5-1995

Metabolism of Selected Antiviral Agents in Cells Infected with Drug-Resistant and Wild-Type Strains of Murine Cytomegalovirus

Kevin M. Okleberry
Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Toxicology Commons

Recommended Citation
Okleberry, Kevin M., "Metabolism of Selected Antiviral Agents in Cells Infected with Drug-Resistant and Wild-Type Strains of Murine Cytomegalovirus" (1995). All Graduate Theses and Dissertations. 4657.
https://digitalcommons.usu.edu/etd/4657

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact dylan.burns@usu.edu.
ABSTRACT

Metabolism of Selected Antiviral Agents in Cells Infected with Drug-Resistant and Wild-type Strains of Murine Cytomegalovirus

by

Kevin M. Okleberry, Master of Science
Utah State University, 1995

Major Professor: Dr. Reed P. Warren
Program: Toxicology

Resistance of human viral pathogens to various antiviral drugs is a serious medical problem. Two modes of drug resistance in cytomegalovirus infections have been observed, the first being altered (decreased) drug metabolism by the infected cells, and the second reduced sensitivity of the viral deoxyribonucleic acid polymerase enzyme to the active form of the drug. Mice infected with the murine cytomegalovirus have been used extensively as an animal model for the human cytomegalovirus, and drug-resistant strains in this model have been identified. To better understand the mode of drug resistance of the virus, the metabolism of two antiviral drugs, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir) and (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir), was studied in cells infected with the virus.

The degree of resistance of the mutant virus strain to these two drugs and also to the drug phosphonoformic acid (foscarnet) was measured in viral plaque reduction assays. The resistant strain was 14-, 4-, and 11-fold less sensitive to the
drugs ganciclovir, foscarnet, and cidofovir, respectively, than a sensitive (wild-type) strain.

Metabolism of the antiviral drugs ganciclovir and cidofovir was studied in C127I mouse mammary tumor cells infected with the mutant strain. Uninfected C127I cells and C127I cells infected with the sensitive strain of murine cytomegalovirus were used as controls. The cells were treated with tritium-labeled ganciclovir or cidofovir and studied under a variety of parameters. Among these were duration of treatment, multiplicity of infection, and concentration of compound. After incubating, the cells were acid extracted and analyzed with high-pressure liquid chromatography. The radioactivity of each sample was measured on a scintillation counter and converted into picomoles of drug per million cells.

No significant difference was observed between the virus strains in terms of metabolism or catabolism of the two drugs. This effect remained constant, even when controlling for parameters such as the amount of virus infecting each cell, duration of treatment, or concentration of drug. Based on these results, it appears that the mode of resistance in this mutant strain of virus to ganciclovir and cidofovir is not due to an alteration in metabolism of these two compounds by infected cells. Thus, it is proposed that drug resistance in this mutant strain of virus is due to altered viral deoxyribonucleic acid polymerase function.
ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Donald Smee, for providing financial support from his contract (NIH Contract #RFA-94-AI-14), as well as general advice and friendship. I also wish to thank my major professor, Dr. Reed Warren, and my other committee member, Dr. David Drown, for their flexibility and support. I would especially like to thank Dr. Robert Sidwell for his support, both financial and moral.

I would like to give special recognition to my wife, Leigh, whose love, support, and faith helped guide me through graduate school, and my son, Jeremy, whose happy smiles made all of my effort worthwhile.

Kevin M. Okleberry
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The cytomegaloviruses—biology and disease</td>
<td>1</td>
</tr>
<tr>
<td>9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG, ganciclovir)</td>
<td>1</td>
</tr>
<tr>
<td>(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC)</td>
<td>3</td>
</tr>
<tr>
<td>Phosphonoformic acid (foscarnet)</td>
<td>5</td>
</tr>
<tr>
<td>Viral resistance to antiviral drugs</td>
<td>6</td>
</tr>
<tr>
<td>JUSTIFICATION OF RESEARCH</td>
<td>8</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>10</td>
</tr>
<tr>
<td>Compounds and solutions</td>
<td>10</td>
</tr>
<tr>
<td>Virus and cells</td>
<td>12</td>
</tr>
<tr>
<td>Preparation of virus pools</td>
<td>12</td>
</tr>
<tr>
<td>Determination of virus resistance</td>
<td>14</td>
</tr>
<tr>
<td>Determination of metabolism of DHPG and HPMPC in cells</td>
<td>15</td>
</tr>
<tr>
<td>Effect of different concentrations of drug on viral metabolism</td>
<td>15</td>
</tr>
<tr>
<td>Effect of multiplicity of infection (MOI) on drug phosphorylation</td>
<td>16</td>
</tr>
<tr>
<td>24-hour time-course metabolism experiment</td>
<td>17</td>
</tr>
<tr>
<td>Dephosphorylation and excretion of drugs</td>
<td>17</td>
</tr>
<tr>
<td>96-hour time-course metabolism experiment</td>
<td>18</td>
</tr>
<tr>
<td>Virus yield assay</td>
<td>19</td>
</tr>
<tr>
<td>High-pressure liquid chromatography analysis of the frozen supernatants</td>
<td>21</td>
</tr>
<tr>
<td>RESULTS</td>
<td>24</td>
</tr>
<tr>
<td>Determination of virus resistance</td>
<td>24</td>
</tr>
<tr>
<td>Drug metabolism experiments</td>
<td>24</td>
</tr>
<tr>
<td>Dose-dependent phosphorylation of drugs</td>
<td>28</td>
</tr>
<tr>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Effect of multiplicity of infection (MOI) on drug phosphorylation</td>
<td>30</td>
</tr>
<tr>
<td>24-hour time-course experiment</td>
<td>32</td>
</tr>
<tr>
<td>Dephosphorylation and excretion of drugs</td>
<td>34</td>
</tr>
<tr>
<td>96-hour time-course metabolism experiment</td>
<td>36</td>
</tr>
<tr>
<td>Virus yield results for 96-hour assay</td>
<td>37</td>
</tr>
<tr>
<td>Correlation of drug phosphorylation to virus titers</td>
<td>41</td>
</tr>
</tbody>
</table>

DISCUSSION | 43

CONCLUSION | 46

REFERENCES | 47
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fifty percent effective concentration (EC50) values (μM) of DHPG, HPMPC and foscarnet against resistant and sensitive virus strains, determined by plaque-reduction assays in C127I cells</td>
</tr>
<tr>
<td>2</td>
<td>Average amounts of phosphorylated species of DHPG (pmol/10^6 cells) produced by cells infected with resistant, sensitive, or no virus at an MOI of 1.0 and treated with 10 μM DHPG for 24 hours</td>
</tr>
<tr>
<td>3</td>
<td>Average amounts of phosphorylated species of HPMPC (pmol/10^6 cells) produced by cells infected with resistant, sensitive, or no virus at an MOI of 1.0 and treated with 1.0 μM HPMPC for 24 hours</td>
</tr>
<tr>
<td>4</td>
<td>Half-lives (in hours) of DHPGppp and HPMPCpp in cells infected with resistant, sensitive, or no virus</td>
</tr>
<tr>
<td>5</td>
<td>Correlation between 96-hour virus production curves and 96-hour phosphorylation curves in cells infected with resistant or sensitive virus and treated with 10 μM DHPG or 1.0 μM HPMPC</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chemical structures of antiviral compounds used</td>
</tr>
<tr>
<td>2</td>
<td>High-pressure liquid chromatography retention times of DHPG, DHPGp, DHPGpp, and DHPGppp</td>
</tr>
<tr>
<td>3</td>
<td>High-pressure liquid chromatography retention times of HPMPC, HPMPC-choline, HPMPCp, and HPMPCpp</td>
</tr>
<tr>
<td>4</td>
<td>Amount of DHPGppp produced in cells infected with resistant, sensitive, or no virus and treated with 30, 10, or 3.0 µM DHPG for 24 hours</td>
</tr>
<tr>
<td>5</td>
<td>Amount of HPMPCpp produced in cells infected with resistant, sensitive, or no virus and treated with 3.0, 1.0, or 0.3 µM HPMPC for 24 hours</td>
</tr>
<tr>
<td>6</td>
<td>Effect of multiplicity of infection (MOI) on production of DHPGppp in cells infected with resistant or sensitive virus and treated with 10 µM DHPG for 24 hours</td>
</tr>
<tr>
<td>7</td>
<td>Effect of multiplicity of infection (MOI) on production of HPMPCpp in cells infected with resistant or sensitive virus and treated with 1.0 µM HPMPC for 24 hours</td>
</tr>
<tr>
<td>8</td>
<td>Production of DHPGppp over a course of 24 hours in cells infected with resistant, sensitive, or no virus and treated with 10 µM DHPG</td>
</tr>
<tr>
<td>9</td>
<td>Production of HPMPCpp over a course of 24 hours in cells infected with resistant, sensitive, or no virus and treated with 1.0 µM HPMPC</td>
</tr>
<tr>
<td>10</td>
<td>Dephosphorylation of intracellular DHPGppp over a course of 24 hours in cells infected with resistant, sensitive, or no virus</td>
</tr>
<tr>
<td>11</td>
<td>Dephosphorylation of intracellular HPMPCpp over a course of 24 hours in cells infected with resistant, sensitive, or no virus</td>
</tr>
<tr>
<td>12</td>
<td>Production of DHPGppp in cells infected with resistant, sensitive, or no virus and treated with 10 µM DHPG for a period of 96 hours</td>
</tr>
</tbody>
</table>
13 Production of HPMPCpp in cells infected with resistant, sensitive, or no virus and treated with 1.0 μM HPMPC for a period of 96 hours ...........38

14 Virus production in cells infected with resistant or sensitive virus and treated with 10 μM DHPG for a period of 96 hours..............................40

15 Virus production in cells infected with resistant or sensitive virus and treated with 1.0 μM HPMPC for a period of 96 hours..............................40
INTRODUCTION

The cytomegaloviruses--biology and disease

The cytomegaloviruses are ubiquitous agents that commonly infect many species of animals as well as humans (Alford and Britt, 1990). In recent years infections due to human cytomegalovirus (HCMV) have grown in significance largely due to the AIDS epidemic. In healthy individuals, HCMV normally causes an inapparent infection. However, in neonates, transplant recipients, and immunocompromised individuals it can cause severe and sometimes fatal complications, including retinitis, gastrointestinitis, and pneumonia (Alford and Britt, 1990; Balfour, 1990). CMV disease has been treated mainly with the drugs phosphonoformate (foscarnet) or nucleoside analogs, the most effective being the guanine analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, called DHPG or ganciclovir (Balfour, 1990; Jacobson et al., 1989; Smee et al., 1983).

Other animal species typically infected with cytomegaloviruses are mice, rats, guinea pigs, cattle, horses, and primates (Staczek, 1990; Freitas et al., 1985). All cytomegaloviruses produce the characteristic enlargement of the infected cell with the presence of intranuclear inclusions (Staczek, 1990). The murine cytomegalovirus (MCMV) model has been used extensively as an in vivo model to test drugs for use against HCMV (Duke et al., 1986; Hudson, 1979; Kern, 1991; Smee et al., 1992; Wilson et al., 1987), and MCMV infection is considered to be predictive for HCMV infection (Bronson et al., 1989; Duke et al., 1986; Kern, 1991).

9-(1,3-dihydroxy-2-propoxymethyl)guanine
(DHPG, ganciclovir)

DHPG is a guanine analog that exerts its antiviral effect by inhibiting the
CMV DNA polymerase (Neyts et al., 1990), similar to the related drug acyclovir (Elion et al., 1977; Smee et al., 1985). Both compounds are taken up by infected cells and phosphorylated by viral and cellular enzymes (Elion et al., 1977; Biron et al., 1985). The 5' triphosphate has been shown to be the active antiviral form (Duke et al., 1986). However, greater concentrations of the DHPG triphosphate are produced in CMV-infected cells than in cells treated with acyclovir (Biron et al., 1985; Frietas et al., 1985). The general mechanism of action is the conversion of DHPG by viral enzymes into its monophosphate form, the metabolism of the monophosphate by cell enzymes to di- and triphosphates, then the subsequent inhibition of the viral DNA polymerase by DHPG triphosphate (Biron et al., 1986; Matthews and Boehme, 1988).

Originally, DHPG activity was thought to be initiated by conversion to the monophosphate form by a cellular enzyme (Smee, 1985). Infection with cytomegalovirus causes a general increase in host cell enzyme activity, which in turn causes an increase in drug phosphorylation (Stinski, 1990). This was initially believed to be the cause of increases in DHPG metabolism in CMV-infected cells (Smee, 1985). Later, Biron et al. (1986) found a DHPG-resistant CMV strain that would not induce increased DHPG metabolism, suggesting the role of a viral enzyme in drug phosphorylation. Littler et al. (1992) and Sullivan et al. (1992) later discovered the viral gene, dubbed UL97, in cells infected with human cytomegalovirus, which produces the enzyme responsible for the initial step of DHPG phosphorylation.

After formation of the DHPG monophosphate (DHPGp), the cellular guanylate kinase enzyme phosphorylates it to form DHPG diphosphate (DHPGpp). DHPG diphosphate is then phosphorylated again to form DHPG triphosphate (DHPGppp) (Matthews and Boehme, 1988). This compound acts as
a 2'-deoxyguanosine triphosphate analog and binds preferentially to the virus-encoded enzyme and inhibits viral DNA synthesis (Mar et al., 1983). Because of the presence of the UL97 gene, HCMV-infected human cells phosphorylate DHPG much more efficiently than do uninfected cells. Thus, DHPG has a high degree of selectivity, with low toxicity to uninfected cells (Andrei et al., 1991; Freitas et al., 1985; Mar et al., 1983).

In spite of the good safety profile of DHPG in cell culture and in mice, toxicity has occurred in humans. The main problem resulting from ganciclovir therapy is the development of bone marrow toxicity following prolonged treatment (Balfour, 1990).

(5)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, cidofovir)

The problem of DHPG toxicity has led to the development of newer types of drugs, including several phosphonylmethoxyalkyl derivatives of nucleosides. This family of drugs has a wide spectrum of activity against several DNA viruses, including herpes simplex virus types 1 and 2, varicella-zoster virus, and the thymidine kinase deficient (TK-) mutants of these strains (De Clerq et al., 1986; Snoeck et al., 1988). These drugs include the compound (S)-9-(3-hydroxy-2-phosphonylmethoxylpropyl)cytosine or HPMPC, which shows activity against human, rat, and murine cytomegaloviruses (Bronson et al., 1989).

HPMPC has several advantages over DHPG. It is almost five times less toxic as measured by selective index in cell culture (Andrei et al., 1991), and persists in the cell much longer than DHPG (Neyts et al., 1990). Since HPMPC is a phosphonate, it does not require initial conversion to the monophosphate form, and thus does not require thymidine kinase activation (De Clerq et al., 1986;
Snoeck et al., 1988). This makes it more effective against thymidine kinase deficient (TK−) strains of herpes simplex viruses as well as DHPG-resistant strains of human cytomegaloviruses (De Clerq, 1993). Inside the cells, HPMPC is converted to three primary species: a choline adduct (HPMPC-choline), a monophosphate form (HPMPCp), and a diphosphate (HPMPCpp) (Ho et al., 1992). The HPMPC diphosphate (HPMPCpp) is thought to be the active form of the drug (De Clerq, 1993; Ho et al., 1992), and persists much longer in infected cells than DHPG triphosphate. This allows for more infrequent treatments. HPMPCpp is a much more efficient inhibitor of the viral DNA polymerase than DHPG triphosphate (Kern, 1991; Neyts et al., 1990). Another advantage of HPMPC is that it has a higher degree of selectivity than DHPG in the sense that it inhibits viral DNA polymerase much more efficiently than cellular DNA polymerase (Andrei et al., 1991).

In rats infected with rat cytomegalovirus (RCMV), one treatment of HPMPC was found to inhibit RCMV infection even when administered 7 days prior to infection (Stals et al., 1991). Stals et al. (1991) also noted that HPMPC was more active than DHPG against RCMV infection in terms of mortality, histopathological analysis, and virus titers. Rabbits were protected from herpes simplex virus type 1 viral retinitis when pretreated with HPMPC (Flores-Aguilar et al., 1994). Otova et al. (1992) found that a single treatment of HPMPC in vitro was sufficient for antiviral effect.

When DHPG and HPMPC were compared against each other in a mouse model of MCMV infection, the virus was found to be more sensitive to HPMPC, with HPMPC being approximately 10 times more effective than DHPG in terms of increasing the number of MCMV-infected mice surviving after treatment (Smee et al., 1992). In MCMV-infected immunosuppressed mice, HPMPC was
shown to have a therapeutic index 50-fold greater than DHPG (Smee et al., 1992). HPMPC was more effective than DHPG in preventing mortality in both immunocompetent and immunosuppressed mice (Neyts et al., 1993). In vitro, several strains of HCMV and one strain of MCMV have been shown to be 5- to 40-fold more sensitive to HPMPC than to DHPG (Kern, 1991).

The main toxic side effect of HPMPC is nephrotoxicity (Neyts and De Clerq, 1993). Kern (1991) noted that HPMPC can accumulate in the kidneys of mice. HPMPC nephrotoxicity has been observed in humans after two weekly doses of 10 mg/kg, and was reversible after cessation of therapy (Neyts and De Clerq, 1993). Nephrotoxicity has been reduced by the conversion of HPMPC into its cyclic cogenor (Hitchcock et al., 1995).

*Phosphonoformic acid (foscarnet)*

Foscarnet is an analog of pyrophosphate, which reversibly and noncompetitively inhibits the cytomegalovirus (CMV) DNA polymerase (Balfour, 1990), as well as other viral DNA and RNA polymerases and reverse transcriptase (Oberg, 1989). As a result, foscarnet has in vitro antiviral activity against several species of viruses, including virtually all herpesviruses, human immunodeficiency virus (HIV), and hepatitis B virus (Oberg, 1989; Fletcher et al., 1994). It is currently approved for use against human CMV retinitis in immunocompromised individuals.

Treatment with foscarnet has been shown to be effective in reducing or stabilizing the number of CMV retinal lesions in immunocompromised patients as well as reducing viruria and viremia (Jacobson et al., 1989). Ringden et al. (1985) reported that after foscarnet treatment, clinical improvement was seen in 69% of transplant patients infected with CMV. Oberg (1989) reported that
several other researchers have noted improvement in CMV-infected AIDS patients. As for treatment of HIV infection, Fletcher et al. (1994) reported that several patients had increased amounts of CD4+ lymphocytes and decreased mean amounts of HIV antigen in the blood. Against murine cytomegalovirus, foscarnet treatment significantly reduced the mortality rate in infected mice, as well as viral titers in infected organs (Overall et al., 1976).

The main adverse effect of foscarnet treatment is renal dysfunction (Balfour, 1990), although hydration may reduce this effect (Taburet et al., 1992). Foscarnet acts as a chelating agent for calcium and has been linked to seizures, arrhythmias, and fatal cases of hypocalcemia (Jacobson et al., 1991). Up to 28% of the cumulative dose is deposited in bone tissue (Balfour, 1990).

**Viral resistance to antiviral drugs**

Prolonged therapy with DHPG has been associated with the formation of DHPG-resistant strains of cytomegalovirus (Drew et al., 1991; Erice et al, 1989). It is estimated that 10-15% of AIDS patients with CMV retinitis who receive long-term ganciclovir therapy develop resistant strains of HCMV (Biron, 1991). Two main modes of cytomegalovirus resistance to DHPG have been reported. Lurain et al. (1992) and Sullivan et al. (1992) reported that point mutations in HCMV gene UL97 produced a resistant viral strain. This is due to decreased DHPG phosphorylation in infected cells. Both investigators reported that resistance due to point mutations in the HCMV DNA polymerase also conferred cross-resistance to DHPG and HPMPC (Lurain et al., 1992; Sullivan et al., 1993). Lurain et al. (1994) reported that HCMV DNA containing a UL97 mutation could be transferred to a sensitive strain, thus conferring resistance to DHPG.
Resistance to HPMPC in human and murine cytomegaloviruses has already been reported both in laboratory strains (Smee et al., 1995; Sullivan et al., 1993) and from CMV-infected patients treated with ganciclovir (Drew et al., 1991; Erice et al., 1989; Stanat et al., 1991). Cross-resistance between DHPG, HPMPC, and other drugs in human strains has also been observed (Lurain et al., 1992).

Resistance to foscarnet has also been noted in clinical (Tatarowicz et al., 1992) as well as in laboratory virus strains (Sullivan and Coen, 1991). Some of these strains have shown cross-resistance to DHPG and HPMPC (Sullivan and Coen, 1991; Tatarowicz et al., 1992). Jacobson et al. (1991) observed that foscarnet therapy was effective in patients with ganciclovir-resistant cytomegalovirus retinitis. Since foscarnet does not require intracellular phosphorylation to exert its antiviral effect (Oberg, 1989), it has been postulated that resistance to foscarnet is indicative of a mutation in the viral DNA polymerase (Tatarowicz et al., 1992).

Smee et al. (1995) developed a murine cytomegalovirus strain that was cross-resistant to DHPG, HPMPC, and foscarnet. In order to better understand the mode of drug resistance of this murine virus, the studies presented in this thesis were conducted. To date, no results have been published on the phosphorylation of DHPG and HPMPC in murine cytomegalovirus-infected cells. The results presented here shed light on the mode of drug resistance of this mutant strain of MCMV.
JUSTIFICATION OF RESEARCH

The problem of the development of viral drug resistance during prolonged therapy has created the need for models to determine the modes of resistance to these drugs, and to develop strategies to overcome or delay the emergence of drug-resistant virus strains. Researchers have determined that HCMV resistance to nucleoside and nucleotide analog drugs is usually due to one or both of two mechanisms: an alteration resulting in the decrease in the uptake or phosphorylation of the compound (Lurain et al., 1992; Sullivan et al., 1993), or a mutation in the viral DNA polymerase which causes lowered affinity for the active form of the drug to this enzyme (Lurain et al., 1992; Ochiai et al, 1992).

The model of HCMV infection chosen for this research is MCMV. Smee et al. (1995) developed a strain of MCMV resistant to DHPG, HPMPC, and foscarnet. The reason why this virus is resistant to these antiviral drugs has not been previously explored. Because HCMV and MCMV are closely related, cross-resistance to DHPG, HPMPC, and other similar drugs by the mutant strain of MCMV is assumed to be due to differences in either drug metabolism or inhibition of DNA polymerization, or both. The first hypothesis can be tested by showing significantly lower amounts of active drug forms are produced in cells infected by the resistant virus as compared to the sensitive virus. To do so, the amounts of DHPG triphosphate (DHPGppp) and HPMPC diphosphate (HPMPCpp) (the active antiviral drug metabolites) that are produced by the infected cells were compared. Negative results, which in this case would be no significant difference between sensitive and resistant virus strains in terms of affecting drug metabolism, indicate a different mode of resistance.
The second hypothesis is that the resistance is due to a mutation in the MCMV DNA polymerase. Lower affinity of the resistant polymerase for active forms of the drugs as compared to the sensitive polymerase would support this hypothesis. Also supporting this hypothesis is the fact that the resistant strain is cross-resistant to foscarnet, which does not require initial phosphorylation to be active (Balfour, 1990), and is a known inhibitor of CMV DNA polymerase (Oberg, 1989).

The first hypothesis, which involved studying drug metabolism in cells infected by resistant and sensitive viruses, was investigated in detail. As this research project progressed, it became apparent that it would not be possible to test this second hypothesis, due to a lack of time and funding. Therefore, only the first hypothesis was tested. As a result, this thesis answers only one part of the question of drug resistance, that of whether the resistance is due to decreases in metabolism of the antiviral drugs by the infected cells.
MATERIALS AND METHODS

Compounds and solutions

Ganciclovir was manufactured by Syntex (Palo Alto, CA) and obtained from a local pharmacy. HPMPC was obtained from Gilead Sciences (Foster City, CA). Foscarnet was obtained from Sigma (St. Louis, MO). 3H-labeled DHPG and HPMPC were purchased from Moravek Biochemicals (Brea, CA). The DHPG was labeled on the guanine methyl group, while the HPMPC was labeled on the hydrogen atom of the fifth carbon of the cytosine. The structures of all three compounds are shown in Figure 1.

The high-pressure liquid chromatography buffer was prepared using HPLC-grade ammonium phosphate (JT Baker, Phillipsburg, NJ). Perchloric acid was obtained from Mallinckrodt (Paris, KY), and potassium hydroxide and imidazole were obtained from Fisher Scientific (Fair Lawn, NJ).

The high-pressure liquid chromatography apparatus consisted of a Waters model 510 high-pressure liquid chromatograph, with the gradient being controlled by a Waters Lambda-Max Model 481 automatic gradient controller (San Francisco, CA). The column used was a Whatman (Clifton, NJ) 25-cm partisil 10X strong anion exchange column. Spectrophotometric data from the liquid chromatographer were obtained from a Waters Lambda-Max model 481 LC spectrophotometer and were graphed using a Shimadzu CR 601 chromatopac integrator (Columbia, MD). Fractions from the HPLC were collected in 7-ml polyethylene scintillation vials (Kimble, Vineland, NJ) using a Pharmacia Frac 100 fraction collector (Piscathaway, NJ), and the liquid fractions were suspended in Beckman Ready-Safe scintillation fluid (Beckman, Fullerton, CA).
9-(1,3-dihydroxy-2-propoxymethyl)-guanine (DHPG, Ganciclovir)

(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC, Cidofovir)

Phosphonoformic Acid (Foscarnet)

Fig. 1. Chemical structures of antiviral compounds used.
The radioactive samples were analyzed using a Beckman LC 5801 scintillation counter.

**Virus and cells**

The sensitive, “wild-type” Smith strain of MCMV (hereafter referred to as the “sensitive” strain) was obtained from American Type Culture Collection (Rockville, MD). The plaque-purified resistant strain, referred to as GCV-r MCMV in Smee et al. (1995), was developed by propagating the sensitive MCMV strain in increasing concentrations of DHPG. This virus shows resistance to DHPG, HPMPC, and foscarnet. C127I cells, derived from mouse mammary tumors, were obtained from ATCC. The cells were grown in 55-cm² petri dishes, the resistance assays were performed in 12-well tissue culture plates, and the labeling experiments were done in 25-cm² tissue culture flasks. All plasticware was obtained from Corning, Inc. (Corning, NY). The cells were grown in Dulbecco’s minimum essential medium (DMEM) with 5 mM HEPES buffer (Sigma) and 10% fetal bovine serum added (HyClone Laboratories, Logan, UT). Virus plaques were counted using a Plaque Viewer (Bellco Labs, Vineland, NJ). SeaPlaque agarose was obtained from FMC Bioproducts (Rockland, ME).

**Preparation of virus pools**

Two large pools of virus, one of the mutant strain (resistant) and one of the wild-type or Smith strain (sensitive), were prepared. Four 150-cm² and two 75-cm² tissue-culture flasks were seeded with C127I cells in DMEM 10% FBS and the cells were grown at 37°C until confluent. When confluent, two 150-cm² flasks and one 75-cm² flask were infected with either the resistant virus or the sensitive virus. Each pool of virus was suspended in 50 ml of DMEM 2% FBS at a
concentration of 1000 plaque-forming units (PFU) per ml, and 20 ml was added to each 150-cm$^2$ flask and 10 ml was added to the 75-cm$^2$ flask.

After 3 days of incubation at 37° C, the media were removed and replaced with fresh DMEM 2% FBS at the same volume per flask. After a total of seven days of incubation, the infected cells were harvested with the media, pooled, and disrupted by sonication for 1 min. The pools were then dispensed into cryovials at 1.0 ml per vial and frozen at -70° C.

The virus pools were titrated using a crystal violet plaque assay. Two 12-well tissue culture plates were seeded with C127I cells, grown to confluency, and infected with 0.5 ml per well of diluted virus, ranging from 10$^{-1}$ to 10$^{-6}$, two wells per dilution. The virus was allowed to adsorb for 1 h and 15 min, removed, and the cells were overlaid with DMEM 2% FBS, 0.1% NaHCO$_3$, with 0.5% agarose added, and the agarose was allowed to harden at 4° C for 5-10 min. After 7-8 days of incubation at 37° C, the cells were fixed for 15 min with 10% phosphate-buffered formalin. The formalin and agarose were removed, and the cells were stained in a solution of 20% ethanol containing 0.2% crystal violet. To obtain the virus titer, the clear plaques in the cell monolayers, each representing one virus particle, were counted on a Plaque Viewer and multiplied by the dilution factor and a conversion factor of 12.65. The conversion factor takes into account the actual volume of liquid that touches the cell monolayer, since most of the virus in the medium does not adsorb to the cell surface, and is used in accurately determining PFU per ml of virus-containing medium. This titration procedure was repeated three times for each virus pool, and the results were averaged to yield the titer.
Determination of virus resistance

The relative sensitivities of both pools of virus to the drugs DHPG, HPMPC and foscarnet were determined using a plaque-reduction assay described by Smee et al. (1995). Three 12-well plates were used for each drug tested, one and one-half plates for each virus strain. The plates were seeded with C127I cells in DMEM 10% FBS and grown until confluent. The cells were infected with enough virus to produce approximately 100 plaques per well. For the resistant virus, a 1:10,000 dilution was used, while the sensitive virus used a 1:5000 dilution. One-half milliliter of virus was added to each well, one and one-half plates being infected with the resistant virus, with the other one and one-half being infected with the sensitive virus. The virus was adsorbed at 37° C for 1 h and 15 min.

After adsorption, the virus was then removed and replaced with DMEM 2% FBS, 0.1 % NaHCO3, 0.5 % agarose containing eight serial 1:2 dilutions of the drug tested. Two wells per concentration were used, with two wells left untreated as a virus control. The concentrations of drug varied, depending on the virus strain: For DHPG, the concentrations used against the resistant virus ranged from 160 to 1.25 μM, while the concentrations used against the sensitive virus ranged from 40 to 0.3125 μM; for HPMPC, the resistant virus was tested against concentrations of 16.0 to 0.125 μM, while the sensitive virus was tested against concentrations of 4.0 to 0.03125 μM; for foscarnet, the resistant virus was tested against concentrations of 1200 to 12.5 μM, and the sensitive virus was tested against concentrations of 400 to 3.125 μM. The plates were incubated for 7 or 8 days, and then fixed, stained, and the plaques counted as described previously. The average plaque count for each virus concentration was expressed as a percentage of the control, and the 50% effective concentration
(EC50) values were estimated by plotting percent of plaques inhibited versus drug concentration on semilog graph paper. This trial was repeated three times for each virus against each drug, and then an average value with standard deviations was obtained for each one.

**Determination of metabolism of DHPG and HPMPC in cells**

To determine the metabolism of these drugs in infected and uninfected C127I cells, a basic high-pressure liquid chromatography (HPLC) procedure similar to those previously described (Freitas et al., 1985; Smee et al., 1985; Smee and Matthews, 1986) was used. Five different experiments were performed that addressed the variables of different concentrations of drug, multiplicity of infection and time on drug metabolism, and the catabolism of phosphorylated drugs inside the cell. The HPLC procedure is described on pages 21 and 22.

**Effect of different concentrations of drug on viral metabolism**

In the first experiment, the effect of different concentrations of drug on virus-induced phosphorylation was examined. Eighteen 25-cm² flasks were seeded with C127I cells and grown to confluency. When confluent, the flasks were separated into three sets of six flasks each. One set was infected with the resistant strain, one with the sensitive strain, and one was left as an uninfected control. The virus was suspended in DMEM 2% FBS, 5 mM HEPES at a multiplicity of infection (MOI) of 2.0 PFU per cell, and 1.2 ml of medium was added to each flask. The virus was adsorbed for 1 h and 15 min.

DHPG was diluted in DMEM to concentrations of 30, 10, and 3.0 μM, and HPMPC was diluted to concentrations of 3.0, 1.0, and 0.3 μM. Twenty μCi/ml of
\(^{3}\text{H}\)-labeled compound was added to each dilution for a total activity of 24 \(\mu\text{Ci}\) per flask, (1.2 ml of medium containing labeled drug was added to each flask), and the cells were incubated for 24 h at 37° C.

The cells were harvested by removing the radioactive media, rinsing 1X with 2.0 ml of DMEM without FBS added, adding 0.5 ml of 3.5\% perchloric acid, and incubating at 4° C for 5 min. After incubation, the flasks were placed on end and the acid was neutralized with 0.25 ml of 1.0 N KOH with 0.2 M imidazole (1,3-diazo-2,4-cyclopentadiene) added. The supernatant was mixed in the flask briefly and then removed and frozen at -70° C.

**Effect of multiplicity of infection (MOI) on drug phosphorylation**

The second experiment dealt with the effect of MOI on drug phosphorylation. MOI refers to the number of infectious virus particles to the number of cells. For example, an MOI of 1.0 means one virus particle per cell. Eighteen 25-cm\(^2\) tissue culture flasks were seeded with C127I cells and grown to confluency. At the time of infection, they were separated into two groups of eight flasks each, with two flasks left over. Both groups were infected with either the resistant virus or the sensitive virus suspended in DMEM containing 10\% FBS and 5 mM HEPES buffer. In each of the infected groups, two flasks were infected at an MOI of 3.0, 1.0, 0.3, or 0.1 PFU/cell, and the virus was allowed to adsorb for 1 h and 15 min. The two remaining flasks were used as uninfected controls.

After adsorption, the virus was removed and the flasks were arranged into two groups of nine flasks each. Each group contained one flask of each virus at each MOI and an uninfected control. One group was treated with 10 \(\mu\text{M}\)
DHPG with 20 μCi/ml $^3$H-labeled DHPG added, while the other was treated with 1.0 μM HPMPC with 20 μCi/ml of $^3$H-labeled HPMPC added. The cells were then incubated for 24 h at 37° C, and then harvested using the perchloric acid-KOH/imidazole procedure and frozen at -70° C.

24-hour time-course metabolism experiment

This experiment was done to determine the differences, if any, between the phosphorylation of DHPG and HPMPC in cells infected with the two virus strains over a period of 24 h. C127I cells were seeded into 36 25-cm$^2$ tissue culture flasks and grown to confluency. Of the 36 total flasks, 12 were infected with the resistant virus strain at an MOI of 1.0 PFU/cell, while 12 were infected with the sensitive virus strain at the same MOI, and 12 were left uninfected as controls. The virus was adsorbed the usual time of 1 h and 15 min.

After adsorption, the virus was removed and flasks were divided into two sets of 18, each set containing 6 flasks from the three infection groups. One set of 18 flasks was treated with DHPG at 10 μM and the other was treated with HPMPC at 1.0 μM. The flasks were incubated at 37° C.

At time points of 1, 2, 4, 6, 12, and 24 h postinfection, three flasks from each drug treatment group (one resistant-infected, one sensitive-infected, and one uninfected) were harvested using the perchloric acid-KOH/imidazole procedure and the supernatants were frozen at -70° C.

Dephosphorylation and excretion of drugs

This experiment described the difference between the catabolism of the drugs. The experiment was organized the same way as the previously described experiment; however, the drugs were left on the cells for 24 h of incubation, and
then the media were removed and the cells were washed 2X with 2.0 ml of DMEM and refed with 1.2 ml of drug-free DMEM 10% FBS. Cell supernatants were harvested using the perchloric acid/KOH method at 0, 1, 2, 4, 6, 12, and 24 h after the removal of the drugs, for a total of six per time point. At the same time points, 600-μl samples of the cell media were removed and analyzed for radioactivity. The cell supernatants were frozen at -70°C.

96-hour time-course metabolism experiment

In this experiment, the phosphorylation of DHPG and HPMPC was measured over a period of 4 days. Because of the possibility of severe cytotoxicity due to the virus infection affecting the amount of phosphorylated drug produced, a duplicate set of flasks was prepared for this experiment, and cell counts were performed at each time point.

Forty-eight 25-cm² flasks were seeded with C127I cells and grown to confluency. The flasks were divided into three groups of 16 flasks each. One group was infected with resistant virus at an MOI of 1.0 PFU/cell, another group was infected with sensitive virus at the same MOI, and the third group was left uninfected as a control. Each of the flasks received 1.2 ml of medium, and the virus was adsorbed for the standard period of time.

After adsorption, the virus was removed and the flasks were divided into four groups of 12 flasks, each with 4 resistant-infected, 4 sensitive-infected, and 4 uninfected flasks. One group was treated with 1.2 ml/flask of HPMPC at 1.0 μM with 20 μCi/ml of ³H-labeled HPMPC added, while another was treated with 1.2 ml/flask of DHPG at 10 μM with 20 μCi/ml of ³H-labeled DHPG added. The two remaining groups were treated either with 1.2 ml of 1.0 μM HPMPC or 1.2
ml of 10 μM DHPG with no radioactive compounds added. All were incubated at 37°C.

At time points of 24, 48, 72, and 96 h postinfection, one set of three flasks from each group, each containing one infected with resistant virus, one infected with sensitive virus, and one uninfected control, was removed. The flasks treated with the 3H-labeled compounds were harvested using the perchloric acid-KOH/imidazole procedure and the supernatants were frozen at -70°C. The controls for determining cell numbers at each time point were harvested by removing the media, adding 1.0 ml of trypsin per flask, allowing the cells to detach and then resuspending them in 4.0 ml of DMEM 10% FBS. The cells were counted microscopically using a hemocytometer, and two counts were performed per sample.

Virus yield assay

To determine the effect of the drugs HPMPC and DHPG on virus yield in cells infected with sensitive or resistant virus, an assay that measured the levels of virus over a period of 4 days was developed, based on an assay previously described by Smee et al. (1995). Four 12-well plates were seeded with C127I cells and allowed to grow to confluency. Each plate was divided in half (6 wells) and one half was infected with 0.5 ml of resistant virus while the other half was infected with 0.5 ml of sensitive virus. Both virus strains were suspended in DMEM containing 10% FBS and 5 mM HEPES, and both were infected at an MOI of 1.0 PFU/ml.

After virus adsorption, the virus was removed and each plate was divided into three sections of four wells, each section with two wells of resistant virus-infected cells and two wells of sensitive virus-infected cells. On each plate, one
section was treated with 10 μM DHPG, one was treated with 1.0 μM HPMPC, and one was placebo-treated with media only. The drugs were suspended in DMEM 10% FBS with 5 mM HEPES added, and 1.0 ml of solution was used per well. The plates were incubated at 37°C.

At 24, 48, 72, and 96 h postinfection, one plate at each time was removed and frozen at -20°C. The virus was harvested by removing the treated plates from the freezer and allowing them to thaw slightly. The frozen media were swirled in the wells to scrape the cells from the bottom, and then the samples were allowed to thaw completely. Each plate had two wells with the same samples, and the contents of these two wells were pooled in a test tube and sonicated for 1 min. From this tube 111 μl was removed and diluted in 1.0 ml of DMEM, and then serially diluted to 10^-6 for the 24-h samples, and 10^-7 for the rest of the time points.

Prior to harvesting the virus from each time point, three 12-well plates were seeded with C127I cells and grown to confluency. Each plate was divided in half and then 0.5 ml of each virus dilution was placed in a well, for a total of six wells per sample, two samples per plate. The virus was adsorbed for 1 h and 15 min, removed, and the cells were overlaid with DMEM 2% FBS, 0.5% agarose. The plates were incubated for 7 days and then fixed with formalin and stained with crystal violet as per previous procedures.

The plaques were counted using the Plaque Viewer, and the titers for each sample were obtained by multiplying the number of plaques by the dilution factor and by the conversion factor as described on page 13.
High-pressure liquid chromatography analysis of the frozen supernatants

HPLC analysis of the samples was performed using a modified procedure of one described in Smee et al. (1985). The frozen radioactive supernatants were thawed, gently vortexed, and filtered using a 0.45-μm syringe-type filter (Gelman Scientific, Ann Arbor, MI) designed for HPLC samples. Five hundred microliters (500 μl) of each sample were injected into the HPLC. The HPLC buffer was prepared from ammonium phosphate (J. T. Baker, Phillipsburg, NJ) at concentrations of 1.0 M and 0.01 M, pH-adjusted to 3.5, and filtered through Whatman 0.45 micron filters. The DHPG-treated cell extracts were separated on a linear gradient starting at 0.01 M ammonium phosphate, pH 3.5, and ending at 1.0 M ammonium phosphate, pH 3.5, at 35 min. The gradient was sustained at 1.0 M ammonium phosphate for another 5 min, and then was reduced to 0.01 M using a linear gradient for 5 min. Total running time for the DHPG gradient was 45 min.

The HPMPC-containing samples were separated using the same concentrations of ammonium phosphate, but different run times. The initial linear gradient lasted for 45 min. The 1.0 M ammonium phosphate plateau was sustained for 5 min, and then the concentration was returned to 0.01 M ammonium phosphate using a linear gradient lasting for 4 min. The flow rate of the HPLC pumps was set at 1.0 ml per min, and 1-min fractions were collected in the scintillation vials.

During the separation, the UV absorbance (280 nm wavelength) of the various fractions was measured by the spectrophotometer and the resulting peaks were recorded by the integrator.
After chromatographic separation of the samples, 4.0 ml of scintillation fluid were added to each vial, mixed with the sample, and then the radioactivity of each fraction was determined using the scintillation counter. The counter provided the counts per minute (CPM) of each 1-min fraction.

The amount of pmol/10^6 cells was determined by a procedure adapted from Smee et al. (1985). Briefly, it was first necessary to determine the average number of cells analyzed and the specific radioactivity of each drug used (CPM/picomole). To obtain cell counts, four 25-cm^2 tissue culture flasks were seeded with C127I cells and grown to confluence. The cells were trypsinized, suspended in DMEM, and counted microscopically using a hemocytometer. Each sample was counted twice, and the counts were averaged to yield 2.7 X 10^6 cells. This number was used for all 0-24 h metabolism experiments as being representative of the amount of cells in a confluent 25-cm^2 flask.

After each sample was counted on the scintillation counter, the peaks were identified and quantified by adding the total counts per minute in each peak, minus a background value of 20 counts per minute. These peak totals were converted into picomoles using a conversion factor. This conversion factor was obtained by removing 10 μl of medium from each sample flask immediately prior to harvesting the cells. The 10 μl were suspended in 5 ml of scintillation fluid and counted. Because the amount of labeled and unlabeled compound added to the media was the same for each sample, the amount of radioactivity in each sample was considered to be directly proportional to the amount of unlabeled compound in the media. Thus, 10 μl of a 10 μM solution had a total of 100 picomoles of unlabeled compound. By counting the amount of radioactivity in each 10-μl sample, and expressing this as CPM/pmol, the amount of picomoles in each peak was determined by multiplying the total CPM/peak by
this ratio. The product was then divided by 2.7 to express it in terms of pmol/10^6 cells. The resulting values of pmol/10^6 cells were plotted versus time, concentration of drug, or multiplicity of infection.
RESULTS

Determination of virus resistance

Plaque reduction assays were conducted to establish that the plaque-purified drug-resistant virus was less sensitive to three antiviral compounds than the sensitive (wild-type) virus. Against the drug DHPG, the resistant virus had an average 50% effective concentration (EC\textsubscript{50}) of 72 \textmu M, while the sensitive virus had an EC\textsubscript{50} of 5.1 \textmu M, a 14-fold difference. For HPMPC, the EC\textsubscript{50} of the resistant virus was 2.7 \textmu M versus an EC\textsubscript{50} of 0.24 \textmu M for the sensitive virus, an 11-fold difference. The EC\textsubscript{50}s for the drug foscarnet against the resistant and sensitive virus strains were 627 and 141 \textmu M, respectively, a 4-fold difference.

The average EC\textsubscript{50} of the resistant and sensitive virus strains were compared for each compound using a two-tailed t-test with the InStat\textsuperscript{®} statistical program, and the differences were determined to be extremely significant \((p<0.001)\). The EC\textsubscript{50}s for the three drugs tested versus both the resistant and sensitive strains were similar to the values reported previously by Smee et al. (1995) (the former drug-resistant virus was not plaque-purified). The resistance data are summarized in Table 1.

Drug metabolism experiments

Phosphorylation of DHPG and HPMPC was initially studied under a defined set of experimental conditions. These parameters were a multiplicity of infection of 1.0, a DHPG concentration of 10 \textmu M or an HPMPC concentration of 1.0 \textmu M, and an incubation time of 24 h. Analysis of drug metabolites was made by HPLC.
Table 1
Fifty-percent effective concentration (EC₅₀) values (µM) of DHPG, HPMPC, and foscarnet against resistant and sensitive virus strains, determined by plaque-reduction assays in C127I cells

<table>
<thead>
<tr>
<th></th>
<th>DHPG</th>
<th>HPMPC</th>
<th>Foscarnet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant virus</td>
<td>72 ± 2.0 a (14.1) b</td>
<td>2.7 ± 0.4 (11.3) b</td>
<td>627 ± 90 (4.4) b</td>
</tr>
<tr>
<td>Sensitive virus</td>
<td>5.1 ± 2.4</td>
<td>0.24 ± 0.04</td>
<td>141 ± 10</td>
</tr>
</tbody>
</table>

a Mean ± standard deviation (three independent assays).
b p<0.001.

For the drug DHPG, the unphosphorylated parent compound was eluted 4 to 8 min postinjection (Fig. 2). The monophosphate form of DHPG (DHPGₚ) was eluted immediately afterwards, at 9-13 min postinjection. The diphosphate form (DHPGₚp) was eluted between 23-27 min postinjection, and its peak was generally larger than the monophosphate peak. The triphosphate form (DHPGₚₚp), which is the active form of the drug, was eluted at 38-42 min postinjection. The averages for three sample sets treated with DHPG showed that the sensitive virus-infected samples produced about the same amount of the active forms of compounds as the resistant-infected samples, while the uninfected control samples consistently produced the lowest amounts (Table 2).

Table 2
Average amounts of phosphorylated species of DHPG (pmol/10⁶ cells) produced by cells infected with resistant, sensitive, or no virus at an MOI of 1.0 and treated with 10 µM DHPG for 24 hours

<table>
<thead>
<tr>
<th></th>
<th>DHPGₚ</th>
<th>DHPGₚp</th>
<th>DHPGₚₚp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant virus</td>
<td>0.221 ± 0.030</td>
<td>0.166 ± 0.099</td>
<td>0.147 ± 0.011</td>
</tr>
<tr>
<td>Sensitive virus</td>
<td>0.037 ± 0.012</td>
<td>0.110 ± 0.047</td>
<td>0.154 ± 0.004</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.019 ± 0.012</td>
<td>0.066 ± 0.013</td>
<td>0.122 ± 0.023</td>
</tr>
</tbody>
</table>
The DHPG monophosphate peak was usually quite small in relation to the other peaks, except in cells infected with resistant virus. The monophosphate peak was approximately 16% the size of the triphosphate peak in uninfected cells, 24% in sensitive-infected cells, and 150% in resistant-infected cells. The resistant-infected cells also produced more of the diphosphate than of the triphosphate. On average, the diphosphate peak was approximately 54% as large as the triphosphate peak in uninfected cells, 71% as large in sensitive-infected cells, and 113% as large in the resistant-infected cells. There was much less variation between experiments in the amount of triphosphate produced by the cells, as compared to the amounts of the other phosphorylated species.

The parent (unphosphorylated) form of HPMPC was eluted between 4 and 9 min postinjection (Fig. 3). Immediately after elution of the parent compound, a relatively large peak of HPMPC-choline adduct was eluted, between 10 and 14 min postinjection. A monophosphate form of HPMPC (HPMPCp) was eluted between 19-23 min, and the diphosphate or active form of the drug (HPMPCpp) was eluted at 33-36 min postinjection.

In general, no significant differences in HPMPC metabolism were noted between cells infected with the two types of virus, and the uninfected control cells (Table 3). Cells infected with the resistant virus produced slightly more of the phosphorylated species than cells infected with the sensitive strain or the uninfected cells. The time of elution for each phosphorylated specie of compound also remained the same. The HPMPC-choline peak was, on average, 3.4, 3, and 3.4 times larger than the diphosphate peak produced by the resistant-infected, sensitive-infected, and uninfected cells, respectively. The monophosphate peak was usually the smallest peak produced, with it being 44%, 36%, and 70% of the diphosphate peaks produced by resistant, sensitive, and
Fig. 2. High-pressure liquid chromatography retention times of DHPG, DHPGp, DHPGpp, and DHPGppp.

Fig. 3. High-pressure liquid chromatography retention times of HPMPC, HPMPC-choline, HPMPCp and HPMPCpp.
Table 3
Average amounts of phosphorylated species of HPMPC (pmol/10^6 cells) produced by cells infected with resistant, sensitive, or no virus at an MOI of 1.0 and treated with 1.0 μM HPMPC for 24 hours

<table>
<thead>
<tr>
<th></th>
<th>HPMPC-choline</th>
<th>HPMPCp</th>
<th>HPMPCpp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant virus</td>
<td>0.108 ± 0.046</td>
<td>0.014 ± 0.001</td>
<td>0.032 ± 0.009</td>
</tr>
<tr>
<td>Sensitive virus</td>
<td>0.108 ± 0.035</td>
<td>0.013 ± 0.002</td>
<td>0.036 ± 0.008</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.093 ± 0.022</td>
<td>0.018 ± 0.006</td>
<td>0.027 ± 0.016</td>
</tr>
</tbody>
</table>

uninfected samples, respectively. Like the HPMPC-choline peak, the resistant-infected, sensitive-infected, and uninfected cells produced roughly equal amounts of HPMPCp and HPMPCpp (Table 3).

*Dose-dependent phosphorylation of drugs*

In this experiment, cells were infected with an MOI of 2, and treated with DHPG at concentrations of 30, 10, or 3.0 μM; or HPMPC at concentrations of 3.0, 1.0 or 0.3 μM and incubated for 24 h. At 30 μM DHPG, the infected cells produced 0.597 pmol of active DHPGppp per 10^6 cells, as compared to 0.418 pmol of DHPGppp produced by cells infected with the sensitive strain and 0.211 pmol of DHPGppp produced by uninfected cells (Fig. 4). The amount of triphosphate produced by the infected and uninfected cells rose linearly in proportion to the concentration of DHPG present in the media.

A similar effect was noted with HPMPC, although the sensitive-infected and uninfected cells produced similar amounts of HPMPC diphosphate. At 3.0 μM of HPMPC, the resistant-infected cells produced 0.255 pmol/10^6 cells, the sensitive-infected produced 0.148 pmol/10^6 cells, and the uninfected cells produced 0.134 pmol/10^6 cells (Fig. 5).

Cells infected with the resistant virus produced slightly more of the active
Fig. 4  Amount of DHPGppp produced in cells infected with resistant, sensitive, or no virus and treated with 30, 10, or 3.0 μM DHPG for 24 hours.

Fig. 5. Amount of HPMPCpp produced in cells infected with resistant, sensitive, or no virus and treated with 3.0, 1.0, or 0.3 μM HPMPC for 24 hours.
forms of both DHPG and HPMPC at all three concentrations used. However, this was not considered significantly larger than amounts of metabolites produced in cells infected with the sensitive virus, based on previous work with resistant strains (Biron et al., 1986). With both DHPG and HPMPC, the increase in the amount of active forms of the drugs was linearly proportional to the increase in concentration.

**Effect of multiplicity of infection (MOI) on drug phosphorylation**

Cells were infected with resistant or sensitive virus at multiplicities of infection (MOI) of 3.0, 1.0, 0.3, or 0.1, and treated with media containing DHPG at 10 μM or HPMPC at 1.0 μM for 24 h. In the cells treated with DHPG, the amounts of DHPGppp produced by cells infected with both strains of virus were almost identical to each other and the uninfected control. The amount of DHPGppp did not increase until the MOI reached 3.0 PFU/cell. The resistant sample had 0.228 pmol/10^6 cells and the sensitive sample had 0.235 pmol/10^6 cells. The uninfected control produced 0.146 pmol/10^6 cells (Fig. 6).

The cells treated with HPMPC followed a similar pattern. There was a slow increase in the amount of HPMPCpp produced by the infected cells as MOI increased, and the amounts produced by the cells infected with the two virus strains were roughly the same. At an MOI of 3.0, the amount of HPMPCpp produced reached 0.033 pmol/10^6 cells for the resistant infection, 0.024 pmol/10^6 cells for the sensitive infection, as compared to 0.016 pmol/10^6 cells for the uninfected control (Fig. 7). It appears there was no significant difference in the metabolism of the compounds in cells infected with sensitive and resistant viruses when the MOI was increased.
Fig. 6. Effect of multiplicity of infection (MOI) on production of DHPGppp in cells infected with resistant or sensitive virus and treated with 10 μM DHPG for 24 hours.

Fig. 7. Effect of multiplicity of infection (MOI) on production of HPMPCpp in cells infected with resistant or sensitive virus and treated with 1.0 μM HPMPC for 24 hours.
24-hour time-course experiment

In this experiment, the cells were infected with resistant, sensitive, or no virus at an MOI of 1.0. The cells were incubated for 24 h, with samples being harvested at 1, 2, 4, 6, 12, and 24 h postinfection. For cells treated with 10 μM DHPG, the metabolic data produced a linear curve. The amounts of DHPGppp at 1 h postinfection was 0.008 pmol/10^6 cells for the resistant virus, 0.008 pmol/10^6 cells for the sensitive virus, and 0.003 pmol/10^6 cells for the uninfected controls. At 24 h, these totals had increased to 0.150 pmol/10^6 cells, 0.152 pmol/10^6 cells, and 0.100 pmol/10^6 cells, respectively (Fig. 8).

With 1.0 μM HPMPC, the metabolic data produced a sigmoidal curve. The rate of production of HPMPCpp increased slowly until 6 h postinfection, when most of the production occurred. At 1 h postinfection, the amounts of HPMPCpp for all three samples were less than 0.001 pmol/10^6 cells. This had increased to only 0.003 pmol/10^6 cells for all samples at 6 h postinfection. By 12 h postinfection, the amounts of HPMPCpp had increased sharply to 0.025 pmol/10^6 cells for the resistant and sensitive virus-infected samples and 0.019 pmol/10^6 cells for the uninfected control. The rate of increase then leveled off again. At 24 h postinfection, the amounts of HPMPCpp were 0.026 pmol/10^6 for the resistant sample, 0.030 pmol/10^6 cells for the sensitive sample, and 0.019 pmol/10^6 cells for the control sample (Fig. 9).

There was no significant difference in the metabolism of the compounds between the two strains of virus and the uninfected cells. With both DHPG and HPMPC, the amount of active form of the drugs produced by the uninfected controls began to level off after 12 h, while the amounts of active forms produced by the two virus strains were higher but virtually identical to each other.
Fig. 8. Production of DHPGppp over a course of 24 hours in cells infected with resistant, sensitive, or no virus and treated with 10 μM DHPG.

Fig. 9. Production of HPMPCpp over a course of 24 hours in cells infected with resistant, sensitive, or no virus and treated with 1.0 μM HPMPC.
Dephosphorylation and excretion of drugs

Cells were infected with resistant, sensitive, or no virus at an MOI of 1.0, and incubated for 24 hours in media containing DHPG at 10 μM or HPMPC at 1.0 μM. After 24 h, the drug-containing media were removed, and drug metabolites were quantified at different times thereafter. At 1 h radioactivity was detected in the cell culture media, the amount of which remained at equilibrium for the rest of the experiment. This indicated that the unphosphorylated form of each drug was rapidly excreted from cells.

At the time of removal of the DHPG-containing medium, the amounts of DHPGppp present were 0.150 pmol/10^6 cells for the resistant sample, 0.152 pmol/10^6 cells for the sensitive sample, and 0.125 pmol/10^6 cells for the uninfected sample. After 24 h, these totals had been reduced by 90% to 0.015, 0.014, and 0.014 pmol/10^6 cells, respectively (Fig. 10). The catabolism curves for the resistant and sensitive samples were nearly identical.

The HPMPCpp was dephosphorylated much more slowly than the DHPGppp. At the time of drug removal, the amounts of HPMPCpp were 0.046 pmol/10^6 cells for the resistant sample, 0.045 pmol/10^6 cells for the sensitive sample, and 0.051 pmol/10^6 cells for the uninfected sample. These totals did not begin to decrease significantly until after 6 h postinfection; at that time, the resistant sample had 0.048 pmol/10^6 cells, the sensitive sample had 0.043 pmol/10^6 cells, and the uninfected sample had 0.036 pmol/10^6 cells. By 24 h postinfection, the totals were 0.022 pmol/10^6 cells for the resistant sample, 0.022 pmol/10^6 cells for the sensitive sample, and 0.018 pmol/10^6 cells for the uninfected sample. Thus, nearly 50% of the initial HPMPCpp was still present in infected cells 24 h later. The catabolism curves for the resistant samples and the sensitive samples closely followed each other (Fig. 11).
Fig. 10. Dephosphorylation of intracellular DHPGppp over a course of 24 hours in cells infected with resistant, sensitive, or no virus.

Fig. 11. Dephosphorylation of intracellular HPMPCpp over a course of 24 hours in cells infected with resistant, sensitive or no virus.
The half-lives of DHPGppp HPMPCpp in the resistant, sensitive, and uninfected samples were calculated using the InStat® statistical program. The data for the dephosphorylation curves were entered and standard curves were obtained for each sample. A value that represented 50% of the starting amount of DHPGppp or HPMPCpp was entered and a time for that value was estimated. The results are summarized in Table 4.

96-hour time-course metabolism experiment

To measure the differences in drug metabolism over 96 h, cells were infected with resistant, sensitive, or no virus and treated with 10 μM DHPG or 1.0 μM HPMPC and incubated for 96 h. Cells infected with each virus type were harvested at 24, 48, 72, and 96 h and the amounts of active forms of drugs were measured.

In the case of DHPG, the total amounts of DHPGppp produced after 24 h were 0.135 pmol/10^6 cells for the resistant sample, 0.152 pmol/10^6 cells for the sensitive sample, and 0.120 pmol/10^6 cells for the control sample. At 96 h post-infection, the amounts had increased to 0.627 pmol/10^6 cells for the resistant sample and 0.232 pmol/10^6 cells for the sensitive sample, and had decreased to 0.086 pmol/10^6 cells for the control sample (Fig. 12).

A similar effect was observed with HPMPC. At 24 h postinfection, the amount of HPMPCpp was 0.030 pmol/10^6 cells for the resistant sample, 0.042 pmol/10^6 cells for the sensitive sample, and 0.022 pmol/10^6 cells for the control sample. After 96 h of incubation, the amounts were 0.241 pmol/10^6 cells in the resistant sample, 0.118 pmol/10^6 cells in the sensitive sample, and 0.048 pmol/10^6 cells in the control sample (Fig. 13).
Table 4  
Half-lives (in hours) of DHPGppp and HPMPCpp in cells infected with resistant, sensitive, or no virus

<table>
<thead>
<tr>
<th></th>
<th>DHPG-treated</th>
<th>HPMPC-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>9.8</td>
<td>24.8</td>
</tr>
<tr>
<td>Sensitive</td>
<td>10.8</td>
<td>25.3</td>
</tr>
<tr>
<td>Uninfected</td>
<td>11.8</td>
<td>16.6</td>
</tr>
</tbody>
</table>

The progression of the cytomegalovirus infection over a period of 96 h causes a concomitant increase in cytotoxicity. Because of this, each sample was adjusted for cytotoxicity by determining cell numbers from the parallel sets of flasks. At 96 h postinfection, the cell numbers for all sensitive and uninfected samples were between 64% and 78% of the 24-h cell totals. However, the cell numbers for the DHPG and HPMPC-treated resistant samples were 47.5% and 41.7%, respectively.

_Virus yield results for 96-hour assay_

To explain why phosphorylation continued to increase in the cells infected with the resistant virus, a virus-yield assay over a period of 96 h was performed. This was done to support the hypothesis that the increase in the levels of DHPGppp and HPMPCpp in resistant-infected cells was related to virus production.

The resistant virus was able to grow when treated with either 10 μM DHPG or 1.0 μM HPMPC. The rates of growth were remarkably similar, although the virus titers were about 2-fold higher in the DHPG-treated cells. At 24 h postinfection, the titer from the DHPG-treated cells was $6.6 \times 10^3$ PFU/ml, the titer from the HPMPC-treated cells was $9.0 \times 10^3$ PFU/ml, and the titer from
Fig. 12. Production of DHPGppp in cells infected with resistant, sensitive, or no virus and treated with 10 μM DHPG for a period of 96 hours.

Fig. 13. Production of HPMPCpp in cells infected with resistant, sensitive, or no virus and treated with 1.0 μM HPMPC for a period of 96 hours.
the untreated cells was $7.5 \times 10^3$ PFU/ml. At 48 h, the resistant virus titers had jumped over 10-fold in the treated cells and over 100-fold in the untreated cells. Between 48 and 72 h postinfection, the titers continued to increase rapidly, and between 72 and 96 h postinfection the titers began to level off. At 96 h postinfection, the resistant virus titers were $8.9 \times 10^5$ PFU/ml from the DHPG-treated cells, $3.5 \times 10^5$ PFU/ml from the HPMPC-treated cells, and $2.9 \times 10^6$ PFU/ml for the placebo-treated sample (three to nine times higher than the drug-treated cells). Although the rate of increase of the virus titers had slowed during the final 24 h of the experiment, they still increased during that time (Figs. 14, 15).

The sensitive virus grew in media containing DHPG and HPMPC at a similar rate as the resistant virus for the first 24 h after infection. At 24 h postinfection, the titers from the DHPG- and HPMPC-treated cells were actually higher than those of the corresponding cells of the resistant virus, at $2.7 \times 10^4$ PFU/ml and $3.9 \times 10^4$ PFU/ml, respectively. However, by 72 h postinfection the titers had began to drop in the HPMPC-treated cells, and the DHPG-treated titer had increased only slightly. By 96 h postinfection, both the DHPG- and HPMPC-treated samples had dropped in titer. Final titers for the sensitive virus samples were $9.0 \times 10^4$ PFU/ml from the DHPG-treated cells and $6.7 \times 10^4$ PFU/ml from the HPMPC-treated cells (Figs. 14, 15). In contrast, the placebo-treated titer was $2.8 \times 10^7$ PFU/ml, and it had increased logarithmically throughout the experiment.

In summary, a general pattern of infection emerged during the 4-day course of the experiment. The resistant virus continued to grow in spite of antiviral treatment, although the rate of growth was slow. The treated sensitive virus grew more quickly at first, but the treatment soon caused a drop in virus
Fig. 14. Virus production in cells infected with resistant or sensitive virus and treated with 10 µM DHPG for a period of 96 hours.

Fig. 15. Virus production in cells infected with resistant or sensitive virus and treated with 1.0 µM HPMPC for a period of 96 hours.
titers by 72 h postinfection. The placebo-treated sensitive virus continued to grow throughout the experiment.

**Correlation of drug phosphorylation to virus titers**

Upon comparing the 96-h phosphorylation data with the 96-h virus yield data (Figs. 12-15), a striking similarity was noted between the phosphorylation curves for the resistant-infected cells and the virus production curves from similar samples. In both the DHPG and HPMPC-treated cells, the data curve of the amounts of active form of the compounds closely matched the virus titers obtained in the 96-h virus time course. In both cases, the resistant-infected cells continued to produce phosphorylated drug/progeny virus for the entire experiment, while the amounts of drug/virus produced by the sensitive infected cells began to drop or level off after 48 h postinfection.

To test this relationship, r- and r²-values for the two curves were calculated using the InStat® program. These values are tests of correlation for linear regression, which is used to calculate the relationship between two different sets of data. The closer the r-value is to 1.0, the more the two sets of data are related, and the further the r-value is from 1.0, the less they are related. (Two data sets that produce a curve with a slope of 1.0 are directly related.) The r²-value is simply the square of the r-value, and is a more robust test of significance. The same tests were performed for the sensitive-infected cell data, and the results are shown in Table 5.

The correlation between the production of DHPGppp and HPMPCpp by resistant-infected cells and virus production is strong. Two-tailed t-tests of the r-value showed significance of p<0.02 for the HPMPC-treated samples and p<0.005
for the DHPG-treated samples. This is not surprising, since the resistant virus continued to grow during the treatment, and any increase in resistant virus was expected to stimulate cellular kinases to phosphorylate more DHPGppp or HPMPCpp. The correlation between virus production and drug phosphorylation by the cells infected with sensitive virus is much weaker and was not found to be significant by t-test of r-values. This is probably due to the fact that virus production was inhibited as the production of DHPGppp or HPMPCpp increased.

Table 5
Correlation between 96-hour virus production curves and 96-hour phosphorylation curves in cells infected with resistant or sensitive virus and treated with $10^{-6}$ M DHPG or $1.0 \mu$M HPMPC

<table>
<thead>
<tr>
<th></th>
<th>DHPG, 10 µM</th>
<th></th>
<th>HPMPC, 1.0 µM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sensitive</td>
<td>resistant</td>
<td>sensitive</td>
<td>resistant</td>
</tr>
<tr>
<td>r-value</td>
<td>0.7445</td>
<td>0.9960\textsuperscript{a}</td>
<td>0.6416</td>
<td>0.9706\textsuperscript{b}</td>
</tr>
<tr>
<td>r\textsuperscript{2}-value</td>
<td>0.5543</td>
<td>0.9920\textsuperscript{a}</td>
<td>0.4117</td>
<td>0.9420\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}p<0.005
\textsuperscript{b}p<0.02
In these studies it was first demonstrated that a plaque-purified virus derived from the GCV-r strain of MCMV (Smee et al., 1995) was resistant to DHPG, HPMPC, and foscarnet (Table 1). This was followed up by a virus growth study over time in which resistant and sensitive viruses were propagated in 10 μM DHPG of 1.0 μM HPMPC. During the virus-yield experiment (Figs. 14, 15), the resistant virus continued to grow, albeit more slowly than the sensitive "wild-type" strain. After 96 h, the virus titer in the resistant sample treated with 10 μM DHPG was 10-fold greater than the titer in the flask infected with the sensitive sample (Fig. 14), while a similar relationship was observed in the flask treated with 1.0 μM HPMPC, although the resistant titer was only five times greater (Fig. 15). These results confirm the ability of the resistant strain to grow in the presence of the two compounds.

The experiments measuring the phosphorylation of DHPG and HPMPC showed that there is no significant difference in the amount of active forms of the drugs produced in infected cells by the resistant and sensitive viral strains. The extent of drug phosphorylation in resistant virus-infected, sensitive virus-infected, and uninfected cells was very similar (Figs. 8, 9). Throughout all the experiments the uninfected samples normally had lower levels of triphosphate production. However, the levels of DHPGppp or HPMPCpp produced by the resistant, sensitive, and uninfected samples were dissimilar only after 24 h of incubation. At earlier time points, the levels produced by the three samples were, in many cases, identical. It appears that there is no metabolic mechanism during the first 24 h of infection, which would account for viral resistance to DHPG and HPMPC. In fact, after 96 h of incubation, there was much more
DHPGppp and HPMPCpp produced in the resistant-infected cells than in the sensitive-infected cells.

A similar observation is made with the dephosphorylation data. The dephosphorylation curves for the three infection categories show that the drugs are dephosphorylated at the same rate, and the half-lives of DHPGppp and HPMPCpp in cells infected with both types of virus are nearly equal (Figs. 10, 11; Table 4). In addition, the amounts of active form of each drug after 24 h are the same for the resistant and sensitive virus. There appears to be no mechanism that would allow the resistant virus to catabolize the active forms of the drugs more rapidly than the cells infected with the sensitive virus or the uninfected cells.

Changing the parameters of multiplicity of infection (MOI) and the concentration of the compound did not appear to demonstrate any significant differences in drug phosphorylation in cells infected by the two strains of virus. If the sensitive virus encoded a unique drug-phosphorylating enzyme, and resistance to DHPG and HPMPC was due to a mutation in this enzyme, one might have observed a major difference in phosphorylation rates in cells infected with this virus as the MOI increased. The slight enhancement in drug phosphorylation correlated with increasing MOI is consistent with the known observation that host cell enzyme activity is stimulated by cytomegalovirus infection (Stinski, 1990).

In light of the results from the virus yield experiment, which showed that the resistant strain continues to produce virus over the course of the infection, the results of the 96-h metabolic experiment are expected: The resistant virus would of course continue to stimulate host cell kinases to produce active forms of the drug, since the virus is less sensitive to inhibition by those compounds. The
sensitive virus, on the other hand, would eventually stimulate the cells to produce enough active compounds that they would inhibit further virus production. The strong correlation between the 96-h virus production curve for the resistant virus and the 96-h drug phosphorylation curve supports the view that the infection stimulates phosphorylation. In addition, since viral infection stimulates host cell enzymes (Stinski, 1990), this explains why the uninfected cells produced the least amount of phosphorylated drugs.

As mentioned previously in this thesis, viral resistance to drugs such as DHPG and HPMPC may be the result of two general mechanisms: decreased phosphorylation of the parent compound in infected cells, or reduced ability of the compound to inhibit the viral DNA polymerase (Sullivan et al., 1993). These experiments were performed to study the possibility that the resistance of the mutant strain was due to a change in a viral enzyme that would cause a decrease in drug phosphorylation in infected cells. Since drug phosphorylation was not reduced in cells infected with the resistant virus, this strengthens the second hypothesis that resistance is due to a mutation in the viral DNA polymerase.

Another piece of data implicating the viral DNA polymerase in drug resistance is that the resistant strain of virus is not only resistant to drugs requiring phosphorylation (DHPG, HPMPC), but also to a drug that does not require phosphorylation (foscarinet). Foscarinet can exert its antiviral effect without modification (Oberg, 1989). Thus, resistance is most likely due to diminished affinity of the MCMV DNA polymerase to these drugs, possibly the result of a point mutation that affects the enzyme's active site.
CONCLUSION

C127I cells infected with a drug-resistant strain of murine cytomegalovirus do not phosphorylate DHPG or HPMPC differently than cells infected with the sensitive cytomegalovirus strain or uninfected C127I cells. Metabolism and catabolism of DHPG and HPMPC in resistant, sensitive, and uninfected cells were essentially similar during the first 24 h of infection. The phosphorylation of DHPG and HPMPC by the resistant and sensitive virus strains was not significantly affected by changing the multiplicity of infection or concentration of compound. Cells infected with the resistant strain of virus continued to produce virus when treated with DHPG or HPMPC at concentrations that caused the sensitive strain to eventually stop replicating. Production of virus in the resistant strain was closely correlated with production of the active forms of DHPG and HPMPC. This correlation was also evident in cells infected with the sensitive strain of virus, but was not as strong. These data are consistent with the fact that cytomegalovirus infection stimulates host cell kinase activity, thus indirectly enhancing drug phosphorylation. Based on these results, the mode of resistance in the mutant strain of virus does not appear to be due to differences in the ability of cells infected with this virus to metabolize or catabolize DHPG or HPMPC. This implies that a mutation in the viral DNA polymerase may be responsible for drug resistance.
REFERENCES


Neyts, J., Snoeck, R., Schols, D., Balzarini, J., and De Clercq, E. (1990) Selective inhibition of human cytomegalovirus DNA synthesis by (S)-1-(3-hydroxy-2-
phosphonylmethoxypropyl)cytosine [(S)-HPMPC] and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Virology 179, 41-50.


