Effect of 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine on Human Cytomegalovirus Replication In Vitro

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We studied the effect of a novel purine acyclic nucleoside, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), on human cytomegalovirus (HCMV) replication. The susceptibility of HCMV to this drug was monitored in cell culture by plaque reduction assay. HCMV replication of various strains was inhibited to the extent of 50% by 1 to 5 μM DHPG. DHPG was highly specific in its anti-HCMV activity, since at concentrations as high as 100 μM it did not exert any detectable inhibitory effect on uninfected cell macromolecular synthesis and cell growth. At concentrations of 2 to 4 μM, the drug inhibited the synthesis of six virus-specific polypeptides with molecular weights of 200,000 (VP200), 150,000 (VP150), 67,000 (VP67), 54,000 (VP54), 32,000 (VP32), and 27,000 (VP27) up to 96 h after infection. HCMV DNA synthesis was also considerably suppressed at concentrations of 2 to 4 μM DHPG. Upon removal of the inhibitor, however, viral DNA synthesis resumed and infectious virus reappeared, indicating that this inhibition was a virostatic reversible-type inhibition.

Ideal antiviral chemotherapy should be specific for the inhibition of viral replication with minimum toxicity to the host. Of the existing antiviral compounds, acyclovir (ACV) was discovered to be a potent and selective inhibitor of herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), with little effect on uninfected cells (5). A virus-encoded thymidine kinase (TK) is required to activate ACV in infected cells (5). ACV has been found to have limited anti-human cytomegalovirus (HCMV) activity (4, 11, 13) since HCMV does not encode virus-specific TK enzyme in infected cells (6, 16).

Recently, four companies (Syntex Inc., Biologics Co., Wellcome Research Laboratories, and Merck Sharpe and Dohme Research Laboratories) have independently developed a novel guanosine analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). Besides its extreme potency against HSV-1 and HSV-2 (1, 3, 12, 14, 15), DHPG has been demonstrated to effectively inhibit HCMV replication in vitro (14). In this communication, we report the in vitro antiviral activity of DHPG and its mode of action on viral DNA replication and virus-specific protein synthesis. Our results confirm and extend the observations made by other investigators (14).

MATERIALS AND METHODS

Chemicals and radiochemicals. DHPG was synthesized and provided by Syntex Inc., Palo Alto, Calif. [3H]-labeled amino acids and [5-3H]UTP were purchased from New England Nuclear, Boston, Mass. Cells and virus. Human fibroblastic diploid cells, WI-38 strain, were obtained from the American Type Culture Collection, Rockville, Md. Cells at passages 20 to 26 were used. HCMV strains Clegg and TW087 (isolated from semen and cervix, respectively, by E.-S. Huang), strain Towne (isolated from a congenitally infected baby), strain BT1493 (isolated from an organ transplant), and strain Major (isolated from a prostate) were used in this study. Strains Towne and BT1493 were provided by S. A. Plotkin, and strain Major was provided by F. Rapp.

Synthesis of [3H]-labeled RNA to HCMV strain Towne DNA. The virus obtained from extracellular fluids was purified by two cycles of ultracentrifugation in sucrose and CsCl gradients, as described elsewhere (9). The purified virus was then subjected to pronase digestion and phenol extraction. Viral DNA which banded in the CsCl equilibrium gradient at a density of 1.716 g/cm3 was used for the preparation of [3H]eRNA, with [3H]UTP as the radiolabeled substrate, according to the procedure of Huang (8).

c-RNA-DNA membrane hybridization. Using slightly modified versions of the procedures of Gillespie and Spiegelman (7) and Huang (8), we determined the effect of DHPG on viral DNA synthesis. Briefly,
Towne virus-infected and mock-infected WI-38 cells were lysed with 10 mM Tris-hydrochloride (pH 8.0)–10mM EDTA–1% sodium dodecyl sulfate and then digested with pronase (1 mg/ml) for 3 h at 37°C. The mixture was extracted with phenol and precipitated with alcohol. To remove RNA, the obtained nucleic acid was incubated for 3 h at 37°C with heat-pretreated pancreatic RNase A (40 μg/ml) in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA. After further purification by one more cycle of phenol extraction and alcohol precipitation, the DNA was dissolved in 1× SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate). For [3H]cRNA-DNA membrane hybridization, the infected or mock-infected cell DNA was first denatured in 0.5 N NaOH for 2 h at 37°C and then neutralized to a final pH of 7.4 with 1.1 N HCl in 0.2 M Tris. The mixture was then adjusted to 6× SSC, and single-stranded DNA was immobilized onto a nitrocellulose filter. After vacuum drying at 80°C for 2 h, the DNA-immobilized filters were prehybridized at 66°C for 1 h in buffer A (6× SSC, 0.1% sodium dodecyl sulfate, and 1 mg of yeast tRNA per ml). Then HCMV [3H]cRNA at 200,000 cpm (specific activity, 1.2 × 10⁷ cpm/μg) in buffer A was applied to each filter in glass scintillation vials. The hybridization was performed at 66°C for 18 h. After thorough washing with 2× SSC, the nitrocellulose filters were treated with RNase A to remove any unhybridized RNA. After the filters were dried, radioactivity was determined with a liquid scintillation counter.

Effect of DHPG on viral protein synthesis in polyacrylamide gel electrophoresis. WI-38 monolayers were mock or virus infected (multiplicity of infection, 1 to 2). After adsorption for 2 h the virus inoculum was replaced with minimum essential medium supplemented with 4% fetal calf serum and various concentrations of DHPG. At various times after infection, the culture cells were pulse-labeled for 2 h with [3H]-labeled amino acids (5 μCi/ml) in minimum essential medium containing 1/20 the strength of regular amino acids. The cells were sonicated postinfection in buffer B (10 mM Tris-hydrochloride, pH 8.0, 10 mM β-mercaptoethanol and 1 mM p-methylenephenylsulfonyl fluoride), and the labeled proteins were precipitated with 10% trichloroacetic acid. After being washed once each with ethanol and acetone, the proteins were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 9% acrylamide gels in a Tris buffer system as described by Laemmli (10). After the gels were subjected to fluorography (2), autoradiography was done by exposing the dried gel to X-ray film at −70°C for 7 days.

Plaque formation assay. The plaque formation assay was used to determine the antiviral activity of DHPG. Confluent WI-38 cells in six-well cluster plates were incubated with 200 PFU of virus in 0.2 ml of minimum essential medium–4% fetal calf serum. After incubation, the nonadsorbed virus was removed and the cultures were overlaid with 1% agarose in minimum essential medium–4% fetal calf serum and appropriate concentrations of DHPG. Four days later, a second agarose layer was added. On day 9 postinfection, the monolayers were fixed (with phosphate-buffered 10% Formalin), stained (with 0.03% crystal violet), and scored for plaques. Then the concentration needed to reduce plaque formation by 50% (ID₅₀) was calculated by counting the number of plaques in the control wells and comparing them with the surviving plaques in the presence of various concentrations of DHPG. We also used this assay to examine the release of infectious virus from drug-treated and post-drug-treated infected cell cultures.

RESULTS

Effect of DHPG on HCMV replication in cell cultures. The antiviral activity of DHPG was measured by plaque reduction assay and expressed in terms of ID₅₀, which was defined as the concentration of DHPG at which viral replication was inhibited by 50%. The results are summarized in Table 1. DHPG exhibited a potent anti-HCMV activity and had ID₅₀s ranging from 1 to 4.8 μM for five HCMV isolates tested.

Effect of DHPG on HCMV DNA synthesis in virus-infected WI-38 cells. The amount of viral DNA synthesis in virus-infected cells was monitored by nuclear acid hybridization with virus-specific [3H]cRNA as a probe. HCMV DNA synthesis was initiated approximately 20 h after infection and increased exponentially for up to 96 h (Fig. 1). In the presence of 2 or 4 μM DHPG, viral DNA synthesis was suppressed substantially compared with that of nontreated infected cells (Fig. 1). However, upon removal of the drug, viral DNA synthesis resumed (Fig. 1).

Comparison of protein synthesis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis between DHPG-treated and nontreated infected cell systems. The toxicity of DHPG for uninfected cells was monitored for 24 h (48 h after treatment) by measuring the inhibition of incorporation of [3H]labeled amino acids into proteins and [3H]thyidine into DNA. At a concentration of 100 μM DHPG, no significant inhibition was detected. Incorporation of radiochemicals into cell macromolecules was inhibited by 50% at 300 μM DHPG (ID₅₀). On the other hand, the synthesis of virus-specific proteins was inhibited at drug concentrations of 2 to 4 μM, which were well below the cytotoxic level (ID₅₀ = 300 μM).

Table 2 shows the inhibition of six virus-specific polypeptides (VP200, VP150, VP67, VP54, VP32, and VP27) by the drug for up to 96 h after infection. However, the expression of VP67 was not completely inhibited. These proteins were detected in non-drug-treated infected cells 48 h

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Virus passage</th>
<th>ID₅₀ (μM)</th>
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<tbody>
<tr>
<td>Towne</td>
<td>P-42</td>
<td>1.5</td>
</tr>
<tr>
<td>Major</td>
<td>P-29</td>
<td>4.8</td>
</tr>
<tr>
<td>BT1493</td>
<td>P-9</td>
<td>1.1</td>
</tr>
<tr>
<td>Clegg</td>
<td>P-4</td>
<td>1.0</td>
</tr>
<tr>
<td>TW087</td>
<td>P-28</td>
<td>1.5</td>
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of genome and _U, concentration of 2 or 4 FM immediately after final drug was adsorption. At various times after infection, virus DNA was extracted from the infected cells for virus replication content. Amounts of DNA and viral DNA was extracted from the infected cells for virus infection. After infection, virus-specific tk gene content of 2 and 4 FM DHPG, respectively; _A_—_A_ and _A_—_A_, recovery of DNA synthesis after removal of 2 and 4 FM DHPG.

after infection. Thus, these polypeptides should be virus-specific late antigens.

**DISCUSSION**

A recently developed ACV congener, DHPG, was demonstrated to be an extremely potent antiviral agent with low toxicity to normal cells (3, 12, 14, 15). The modes of action of DHPG and ACV are similar in that the herpes TK specifically phosphorylates each nucleoside and both compounds selectively inhibit viral replication over cell proliferation (1, 3, 5, 12, 14, 15). It has been observed that ACV and DHPG are totally inactive against HSV mutants which do not induce virus TK in infected cells (3, 5, 14, 15), indicating that a herpes-encoded TK enzyme is required to activate DHPG as well as ACV in infected cells.

Recently, DHPG was reported to have a marginal activity against Epstein-Barr virus (ID<sub>50</sub> = 32 μM) and to have no activity against HCMV even at a concentration as high as 390 μM (15). In contrast, Smee and colleagues (14) demonstrated that, besides its potency against HSV-1 and HSV-2, DHPG effectively inhibited a strain of HCMV (AD169) by 50% at 7 μM. In addition, at concentrations as low as 0.05 μM, DHPG reduced Epstein-Barr virus genome numbers in P3HR-1 cells by 50% (J. S. Pagano, J. W. Sixbey, and J.-C. Lin, J. Antimicrob. Chemother., in press; J.-C. Lin, M. C. Smith, and J. S. Pagano, submitted for publication). In the present investigation, we also demonstrate that DHPG is a potent and selective anti-HCMV agent with viral ID<sub>50</sub>s ranging from 1 to 5 μM, depending upon the virus strain used (Table 1). The ID<sub>50</sub> for cell growth was 300 μM. Our data are in reasonable agreement with the data reported by Smee and co-workers (14). The discrepancy between positive and negative results of anti-Epstein-Barr virus and anti-CMV activities of DHPG as reported by others may be due to the use of different methodologies. Smith et al. (15) examined the effect of DHPG on the expression of virus-specific antigens which were expressed despite the inhibition of viral DNA synthesis, whereas in this study and in the

![Time-course synthesis of virus-specific proteins in HCMV-infected WI-38 cells. Protein synthesis was studied by incorporation of 3H-labeled amino acids (5 μCi/ml) at various times after infection. The polyacrylamide gel was subjected to fluorography and autoradiography by exposing the dried gel to X-ray film at -70°C for 7 days. Numbers in column MW indicate molecular weight × 10<sup>3</sup>. M = mock infected. Arrows indicate inhibition of the virus-specific proteins by DHPG at the designated concentration.](http://aac.asm.org/)

![Effect of DHPG on HCMV strain Towne DNA replication in virus-infected WI-38 cells. The drug was added to the virus-infected cell cultures at a final concentration of 2 or 4 μM immediately after virus adsorption. At various times after infection, DNA was extracted from the infected cells for virus genome quantitation by [3H]cRNA-DNA membrane hybridization. Amounts of DNA and HCMV-specific [3H]cRNA applied to each filter were 20 μg and 4 × 10<sup>5</sup> cpm, respectively. The nucleic acid hybridization was performed as described previously (8). Symbols: ○, viral DNA content of cultures without drug treatment; _A_—_A_ and _A_—_A_, viral DNA content in the presence of 2 and 4 μM DHPG, respectively; _A_—_A_ and _A_—_A_, recovery of DNA synthesis after removal of 2 and 4 μM DHPG.](http://aac.asm.org/)
studies of Smee et al. (14), Pagano et al. (in press), and Lin et al. (submitted) the inhibition of HCMV and Epstein-Barr virus replication was determined by the inhibition of viral DNA and virus-specific protein syntheses and the reduction of virus progeny yield.

Quantitation of viral DNA synthesis, using cRNA-DNA hybridization, demonstrated a dramatic inhibition of HCMV DNA replication at 2 to 4 μM DHPG (Fig. 1). At these drug concentrations, the synthesis of five virus-specific late polypeptides (VP200, VP150, VP54, VP32, and VP27) was markedly inhibited (Fig. 2). The additional virus-specific polypeptide (VP67) which was inhibited substantially at an early stage of infection accumulated to a certain extent at a late stage of infection (Fig. 2). The resumption of VP67 (Fig. 2) and a gradual and low-level increase in the concentration of viral DNA sequences (Fig. 1) at a later stage of drug treatment may be due to degradation of the drug in cell cultures. After removal of the inhibitor from cell cultures, viral DNA synthesis resumed (Fig. 1) and infectious virus became detectable. These phenomena suggest that the inhibitory effect by DHPG of HCMV replication was a virostatic reversible type of inhibition.

In spite of the structural resemblance of DHPG to ACV, the therapeutic index of DHPG against HCMV is much higher in cell culture than that of ACV. The broader antiviral spectrum, greater inhibitory effect, lower cytotoxicity, and higher solubility of DHPG (14, 15) compared with other existing antitherpepetic compounds make DHPG a potential candidate for antitherpes chemotherapy.

The ineffectiveness of DHPG on TK− mutants of HSV-1 and HSV-2 (3, 14, 15) suggests the importance of the virus-encoded TK enzyme in the molecular action of this drug. Since HCMV does not encode a virus-specific TK, the mechanism by which DHPG exhibits the inhibitory effect against HCMV in vitro is still unclear. It is one of our major interests to study the possible role of virus-stimulated cellular TK enzyme (6, 16) in regard to the anti-HCMV activity of DHPG.

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LITERATURE CITED