Metabolism of Cyclopropavir and Ganciclovir in Human Cytomegalovirus-Infected Cells

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Human cytomegalovirus (HCMV) is a widespread pathogen that can cause severe disease in immunologically immature and immunocompromised patients. The current standard of therapy for the treatment of HCMV infections is ganciclovir (GCV). However, high incidence rates of adverse effects are prevalent and limit the use of this drug. Cyclopropavir (CPV) is 10-fold more effective against HCMV in vitro than GCV (50% effective concentrations [EC_{50}] = 0.46 and 4.1 μM, respectively) without any observed increase in cytotoxicity (S. Zhou, J. M. Breitenbach, K. Z. Borysko, J. C. Drach, E. R. Kern, E. Gullen, Y. C. Cheng, and J. Zemlicka, J. Med. Chem. 47:566–575, 2004, doi:10.1021/jm030316s). We have previously determined that the viral protein kinase pUL97 and endogenous cellular kinases are responsible for the conversion of CPV to a triphosphate (TP), the active compound responsible for inhibiting viral DNA synthesis and viral replication. However, this conversion has not been observed in HCMV-infected cells. To that end, we subjected HCMV-infected cells to equivalently effective concentrations (~5 times the EC_{50}) of either CPV or GCV and observed a time-dependent increase in triphosphate levels for both compounds (CPV-TP = 121 ± 11 pmol/10^6 cells; GCV-TP = 43.7 ± 0.4 pmol/10^6 cells). A longer half-life was observed for GCV-TP (48.2 ± 5.7 h) than for CPV-TP (23.8 ± 5.1 h). The area under the curve for CPV-TP produced from incubation with 2.5 μM CPV was 8,680 ± 930 pmol·h/10^6 cells, approximately 2-fold greater than the area under the curve for GCV-TP of 4,520 ± 420 pmol·h/10^6 cells produced from incubation with 25 μM GCV. We therefore conclude that the exposure of HCMV-infected cells to CPV-TP is greater than that of GCV-TP under these experimental conditions.

H uman cytomegalovirus (HCMV) is a widespread pathogen infecting between 40% and 80% of the population worldwide (1). Although individuals with competent immune systems rarely manifest any symptoms, HCMV infections can result in severe interstitial pneumonia, encephalitis, and gastroenteritis in immunocompromised individuals (2). HCMV is also the most common congenital infection in the United States and results in over 4,000 cases of severe mental disabilities, hearing, and/or vision loss in infants each year (3, 4). Drugs currently approved by the FDA for the treatment of systemic HCMV infections are ganciclovir (GCV; Fig. 1) and its oral produg valganciclovir, foscarnet (PFA), and cidofovir (5–8). The mechanism of action for each of these drugs involves inhibition of the viral DNA polymerase, resulting in inhibition of HCMV DNA synthesis and viral replication (5). However, long-term chemotherapy for HCMV is generally required due to recurrence of infection upon cessation of treatment. As such, the selection of strains with decreased drug susceptibility is common (6, 9–11). Because adverse effects occur with a high rate of incidence (up to 30% of patients) (12), there is a need for new compounds with a greater therapeutic index for the treatment of systemic HCMV infection.

We have previously demonstrated that cyclopropavir (CPV; Fig. 1), a methylenecyclopropane guanosine nucleoside analog, is approximately 10-fold more active in vitro (50% effective concentration [EC_{50}] = 0.46 μM) than GCV (EC_{50} = 4.1 μM) without any observed increase in cytotoxicity (13). In addition, CPV is also active against several HCMV strains that are resistant to GCV or PFA (14). Further experimentation in vivo with CPV demonstrated a 2 to 5 log_{10} reduction in titers of murine cytomegalovirus, resulting in reduced mortality in severe combined immunodeficient (SCID) mice and reduced viral replication in human fetal tissue implanted in SCID mice infected with HCMV (15). Toxicology studies performed in vivo demonstrated few to no adverse effects at therapeutic concentrations, making CPV a good clinical candidate for the treatment of systemic HCMV infections (16).

We and others have previously established that the mechanism of action of CPV is similar to that of GCV, namely, phosphorylation to a monophosphate (MP) by the viral pUL97 protein kinase (17–19), additional phosphorylation to a triphosphate (TP) by an endogenous cellular kinase (20), and viral DNA synthesis inhibition resulting in inhibition of viral replication (14, 21). Although enzymatic conversion of CPV to a triphosphate by the pUL97 viral protein kinase and an endogenous cellular kinase has been established (17, 20), this conversion has not been observed in virus-infected cells. Therefore, the goal of this study was a comparison of the metabolism of CPV and the current standard for HCMV chemotherapy, GCV, in HCMV-infected cells.

MATERIALS AND METHODS

Viral strain and chemicals. HCMV strain Towne was kindly provided by M. F. Stinski, University of Iowa. GCV was kindly provided by Hoffman La Roche (Palo Alto, CA). Cyclopropavir ([Z]-9-[2,2-bis-(hydroxymethyl)cyclopropylidene]-methyl)guanine; CPV), along with its monophosphates, diphosphates (DP), and triphosphates (TP), was kindly provided by Jiri Zemlicka (Karmanos Cancer Center, Wayne State University, Detroit, MI) (13, 22). 8-[^3H]GCV (19 Ci/mmole) and 8-[^3H]CPV (0.3 Ci/mmole) were synthesized by New England Nuclear Corporation, Boston, MA. [6-^-3H]-L-serine (0.1 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. 13H]-thymidine (2.9 Ci/m mole) was purchased from ICN Biomedicals (Irvine, CA). The nucleosides 2′-deoxyguanosine-5′-monophosphate (GMP), 2′-deoxyadenosine-5′-monophosphate (AMP), and 2′-deoxythymidine-5′-monophosphate (TMP) were obtained from Sigma Chemical Company (St. Louis, MO). Other solutions and reagents were of the highest purity available.

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Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA) and provided through the courtesy of Microbiotix, Inc. (Worcester, MA).

**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 in a humidified atmosphere of 3% to 5% CO₂ and 97% to 95% air and were regularly passaged at 1:2 dilutions using conventional procedures with 0.05% trypsin–0.02% EDTA–HEPES-buffered saline (23).

Nucleoside analog triphosphate biosynthesis. HFF cells were seeded at 250,000 cells per well in a 6-well cluster dish and infected the following day at a multiplicity of infection (MOI) of ~5 PFU per cell. At 2 h postinfection, 2.5 μM [³H]CPV or 25 μM GCV (both [³H]GCV [~2%] and unlabeled GCV) was added to the cells. At designated times, cells were removed from plates using trypsin (0.05% with 0.02% EDTA), counted, and lysed using water, and proteins were precipitated using perchloric acid (final concentration, 0.4 N). Samples were neutralized using 10 N potassium hydroxide and stored at 20°C until analysis by high-pressure liquid chromatography (HPLC).

Nucleoside analog triphosphate half-lives. HFF cells were seeded at 250,000 cells per well in a 6-well cluster dish and infected the following day at a MOI of ~5 PFU per cell. At 2 h postinfection, 2.5 μM [³H]CPV or 25 μM GCV (both [³H]GCV [~2%] and unlabeled GCV) was added to the cells. Following 5 days of drug incubation, media containing drug were removed and replaced with fresh media without drug. Samples were collected at the time of and at designated times following replacement of media and processed as described above.

Reverse-phase HPLC. CPV and its phosphorylated derivatives (CPV-MP, CPV-DP, and CPV-TP) were separated by reverse-phase HPLC (System Gold Programmable Solvent Module 125 and System Gold Programmable Detector Module 166 controlled by 32 Karat software (version 7.0); Beckman Coulter, Inc., Indianapolis, IN). Before injection, each sample was centrifuged at 14,000 rpm for 10 min to remove any remaining particulate matter. Samples were loaded onto a 10-μm-pore-size Alphabond C₈ reverse-phase column (Alttech, Deerfield, IL) (300 by 3.9 mm) at a flow rate of 1.0 ml/min. Baseline separation of CPV and its phosphorylated derivatives was achieved by elution with 150 mM ammonium hydroxide and stored at ~20°C until analysis by high-pressure liquid chromatography (HPLC).

Strong anion exchange HPLC. GCV and its phosphorylated derivatives (GCV-MP, GCV-DP, and GCV-TP) were separated by strong anion exchange HPLC (System Gold Programmable Solvent Module 125 and System Gold Programmable Detector Module 166 controlled by 32 Karat software (version 7.0); Beckman Coulter, Inc., Indianapolis, IN). Before injection, each sample was centrifuged at 14,000 rpm for 10 min to remove any remaining particulate matter. Samples were loaded onto a 5-μm-pore-size Hypersil strong anion exchange column (Thermo Scientific, Waltham, MA) (250 by 4.6 mm) at a flow rate of 1.0 ml/min. Baseline separation of GCV and its phosphorylated derivatives was achieved by elution with 10 mM ammonium phosphate (pH 3.0) and 500 mM ammonium phosphate (pH 3.0) (10 mM ammonium phosphate isocratic conditions for 12 min followed by 25% gradient of 500 mM ammonium phosphate over 24 min). One-minute fractions were collected and analyzed and triutium levels quantified by liquid scintillation spectrometry using a Beckman LS 6500 scintillation counter (Beckman Coulter, Inc., Indianapolis, IN). Concentrations of GCV-TP were calculated as described above for CPV-TP.

Data analysis. Upon collection of data and calculation of triphosphate concentrations, results were graphed and analyzed using Prism (version 5.0; GraphPad Software, San Diego, CA) to determine standard deviations, linear regressions, statistical significance (Student’s t test), and areas under the curve.

**RESULTS**

Metabolism of CPV and GCV to their respective triphosphates in HCMV-infected HFF cells. We have hypothesized that the mechanism of action of CPV involves phosphorylation to a triphosphate, the active compound that inhibits the viral DNA polymerase (14, 17, 20). However, the production of CPV-TP in HCMV-infected cells has not been demonstrated. Therefore, to test for the biosynthesis of CPV-TP in vitro, HFF cells infected with the Towne strain of HCMV (MOI, ~5) were incubated with either 2.5 μM CPV or 25 μM GCV (positive control) and cell extracts were analyzed for the presence of nucleoside analog triphosphates (Fig. 2 and Table 1). HCMV-infected cells incubated with 2.5 μM CPV demonstrated a time-dependent increase in CPV-TP levels resulting in a maximum of 121 ± 11 pmol/10⁶ cells at 120 h. HCMV-infected cells incubated with 25 μM GCV demonstrated a similar time-dependent increase in triphosphate levels. However, the maximum quantity of GCV-TP (43.7 ± 0.4 pmol/10⁶ cells) was significantly lower than that seen with CPV-TP.
pmol) was 2.5-fold lower and occurred earlier (96 h) than that of CPV-TP even though the concentration of GCV with which HCMV-infected cells were incubated was 10-fold greater than that of CPV. No mono- or diphosphates of CPV or GCV were detected, indicating that the rate-limiting step in the biosynthesis of both triphosphates is the initial phosphorylation step. Uninfected HFF cells incubated with 2.5 μM CPV demonstrated no measurable level of CPV-TP (limit of detection, ~0.6 pmol/10^6 cells), indicating that the presence of virus, and, more specifically, of the viral protein kinase pUL97 (17), is necessary for the conversion of CPV to CPV-TP.

**Half-life of CPV-TP and GCV-TP in HCMV-infected HFF cells.** Enzymatic conversion of CPV to CPV-TP, while necessary for the drug to elicit an antiviral effect, is not the sole determinant of efficacy. Drug half-life, or the length of time that the virus is exposed to the active compound, is another major determinant of efficacy. Therefore, we measured the half-life of CPV-TP and GCV-TP in HCMV-infected cells following 5 days of exposure to 2.5 μM CPV and 25 μM GCV, respectively (Fig. 3 and Table 1). The results for both compounds demonstrated first-order kinetics, and the half-lives were calculated from their respective linear regression lines. Although triphosphate levels for both compounds persisted through 96 h, GCV-TP had an approximately 2-fold-longer half-life (48.2 ± 5.7 h) than CPV-TP (23.8 ± 5.1 h). Thus, while the accumulation of GCV-TP was not as large as that of CPV-TP, GCV-TP appears to have persisted longer than CPV-TP. In fact, even though the dose of GCV was 10 times greater than that of CPV, the level of GCV-TP (10.2 ± 1.3 pmol/10^6 cells) at 96 h post-wash out was statistically greater than that of CPV-TP (6.5 ± 1.0 pmol/10^6 cells) (P < 0.05), although this difference is less than 2-fold.

**Total exposure of CPV-TP and GCV-TP to HCMV-infected HFF cells.** Since the administration of CPV resulted in greater biosynthesis of triphosphate when compared to GCV but with a shorter half-life, combining the data into a single plot and measuring areas under the curve was used to determine which combination of properties resulted in the greatest exposure of HCMV-infected cells to active compound (Fig. 4 and Table 1). The results demonstrate that, under the conditions used in these experiments, the area under the curve for CPV-TP (8,680 ± 930 pmol · h/10^6 cells) is approximately 2-fold greater than the area under the curve for GCV-TP (4,520 ± 420 pmol · h/10^6 cells) even though cells were exposed to 10 times more GCV than CPV. We therefore conclude that the exposure of HCMV-infected cells to CPV-TP is greater than that to GCV-TP under equivalently effective concentration (EC) conditions—approximately five times the EC_{50} for both drugs (13).

**DISCUSSION**

The formation of CPV-TP has been assumed to be an essential element for CPV to inhibit viral replication and elicit an antiviral effect (14, 17, 18, 20). Since our previous experiments demonstrated that CPV is a better substrate for the pUL97 viral protein kinase than GCV and that the initial phosphorylation to a monophosphate catalyzed by this enzyme is the rate-limiting step in the formation of triphosphate (17), we hypothesize that the production of CPV-TP would be greater than that of GCV-TP under equivalent concentrations. Consistent with this hypothesis, the conversion of CPV to CPV-TP occurred to a greater extent than that of GCV to GCV-TP despite being administered at a lower concentration (2.5 μM CPV versus 25 μM GCV, equivalently effective concentrations [approximately five times the EC_{50}]).

TABLE 1 Comparison of CPV-TP and GCV-TP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peak triphosphate level (pmol/10^6 cells)</th>
<th>Half-life (t_{1/2}) (h)</th>
<th>Area under the curve (pmol · h/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPV-TP</td>
<td>121 ± 11</td>
<td>23.8 ± 5.1</td>
<td>8,680 ± 930</td>
</tr>
<tr>
<td>GCV-TP</td>
<td>43.7 ± 0.4</td>
<td>48.2 ± 5.7</td>
<td>4,520 ± 420</td>
</tr>
</tbody>
</table>

* Values represent mean ± standard deviation from at least two experiments.

* Values were calculated as a result of combining the data from the triphosphate biosynthesis and half-life studies.
Our results demonstrated a significant difference between the half-lives of CPV-TP (23.8 ± 5.1 h) and GCV-TP (48.2 ± 5.7 h) (Fig. 3 and Table 1). In these experiments, GCV-TP appeared to reach equilibrium within the HCMV-infected cell (a point at which the rate of dephosphorylation is equal to the rate of phosphorylation) at 48 h post-drug wash out. The half-life of GCV-TP before this state of equilibrium occurred was approximately 27 h and is not statistically different from that observed for CPV-TP. It also appears that this state of nucleoside analog triphosphate equilibrium between the two compounds is common; the levels of CPV-TP toward the end of the half-life experiment (84 h post-wash out) also appear to have reached an equilibrium state. We hypothesize that if the duration of the experiment had been longer than 96 h post-wash out, we would have also observed a persistent, stable level of CPV-TP.

In contrast to our results, a previous study by Biron et al. reported that the half-life of GCV-TP in HCMV-infected cells was approximately 12 h (24), a significant variance from what we have observed (48.2 h). There is, however, a significant difference between the two studies that can partially account for the variance in results. Biron et al. conducted their experiments using a MOI of 0.5 PFU per cell, which would result in an HCMV infection rate of approximately 50% (24). We conducted these experiments using a MOI of >5 PFU per cell, which would result in nearly 100% of the cells being infected with HCMV. This difference in MOI not only would result in different intracellular concentrations of GCV-TP but also could affect half-life. In fact, Gentry et al. previously demonstrated that a culture in which only 50% of the cells were able to produce GCV-TP resulted in about a 2-fold reduction in half-life compared to a culture in which 100% of the population of cells could produce GCV-TP (25). This reduction in half-life was hypothesized to be the result of two cells dephosphorylating GCV-TP for every cell that could produce GCV-TP in comparison to a culture in which all cells produce and dephosphorylate GCV-TP. The presumed mechanism by which this occurs is transfer of phosphorylated GCV metabolites from virus-infected cells to uninfected cells through gap junctions, intracellular communication channels capable of direct transfer of small molecules from the cytoplasm of one cell to that of another (26). In addition, if you remove the latter time points from our experiment in which the level of GCV-TP was stable (48 h postdrug removal), the calculated half-life would be 27 h, or approximately 2-fold greater than what was previously observed. Therefore, this combination of factors could account for the variance between the GCV-TP half-life reported here and that reported by Biron et al.

By performing these experiments at equivalently effective concentrations, we are able to speculate about the relative efficacy for each compound acting at the enzymatic and whole-cell levels. Regarding the action of the triphosphates against HCMV DNA polymerase and assuming a cell volume of ~5 pl (27), our determination of 121 pmol CPV-TP/10^6 cells and 43.7 pmol GCV-TP/10^6 cells would translate to intracellular concentrations of 5.3 µM and 1.2 µM, respectively. For a whole-cell comparison, the area under the curve for CPV-TP (8,680 ± 930 pmol · h/10^6 cells) is approximately 2 times greater than the area under the curve for GCV-TP (4,520 ± 420 pmol · h/10^6 cells). The inference is that it requires two to four times the amount of CPV-TP compared to GCV-TP to elicit the same antiviral effect. However, it has been previously reported that CPV (EC_{50} = 0.46 µM) is more efficacious than GCV (EC_{50} = 4.1 µM) since it requires less drug to elicit the same antiviral response (13). We therefore hypothesize that the superior efficacy of CPV when compared to GCV does not come from better efficacy of their respective active compound (CPV-TP versus GCV-TP) but stems from the fact that CPV is a better substrate for the viral protein kinase pUL97 and thus is phosphorylated to a monophosphate (rate-limiting step) to a much greater extent than GCV. This would result in a greater production of CPV-TP than GCV-TP under equivalent concentration conditions. Consistent with this hypothesis, we have previously determined that the HCMV protein kinase pUL97 phosphorylates CPV to CPV-MP at a rate 45 times greater than that of GCV to GCV-MP at equivalent concentrations (17).

Our current results demonstrate that at equivalently effective concentrations of CPV and GCV, HCMV-infected cells are exposed to CPV-TP to a greater extent than GCV-TP, the active compounds that elicit an antiviral response. In addition, the greater efficacy of CPV observed in vitro and in vivo without any increase in toxicity (13, 16) and the ability to achieve therapeutic concentrations in vivo without produg modification (7, 16) are two reasons why CPV appears to be superior to GCV for the treatment of HCMV disease.

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