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Short communication

Single and multiple mutations in the human cytomegalovirus UL97 gene and their relationship to the enzymatic activity of UL97 kinase for ganciclovir phosphorylation

Silvia I. Sanchez Puch[a,b,*], Verónica L. Mathet[c], Margarita Porta[d], María L. Cuestas[c], José R. Oubiña[c], Cristina M. Videla[b], Horacio E. Salomón[a]

[a] Centro de Referencia para el SIDA, Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina-Universidad de Buenos Aires. Paraguay 2155, piso 11, (C1121ABG)-CABA, Argentina
[b] Laboratorio de Virología, Hospital Universitario CEMIC. Galván 4102, (C1431FWN)-CABA, Argentina
[c] Centro para el Estudio de las Hepatitis Virales, Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina-Universidad de Buenos Aires. Paraguay 2155, piso 11, (C1121ABG)-CABA, Argentina
[d] Centro de Investigación en Reproducción Humana y Experimental, Hospital Universitario CEMIC. Galván 4102, (C1431FWN)-CABA, Argentina'

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ABSTRACT

In this study we determined that the double mutant M460V/D605E in the UL97 gene of an HCMV isolate from an immunocompromised patient (MMT isolate) is related to resistance to ganciclovir (GCV) therapy. Our results suggest that the aspartic acid-to-glutamic acid substitution at codon 605 may be associated with a natural polymorphism of the UL97 gene, and not with positive selection pressure exerted by the antiviral drug. We also determined that GCV resistance due to the M460V mutation in the HCMV UL97 gene is not offset by a second mutation (D605E) at codon 605. Furthermore, we showed that when the two mutations related to GCV resistance were simultaneously detected in the same HCMV construct, virus-drug resistance might be enhanced in comparison to that of the single mutants studied separately. To our knowledge for the first time, seven of 12 amino acid changes (F102L, D118V, M330T, T400A, R507P and C511R and I533V) in the UL97 gene of an isolate are herein reported.

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Human cytomegalovirus (HCMV)-encoded protein kinase UL97 plays an important role in viral replication and fulfills several regulatory functions. The majority of GCV-resistant clinical isolates show mutations in UL97 region at codons 594–607 (Chou et al., 1995a,b; Erice et al., 1989; Spector et al., 1995; Wolf et al., 2001). Almost all mutations in that region have been shown to impair GCV phosphorylation (Baldanti et al., 1995; Chou et al., 1995a; Lurain et al., 1994; Wolf et al., 1995a; Wolf et al., 1995b). Nevertheless, Ijichi et al., 2002 have postulated a compensatory role for the D605E mutation in a strain with A594P substitution. In a previous study, we reported the presence of two point mutations, M460V and D605E, in the UL97 gene of an HCMV isolate recovered from an immunocompromised patient (MMT) after prolonged treatment with GCV for 151 days (Sánchez Puch et al., 2004). The D605E mutation was also detected in HCMV UL97 isolated from leukocytes of the same patient before GCV treatment.

* Corresponding author at: Escuela Superior en Ciencias de la Salud, UNICEN. Av. Pringles 4375, (7400)-Olavarría. Provincia de Buenos Aires. Argentina.
Fax: +54 11 49429706.
E-mail address: ssp@fibertel.com.ar (S.I. Sanchez Puch).

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The aim of this work was to study the impact of the HCMV M460V/D605E-mutant on GCV phosphorylation and to assess the polymorphism of the complete HCMV UL97 gene of the MMT isolate. We show that GCV phosphorylation impairment due to M460V is not compensated by D605E.

Viral isolation was performed on human foreskin fibroblast cultures prepared at the Laboratory of Virology at CEMIC University Hospital. The AD169 strain was used as a reference strain. Viral DNA was extracted using QIAamp columns (QIAGEN, Inc.).

For transfection experiments, 293T cells, kindly provided by Silvia González (Laboratory of Virology, University of Belgrano, Argentina) were used throughout.

The full-length UL97 gene was amplified by PCR, yielding an amplicon of 2123 bp using primers previously described by Marschall et al., 2001. Plasmid pcDNA-UL97 or pMMrwt that contained the UL97 gene of the HCMV AD169 strain (kindly provided by Manfred Marschall of the Institute für Klinsche und Molekulare Virologie, Universität Erlangen-Nürnberg, Germany) was used as positive control.

PCR amplicons of expected size were obtained with Vent Pol_R DNA polymerase® (New England BioLabs) and detected in a 2% agarose gel stained with ethidium bromide and visualized under

UV light. For sequencing and cloning, PCR products were purified with the QIAquick gel extraction kit (QIAGEN Inc.).

For *in vitro* site-directed mutagenesis, different constructs - pD605E, pM460V, pA594P, pM460V/A594P, pA594P/D605E and pM460V/A594P/D605E – were obtained by using pMMrwt as template and 2.5 U/μl of *Pfx* DNA polymerase (Invitrogen Inc.). Primers were synthesized and purified by HPLC methods with different mutations for both chains. For mutagenesis of methionine to valine within the 460 codon, oligonucleotides GCCACTTTGACAT***C***ACACCCATGAACG (UL97-460F) and CGTTCATGGGT***G***TGATGTCAAA-GTGGC (UL97-460R) were used. For mutagenesis of codons 594 and 605, oligonucleotides UL97-594F and UL97-605F, previously described by Ijichi et al., 2002, were utilized. All constructs were verified by bidirectionally DNA sequencing with an ABI PRISM 3100 Genetic. The amplicon corresponding to the UL97 gene from the MMT isolate was cloned into the pGEM®-T Easy vector system (Promega) and subcloned into the pTARGET vector (Promega) for protein expression, according to the Manufacturer's instructions. Transformation was performed by using *Escherichia coli DH5α* (Invitrogen Inc.) for pGEM®-T Easy vector and *E. coli* JM109 High Efficiency Competent Cells (Promega) for pTARGET vector. Transformants were identified in IPTG/X-GAL (100 mM/50 mg/ml) plates. To verify the presence of the insert, enzymatic digestion with the restriction enzymes *Eco*RI and *Xho*I was done. The construct carrying MMT's UL97 gene was named pTMMT (M460V/D605E). Plasmids were purified by using QIAprep Spin Miniprep or HiSpeed Plasmid Purification kits (QIAGEN Inc.). For transient expression experiments, 293T cells were transfected with Lipofectamine 2000 (Invitrogen, Inc.). Eight microliters of DNA were used following the Manufacturer's instructions. After transfection, cells were maintained with MEMα containing 5% horse serum. Proteins were extracted from cellular lysates using 100 μl M-PER mammalian protein extraction reagent (PIERCE) with 5 μl Halt Protease Inhibitor Cocktail (PIERCE) and they were quantified by photometry using an ELISA plate reader (OD_{562}) and by the Micro BCA Protein Assay (PIERCE). Protein samples were analyzed by SDS-PAGE (Maniatis et al., 1989). Western blotting was performed by using a MBP-UL97 polyclonal antiserum, (He et al., 1997) kindly provided by Donald M. Coen (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts), and subsequently a horseradish-peroxidase-conjugated secondary anti-rabbit IgG antiserum. Western blot was developed in an enhanced chemiluminescent reaction (ECL Western Blotting Systems; Amersham Biosciences), according to the Manufacturer's instructions. We determined GCV phosphorylation in transfected cells by separation of its phosphorylated metabolites by means of [8-^{3}H]-GCV. The 293T cells were transfected with different constructs [pMMrwt, pTMMT (M460V/D605E), pD605E, pM460V, pA594P, pM460V/A594P, pM460V/A594P/D605E and pA594P/D605E] for transient expression experiments, as described above. Cells were first treated with unlabeled GCV, gently provided by Productos Roche S.A.Q e I (Argentina) and then with 5 μCi of [8-^{3}H]-GCV (Moravek Biochemicals). After 24 h incubation, cells were harvested with PBS/EDTA and washed three times with 1× PBS. Cell pellets were then resuspended in ice-cold 60% methanol and incubated 30 min at −80° C. Then, cellular extracts were clarified by centrifugation at 770 × *g* for 15 min at 4 °C and supernatants were evaporated to dryness under vacuum and stored at −70 °C until use. Immediately before starting HPLC analysis, they were resuspended in HPLC-grade water (Lichrosolv, Merck). Three independent transfection events were performed for each of the constructs prepared herein for transient expression experiments.

HPLC was then performed using a C_{18} reverse phase column (Lichrospher 100 RP-18 5 μm) provided by the Research Center for Human and Experimental Reproduction of Argentina with a mobile phase of 20 mM KH_2PO_4 (pH 6.0). Isocratic elution was performed at a flow rate of 0.4 ml/min. Eighty microliters of the samples were directly injected into the HPLC system, and fractions were collected at 1 min intervals for 25 min. Total radioactivity in each fraction was measured by liquid scintillation counting (LKB Wallac 1214 Rack Beta). Retention times for GCV metabolites were obtained using guanosine mono-, di-and tri-phosphate (Sigma), since no standards for each GCV metabolite were available, except for the non-phosphorylated GCV form. Since the acyclovir drug (ACV) is a guanosine analog like GCV, we used ACV (LCA, Filaxis) to estimate the retention time of both native GCV and its metabolites by using a mixture of GMP, GDP, GTP, and unlabeled GCV. A retention time of around 15 min was obtained for both labelled and unlabelled GCV. The retention time for GMP was approximately 4 min, whereas for GDP and GTP, it was approximately 2.5 min, probably due to running conditions.

Based on previous results where we had amplified and sequenced two regions of 133 bp and 255 bp (nt positions 1328–1460 nt and 1708–1962, respectively) of the UL97 gene of the MMT isolate (Sánchez Puch et al., 2004), we have now extended the sequenced area that included the intermediate region. Viral DNA was extracted from viral isolates and peripheral blood leukocyte samples. All these samples were collected at different time points during the course of infection. Viral DNA was then amplified, yielding a 613 bp amplicon. In all cases, in addition to the reportedly known mutations M460V/D605E, amino acid substitutions of methionine for isoleucine at position 526 and of isoleucine for valine at position 533 were detected. These amino acid changes had been previously described as naturally occurring polymorphisms of the UL97 gene (Lurain et al., 2001). We also recovered the mutant D605E from plasma samples and viral isolates from two AIDS patients as well as from two peripheral blood leukocyte samples from patient MMT. All these samples had been obtained before intravenous treatment with GCV.

The full-length UL97 gene of the MMT isolate was inserted into the expression vector pTARGET. This construct was named pTMMT (M460V/D605E) and was then fully sequenced to corroborate its integrity, to compare the nucleotide sequence of the UL97 gene with the reference strain AD169 and for subsequent experiments. Transitions, transversions, synonymous and non-synonymous substitutions observed in the UL97 gene of the MMT isolate are depicted in Table 1. Of the 29 nucleotide changes observed, 12 resulted in amino acid substitutions. Twelve amino acid changes were observed throughout the sequence of the UL97 protein of the MMT isolate as compared with the reference strain AD169: N68D, F102L, D118V, I244V, M330T, T400A, M460V, R507P, C511R, M526I, I533V and D605E. One of them (M460V) is known to be associated with GCV resistance, whereas seven (F102L, D118V, M330T, T400A, R507P, C511R and I533V) have been herein reported, to our knowledge for the first time. Nucleotide sequence variability resulted in 29 point mutations, most of them being synonymous for the amino acid substitutions. Point mutations observed at codons 507, 511, and 533 occurred within subdomains VII and IX of the UL97 protein, which are part of the catalytic domain and the ATP-binding sites of the enzyme. The impact of these amino acid changes on antiviral drug resistance is still a matter of concern. A study using site-directed mutagenesis showed that the M526I mutant is not GCV-resistant (Bodaghi et al., 1999). Sequence homology between the UL97 gene of the MMT isolate and the UL97 gene of the reference HCMV strains AD169, Towne, Davis, Merlin and Toledo, was 98.8%.

To investigate if the double mutant M460V/D605E behaves like the already reported A594P/D605E, we obtained the following constructs by site-directed mutagenesis using plasmid pMMrwt as template, pM460V, pA594P, pD605E, pM460V/A594P, pA594P/D605E, and pM460V/A594P/D605E. In addition, we also

Table 1
Nucleotides and amino acid changes on UL97 sequence of the MMT strain.

Nt #	Nt change		Mutation type	Nt position at the affected codon	Codon #	Substitution type	Amino acid change
	UL97 wt	pTMMT (M460V/D605E)					
108	G	A	Transition	3	36	Synonymous	None
180	C	T		3	60		
195	T	C		3	65		
202	A	G		1	68[a]	Non Synonymous	N → D
234	C	T		3	78	Synonymous	None
306	T	G	Transversion	3	102[b]	Non Synonymous	F → L
353	A	T		2	118[b]		D → V
390	A	G	Transition	3	130	Synonymous	None
462	T	C		3	154		
648	T	C		3	216		
730	A	G		3	244[a]	Non Synonymous	I → V
831	C	T		3	277	Synonymous	None
933	G	A		3	311		
972	T	C		3	324		
989	T	C		2	330[b]	Non Synonymous	M → T
1198	A	G		1	400[b]		T → A
1213	C	T		1	404	Synonymous	None
1378	A	G		1	460[c]	Non Synonymous	M → V
1410	C	T		3	470	Synonymous	None
1455	C	T		3	485		
1509	T	C		3	503		
1520	G	C	Transversion	2	507[b]	Non Synonymous	R → P
1531	T	C	Transition	1	511[b]		C → R
1575	C	T		3	525	Synonymous	None
1578	G	A		3	526[b]	Non Synonymous	M → I
1599	A	G		1	533[b]		I → V
1794	T	C		3	598	Synonymous	None
1815	C	G	Transversion	3	605[c]	Non Synonymous	D → E
2106	C	T	Transition	3	702	Synonymous	None

Nt: nucleotide. Aspargine(N); Aspartic acid(D); Pheylalanine (F); Leucine (L); Isoleucine (I); Methionine (M); Arginine (R);Proline (P); Cysteine (C); Glutamic acid (E).
[a] Previously described by Chou et al., 1995a,b and by Lurain et al., 2001.
[b] Mutations in this study.
[c] Amino acid changes in this study.

used the pTMMT (M460V/D605E) from the MMT isolate. All of them were sequenced to corroborate the presence of mutations in a 613 bp-region placed at nucleotide positions 1328 to 1940 of the UL97 gene. The indicated plasmids were transiently transfected. Proteins were extracted 24 h later and Western blot analysis was performed to verify expression of the 80 kDa-protein encoded by the UL97 gene. Of the three independent transfection events (A, B and C) carried out with the control plasmid pMMrwt (Fig. 1, panel 1), there was protein expression only in C. Once this protocol had been standardized, the remaining plasmids were used for transfection experiments. Non-transfected 293T cells were used as negative controls (QC).

Cells transfected with plasmid pM460V/A594P/D605E produced a truncated protein of approximately 55–60 kDa (Fig. 1, panel 2). In order to investigate if the nucleotide sequence of the construct pM460V/A594P/D605E had suffered any modification responsible for the expression of such truncated protein, a full-length sequencing was performed. Apart from the mutations introduced by site-directed mutagenesis, no changes were observed. However, we currently do not know if this triple mutant might naturally exist within a given isolate *in vivo*.

The effect of different mutations on the UL97 kinase activity can be measured by the ability to convert GCV into its mono-(GCV-M), di-(GCV-D), and tri-(GCV-T) phosphorylated metabolites. Although GCV susceptibility assays were not performed in this study, the terms "GCV resistance" indirectly refers throughout the text to the already known association between the phosphorylation state of GCV and the lack of activity of the pUL97 kinase.

The different chromatography profiles and radioactivity results for each of the pUL97 constructed variants was compared with the positive control pMMrwt. A significant reduction of the total GCV phosphorylation was observed for plasmids pM460V, pA594P and pA594P/D605E, whereas for plasmids pM460V/A594P, pTMMT (M460V/D605E) and pM460V/A594P/D605E, GCV anabolism was minimal and similar to that of the negative control QC. Interestingly, it was observed that phosphorylation levels for plasmid pD605E were the same as or even higher than those for the positive control pMMrwt, suggesting that the aspartic acid-to-glutamic acid substitution at codon 605 might neither affect the enzymatic activity nor the phosphorylation of the drug. The latter result was unexpected, since it has been previously reported that mutations at adjacent positions, namely at codon 601 and 607 are associated with GCV resistance and phosphorylation impairment (Baldanti et al., 1995; Baldanti et al., 1998a,b,c; Cherrington et al., 1998; Chou et al., 1999; Chou et al., 1997; Chou et al., 2002; Wolf et al., 1998).

Previous reports have demonstrated that the UL97 point mutations M460V and A594P significantly reduce GCV phosphorylation, conferring resistance to this drug (Bourgeois et al., 1997; Chou et al., 1995a; Ijichi et al., 2002; Wolf et al., 1998). Our findings using plasmids pM460V and pA594P are consistent with those described above.

We observed that the double mutant M460V/A594P has lower kinase activity in comparison with the single mutants M460V and A594P. As it is shown in Fig. 2, GCV phosphorylation levels of the double mutant M460V/A594P were 5–29% lower than those of the single mutants M460V and A594P. Therefore, this double mutation would be associated with a higher degree of resistance to GCV.

However, we could not detect reversion of UL97 kinase activity for plasmid pA594P/D605E (26 ± 9% GCV phosphorylation). We found that GCV phosphorylation levels of this double mutant were

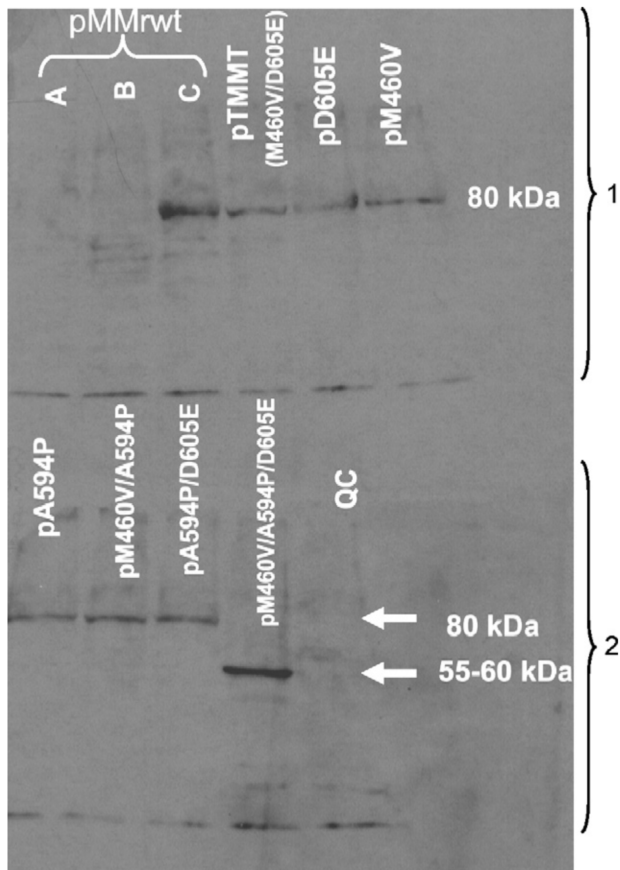

Fig. 1. Transient protein expression in 293T cells transfected with plasmid pUL97. pMMrwt were used as positive control. Three independent transfection events were carried out (lanes A, B and C). However, protein expression was only evident in C. Arrows show both the full-length 80 kDa and the truncated 55–60 kDa UL97 proteins. Panels 1 and 2 correspond to two SDS-PAGE gels from the same electrophoretic event.

10% lower than those of the single mutant A594P (36 ± 13% GCV phosphorylation). This difference was considered to be within the standard error of the assay, and therefore not significant (Fig. 2).

Finally, when we analyzed the effect of transfected cells with plasmid pM460V/A594P/D605E on GCV anabolism, we observed the expression of a truncated UL97 protein of approximately 55–60 kDa that impaired GCV phosphorylation, conferring resistance to this antiviral drug. Since its nucleotidic sequence has not shown any other changes apart from the resulting amino acid substitutions introduced by site-directed mutagenesis, the above mentioned result might be directly or indirectly associated with such mutations.

In this study we determined the presence of multiple amino acid substitutions in the protein encoded by the UL97 gene of an HCMV strain recovered from viral isolates and peripheral blood leukocytes of an immunocompromised patient identified as MMT, including the mutations M460V and D605E. The aspartic acid-to-glutamic acid substitution at codon 605 may be associated with a natural polymorphism of the UL97 gene (Chou et al., 2005), and not with positive selection pressure exerted by the antiviral drug GCV. "Compensatory mutations" usually appear at later stages throughout viral infections (Page and Holmes, 1998) and accumulate to overcome the generally reduced replicative capacity of resistant variants. We have found that in leukocyte samples as well as in viral isolations from the MMT isolate, the D → E mutation at codon 605 was already present before starting GCV treatment. This suggests that the mutation at codon 605 occurs by a natural polymorphism of the gene and not as a compensatory mechanism of changes before the onset of a mutation associated with GCV resistance.

Chou et al. have recently described a phenotypic method to assess HCMV drug resistance, based on the incorporation of a reporter gene by using recombinant viruses (Chou et al., 2005). They have also suggested that the point mutation D605E in the UL97 protein did not confer resistance to GCV. However, they could not determine if this polymorphism had any significant reversion effect on the resistance levels conferred by the point mutation A594V and by other common mutations related to antiviral drug resistance. They assumed that the effect of the amino acid substitution D605E was probably too low to be measured with the system they described for phenotyping HCMV resistance. The study carried out by Chou et al., 2005 —in comparison with our present study and Ijichi et al., 2002— analyzes the most usual mutation which is the well-known alanine-to-valine substitution at codon 594.In contrast, and since the A594P mutation is less frequently recovered from clinical HCMV isolates (Ijichi et al., 2002; Lurain et al., 2002), there have been very few *in vitro* studies of this A594P mutant.

The observed effects on GCV phosphorylation for the MMT isolate pM460V/D605E were very similar to those herein described for the pA594V/D605E. Furthermore, we found that the pM460V/D605E is 8% less efficient in phosphorylating GCV than

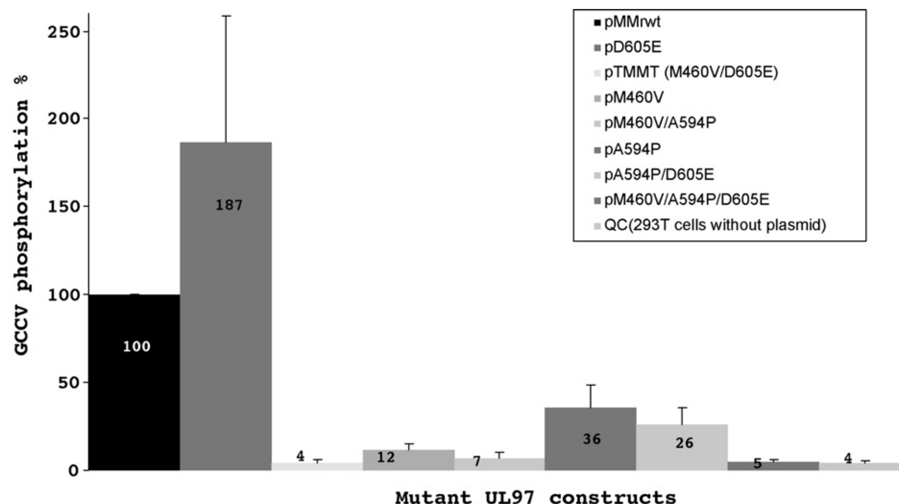

Fig. 2. Percentage of GCV phosphorylation in 293T cells transfected with different plasmids. 100% corresponds to the level of phosphorylation observed with the wild type positive control (containing the UL97 gene of the AD169 strain). Error standard values correspond to three independent assays.

the pM460V (4 ± 2 and $12 \pm 3\%$ GCV phosphorylation, respectively), this difference was considered to be within the standard error of the assay, and therefore not significant. In a previous study, Chou et al., 2005 reported that the differences in IC_{50} values for the mutations with and without the D605E did not exceed the standard errors of the assays (11.5 ± 3.1 μM for M460V/D605E [T2256] and 9.8 ± 2.4 for M460V [T2259]). These results are very similar to those obtained in our study for plasmids pTMMT (M460V/D605E) and pM460V, despite the different methodology used (viral phenotyping method vs HPLC).

When two mutations related to GCV resistance are simultaneously detected in the same HCMV genome (pM460V/A594P), such viral resistance might be enhanced when compared to that of the single mutants studied separately (pM460V or pA594P). However, the simultaneous existence of both mutations within the same DNA molecule might not necessarily be a naturally occurring phenomenon. Also, we found that the UL97 gene from the MMT isolate exhibits amino acid substitutions reported to our knowledge for the first time. At present, we do not know if these amino acid substitutions contribute or not to antiviral resistance, in particular those at codons 507, 511 and 533, between subdomains VII and IX of the catalytic site of the UL97 viral kinase. Therefore, further studies are necessary to elucidate this point and to assess if the UL97 kinase activity encoded by the MMT isolate is affected by such amino acid changes. Moreover, the polymorphism of the UL97 gene should be further investigated, as a way to determine which amino acid changes are really implicated in conferring viral resistance to GCV, and to assess if the novel point mutations reported herein are involved in the interaction of pUL97 with the protein encoded by the UL27 gene (Chou et al., 2004). The impact of these novel mutations on the mechanisms of action of another anti-HCMV drug, maribavir, (phase III study) should also be analyzed.

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