Patient-Derived Cytomegaloviruses with Different Ganciclovir Sensitivities from UL97 Mutation Retain Their Replication Efficiency and Some Kinase Activity *In Vitro*

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ABSTRACT Mutations in the cytomegalovirus UL97 kinase gene contribute to antiviral resistance. Mutations A594S and G598D from two clinical isolates were analyzed, and bacterial artificial chromosome (BAC)-engineered A594S recombinant cytomegalovirus exhibited a ganciclovir-resistant phenotype on plaque reduction. Viral replication was comparable to that of the wild type. Cell-based kinase activity and autophosphorylation of ectopically expressed proteins showed that mutants retained some kinase activity. This study showed that patient-derived cytomegalovirus with different ganciclovir sensitivities retained replication efficiency and exhibited some kinase activity *in vitro*.

KEYWORDS UL97, cytomegalovirus, drug resistance, ganciclovir, mutation, phosphorylation, protein kinase

Human cytomegalovirus (CMV) represents a clinically important pathogen that causes severe infections in immunocompromised patients such as allograft recipients or AIDS patients (1, 2). Infection or reactivation during pregnancy can lead to adverse outcomes for the developing fetus (3). Management of CMV infection in clinical practice, i.e., antiviral treatment, prophylaxis, or preemptive therapy, includes the use of antivirals such as ganciclovir (GCV), less frequently, foscarnet, and cidofovir. Letermovir has currently been approved for prophylaxis in allogeneic stem cell transplant recipients (4), while another investigational drug, maribavir, is currently not approved for clinical use.

Infection with viral strains of decreased drug susceptibility remains a significant problem in immunocompromised hosts, especially after prolonged or repeated therapy (5). For example, in patients with symptomatic infection by human immunodeficiency virus type 1 (HIV-AIDS), resistance was associated with increased risk of progression of retinitis (6). Sequence-specific alterations in the *UL97* or *UL54* gene of CMV provide a molecular basis for GCV resistance. In this study, two CMV isolates from patients with HIV-AIDS and low CD4 counts (<50) were found on routine blood testing during 2014 and 2015 in our center to carry mutations in *UL97*, with an unknown antiviral resistance phenotype (7). PCR sequencing showed sample one carried the mutations G598D (unknown phenotype) and L595S (ganciclovir resistance) in the *UL97* gene and A505T (unknown phenotype) in the *UL54* gene (7). Sample two carried a single A594S mutation (unknown phenotype) in the *UL97* gene, and no mutations were detected in the *UL54* gene. Marker transfer of the *UL97* mutations G598D and A594S to a recom-

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FIG 1 Replication efficiency of recombinant CMV wild-type and corresponding *UL97* mutants in dividing and nondividing cells. (A) Dividing cells, maintained in the presence of 10% FBS, were infected at an MOI of ~ 0.001 . (B) Serum-deprived cells, maintained in medium containing 0.1% FBS, were infected and compared for DNA yield via MIE quantitative PCR (qPCR). Mean ($\pm$ standard deviation [SD]) $\log_{10}$ values of experimental triplicate data points are shown.

binant wild-type CMV was performed. In addition, the A594V mutation that confers resistance to GCV was included to serve as a control. Testing *in vitro* showed one of the mutations (A594S) conferred resistance to GCV, without significantly affecting viral replication, and retained some pUL97 protein kinase activity.

Primary HFF (Sigma) and MRC-5 fibroblasts (ECACC) were maintained in minimal essential medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HEK293T and HEK293 (ATCC) cells were maintained in supplemented Dulbecco's modified Eagle medium (DMEM; Invitrogen). CMV mutants were generated from bacterial artificial chromosome pAL1111 (strain Merlin; kindly provided by Barry Slobedman, The University of Sydney) with a recombineering protocol described previously (8, 9) (see Table S1 in the supplemental material). The *UL97*, *UL54*, and *UL27* genes of all viral stocks were verified by sequencing analysis.

Viral replication was assayed in confluent HFF cells at a multiplicity of infection (MOI) of ~ 0.001 PFU/cell, and supernatant samples were subjected to quantitative PCR targeting the CMV major immediate early (MIE) promoter region as previously described (10, 11). A previous study showed pUL97 is required for efficient viral DNA synthesis in quiescent cells (12). Thus, we tested whether the studied point mutations in *UL97* affected viral replication in serum-deprived cells. Serum-deprived HFF cells were maintained in medium containing 0.1% FBS for 48 h prior to and during the next 14 days of infection. Cell proliferation was tested on mock and wild-type treated cells using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Susceptibility of recombinant viruses to GCV (G2536; Sigma) was tested in MRC-5 in 24-well plates using a plaque reduction assay aimed at ~ 70 PFU/well (7). The GCV concentrations were 0, 0.75, 1.5, 3, 6, 12, 24, 48, and 96 μ M. A normalized dose-response inhibition curve was fitted with a least-squares method using GraphPad Prism 5 software (GraphPad Inc., La Jolla, California).

The *UL97* open reading frame (ORF) of strain Merlin was amplified using published primers 5-UL97-EcoRI and 3-UL97-XhoI (13) and cloned into pcDNA 3.1 (Invitrogen). The mutations G598D, A594S, and K355M were introduced by site-directed mutagenesis (Agilent Technologies) according to the manufacturer's instructions and verified by sequencing analysis. The combined coimmunoprecipitation-*in vitro* kinase assay (CoIP-IVKA) was performed using immunoprecipitated proteins from lysates of transiently transfected 293T cells using pAb-UL97 as previously described (14). The pUL97 in-cell activity assay (ICAA) was performed in HEK293 cells as previously described (13). Red fluorescent protein (RFP) (pDsRed1-N1; BD Clontech) served as a negative control.

The viral replication kinetics results showed similar efficiency for all three mutants compared to that of the wild type in cycling fibroblasts (Fig. 1A). This suggests the

TABLE 1 Susceptibilities of recombinant viruses to ganciclovir by plaque reduction assay

Recombinant CMV virus	EC ₅₀ ganciclovir (μM) ^a	Fold-change relative to wild type
UL97-wild type	1.6 ± 0.2	
UL97-A594V	9.2 ± 1.5	5.7
UL97-A594S	6.8 ± 2.0	4.2
UL97-G598D	1.8 ± 0.2	1.1

^aMean (± SD) derived from 3 independent experiments.

single mutation A594V, A594S, or G598D at the *UL97* locus did not contribute to a compromised viral replication *in vitro*. This finding is consistent with previous marker transfer studies showing GCV resistance-conferring *UL97* mutations (e.g., M460V) or deletions (e.g., 591 to 607) did not impair viral growth (15, 16). In serum-deprived cells, the MTT results showed signals for the mock and wild type did not increase over the 14 days of treatment compared to baseline (see Fig. S1). This supports the idea that the inocula and infection medium used did not trigger cell proliferation. The recombinant viruses were then tested in the low-serum setting, and results showed that viruses grew comparably throughout the time course (Fig. 1B).

Plaque reduction assays were conducted on the four recombinant viruses in parallel under the same testing conditions and repeated on three different days. The initial 50% effective dose (EC₅₀) cutoff proposed for classifying GCV resistance was >6 μM (17). Our results revealed EC₅₀ values of 1.6 ± 0.2 μM, 9.2 ± 1.5 μM, 6.8 ± 2.0 μM, and 1.8 ± 0.2 μM for UL97 wild type and A594V, A594S, and G598D mutants, respectively (Table 1). The inclusion of a drug-sensitive control enabled more precise cutoffs, defined as the ratio of EC₅₀ relative to the wild-type control. Resistance to GCV was suggested when a 2-fold or greater increase over the wild type was observed (18). The results showed UL97-A594V led to the highest EC₅₀ increase for GCV compared to that for the wild-type virus (5.7-fold), followed by UL97-A594S (4.2-fold), whereas UL97-G598D did not show an increase (Table 1). A recent study systematically assessed 17 mutants over the codon range 591 to 603 with high replicate numbers to demonstrate mutations in *UL97* resulted in different levels of GCV resistance (19). The reported fold change between wild-type and pUL97 A594V-carrying virus was 6.4-fold, therefore slightly higher than our findings (19). Together, our results suggest the A594S mutation but not the G598D mutation confers resistance to GCV. Other amino acid substitutions at position 594 that were previously shown to confer resistance to ganciclovir include A594T with 2.7-fold (15) and A594P/D605E with 6-fold resistance (20).

Expression plasmids of ORF *UL97* carrying the mutations of interest, G598D and A594S, were analyzed for their activity in phosphorylating (i) pUL97 protein (autophosphorylation) and human histones (exogenously added substrate) via assaying in ColP-IVKA and (ii) phosphorylating GCV via assaying the pUL97-specific ICAA. The catalytically inactive mutant K355M was included as a negative control. All *UL97*-encoding plasmids expressed detectable levels of pUL97 as shown by Western blot analysis (Fig. 2A). The pUL97 versions were immunoprecipitated (Fig. 2B) and subjected to IVKA. Results showed signals corresponding to the autophosphorylation of pUL97 and histones on the autoradiogram for wild-type, A594S, and G598D versions of pUL97 (Fig. 2C). Complete loss of protein phosphorylation was observed for the K355M mutant (Fig. 2C). The phosphorylation activity of ectopically expressed pUL97 was assessed via ICAA using GCV as the substrate. Briefly, 293 cells were transiently transfected with expression plasmids and incubated with increasing concentrations of GCV. At 5 days post-transfection, pUL97 kinase-mediated phosphorylation of GCV was quantitated by measuring cytotoxicity signals (lactate dehydrogenase [LDH] activity) in the residual cell layers. A two-way repeated measures analysis of variance (ANOVA) with Bonferroni's posttest analysis was performed to compare mean LDH activities at each drug concentration between wild-type and mutant proteins (GraphPad Inc., La Jolla, California). While the K355M mutant and RFP negative control showed a clear lack of converting

FIG 2 Coimmunoprecipitation (CoIP) and *in vitro* kinase assay (IVKA) of ectopically expressed pUL97 showed A594S and G598D mutants retained some kinase activity. (A) Western blot image of transfected 293T cell lysate controls. (B) Western blot image of pUL97 CoIP control. (C) Autoradiogram image for IVKA.

GCV (5 to 320 μ M), signals obtained for A594S and G598D pUL97 showed a smaller trend of decreased reduction in LDH activity across all tested GCV concentrations. Statistical significance ($P < 0.05$) was observed for A594S at 40 μ M (Fig. 3). Therefore, the mutants retain some ganciclovir kinase activity and a trend toward that activity being reduced by the mutation.

Rapid detection of resistant viruses facilitates clinical decisions to switch to alternative therapies such as the use of second-line anti-CMV agents. This is the first case of the A594S mutation from a clinical sample. The G598D mutation in *UL97* did not result in GCV resistance; however, future work investigating its potential role on resistance or fitness in viruses that already carry a known antiviral mutation is warranted. Although impairment of phosphorylation is the preferred mechanism of GCV resistance, previous cases of resistance mutations (e.g., F342S and V356G) were compatible with unimpaired viral growth and pUL97 autophosphorylation (21). A limitation of the kinase assays applied in this study is the variability in pUL97 expression following transfection of the different genes (Fig. 2A). Therefore, the results permit a qualitative assessment that the mutant retains some kinase activity.

In summary, transfer of A594S or G598D mutations to a recombinant CMV wild-type *UL97* sequence showed that both viruses were able to replicate efficiently in cell culture and that the A594S mutant conferred resistance to GCV. Notably, some kinase activity of ectopically derived versions of these mutants was demonstrated via pUL97 auto-

FIG 3 The pUL97 in-cell activity assay demonstrates levels of GCV phosphorylation. All measurements were based on biological triplicate transfections ($n = 3$). Mean ($\pm$ SD) values are shown.

phosphorylation or GCV phosphorylation, and this likely helps explain the efficient replication of the mutant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02425-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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