initial phosphorylation of GCV that is required for antiviral activity, and UL97 mutations that impair this phosphorylation are the preferred mechanism of GCV resistance. One of 7 canonical UL97 mutations (M460V/I, H520Q, C592G, A594V, L595S and C603W) is found in over 80% of GCV-resistant clinical CMV strains [3], representing mutations that selectively impair GCV phosphorylation without seriously damaging normal kinase function. The dominance of these mutations as laboratory markers of GCV resistance is confirmed by all recent clinical studies [6–9], although a variety of uncommon resistance mutations are known to occur at UL97 codons 405, 460, 466 and 590–607 [3,10–12]. While UL97 mutations do not affect susceptibility to FOS or CDV, UL54 mutations can confer resistance to all current drugs and may emerge after prolonged GCV therapy to increase the level of resistance conferred by a pre-existing UL97 mutation [3,6]. Dual GCV-CDV resistance commonly maps to UL54 exonuclease domains and codon 987, while FOS mutations range widely over the polymerase catalytic domain, including in region III where low-grade GCV and/or CDV cross-resistance is common (Figure 1) [3].

CMV drug resistance in the clinical setting

Drug resistance generally occurs after prolonged drug exposure (weeks to months) with incomplete viral suppression, manifesting as increasing viral loads or disease despite therapy [1–3]. Risk is greatest in solid-organ transplant recipients who are previously CMV seronegative and receive an organ from a seropositive donor (D+/R–), resulting in a post-transplant primary CMV infection exacerbated by immunosuppression [3]. Recent reports [6,7] confirm that ~90% of cases of GCV resistance occur in this recipient subset, and that the frequent occurrence of symptomatic disease and prolonged treatment leads to CMV drug resistance in 5%–10% of this population [2]; it was higher at 12.5% of 209 D+/R– kidney recipients in one large study [7], and also tends to be higher in lung transplant cases [3].

Prophylaxis with GCV and vGCV for 100–200 days post-transplant has become routine in solid organ transplantation [2], while shorter periods of pre-emptive therapy guided by the detection of PCR-detected viremia remains a valid alternative. Although the more selective pre-emptive treatment approach might be expected to reduce the incidence of resistance, some recent studies actually showed a higher incidence of resistance in those on pre-emptive management than those given prophylaxis [7,8].

Concern for late recurrent CMV disease has prompted clinical trials showing that extending vGCV prophylaxis from 100 to 200 days in D+/R– kidney recipients significantly reduced the cumulative incidence of CMV disease at 12 months [13]. Similar results were reported in lung transplant recipients with vGCV prophylaxis extended to 12 months [14]. Prolonged prophylaxis did not appear to increase the incidence of genotypic drug resistance significantly [14,15], presumably because of effective suppression of viral replication in most cases.

Laboratory assays for drug resistance

Routine susceptibility testing of CMV culture isolates against antiviral drugs is not feasible for technical reasons, including the unavailability of culture isolates in the current molecular diagnostic era. Laboratory confirmation of drug resistance is now based on sequence analysis of PCR-amplified CMV DNA from clinical specimens for the presence of diagnostic mutations. Standard dideoxy sequencing can detect an emerging resistance mutation when it exceeds ~20% of the sequence population [3]. Pyrosequencing has been reported to detect mutant subpopulations at a ~6% level at codons 460, 520, and 592–607 of UL97 [16], but the short sequence reads are unsuitable for analyzing the full range of codons needed for genotypic diagnosis (e.g. UL97 codons 335 to 670 and UL54 codons 300 to 1000). Ultra-deep pyrosequencing shows promise in identifying small (~1%) subpopulations of variant CMV sequences in clinical samples [17], which may signal impending drug resistance and elucidate the sequential mutations that confer increasing resistance to various drugs. The technique is currently too elaborate and expensive for routine use, and will require much future research to validate. There is continued interest in other sequencing technologies with potential for general use in clinical diagnostic genotyping [18].

Quality control initiatives for CMV resistance genotyping are underdeveloped. For example, there are no standardized proficiency testing templates containing controlled mixtures of CMV genotypes that simulate clinical specimens. Genotype reports of unusual and mixed sequence populations raise issues of technical artifact resulting from the PCR and sequencing techniques used, especially when the viral load in the specimen is low. Such cases may benefit from re-sequencing of additional specimens from the same subject.

Recombinant phenotyping

With the ongoing detection of many sequence changes unaccompanied by a viral culture isolate, the corresponding drug susceptibility phenotypes are now determined by introduction of specific mutation(s) into a baseline CMV strain to create a recombinant virus that can be cultured under drug to determine the concentration that reduces viral growth by 50% (EC50 value) [3]. Site-specific viral mutagenesis has been greatly facilitated by the recent widespread adoption of bacterial artificial chromosome (BAC) clones of CMV, some of which are engineered to express reporter genes for rapid viral quantitation [10,12,19–21]. Mutagenesis of BAC clones by recombination in *E. coli* is followed by sequence and integrity checks, and generation of live virus by transfection of BAC DNA into fibroblast cultures [5,8]. Since publication of the recent review [3], BAC-based recombinant phenotyping information has newly validated some unusual resistance mutations (Table 1) while showing many other sequence variants to have no detectable effect on drug susceptibility (Table 2).

Drug susceptibility phenotypes determined using BAC-derived recombinant strains and reporter-based viral growth quantitation generally correlate well with results from older studies reporting plaque reduction assays of viruses containing the same mutations [10,19–21]. Some minor inconsistencies have been reported [19,20] usually involving mutants with low-grade resistance or borderline cross-resistance phenotypes. Growth quantitation under drug may vary depending on the viral strain, multiplicity of infection, cell type and physiology, and the method of quantitation (plaque counting, PCR, or various reporter gene systems). Repeated EC50 assays in the same assay system commonly have a standard deviation of 30% of the mean EC50, and thus a 2-fold increase in EC50 values has been a traditional cutoff for defining phenotypic resistance [3]. Given enough independent replicates, newer recombinant phenotyping assays may permit recognition of reduced

susceptibility at an EC50 ratio of 1.5 to 2-fold over baseline [21]. The canonical UL97 mutations confer 5- to 10-fold GCV EC50 increases, except 3-fold for C592G [3,10].

Distinguishing sequence polymorphism from resistance-related mutations

Considerable baseline interstrain sequence variation, unrelated to prior drug exposure, is known to exist in the CMV UL54 polymerase gene. Dozens of variations in amino acid encoding occur, often at nonconserved residues such as 614–697 where little homology exists among herpesvirus polymerases [3]. A lesser extent of polymorphism exists in UL97 [3], where a recent study identified an additional 9 uncommon amino acid variants between codons 7 and 249, which are not usually sampled for clinical diagnosis, and one noteworthy variant N467D [22] located close to canonical resistance mutations M460V/I.

Even after filtering out sequence variation in baseline isolates, many uncharacterized amino acid sequence variants are found in specimens collected after antiviral drug exposure in clinical practice and during clinical trials, creating uncertainty as to their relevance for drug resistance [6,7,9,15,23]. In the absence of phenotypic information, circumstantial evidence may be used to suggest a role in resistance for specific variants if they are at conserved codons in known functional domains, are similar to known resistance mutations at the same or nearby codons, or if they newly developed after drug exposure, but none of these criteria has proven to be reliable [3,11,20]

Growing technical capabilities for recombinant phenotyping have enabled an increasing number of sequence variants to be classified as resistance related or not. For example, UL97 variants (A440V and A442G), and 15 UL54 variants detected in a vGCV prophylaxis trial conferred no significant GCV resistance (Table 2) [15]. Unexpectedly UL54 G698D and Y818C, amplified from plasma specimens as partial sequence populations, yielded either no or poorly viable virus from the corresponding recombinant BACs [15]. These conserved residues probably have a critical function based on the homologous herpes simplex virus polymerase structure [24], leaving open the question of whether such nonviable mutations represent technical artifacts of genotyping assays or recombinant phenotyping, or a previously unrecognized pathway of drug resistance.

Surprising findings resulted from recombinant phenotyping of UL54 mutations selected *in vitro* under GCV and FOS (Table 1) [21]. Extensive information is available from clinical isolates studied after treatment with these drugs [3,6,20]. The mutations selected *in vitro* were not among those commonly reported in clinical isolates; in fact most of them were previously unknown, such as L862F, and L957F (1.7-fold to 2.7-fold increased GCV EC50) or T552N, F595I and V946L (2.0-fold to 2.6-fold increased FOS EC50). These mutations are distant from those previously confirmed to confer resistance to these drugs. Curiously, the FOS-resistant mutations V946L and F595I were selected under GCV, and the GCV-resistant mutations N408D, K500N and L957F were selected under FOS, yet none of these mutations individually conferred GCV-FOS cross-resistance. Certain combinations, such as N408D+L957F and L545S+P829S showed FOS resistance whereas the single mutations did not. The authors suggest by use of computational modeling that mutations outside of areas that are directly involved in catalytic activity may result in structural changes that lead to drug resistance [21].

Experimental CMV DNA polymerase inhibitors

The UL54 DNA polymerase continues to be a target for antiviral drug development to improve potency and lessen cross-resistance or toxicity. An orally bioavailable lipid conjugate of CDV (CMX001) is very active *in vitro* against various DNA viruses [25] and is undergoing clinical trials for prevention of CMV infection after stem cell transplantation.

CMV resistance to CMX001 has not been characterized but is expected to involve the same UL54 exonuclease and codon 987 mutations most relevant to CDV resistance.

Cyclopropavir (CPV) is a methylenecyclopropane nucleoside analog that is similar to GCV in requiring initial phosphorylation by the UL97 kinase [26], and clinical trials are anticipated based on a favorable potency and toxicity profile *in vitro*. There is partial cross-resistance between GCV and CPV. While the common UL97 GCV resistance mutation L595S confers no CPV resistance, mutations M460I/T, H520Q and C603R confer definite GCV-CPV cross-resistance, with 8- to 20- fold elevation of CPV EC50 values [27]. Some cross-resistance between CPV and GCV or FOS is expected on the basis of UL54 mutations as well.

Antivirals with novel CMV targets

Maribavir (MBV) is a benzimidazole L-riboside ATP-competitive inhibitor of the UL97 kinase [4,5,28]. Consistent with this mechanism, propagation of CMV *in vitro* under MBV selects for UL97 mutations (V353A, L397R, T409M, and H411L/N/Y), mapping to the kinase ATP binding site, that confer moderate-to-high level (9-fold to > 200-fold) MBV resistance [3,29]. There is no overlap of these mutations with UL97 mutations conferring GCV resistance in clinical isolates [3]; the GCV-resistance mutations M460V/I actually confer MBV hypersensitivity [28,29]. GCV-MBV cross-resistance is expected for UL97 mutations that severely impair or delete its overall kinase function (such as K355M), but have not thus far been observed in clinical isolates presumably because of the associated severe growth defect [3]. A possible exception is V466G, which was detected as a partial population in PCR-amplified material from a blood sample that also contained M460V and C592G [12]. A GCV-resistant recombinant virus containing V466G showed poor growth and abnormal cytopathic effect similar to UL97 kinase-defective viruses [11], suggesting a good probability of MBV cross-resistance.

Mutations in a little known CMV gene UL27 that confer low-grade MBV resistance emerge under drug *in vitro*, and appear spontaneously in genetically UL97-defective CMV strains without added drug, suggesting a functional compensation for loss of UL97 kinase activity [30]. UL27 is dispensable for CMV replication in cell culture. Recent evidence indicates that UL27 expression up-regulates the cellular cyclin-dependent kinase (CDK) inhibitor p21^{cip} by promoting the degradation of the Tip60 acetyltransferase, ultimately resulting in G0/1 cell cycle arrest [31]. However, Tip60 degradation and p21^{cip} up-regulation are early and transient events during CMV replication, while pUL27 levels are more persistent [31]. Further study of pUL27 function in MBV resistance may elucidate cellular factors affecting its antiviral potency.

After early studies showing promising antiviral activity and minimal toxicity, highly anticipated Phase III MBV trials showed a disappointing lack of efficacy in prevention of CMV viremia after stem cell transplantation [32]. Proposed explanations for this outcome include an inadequate dosing regimen, choice of study endpoint [33], and a possibility that *in vivo* antiviral potency may not be as high as shown *in vitro* using some cell culture systems but not others [34]. At a higher dose of MBV, four out of six transplant recipients cleared their viremia within six weeks after failing conventional therapy [35]. A heart transplant recipient with multi-resistant high-grade CMV viremia became the first known patient to develop MBV resistance during therapy [36], associated with UL97 mutations T409M and H411Y in plasma specimens and a urine culture isolate; no UL27 mutations were found.

Small molecule inhibitors targeting the CMV UL56/UL89 terminase complex required for viral replication have been studied for over a decade [4]. AIC246 is a 3,4- dihydro-

quinazoline derivatives discovered by high-throughput screening [37] and subsequently confirmed to be a terminase inhibitor, including the identification of resistance mutations mapping to UL56 that do not confer cross-resistance to older terminase inhibitors tomeglovir (BAY 38-4766) or BDCRB [38]. AIC246 has been used to treat a case of multidrug resistant CMV infection [39], and is currently in phase II clinical trials for prevention of CMV infection after stem cell transplantation.

Conclusion

CMV drug resistance occurs most frequently in D+/R– solid organ transplant recipients receiving prolonged GCV treatment, and seven UL97 kinase mutations are dominant laboratory markers of GCV resistance. An increasing volume of viral genotyping data from clinical studies and drug trials, combined with advances in recombinant phenotyping, are helping to resolve the functional significance of uncharacterized sequence variants (mostly in UL54), some of which represent newly identified resistance pathways. These data improve the diagnosis and management of current antiviral therapy and are important in the development of experimental antiviral compounds with improved toxicity and cross-resistance profiles.

Key Points

- CMV drug resistance results from continued viral replication after prolonged drug exposure, usually in patients treated for post-transplant primary CMV infection.
- In the vast majority of cases, ganciclovir resistance in clinical practice results from one of seven mutations in the viral UL97 kinase gene.
- Increasingly diverse viral UL54 DNA polymerase mutations have been shown to confer various combinations of resistance to ganciclovir, foscarnet or cidofovir.
- Since genotypic assays are the principal means of laboratory confirmation of CMV drug resistance, reliable correlation of genotypes with resistance phenotypes is needed.
- Technical advances in recombinant phenotyping are helping to resolve the status of many uncharacterized sequence variants encountered in clinical genotyping assays.
- Mechanisms of resistance to existing and experimental antiviral agents are an important aspect of the development of improved anti-CMV treatments.

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Figure 1.

Map of the CMVUL54 DNA polymerase gene showing conserved functional domains, ranges of codons containing drug resistance mutations, and the locations of drug resistance mutations and non-resistant sequence variants newly described since the recent review [3]. Phenotype details are in Tables 1 and 2.

▲ denotes the D301N and A987G mutations which confer dual GCV/CDV resistance.

● denotes the 981–982 deletion and Q578H mutations which confer triple GCV/CDV/FOS resistance.

Table 1

Newly identified UL54 drug resistance mutations¹

UL54 mutation	Fold increase in drug EC50 ²			Mutant source ³		Reference
	GCV	CDV	FOS	Clinical	in vitro	
F412L	4.6	9.4	–	x		20
F412S	5.3	13	–	x		20
P488R	3.5	7.9	–		GCV	21
K500N	3.2	3.0	–		FOS	21
C539R	3.2	13.3	–		GCV	21
L545W	4.9	6.3	–	x		20
T552N	1.9	–	2.6		FOS	21
Q578H	3.3	2.3	4.5	x		20
S585A	–	–	2.7		FOS	21
F595I	–	–	2.0		GCV	21
L802V	1.8	–	–		FOS	21
P829S	2.0	–	–		GCV	21
L862F	1.7	–	–		GCV	21
V946L	–	–	2.4		GCV	21
L957F	2.7	–	–		FOS	21

¹Updated from UL54 resistance mutation list published in reference 3

²Compared with baseline strain. No increase in drug EC50 is denoted by (–).

³Mutation detected in a clinical specimen or after selection in vitro with either GCV or FOS.

Table 2Newly phenotyped UL54 sequence variants¹

Drugs tested		Reference
GCV ²	GCV, FOS, CDV	
	S291P	19
	C304S	20
E315D		15
F357L		15
V377A		15
	K415R	19
P448S		15
R512H		15
C524R		15
E530G		15
	V544A	20
D576G		15
A635T		15
P648S		15
S649P		15
	A692V	19
V694I		15
	S695T	19
	A714V	20
	N757K	21
I837V		15
	D879G	21
S897P		15
	L926V	21
	A972V	19
G993D		15

¹ Not conferring drug resistance, updated from recent list [3]² Sequence variants observed after vGCV therapy