9-1-2013

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Resistance of Human Cytomegalovirus to Cyclopropavir Maps to a Base Pair Deletion in the Open Reading Frame of UL97

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Human cytomegalovirus (HCMV) is a widespread pathogen in the human population, affecting many immunologically immature and immunocompromised patients, and can result in severe complications, such as interstitial pneumonia and mental retardation. Current chemotherapies for the treatment of HCMV infections include ganciclovir (GCV), foscarnet, and cidofovir. However, the high incidences of adverse effects (neutropenia and nephrotoxicity) limit the use of these drugs. Cyclopropavir (CPV), a guanosine nucleoside analog, is 10-fold more active against HCMV than GCV (50% effective concentrations [EC_{50}] = 0.46 and 4.1 μM, respectively). We hypothesize that the mechanism of action of CPV is similar to that of GCV: phosphorylation to a monophosphate by viral pUL97 protein kinase with further phosphorylation to a triphosphate by endogenous kinases, resulting in inhibition of viral DNA synthesis. To test this hypothesis, we isolated a CPV-resistant virus, sequenced its genome, and discovered that bp 498 of UL97 was deleted. This mutation caused a frameshift in UL97 resulting in a truncated protein that lacks a kinase domain. To determine if this base pair deletion was responsible for drug resistance, the mutation was engineered into the wild-type viral genome, which was then exposed to increasing concentrations of CPV. The results demonstrate that the engineered virus was approximately 72-fold more resistant to CPV (EC_{50} = 25.8 ± 3.1 μM) than the wild-type virus (EC_{50} = 0.36 ± 0.11 μM). We conclude, therefore, that this mutation is sufficient for drug resistance and that pUL97 is involved in the mechanism of action of CPV.

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Received 31 January 2013 Returned for modification 15 February 2013 Accepted 18 June 2013
Published ahead of print 1 July 2013
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Human cytomegalovirus (HCMV), a betaherpesvirus, is a widespread pathogen affecting a majority of the world’s population (1). Individuals at risk for symptomatic HCMV disease—which includes interstitial pneumonia, encephalitis, and retinitis—are those with deficiencies in T-cell immunity. In addition, with more than 4,000 cases reported each year, HCMV is the most common congenital infection in the United States and can result in severe mental retardation, hearing loss, and/or vision loss (2).

The drugs currently approved by the FDA for the prophylaxis or treatment of systemic HCMV infections are ganciclovir (GCV) (Fig. 1) and its oral prodrug valganciclovir, foscarnet, and cidofovir (3–5). GCV is the most common therapy option for the treatment of HCMV infections among patients with impaired immunity, particularly those with advanced HIV/AIDS, those undergoing cancer chemotherapy, and recipients of solid-organ or bone marrow transplants (6–8). GCV, an acyclic analog of the nucleoside guanosine, is converted intracellularly to a monophosphate by a viral protein kinase encoded by the HCMV gene UL97 (9, 10). Upon further phosphorylation by cellular enzymes (11, 12), GCV triphosphate competes with endogenous dGTP for incorporation into progeny viral genomes, resulting in pUL54 (viral DNA polymerase) inhibition (13, 14). Once incorporated, GCV also acts as a chain terminator, further inhibiting viral DNA synthesis (15, 16). However, long-term therapy is typically required due to recurrence of infection and reactivation of latent HCMV upon cessation of therapy. Drug resistance and adverse effects, such as neutropenia (affecting as many as 30% of patients) and nephrotoxicity (affecting as many as 50% of patients), are prevalent and limit the use of GCV, foscarnet, and cidofovir (3, 17–19). With the increased use of immunosuppression for cancer chemotherapy and organ transplantation, there is an increasing need for more-effective and less-toxic drugs to treat HCMV.

Cyclopropavir (CPV) (Fig. 1), a methylene cyclopropane guanosine nucleoside analog, is currently being explored as a viable chemotherapy option for HCMV due to its high potency and low incidence of adverse effects in vivo (20, 21). Previous studies have demonstrated that CPV is approximately 10-fold more active in vitro (50% effective concentration [EC_{50}] = 0.46 μM) than GCV (EC_{50} = 4.1 μM), with no observed increase in cytotoxicity (22). Furthermore, CPV elicits a 2- to 5-log reduction in murine cytomegalovirus titers in vivo, resulting in reduced mortality in SCID mice (21). Although the mechanism of action of CPV has been hypothesized in previous studies, the exact mechanism by which CPV elicits an antiviral effect has not been determined (23). We have established that both CPV and GCV are phosphorylated to monophosphates by viral pUL97 protein kinase (24). Additional phosphorylation to a triphosphate by endogenous kinases is also similar for CPV and GCV (25). However, viral DNA polymerase inhibition and incorporation into HCMV are known for GCV (13–16) but have not been established for CPV. Furthermore, the necessity of phosphorylation by pUL97 for antiviral activity is unknown. Thus, the goal of this study is to determine whether the
viral protein kinase pUL97 is involved in the mechanism of action of CPV.

**MATERIALS AND METHODS**

**Chemicals.** CPV was synthesized in the laboratory of J. Zemlicka as described previously (22). GCV was kindly provided by Hoffmann-La Roche (Palo Alto, CA).

**Cell culture procedures.** Human foreskin fibroblasts (HFF) were grown in minimal essential medium with Earle’s salts and 10% fetal bovine serum. They were grown at 37°C under a humidified atmosphere of 3% CO₂ and 95% air and were regularly passaged at 1:2 dilutions using conventional procedures with 0.05% trypsin and 0.02% EDTA in HEPES-buffered saline (26).

**Viral strain.** The HCMV strain Towne was kindly provided by M. F. Stinski, University of Iowa. A bacterial artificial chromosome (BAC) clone of strain AD169 (AD169rv) was generously provided by U. H. Koszinowski, Ludwig-Maximilians University (Munich, Germany) (27).

**HCMV plaque assay reduction assay.** HFF were seeded at 85,000 per well in a 24-well cluster dish and were infected 3 days later with HCMV at 100 PFU per well. Two hours postinfection, media containing serial dilutions of drug, fetal bovine serum (final concentration, 3%), and 0.5% methylcellulose were added. After incubation at 37°C for 9 to 11 days, cell monolayers were stained with crystal violet, and plaques were enumerated by light microscopy. The number of plaques observed in the presence of each drug concentration was plotted against the log of drug concentration, and samples were collected every 24 h over the course of 10 days (collected at an MOI of approximately 1.0 PFU/ml and were grown in a 25-cm² tissue culture flask. After 4 days, cells were harvested and were lysed using lysis buffer (2% NP-40, 0.1 M Tris [pH 8.8], 0.1% SDS, 0.3 M NaCl), and protein levels were determined using the DC protein assay (Bio-Rad Corp., Hercules, CA). Fifty micrograms of protein per sample was electrophoresed under reducing conditions and was blotted electrophoretically onto nitrocellulose paper (Bio-Rad Corp., Hercules, CA). pUL97 was detected using a previously characterized polyclonal antibody developed against full-length pUL97 (a generous gift of Don Coen, Harvard University, Boston, MA) (29) and the SuperSignal West Pico chemiluminescent substrate imaging system (Thermo Scientific, Rockford, IL). A monoclonal antibody against human actin was used as a loading control.

**Marker transfer studies.** “En passant” mutagenesis of BACs was performed as described elsewhere (30–32) to remove bp 498 of the UL97 open reading frame from a BAC clone of HCMV strain AD169, AD169rv (a generous gift of Ulrich Koszinowski) (27). All manipulations were performed in *Escherichia coli* strain GS1783 (a generous gift of Greg Smith, Northwestern University, Chicago, IL). Briefly, an excisable kanamycin resistance marker (I-SceI-aphAI) was amplified from a plasmid template by using oligonucleotide primers d bp498_F (5′-AAA CTT CGG CCA TGT GGT GGT TCG AGT ACG ATC GCG ACG GGA CGT GAC CAG 3′) and d bp498_Rv (5′-TGC CCG CGG TTA ACG ATG GTA TT-3′) and was then electroporated into *E. coli* containing the AD169rv BAC. Integrated colonies were resolved to remove the kanamycin resistance marker. The resulting recombinant BAC, AD169rv d498, was confirmed by restriction enzyme digestion analysis and by DNA sequencing of the modified region (Genewiz, Inc., South Plainfield, NJ).

**Virus reconstitution.** Infectious virus was reconstituted from BAC DNA as described previously (32), by cotransfection of ~7.1 × 10⁶ human foreskin fibroblasts in a 24-well cluster plate (Corning, Inc.) with total of 1 µg DNA containing 750 ng BAC DNA, 200 ng of the pp71 expression plasmid pSG5-pp71 (33) (a gift of Robert Kalejta, University of Wisconsin, Madison, WI), and 50 ng of the Cre recombinase expression plasmid pCAGGS-nsCre (34) (a gift of Michael I. Kotlikoff, Cornell University, Ithaca, NY), by using 7 µl of Superfect transfection reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer’s recommendations.

**HCMV growth assay.** HFF were seeded and grown to confluence in a 25-cm² tissue culture flask at 37°C. Cells were then infected with virus at an MOI of 0.02. Two hours postinfection, medium was added to the flask, and samples were collected every 24 h over the course of 10 days (collected samples were stored at −80°C until titers could be determined). The titer of virus for each sample was determined by first plating a 96-well plate with HFF grown to confluence. Serial dilutions (1:3) of samples (total volume, 200 µl) were then plated, and the virus was allowed to grow for 7 to 9 days at 37°C. Finally, cell monolayers were stained with crystal violet and plaques were enumerated by light microscopy. The virus titer was calculated as 5 × 3ⁿ of the number of plaques, where n is the serial dilution in which the plaques were enumerated.

**RESULTS**

**Initial isolation and characterization of 2696” (CPV-resistant HCMV).** We hypothesize that the mechanism of action of CPV is a multistep process: initial phosphorylation by viral pUL97 to a monophosphate, followed by phosphorylation by endogenous kinases to a triphosphate, resulting in the inhibition of viral DNA synthesis. Although we have demonstrated previously that CPV is phosphorylated by pUL97 to a monophosphate (the initial step of our hypothesis) (24), it has not been determined whether this enzymatic conversion is necessary for CPV to elicit an antiviral effect. Therefore, to test the first step of our hypothesis, HFF in-
fected with a CPV-resistant HCMV strain (2696') were exposed to increasing concentrations of either CPV (■) or GCV (▲) and were compared to HFF infected with wild-type virus (solid lines) exposed to the same concentrations of drugs. Values are means ± standard deviations from at least two experiments. Asterisks indicate that the replication of 2696' is significantly different (P < 0.01) from that of the wild-type virus (with both drugs).

DNA sequencing of 2696'. Since 2696' was resistant to GCV, and GCV resistance has been mapped to mutations in UL54 and UL97 (17, 18), the open reading frames of UL54 and UL97 in 2696' were sequenced. The results demonstrated that the open reading frame of UL54 in 2696' contained no mutations, whereas bp 498 of the 2696' gene UL97 was deleted (Fig. 3). This deletion results in a codon frameshift and the introduction of a stop codon at bp 502 to 504 (corresponding to bp 503 to 505 of wild-type HCMV UL97). The protein product of this mutated gene would be considerably truncated (167 amino acids versus 707 amino acids for wild-type [Towne strain] pUL97), lacking both an ATP binding site and a substrate pocket (10, 35, 36). To confirm that pUL97 is truncated in 2696', Western blot analysis was performed (Fig. 4). The results depict a significantly lower molecular mass reactive species (molecular mass, 17.8 kDa) in the 2696' lane that is absent from the wild-type lane. In addition, the reactive species corresponding to pUL97 in the wild-type lane (molecular mass, 78.3 kDa) is absent from the 2696' lane. These results demonstrate that pUL97 is indeed truncated in 2696'.

Recombinant HCMV UL97Δ498 exhibits resistance to both CPV and GCV. To confirm that the bp 498 deletion of 2696' is the mechanism by which this virus resists the antiviral effects of CPV, this mutation was engineered into a wild-type virus (producing HCMV UL97Δ498). Since previous studies have demonstrated that UL97-null mutants have a 1- to 2-log growth defect that is attributable to the loss of kinase activity (29, 37), and thus the mutation discovered in 2696' would result in a virus without an active pUL97 kinase, both 2696' and HCMV UL97Δ498 were assayed for replication competency, and their growth was compared to the growth of a wild-type virus. As shown in Fig. 5, in agreement with previous results, 2696' (1.0 × 10^5 ± 0.17 × 10^5 PFU/ml) and HCMV UL97Δ498 (1.5 × 10^5 ± 0.11 × 10^5 PFU/ml) exhibited growth defects in excess of 1 log unit compared to wild-type (BAC-derived AD169rv) virus (2.8 × 10^6 ± 0.17 × 10^6 PFU/ml).

Following confirmation that the engineered virus replicated (albeit at a lower rate than that of the wild-type virus), cells infected with HCMV UL97Δ498 were exposed to increasing concentrations of either CPV or GCV and were compared to wild-type virus under the same conditions. The results, shown in Fig. 6 and Table 1, demonstrate that HCMV UL97Δ498 was approximately 72-fold more resistant to CPV (EC_{50} = 25.8 ± 3.1 μM) and 14-fold more resistant to GCV (EC_{50} = 28.1 ± 6.6 μM) than the wild-type virus (BAC-derived AD169rv) (EC_{50} = 0.36 ± 0.11 and 2.0 ± 0.24 μM, respectively). We conclude, therefore, that the base pair deletion mutation in the open reading frame of the HCMV gene UL97 is sufficient for drug resistance and that the protein product of this gene is involved in the mechanism of action of CPV.

### Table 1 Inhibition of 2696' and HCMV UL97Δ498 replication by ganciclovir and cyclopropavir

<table>
<thead>
<tr>
<th>Compound</th>
<th>Towne</th>
<th>2696'</th>
<th>Fold resistance of 2696'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCV</td>
<td>1.5 ± 0.16</td>
<td>41.3 ± 2.8</td>
<td>28</td>
</tr>
<tr>
<td>CPV</td>
<td>0.91 ± 0.15</td>
<td>22.5 ± 3.5</td>
<td>25</td>
</tr>
</tbody>
</table>

\* Values are means ± standard deviations from at least two experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AD169rv</th>
<th>HCMV UL97Δ498</th>
<th>Fold resistance of HCMV UL97Δ498</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCV</td>
<td>2.0 ± 0.24</td>
<td>28.1 ± 6.6</td>
<td>14</td>
</tr>
<tr>
<td>CPV</td>
<td>0.36 ± 0.11</td>
<td>25.8 ± 3.1</td>
<td>72</td>
</tr>
</tbody>
</table>

\* Values are means ± standard deviations from at least four experiments.
CPV is a known inhibitor of HCMV replication (23). The hypothesized mechanism by which CPV elicits this effect involves the phosphorylation of CPV to a monophosphate by the viral enzyme pUL97. Our current results demonstrate that a previously characterized mutation in the open reading frame of UL97, which would result in the expression of a truncated pUL97 lacking an active kinase domain, renders the virus resistant to both CPV and GCV. These results establish that pUL97 is necessary for CPV to elicit an antiviral effect and, taken together with our previous results (24), establish that pUL97 is the enzyme responsible for the initial phosphorylation of CPV to a monophosphate.

Previous studies have identified mutations within the HCMV genome that confer resistance to CPV on the virus. The first example is a frameshift mutation in UL27, developed as a result of exposure to CPV, in a virus with wild-type UL97 (38). This mutation, presumably, compensates for the loss of pUL97 enzymatic activity, since CPV exhibits some pUL97-inhibitory activity, and a mutation responsible for HCMV resistance to maribavir, a potent pUL97 inhibitor, occurs in UL27 (38–40). Next, a recombinant virus lacking a functional pUL97 kinase is 21-fold more resistant to CPV than wild-type virus (23). The previously uncharacterized mutation that we present in this study, which causes a frameshift with a concomitant premature translational termination of the viral kinase pUL97 and, presumably, a complete loss of enzymatic function, behaves similarly to the previously characterized UL97-null mutant with regard to growth characteristics (Fig. 5) and CPV resistance (Fig. 6). Finally, some mutations generated as a result of exposure to CPV confer resistance to the drug without the complete loss of enzymatic function (41–43). These mutations confer resistance not only to CPV but to GCV as well. The mutation described here (UL97Δ498) also confers cross-resistance with GCV, since GCV requires initial phosphorylation by pUL97 as part of its mechanism of action (9).

Although there are many mutations that confer cross-resistance between GCV and CPV, including the mutation we present above, several mutations confer resistance to GCV but not to CPV (41, 44, 45). This suggests that these two drugs either interact differently or occupy different, though close, sites within the substrate binding pocket of the pUL97 kinase. We therefore cannot rule out the possibility that certain mutations in UL97 may confer resistance to CPV without conferring resistance to GCV. In addition, mutations that confer resistance to maribavir (46), a potent pUL97 inhibitor (47), on HCMV demonstrate no cross-resistance with GCV (48). This suggests that these two nucleoside analogs either interact differently or occupy distinct sites within the substrate binding pocket of pUL97. Given that CPV demonstrates characteristics of both GCV (substrate of pUL97 [24]) and maribavir (inhibitor of pUL97 [38]), we hypothesize that CPV binds to a site central to these two nucleoside analogs, occupying space that would otherwise be occupied by either GCV or maribavir. We further speculate that, given the possibility that CPV and
marivabir occupy similar sites within the substrate binding pocket of pUL97, there could be mutations that confer resistance to both CPV and marivabir. Given that marivabir and CPV are currently being evaluated as candidates for clinical trials, an examination of mutations that could confer cross-resistance to these nucleoside analogs is warranted.

The use of CPV for the treatment or prophylaxis of HCMV disease is promising. Our current results demonstrate that pUL97 is an integral part of the mechanism of action of CPV. As such, it is possible that cross-resistance between CPV and currently approved chemotherapeutics for the treatment of HCMV may occur. However, this has not been observed in clinic. In fact, clinical strains of HCMV resistant to GCV were sensitive to earlier analogs of CPV (49). Also, the increased antiviral activity with no observed increase in toxicity (22) and the ability to achieve therapeutic concentrations in vivo without prodrug modification (5, 20) are two reasons why CPV should be superior to GCV for the treatment of HCMV disease. Further examination of the mechanism of action and preclinical development of this compound is warranted.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (CA32779, AI26077, GM102433-10), Microbiotix, Inc., and the American Heart Association (22GNT11890012), as well as by funds from the University of Michigan and Drake University.

REFERENCES

(3-dihydroxy-2-propoxymethyl)guanine in human diploid fibroblasts infec-
12. Boehme RE. 1984. Phosphorylation of the antiviral precursor 9-
(3-dihydroxy-2-propoxymethyl)guanine monophosphate by guanylate ki-


