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Novel UL97 drug resistance mutations identified at baseline in a clinical trial of maribavir for resistant or refractory cytomegalovirus infection

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ABSTRACT

In a Phase 2 clinical trial, 120 subjects with cytomegalovirus (CMV) infection refractory or resistant to standard therapy were randomized equally to 3 doses of oral maribavir treatment, and 70% achieved undetectable plasma CMV DNA within 12 weeks. At study entry, standard diagnostic UL97 genotyping was available for 71 subjects, with 60 (85%) revealing well-characterized ganciclovir resistance mutations that did not preclude a therapeutic response to maribavir. Central laboratory testing of a range of UL97 codons (288–468) not fully covered by standard genotyping was done on 93 subjects at baseline. This detected no previously known maribavir resistance mutations, but identified atypical mutations in 3 subjects, including a P-loop substitution F342Y, and ATP-binding region substitutions K359E/Q. By recombinant phenotyping, K359E and K359Q each conferred a nearly 4-fold increased ganciclovir 50% inhibitory concentration (EC50) without maribavir resistance, whereas F342Y conferred a 6-fold increased ganciclovir EC50 and a 4.5-fold increased maribavir EC50. The subject with F342Y detected at baseline did not achieve plasma CMV DNA clearance after 12 weeks of maribavir therapy and later developed an additional UL97 substitution H411Y known to confer 12- to 20-fold increased MBV EC50 by itself. The combination of F342Y and H411Y was shown to increase the maribavir EC50 by 56-fold. Diagnostic genotyping of UL97 should be expanded to cover the ATP-binding region beginning at codon 335 to enable the detection of atypical resistance mutations and further correlation of their clinical significance.

1. Introduction

The prevention and treatment of human cytomegalovirus (CMV) infection and disease is an important aspect of medical care after solid organ (SOT) and hematopoietic stem cell transplantation (HSCT). Prophylactic approaches aimed at suppressing CMV replication include use of valganciclovir in SOT (Kotton et al., 2018) and the recently approved use of the terminase inhibitor letermovir after HSCT (Marty et al., 2017). Active CMV infection is best treated pre-emptively before the onset of symptomatic disease, by early detection of CMV DNA in clinical specimens. Prolonged treatment may lead to the development of drug resistance, sometimes limiting the utility of currently approved CMV therapies (ganciclovir, foscarnet and cidofovir) that have the same viral DNA polymerase target (Kotton et al., 2018).

Maribavir (MBV) is a CMV UL97 kinase inhibitor that has been evaluated in several randomized clinical trials. The antiviral drug target is the same kinase that initially phosphorylates ganciclovir in the process of its conversion to the active triphosphate form but is also biologically important for normal viral replication (Prichard, 2009). Suppression or genetic knockout of UL97 kinase results in severe viral growth impairment. Phase 3 trials of low-dose MBV as prophylaxis in HSCT or SOT were unsuccessful (Marty et al., 2011; Winston et al., 2012). However, more recently, a single arm open label Phase 2 trial of MBV at higher doses for treatment of CMV infection refractory or resistant to therapy with standard CMV polymerase inhibitors reported that 67% of cases responded to treatment as measured by clearance of plasma viral DNA within 6 weeks (Papanicolaou et al., 2019). Rebound of viral DNA after initial clearance occurred in about 30% of responders who remained on MBV treatment, along with frequent detection of established UL97 mutations conferring MBV resistance (T409M and H411Y) in those who experienced viral DNA rebound. Extending the genotypic data published for this clinical trial (Papanicolaou et al., 2019), this report describes the characterization of unusual UL97 mutations detected at baseline that have implications for the diagnosis of drug resistance and cross-resistance.

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2. Materials and methods

2.1. Clinical trial subjects and specimens

As published, this was an open label, dose-ranging randomized Phase 2 trial of MBV treatment of CMV infection refractory or resistant to standard CMV therapy (Papanicolaou et al., 2019). Forty subjects per arm were randomly assigned to each of the MBV doses of 400, 800 and 1200 mg orally twice daily. Subjects were periodically monitored for their plasma CMV DNA load according to protocol (weekly through week 1–6, biweekly through week 12, and every 4 weeks through week 24). Although not a condition for study entry, the majority of subjects were screened for UL97 mutations as evidence of drug-resistant CMV in blood specimens. This was done by participating sites through their preferred diagnostic laboratories, and the results were recorded in study case reports as UL97 amino acid substitutions proven or suspected to confer ganciclovir resistance. Additionally, a baseline plasma specimen was obtained at the start of MBV therapy and stored for subsequent genotyping at a central laboratory designated for the study.

2.2. Central laboratory CMV UL97 genotyping

The purpose of this genotyping was to extend the analyzed codon range in the UL97 gene further upstream than is currently performed by most diagnostic laboratories screening for ganciclovir resistance. The analyzed codon range 288–468 also covers the loci of most (but not all) known MBV resistance mutations, notably substitutions T409M and H411Y that have been repeatedly observed *in vitro* and in vivo (Chou and Marousek, 2008; Papanicolaou et al., 2019). UL97 DNA sequencing was performed from nested PCR products using standard Sanger dideoxy sequencing (BigDye 3.1, Applied Biosystems) and primers targeting the specified codon range. Sequencing reactions were set up for both DNA strands, and mutations resulting in amino acid substitutions were confirmed by a consistent finding for both strands, each with a quality value score of > 35 as determined by the base-calling software (Applied Biosystems).

2.3. Recombinant phenotyping

UL97 mutations of interest were transferred to a bacterial artificial chromosome (BAC) clone BD1 of human CMV strain AD169 modified to express a secreted alkaline phosphatase (SEAP) reporter gene for quantitation of viral growth, as previously described (Chou et al., 2017). Mutant or control baseline BAC-cloned viral DNA was transfected into ARPEp cell cultures to produce cell-free CMV stocks, which were sequenced throughout UL97 gene to confirm the intended mutation(s) and absence of introduced errors. Phenotypic assays for ganciclovir and MBV susceptibility were performed in ARPEp cell cultures, which are ARPE-19 cells overexpressing the platelet-derived growth factor receptor alpha, as recently detailed (Chou et al., 2017). ARPEp cells were found to give MBV EC50 values similar to those determined in human embryonic lung fibroblasts (Chou et al., 2018). The drug concentration required to reduce viral growth by 50% (EC50), as measured by supernatant SEAP activity at 6 days, was determined by assaying growth under no drug and at 5 two-fold increasing concentrations centered on the estimated EC50 value. Multiple replicates of testing were performed on at least 7 setup dates per variant. All mean EC50 values interpreted as drug-resistant (≥2-fold increased over wild type) (Kotton et al., 2018) were significantly different from matching wild type EC50 values with a p value of $< 10^{-12}$ by the Student *t*-test (two-tailed, unequal variance). Growth fitness of mutant viruses was compared using growth curves resulting from assay of culture supernatant SEAP activity at each of days 4–8 after inoculation of ARPEp cells at equivalent low multiplicity of infection of 0.02. A genetic knockout UL97 mutant, with an in-frame deletion of the critical lysine residue 355 (K355del) was used to represent the maximal viral growth impairment resulting from inactivation or MBV inhibition of the UL97 kinase (Chou et al., 2013).

Table 1
Known UL97 mutations reported at baseline by study sites.

Mutation[a]	N[b]
M460I	1
M460V	2
H520Q	5
C592G	3
A594V	12
L595F	4
L595S	16
C603W	16
All others[c]	6

[a] UL97 amino acid substitution.
[b] Subjects with indicated mutation.
[c] A594 P/T, L595del/W, N597del3, C607Y, in one subject each.

3. Results

3.1. UL97 mutations detected at screening by participating sites

Baseline UL97 genotypic data were submitted by study sites for 71 (59%) of the 120 subjects. Of these, 11 (15%) had no detectable mutation, while 60 (85%) were reported to have one or more known ganciclovir resistance mutations, as listed in Table 1. Five subjects had more than one of the listed mutations. As expected, the UL97 mutations most characteristically associated with ganciclovir resistance, such as substitutions A594V, L595S and C603W were heavily represented (Lurain and Chou, 2010), and these do not confer maribavir cross-resistance (Drew et al., 2006). The proportion of subjects responding to MBV with viral clearance within 6 weeks among the overall study population was 80/120 (67%). Within this population, the proportion of responding subjects with UL97 mutation(s) as listed in Table 1 was 37/60 (62%), and in those without detected UL97 mutation was 6/11 (55%).

3.2. UL97 mutations detected at baseline by the central laboratory

Genotyping at the central laboratory (UL97 codons 288–468) did not cover the canonical ganciclovir resistance loci at codons 520 or 590–607, but did corroborate the detection of the M460V/I substitutions in the two cases where results were reported from both the participating sites and the central laboratory. Previously characterized maribavir resistance mutations in UL97 (such as T409M and H411Y) were not detected in any of the baseline specimens. Novel and atypical UL97 mutations detected with sequencing quality standards specified in the methods section included the single amino acid substitutions F342Y, K359E and K359Q in three separate subjects at baseline. Each case was confirmed by detection of the same mutation in a second specimen from the same subject.

3.3. Maribavir treatment history of cases with atypical UL97 mutations

The subject with atypical UL97 substitution F342Y at baseline was a renal transplant recipient who developed CMV infection 4 months post-transplant and entered the trial 17 months later with a treatment resistant/refractory plasma CMV load of 500,000 copies/mL. The participating site did not provide UL97 genotypic data at baseline, but testing at day 8 revealed the ganciclovir resistance mutation H520Q. Over a 10-week period of MBV 800 mg twice daily, the plasma viral load slowly declined to 5000 copies/mL (not meeting study criteria for treatment success) but then rebounded to 20,000 copies/mL at week 12

and 40,000 copies/mL at week 16 while remaining on MBV. UL97 genotyping at 16 weeks revealed, in addition to persistence of the F342Y that was detected at baseline, the substitution H411Y, which is known to confer 12- to 20-fold increased MBV EC50 by itself (Chou et al., 2018). At 16 weeks, a switch of therapy from MBV to foscarnet was successful at reducing the plasma CMV load to < 200 copies/mL after 4 weeks.

The subject with atypical UL97 substitution K359E at baseline developed CMV infection more than 10 years after a kidney-pancreas transplant and was enrolled as resistant/refractory to standard therapy 2.5 months later with a plasma viral load of 40,000 copies/mL. Locally acquired baseline genotyping reported no UL97 mutations but did report UL54 substitution A834P that confers multi-drug resistance, as well as UL54 substitution V823A of indeterminate significance. On a treatment regimen of MBV 400 mg twice daily, the plasma viral load decreased to 1000 copies/mL by week 3 but the study drug was stopped because of the adverse event of nausea. Therefore, the subject did not meet protocol criteria for MBV treatment success. Without interim antiviral therapy, follow-up genotyping at study day 52 (plasma CMV DNA 100,000 copies/mL) showed persistence of the UL97 K359E substitution in 70% of the DNA sequence population with the remainder being wild type sequence. Treatment with foscarnet at study day 81 was successful at reducing the plasma CMV DNA from 100,000 to 600 copies/mL within 3 weeks.

The subject with atypical UL97 substitution K359Q developed asymptomatic CMV infection 6 months after a multi-visceral organ transplant and was enrolled in the trial 2.5 months later with a plasma viral load of 2000 copies/mL. No CMV genotyping was done by the participating site. With MBV dosed at 1200 mg twice daily, the plasma viral load decreased to < 200 copies/mL at week 3 and remained at that level until week 8 (meeting protocol criteria for treatment success), but the load rebounded to 40,000 copies/mL at week 10. At this point, MBV therapy was switched to ganciclovir and CMV immune globulin. UL97 genotyping showed persistence of K359Q but absence of known maribavir resistance mutations in the codon range examined (288–468). Four weeks later the plasma viral load had decreased to 300 copies/mL.

3.4. Phenotypic analysis of novel UL97 mutations

Recombinant viruses were constructed from derivatives of the standard strain AD169 BAC clone BD1 incorporating a SEAP reporter gene for viral quantitation, and all transferred mutations were verified by sequence analysis of the full UL97 gene. The ganciclovir and MBV EC50 values for mutant and control strains, and fold-change from baseline are as shown in Table 2. All 3 substitutions F342Y and K359E/Q conferred ganciclovir resistance, with F342Y at levels comparable to the canonical resistance mutations M460I or A594V, and K359E/Q at lower levels similar to A591V or C592G as reported in ganciclovir-treated individuals (Chou et al., 2017; Lurain and Chou, 2010). Among these substitutions, only F342Y located within the kinase P-loop conferred MBV resistance at 4.5-fold EC50 increase. This is less than the 18-fold MBV EC50 increase reported for the *in vitro* selected substitution F342S (Chou et al., 2013). When F342Y was combined with H411Y, as occurred in the study subject (see Section 3.3), it resulted in a 56-fold MBV EC50 increase, without significantly affecting the ganciclovir resistance conferred by F342Y alone.

All 3 new mutant viruses (F342Y, K359E and K359Q) grew similarly as wild type virus or the C592G mutant, and far better than the UL97 kinase functional knockout mutant K355del included as a control (Fig. 1).

4. Discussion

Genotypic analyses of the CMV UL97 kinase at baseline showed the expected high frequency of canonical ganciclovir resistance mutations,

Table 2
Genotypes and phenotypes of recombinant CMV strains.

Strain[a]	UL97 Genotype[b]	Maribavir EC50, μM				Ganciclovir EC50, μM			
		Mean	SD	N	Ratio	Mean	SD	N	Ratio
Control strains									
4200	WT	0.11	0.020	55		1.2	0.24	44	
4207	C592G					3.6	0.65	38	**3.0**
4352	T409M	10	2.2	41	**90**				
4353	H411Y	2.1	0.45	40	**18**				
New recombinants									
4338	F342Y	0.51	0.10	43	**4.5**	7.2	1.57	30	**6.0**
4440	K359E	0.13	0.027	18	1.2	4.5	0.76	18	**3.8**
4441	K359Q	0.15	0.027	23	1.3	4.4	0.74	18	**3.7**
4458	F342Y H411Y	6.4	0.78	16	**56**	7.0	1.22	15	**5.9**

SD = standard deviation; N = number of replicates. Ratio = EC50 of mutant virus/EC50 of wild type control. Boldface indicates drug resistance (≥2-fold increased EC50)_.

[a] Serial number of recombinant virus.

[b] UL97 amino acid substitution indicated.

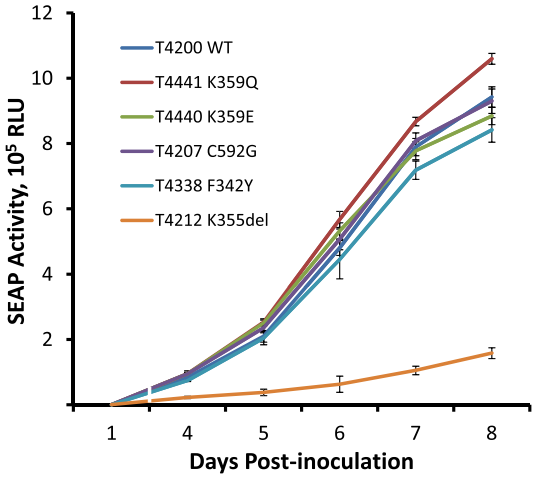

Fig. 1. Comparative growth curves of viral strains. Wild type control virus and UL97 mutants were inoculated at equal multiplicity of infection of 0.02 as calibrated by culture supernatant SEAP activity at 24 h. At each of 4–8 days post-inoculation, culture supernatants were assayed for SEAP activity (relative light units, RLU) as a measure of viral growth. Data points are the mean and standard deviation of 4 replicates set up simultaneously.

which did not prevent plasma viral load clearance within 6 weeks of MBV treatment in the majority of treated subjects. However, three atypical UL97 mutations, presumably selected by prior antiviral therapy, were identified at baseline, which extends the known genetic loci of ganciclovir resistance within the UL97 gene significantly upstream of what was previously documented in clinical practice. Specifically, an atypical substitution F342Y was identified within the P-loop that conferred increased ganciclovir and MBV EC50 without major impact on viral growth fitness. These findings require further clinical correlation and suggest that routine diagnostic genotyping of CMV UL97 needs to be expanded to cover these upstream loci.

During the development of MBV as an alternative therapy for CMV, it has been observed that the resistance mutations most commonly selected after exposure in vivo to either MBV or ganciclovir do not confer cross-resistance to the other (Drew et al., 2006). Mutations at codons 460, 520, and 590–607 are commonly associated with ganciclovir resistance and those at codons 409 and 411 are commonly associated with MBV resistance, without cross-resistance (Lurain and Chou, 2010; Papanicolaou et al., 2019). Observations of ganciclovir and MBV cross-resistance in unusual UL97 mutants reportedly detected in clinical specimens, such as V466G (Martin et al., 2010) and P521L (Eckle et al.,

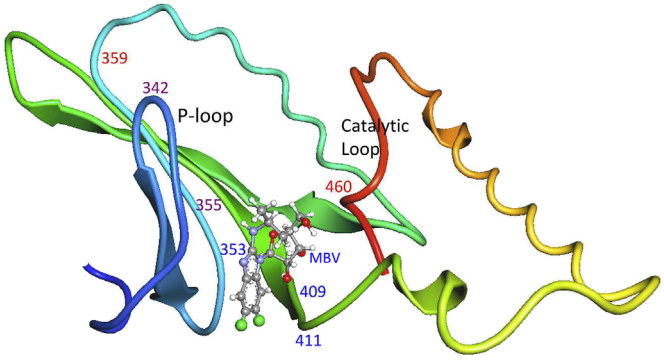

Fig. 2. UL97 kinase structure model. A published structure model of the UL97 kinase ATP-binding region based on yeast GCN2 kinase (Chou and Marousek, 2008) is updated to show residue 342, a locus of cross-resistance, in the central portion of the P-loop (residues 335–346), and residue 359 located downstream of the functionally critical K355 residue. The ATP-competitive site of MBV binding is shown. Mutations conferring MBV resistance (substitutions V353A, T409M and H411Y) are expected to disrupt this binding. The common ganciclovir resistance mutations at residue 460 are in a separate catalytic loop. Original figure copyright © American Society for Microbiology, *Journal of Virology*, 82, 2008, 246–253, DOI: 10.1128/JVI.01787–07.

2000), are potentially less significant because these mutants are severely defective in UL97 kinase activity and growth fitness (Chou et al., 2013), and have not been corroborated by detection in other treated patients. Genotyping artifacts must be considered when interpreting these results. The three novel UL97 mutations identified in this study appear more credible in that they were detected in independent samples with high viral DNA copy numbers from the same subjects and the mutants have well-preserved growth fitness.

The role of UL97 P-loop and other ATP-binding site mutations in drug resistance (Fig. 2) was previously discussed in connection with the *in vitro* selection of substitutions F342S and V356G (Chou et al., 2013), including the observation of P-loop mutations as a common mechanism of resistance to ATP-competitive kinase inhibitors. The current findings provide important clinical context. The K359E/Q mutants have equal growth fitness *in vitro* as the common C592G mutant (Fig. 1) and confer comparable low-grade ganciclovir resistance without MBV cross-resistance. Low-grade ganciclovir resistance mutations may remain amenable to short-term treatment with higher dose ganciclovir (Kotton et al., 2018), as in the case with K359Q after MBV was discontinued. The study subjects with K359E/Q appeared to respond initially to MBV as expected, although in one case the delayed recurrence of a CMV load suggests the possibility of a MBV resistance mutation emerging outside the range of UL97 codons covered by the genotyping (Houldcroft et al., 2016; Komazin-Meredith et al., 2014). The cross-resistant P-loop mutant F342Y detected clinically has better growth fitness than the F342S mutant selected *in vitro* (Chou et al., 2013) but confers a lower level of MBV resistance (4.5-fold) by itself. Although the case synopsis shows that partial viral load reduction may still be possible with MBV in the presence of this mutation, the F342Y–H411Y double mutant that evolved in the treated subject illustrates the availability of additional genetic pathways to high-level MBV resistance and eventual treatment failure when F342Y has been selected under prior therapy.

The frequency with which ganciclovir therapy selects for atypical mutants such as UL97 F342Y with preserved growth fitness and MBV cross-resistance remains unclear because current UL97 diagnostic genotyping has not usually covered the ATP-binding region upstream of codon 380. The central laboratory sequencing survey of baseline specimens in this study would suggest that such mutations are unusual compared with the well-known ganciclovir resistance mutations, but the scope of standard diagnostic genotyping in UL97 needs to be expanded to gain additional insight. Considering homologies to functional domains of other kinases (Hanks et al., 1988), a reasonable proposal is for the sequencing coverage of UL97 to start at codon 335, the beginning of the P-loop, and continue to the end of the gene. Increased sequencing scope could expand the number of cases of genetically confirmed drug resistance in cases of poor response to antiviral treatment.

Declaration of competing interest

SC is principal investigator of Collaborative Research and Development agreements between the Department of Veterans Affairs (VA) and Merck for recombinant phenotyping. JW, KS, and TB are employees of, and hold stock/stock options in, Shire.*

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*A member of the Takeda group of companies.

Data sharing: The datasets, including redacted study protocol, redacted statistical analysis plan, and individual participants' data behind the results reported in this article, will be available 3 months after manuscript publication, to researchers who provide a methodologically sound proposal after de-identification, in compliance with applicable privacy laws, data protection and requirements for consent and anonymization. Data requests should follow the process outlined in the Data Sharing section on Shire's website: http://www.shiretrials.com/en/our-commitment-to-transparency/data-sharing-with-researchers and should be directed to clinicaltrialdata@shire.com.

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