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Research paper

In-depth genomic analyses identified novel letermovir resistance-associated substitutions in the cytomegalovirus UL56 and UL89 gene products

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ABSTRACT

Letermovir is a human cytomegalovirus (HCMV) terminase inhibitor recently approved in the United States for prophylaxis of HCMV infection or disease in adult HCMV-seropositive recipients [R+] of an allogeneic hematopoietic stem cell transplant. In the registrational trial, the rate of clinically significant HCMV infection, defined as the development of HCMV DNAemia leading to preemptive antiviral therapy or the diagnosis of HCMV end-organ disease, through 24 weeks post-transplant, was significantly lower among subjects who received letermovir prophylaxis through 14 weeks post-transplant compared to those who received placebo. We performed independent analyses of the HCMV nucleotide sequencing data generated by next-generation sequencing from this phase 3 registrational trial of letermovir to identify viral genetic characteristics associated with virologic failure during and following letermovir prophylaxis. The pUL56 substitutions V236M, E237G, and C325W, identified at previously known resistance-associated positions, were detected in the virus of subjects who were treated with letermovir and failed letermovir prophylaxis. Several additional substitutions were detected in pUL56 and pUL89, and further characterization is needed to determine if any of these substitutions are clinically relevant. The analyses reported herein were conducted to confirm sponsor-reported drug-resistance pathways, to assess the frequency of resistance, and to better understand the risk of prophylaxis failures and treatment-emergent drug resistance.

1. Introduction

Due to recent advances in human cytomegalovirus (HCMV) detection and treatment strategies, the incidence of HCMV disease (i.e., end-organ disease and HCMV syndrome) has decreased but has not been eliminated in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT) and solid organ transplantation (SOT) (Hodowanec et al., 2019; Kotton et al., 2018; Meijer et al., 2003). Letermovir (marketed as Prevymis®) was recently approved in the United States for prophylaxis of HCMV infection and disease in adult HCMV-seropositive recipients [R+] of an allo-HSCT. Letermovir is the only drug currently approved for HCMV prophylaxis in this population. The clinical development to support the efficacy of letermovir prophylaxis included evaluations in two completed clinical trials; one phase 2 trial (Chemaly et al., 2014) and one phase 3 trial (Chemaly et al., 2014; Ljungman et al., 2019; Marty et al., 2017). In the pivotal phase 3 trial, P001, letermovir prophylaxis was associated with a significant reduction in HCMV infection through Week 24 post-transplant (Marty et al., 2017).

Letermovir is an HCMV terminase complex inhibitor (Goldner et al., 2011, 2014, 2015; Lischka et al., 2010, 2016; Marschall et al., 2012; Wildum et al., 2015). The HCMV terminase complex is comprised of three proteins, pUL51, pUL56, and pUL89 (Borst et al., 2013; Hwang and Bogner, 2002; Neuber et al., 2017). The main function of the terminase complex is to cleave HCMV genome concatamers into functional monomers of HCMV genomes (Gentry et al., 2018; Griffiths and Emery, 2014; Ligat et al., 2018). Nonclinical resistance studies confirmed the

Abbreviations: HCMV, human cytomegalovirus; allo-HSCT, allogeneic hematopoietic stem cell transplantation; SOT, solid organ transplantation; ASAT, all subjects as treated; FAS, full analysis set; PCR, polymerase chain reaction; NGS, next-generation sequencing; HIVE, High-performance Integrated Virtual Environment

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mechanism of action of letermovir and identified target genes to be evaluated in the virus of patients who fail treatment. HCMV mutants with reduced susceptibility to letermovir have been selected in cell culture (Chou, 2015; Goldner et al., 2011, 2014). Substitutions that were selected in cell culture studies mapped to the HCMV pUL56, pUL89, and pUL51 proteins, with the majority of the substitutions mapping to the pUL56: C25F, S229F, V231 A/L, N232Y, V236 A/L/M, E237D, L241P, T244 K/R, L254F, L257F/I, K258E, F261 C/L/S, Y321C, C325 F/R/W/Y, L328V, M329T, A365S, N368D, and R369 G/M/S (Chou, 2015, 2017a, b; Chou et al., 2018; Goldner et al., 2014; Piret and Boivin, 2019). Substitutions V236M and C325F/Y have also been observed clinically (Cherrier et al., 2018; Frietsch et al., 2019; Lischka et al., 2016; Turner et al., 2019).

This report summarizes the independent U.S. Food and Drug Administration (FDA) analyses of drug-resistance data from the phase 3 trial of letermovir prophylaxis that was submitted in the new drug application (NDA) for Prevymis®. These analyses were conducted to confirm sponsor-reported drug-resistance pathways, assess the frequency of resistance, and identify potential approaches to minimize the risk of prophylaxis failures and treatment-emergent drug resistance in clinical practice.

2. Materials and methods

2.1. Study design

We independently analyzed HCMV drug-resistance results reported from the phase 3 clinical trial of letermovir (P001) (Marty et al., 2017). In this trial, HCMV seropositive recipients [R+] of an allo-HSCT were randomized 2:1 to receive letermovir or placebo through 100 days (14 weeks) post-transplant. Subjects were then followed through 48 weeks post-transplant. Routine clinical evaluation, which included safety, HCMV disease, and HCMV DNA polymerase chain reaction (PCR) assessments, was performed weekly through Week 14, then every other week through Week 24, then at Weeks 32, 40, and 48. Of the 565 subjects who received at least one dose of study medication (all subjects as treated [ASAT] population), 70 had detectable HCMV DNA at the time of randomization (48 in the letermovir group and 22 in the placebo group) and were excluded from efficacy analyses but included in the resistance analysis. Of note, of the 48 subjects in the letermovir group who had detectable DNA at the time of randomization, the DNA levels were 137 IU/mL (n = 44), 143 IU/mL (n = 1), 286 IU/mL (n = 1), and 654 IU/mL (n = 1). The remaining 495 subjects without detectable HCMV DNA at the time of randomization comprised the full analysis set (FAS) population used in the primary efficacy analysis.

This clinical trial showed that letermovir administered prophylactically for 14 weeks after allo-HSCT was superior to placebo in preventing clinically significant HCMV infection through Week 24 post-transplant in the FAS population (18% versus 42%, $p < 0.001$). Clinically significant HCMV infection was defined as the development of HCMV DNAemia leading to preemptive antiviral therapy or the diagnosis of HCMV end-organ disease (Table 1). Among letermovir-treated subjects in the FAS population, 8% developed HCMV infection during the Week 0 through Week 14 prophylaxis period and an additional 10% developed HCMV infection after the completion of letermovir prophylaxis.

These data were submitted to the FDA Division of Antiviral Products by Merck & Co., Inc., Kenilworth, NJ USA (the sponsor) as part of the recently approved NDA for Prevymis®. Detailed clinical study protocols and efficacy analyses have been described elsewhere (Marty et al., 2017).

2.2. Genotypic analysis

HCMV drug-resistance analyses for the phase 3 trial were conducted by the FDA using next-generation sequencing (NGS) data submitted by the sponsor. The analysis approach reportedly used by Merck is briefly described here. The genes encoding pUL56 and pUL89 were amplified in three different nested PCR assays. The amplicons generated by the nested PCR were analyzed by NGS using the Illumina MiSeq system. NGS Quality Control analysis and variant calling of amino acid differences for sequences derived from subjects at or near the time of prophylaxis failure compared to a reference sequence for each gene was performed using the Athena pipeline, which is a custom NGS bioinformatics pipeline developed by DDL Diagnostic Laboratory (Rijswijk, The Netherlands) specifically for the analysis of amplicon-based deep sequencing data. The reference sequences used were from the HCMV strain Merlin (GenBank accession number **HCMV_Merlin_NC006273_rev2**). The limit of detection value was 108 copies/mL for UL56, 37 copies/mL for UL89A (Exon 1), and 92 copies/mL for UL89B (Exon 2). The limit-of-detection values were obtained during assay validation experiments using an HCMV virus grown in cell culture and "spiked" into HCMV-naïve human plasma. For the sponsor's resistance analyses, differences detected at a frequency ≥5% at a given position were identified as HCMV genotypic variants.

Genotypic drug-resistance data that incorporated the summarized NGS data generated by DDL, which was provided in resistance table format, were analyzed by the FDA using JMP 11.1.1 software (SAS, Cary, NC). The High-performance Integrated Virtual Environment (HIVE), an NGS bioinformatics analysis platform developed by the FDA (Simonyan et al., 2017) and approved for the analysis of confidential regulatory data, was used by the Division of Antiviral Products reviewers in collaboration with the Center for Biologics Evaluation and Research to perform most of the independent assessments of NGS data for this NDA (Fig. 1). For the independent assessment, results were generated in HIVE and compared to the results reported by the sponsor. If the two analysis approaches generated results with any significant differences in the amino acid variants detected, the frequency of each substitution, or the read coverage at the variant nucleotide loci, a third optimized NGS analysis platform, the CLC Genomics Workbench (Qiagen, Redwood City, CA), was used to determine if there was agreement in results from any two analysis pipelines in a three-way comparison (Fig. 1). Of note, the CLC Genomics Workbench pipeline was only used when there was disagreement between the Athena and HIVE results.

2.3. Resistance analysis definitions

Generating resistance analysis tables. The HIVE Viral Comparator tool and Excel macros were used to convert the frequency tables generated by HIVE or CLC Genomics, respectively, into resistance analysis tables, allowing the resistance tables to be populated using different frequency thresholds. For example, the frequency tables

Table 1
Summary of primary efficacy endpoint for study P001 (FAS population).

	Weeks 0–14		Weeks 14–24		Weeks 0–24	
	Letermovir	Placebo	Letermovir	Placebo	Letermovir	Placebo
Clinically significant HCMV infection (overall)	25/325 (8%)	67/170 (39%)	32/325 (10%)	4/170 (2%)	57/325 (18%)	71/170 (42%)
DNAemia with PET	24/325 (7%)	65/170 (38%)	28/325 (9%)	3/170 (2%)	52/325 (16%)	68/170 (40%)
End-organ disease	1/325 (< 1%)	2/170 (1%)	4/325 (1%)	1/170 (< 1%)	5/325 (2%)	3/170 (2%)

HCMV, human cytomegalovirus; PET, preemptive therapy with ganciclovir or valganciclovir; FAS, full analysis set.

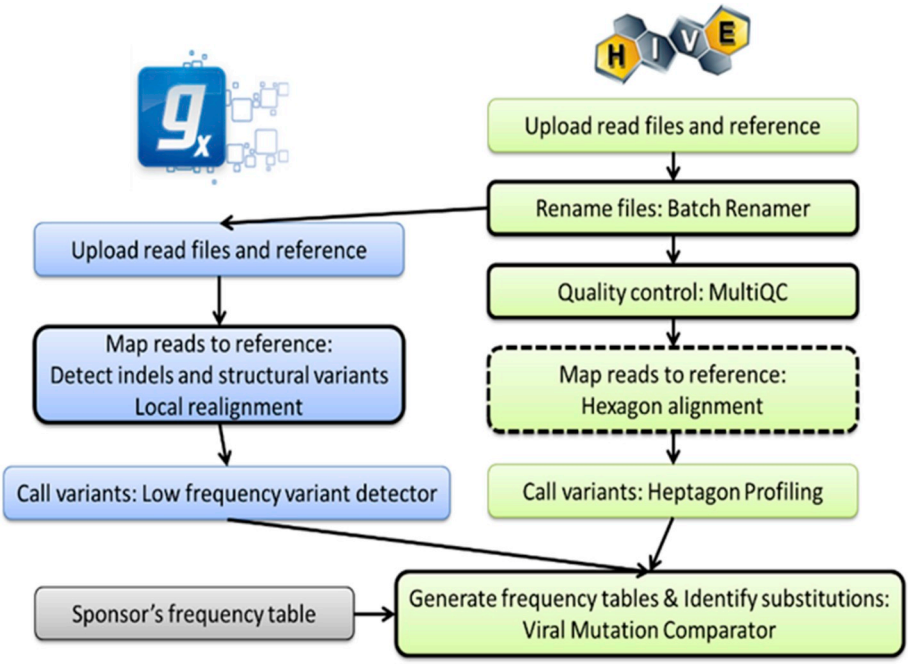

Fig. 1. Overview of NGS analysis pipeline using HIVE with CLC Genomics Workbench.
Specific tools were developed to allow FDA reviewers to batch rename files to meet nomenclature rules for the analysis pipeline, to assess quality control on all sequence files, to convert variant call files (VCF) to frequency tables at the amino acid level, and to generate tables comparing results submitted by the sponsor and those generated by HIVE or CLC Genomics. Of note, CLC Genomics Workbench was only used to clarify discrepancies in the data between Athena and HIVE.
NGS, next-generation sequencing; HIVE, High-performance Integrated Virtual Environment.

generated from HIVE, CLC Genomics Workbench output, or submitted by the sponsor contained all variants with a frequency ≥1%, and this tool allowed resistance analysis tables to be generated showing variants at different levels of sensitivity (2%, 5%, 10%, 15%, 25%, etc.) as defined by the user.

Conducting independent resistance analysis. The frequency tables and resistance analysis tables were analyzed to identify substitutions that occurred at or above a defined frequency threshold of 5%. The criteria used for determining potential resistance-associated substitutions are described in the Supplement.

3. Results

An analysis of amino acid substitutions was conducted using data collected from subjects in the ASAT population who had detectable HCMV DNAemia through Week 24 (on prophylaxis or post-prophylaxis) in the phase 3 prophylaxis trial. Among the ASAT population (as noted, this analysis was not conducted using the FAS population used for the primary efficacy analysis), there were 79/373 (21%) and 88/192 (46%) subjects in the letermovir and placebo arms, respectively, who had clinically significant HCMV infection by Week 24. Table 2 summarizes the trial arm and available NGS sequence files (provided in fastq format) for the subjects with submitted NGS data. Next generation sequencing of the HCMV UL56 and UL89 genes was performed for 84 subjects, 54 in the placebo group (52 subjects during prophylaxis and 2 subjects after prophylaxis) and 30 in the letermovir group (8 subjects during prophylaxis and 22 subjects after prophylaxis). The median viral load for subjects who had UL56 and UL89 amplified was 985 IU/mL (range 137-25,661 IU/mL) and 859 IU/mL (range 137-36,422 IU/mL) in the letermovir and placebo arms, respectively.

Table 2
Subjects for whom NGS data were submitted.

Arm	Study-Phase	Subjects	Fastq Files
Letermovir	OP	8	20
	FU	22	46
Placebo	OP	52	116
	FU	2	4
Totals		84	186

NGS, next-generation sequencing; OP, on prophylaxis; FU, post-treatment follow up.

For the resistance analysis (ASAT population), we used the resistance criteria presented in the Supplement. For pUL56, there were 36 amino acid positions that met the criteria for potential resistance with four substitutions occurring in two or more subjects and 32 occurring in one subject each (Table 3). Substitutions at amino acid positions previously associated with resistance were detected in the virus of three subjects, including V236M (0.99 frequency), C325W (0.99 frequency), and E237G (0.04 frequency). One substitution that was detected in the virus of two subjects required two changes to the codon to result in an E (GAA) to G (GGG) change at position 485.

Substitutions detected in the virus of subjects who failed (i.e., developed clinically significant HCMV infection) during the letermovir prophylaxis phase are shown in Table 4. Among the subjects with evaluable genotypic data in the ASAT population, eight failed letermovir prophylaxis while on study drug, and the virus from three of the eight subjects (approximately 38%) contained known letermovir resistance-associated substitutions at positions V236M (0.99 frequency), C325W (0.99 frequency), and E237G (0.04 frequency). A substitution, E485G, and a deletion at 445-SNS-447, were detected in the virus of two subjects from the letermovir arm who failed while on prophylaxis.

For pUL89, there were 23 amino acid positions that met the criteria for potential resistance with one substitution occurring in two or more subjects who received letermovir and failed prophylaxis and 22 substitutions occurring in one prophylaxis-failure subject each (Table 5). Substitutions previously associated with resistance (at positions 256 and 345) were not detected in the virus of any subjects in the letermovir arm or placebo arm. Two different substitutions were detected at amino acid position 531 (I531V and I531T) in the virus of two subjects who received letermovir and failed prophylaxis, and were seen at frequencies of 1.0 and 0.04, respectively. Substitutions at this position were also observed at low frequency in approximately 4% of subjects from the placebo group.

Substitutions in pUL89 that were detected in the virus of subjects who were on letermovir prophylaxis at the time of failure are shown in Table 6. None of these substitutions occurred at positions previously associated with resistance to letermovir and no substitutions occurred in more than one subject.

Table 3
pUL56 substitutions from letermovir arm meeting potential resistance criteria.

Amino Acid Substitution	% Identity[a]	Polymorphic[b] Amino Acid at This Position	Letermovir Subjects (n = 28)	Visit Day(s) Post-Transplant	Amino Acid Frequency
M3I/M3V	100	M	2	108, 128	0.03, 0.03
L26P	100	L	1	160	0.04
F41L	100	F	1	128	0.03
I48M	100	I	1	62	0.04
A103V	100	A	1	132	0.04
E141stop	100	E	1	128	0.03
N148D	100	N	1	108	0.0227
E157G	100	E	1	128	0.07
Q182K	100	Q	1	128	0.03
Q213R	100	Q	1	9	0.0234
V236M	100	V	1	62	0.99
E237G	100	E	1	42	0.04
S255L	100	S	1	160	1
S269G	100	S	1	144	0.05
E276G	100	E	1	146	0.0204
I313V	100	I	1	162	0.18
C325W	100	C	1	46	0.99
S378N	100	S	1	46	0.024
S445–S447	95.7	S, N, A, M; N, S, T; S, A	2	62, 66	0.7, 0.7
E485G	99.5	E, G	2	62, 66	0.99, 0.99
I535V	100	I	1	125	0.05
E542DEL	100	E	1	128	0.03
Y575C	100	Y	1	120	0.99
M641T	100	M	1	134	0.04
L658S	100	L	1	132	0.15
Y667H	99.5	Y, H	1	134	0.03
S705F	100	S	1	146	0.09
V706A	100	V	1	131	0.04
L750P	100	L	1	162	0.03
Y757H	100	Y	1	130	0.03
T775I	99.5	T, R	1	66	1
R816W	100	R	1	145	0.99
P846L	100	P	1	281	0.11

HCMV, human cytomegalovirus.

[a] Conserved is defined as 100% identity at the amino acid position based on the sponsor's analysis of 187 unique HCMV whole genome sequences available from National Center for Biotechnology Information.

[b] Polymorphic is defined as an observation of more than one unique amino acid residue at any position among the 187 whole genome sequences or among subjects who received placebo.

Table 4
pUL56 substitutions in subjects who failed while on letermovir prophylaxis.

Subject	Timepoints[a]	Resistance Conclusion[b]
A[e]	D85, D89, D157	No substitutions met the resistance criteria
B[e]	D66	445-447 SNS deletion[d] (0.7), E485G[d] (0.99), T775I (1)
C[e]	D42	E237G[c] (0.04) and R826L (0.03)
D	D9	Q213R (0.02)
E[e]	D62	I48M (0.04), V236M[c] (0.99), 445–447 SNS deletion[d] (0.7), E485G[d] (0.99)
F[e]	D95	No substitutions met the resistance criteria
G	D46	C325W[c] (0.99) and S378N (0.02)
H[e]	D46	No substitutions met the resistance criteria

Values in parenthesis represents the frequency at which the amino acid substitution was detected in the NGS data.

[a] Timepoint measured in study day (D); letermovir treatment ended on Day 100.

[b] Substitutions met resistance criteria described in the Supplemental section of this manuscript.

[c] Known resistance site.

[d] Detected in two subjects.

[e] Subjects in the FAS population.

Overall, substitutions at previously identified resistance-associated positions occurred in 3/8 (38%) of the subjects who failed during the letermovir 14 week prophylaxis period and in 0/22 (0%) of the subjects who failed after the 14 week post-prophylaxis period, for a total of 3/30 (10%) letermovir subjects developing substitutions at previously identified positions at any time during the trial.

4. Discussion

The phase 3 prophylaxis trial demonstrated the efficacy of letermovir in preventing clinically significant HCMV infection in adult HCMV-seropositive recipients [R+] of an allo-HSCT (PREVYMIS® Package Insert, 2017). While robust efficacy of letermovir through Week 24 post-transplant was demonstrated, clinically significant HCMV infection was still observed in 57/325 (18%) and 71/170 (42%) of letermovir and placebo subjects (FAS population), respectively, by Week 24 post-transplant. To verify the resistance results reported by the sponsor and to better characterize potential resistance pathways, we performed independent analyses of HCMV nucleotide sequencing data generated by next-generation sequencing from the registrational phase 3 clinical trial (ASAT population) of letermovir. We used the HIVE NGS analysis pipeline to perform the independent analysis of the NGS data provided by Merck and our results were then compared to the results provided by the sponsor. In general, there was good agreement between the results generated by the HIVE and Athena pipelines, with a few minor discrepancies analyzed with a third analysis pipeline, CLC Genomics Workbench, showing that the Athena pipeline generated robust data that were reproducible.

The known pUL56 substitutions V236M, E237G, and C325W were detected in the virus of subjects from the letermovir arm who developed clinically significant HCMV infection while on letermovir (ASAT population). Substitution pUL56 V236M is a known resistance-associated substitution that was also observed in the sponsor's phase 2 study P020 as well as in cell culture (Goldner et al., 2014; Lischka et al., 2016). V236M was detected at a high frequency of 0.99 on Day 62 in one subject. Substitutions pUL56 E237G and C325W occurred at known cell

Table 5
pUL89 substitutions from letermovir arm meeting resistance criteria.

Amino Acid Substitution	% Identity[a]	Polymorphic[b] Amino Acid at This Position	Letermovir Subjects (n = 28)	Visit Day Post-Transplant	Amino Acid Frequency
K41E	100	K	1	175	0.0206
N74S	100	N	1	175	0.06
S102F	100	S	1	145	0.1
F124L	100	F	1	132	0.04
T132A	100	T	1	281	0.03
V146I	100	V	1	125	0.03
P176S	100	P	1	132	0.12
H243R	100	H	1	160	0.04
H246R	100	H	1	66	0.03
D309G	100	D	1	175	0.05
L323P	100	L	1	157	0.0216
T331A	100	T	1	46	0.0207
S373G	99.5	S, N	1	160	0.04
M406V	100	M	1	132	0.09
N426D	100	N	1	175	0.0236
L458P	100	L	1	281	0.04
S521G	100	S	1	175	0.0239
L522P	100	L	1	95	0.07
A532T	100	A	1	146	0.07
I572V	100	I	1	132	0.0249
Q625stop	100	Q	1	66	0.16
T637A	99.5	T, A	1	95	0.0231
V656A	100	V	1	160	0.0222

[a] Conserved is defined as 100% identity at the amino acid position based on the sponsor's analysis of 187 unique HCMV whole genome sequences available from National Center for Biotechnology Information.
[b] Polymorphic is defined as observation of more than one unique amino acid residue.

Table 6
pUL89 substitutions in subjects who failed while on letermovir prophylaxis.

Subject	Timepoints[a]	Resistance Conclusion[b]
A[c]	D85, D89, D157	L323P (0.02)
B[c]	D66	H246R (0.03) and Q625stop (0.16)
C[c]	D42	No substitutions met the resistance criteria
D	D9	No substitutions met the resistance criteria
E[c]	D62	No substitutions met the resistance criteria
F[c]	D95	L522P (0.07) and T637A (0.02)
G	D46	No substitutions met the resistance criteria
H[c]	D46	T331A (0.02)

Values in parenthesis represents the frequency at which the amino acid substitution was detected in the NGS data.
[a] Timepoint in days (D); letermovir treatment ended on Day 100.
[b] Substitutions met the resistance criteria described in section 2.3.
[c] Subjects in the FAS population.

culture–resistance positions and, therefore, were considered resistance-associated substitutions. The pUL56 E237G substitution was observed at a low frequency (~4%) and was also observed in one placebo subject (at a frequency of ~2%) so the clinical significance of this substitution, or any letermovir resistance-associated substitution, at this low frequency is unknown. However, the sample used for genotypic-resistance analysis was taken 4 weeks after prophylaxis failure on Day 10 and after the start of preemptive therapy. The low frequency may indicate that (1) the resistant population was suppressed by ganciclovir preemptive therapy, (2) amino acid replacements at this position may have been of low fitness and therefore were rapidly overgrown by wildtype virus in the 4 weeks after letermovir treatment was stopped, (3) the initial letermovir treatment may have been too short in duration to sufficiently enrich the resistant population, or (4) the amino acid substitution was an artifact resulting from PCR error given the low copy number. This substitution was reported to confer approximately 13-fold reduced susceptibility to letermovir in cell culture (Bray et al., 2018). C325W was detected at a high frequency of 0.99 at Day 46 in one subject who failed while on letermovir prophylaxis. Of note, nearly normal growth fitness was retained in cell culture for viruses with amino acid substitutions within pUL56 positions 231 to 369, including substitutions at positions 236, 237, and 325 (Chou, 2015; Chou et al., 2018; Goldner et al., 2014; Lischka et al., 2016). We did not have longitudinal samples to determine the fitness of these variants in natural isolates in patients.

In addition, the pUL56 445-SNS-447 deletion in combination with an E485G substitution occurred at high frequency (> 70%) in 2/8 (25%) subjects who failed prophylaxis while on letermovir. These substitutions are located outside the functional domains of pUL56 involved in DNA-packaging and do not impact viral replicative capacity (Chou, 2015; Goldner et al., 2014; Lischka et al., 2016). E485G has been described in an independent study as a pUL56 polymorphism, which did not have an impact on letermovir susceptibility (Champier et al., 2008; Lischka et al., 2016). However, given that this substitution at a conserved amino acid position emerged in the virus from two subjects who failed treatment while on letermovir, it was considered a potential resistance-associated site. The 445-SNS-447 deletion has been observed in pUL56 for another terminase complex inhibitor and was not observed in any of the patients in the placebo arm (Champier et al., 2008). While this deletion alone did not confer an obvious reduction in susceptibility to letermovir, its clinical impact, particularly in combination with E485G, is unknown. There were no pUL89 resistance-associated substitutions that previously had been identified in cell culture selection studies with letermovir or at positions that were selected with other terminase complex inhibitors (Chou, 2017a).

Other substitutions were detected in both pUL56 and pUL89 in addition to the substitutions identified at previously known resistance-associated amino acid positions in pUL56. Further characterization is needed to determine whether any of these are clinically relevant. The phenotypic assessment of many of these substitutions and those described previously were part of a post-marketing requirement agreed to by Merck and the FDA. Typically, “resistance-associated substitutions” for HCMV antiviral drugs are defined as substitutions that decrease susceptibility to one or more antiviral drugs (Chemaly et al., 2019). However, substitutions associated with clinical drug resistance do not always confer obvious reductions in phenotypic susceptibility in cell culture or biochemical assays. Therefore, absence of a major phenotypic impact in cell culture assays does not preclude its clinical relevance (Chou et al., 2002; Huang et al., 1999; Komatsu et al., 2017; Kumada et al., 2014; Lontok et al., 2015). We typically consider both clinical

efficacy and phenotype data to identify amino acid substitutions potentially associated with drug resistance. This includes amino acid substitutions that have been clinically associated with resistance to the drug where "clinically associated" refers to substitutions reported at conserved positions that have been observed in subjects who failed drug treatment in multiple independent studies. We have used this approach for resistance analyses of other antiviral drugs (Donaldson et al., 2015; Komatsu et al., 2014).

Overall, the majority of amino acid substitutions that met the criteria for potential resistance in subjects in the letermovir arm with available genotype data were detected after the completion of prophylaxis (see Table 3). Some of these late substitutions could be due to low level HCMV replication during prophylaxis and could confer letermovir resistance. Alternatively, the occurrence of these substitutions in the post-prophylaxis period could indicate that the substitutions are not clinically relevant. As noted above, phenotypic assessments should provide a better understanding of the potential clinical implications of the previously unknown substitutions. Conversely, known resistance-associated substitutions occurred exclusively in subjects who failed during the letermovir 14-week prophylaxis period (3/8 [37.5%]). The absence of any known resistance-associated substitutions in the post-prophylaxis failures suggests that something other than resistance (e.g., ongoing immunosuppression) may be driving these late failures. Therefore, a longer duration of letermovir prophylaxis may be effective in preventing late HCMV in some cases. This is of particular interest as the failures occurring after the completion of letermovir prophylaxis included cases of HCMV end-organ-disease, highlighting the significant ongoing threat that HCMV poses to some HSCT recipients.

There are several limitations to this study. First, only the pUL56 and pUL89 viral proteins were assessed for the development of resistance while letermovir resistance-associated substitutions have also been identified in the pUL51 (Chou, 2017b). Second, based on cell culture–-resistance selection studies, multiple resistance pathways to letermovir are possible as seen with other approved drugs for HCMV (Campos et al., 2016; Komatsu et al., 2014; Lurain and Chou, 2010), complicating the identification of resistance pathways from clinical samples. Third, the low copy number of HCMV DNA in many of these samples may have confounded the detection of low-frequency variants and introduced genotyping artifacts that appeared as potentially resistant substitutions. Fourth, many of the potential resistance-associated substitutions identified were of low frequency. We recognize that substitutions that confer resistance are typically detected in multiple subjects at frequencies much higher than 10%; however, we prefer to err on the conservative side by identifying potential resistance-associated substitutions to monitor across different clinical trials. Finally, resistance data were not available for many subjects who experienced virologic failure. Despite these limitations, this study represents the first NGS resistance analyses that the FDA has conducted for an HCMV clinical trial as well as for a prophylaxis study design. We believe that the knowledge gained from these analyses such as the methodologies used to generate the NGS data and criteria used for data analyses will serve as the basis for designing future studies.

The data presented here represent our current understanding of resistance in the setting of letermovir prophylaxis. However, additional resistance data from clinical trials and real-world clinical experience are needed. Variable results have been reported for the use of letermovir for treating HCMV infection that is resistant or refractory to treatment with other available anti-HCMV therapies (Kaul et al., 2011; Phoompoung et al., 2019; Turner et al., 2019). As additional indications for letermovir use are explored, a better understanding of letermovir's resistance pathways and the robustness of its barrier to resistance are crucial for informing appropriate treatment guidelines.

Disclaimer

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Appendix A. Supplementary data

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