Metabolism of 9-(1,3-Dihydroxy-2-propoxymethyl)guanine, a New Anti-herpes Virus Compound, in Herpes Simplex Virus-infected Cells*

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The metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), one of the most promising new anti-herpes virus compounds, in HeLa cells infected with herpes simplex virus type 1 was compared with that in the uninfected HeLa cells. In the virus-infected cells, the uptake of DHPG was enhanced and the major metabolites were found to be the mono-, di-, and triphosphate derivatives. The formation of these metabolites was dependent on the extracellular concentration of DHPG (0.5 to 5.0 µM). Virus-induced thymidine kinase was capable of phosphorylating DHPG to its monophosphate which could be further phosphorylated to the di- and triphosphate derivatives by the host cellular enzymes. Incorporation of the DHPG into DNA was observed in virus-infected cells. In contrast with 9-(2-hydroxyethoxymethyl)guanine, DHPG seemed not to serve as a chain terminator, but to be incorporated internally into DNA strands.

DHPG, a newly synthesized guanosine analog, was found to have potent activity against HSV (1-3), varicella-zoster virus (1), cytomegalovirus (2, 3), and Epstein-Barr virus (3). DHPG is more potent and the spectrum of susceptible viruses to the compound is broader than that to acyclovir. Furthermore, HSV mutants, which are resistant to acyclovir because of altered thymidine kinase or DNA polymerase, were as sensitive to DHPG as the parental HSV (3). This unique spectrum of activity suggests the potential use of DHPG in the clinic for the treatment of herpesvirus infection. In order to have a better understanding of the mechanism of action of DHPG, it is important to explore its metabolism in virus-infected cells. This paper presents evidence of phosphorylation of DHPG, leading to the formation of DHPGTP, and of incorporation of the nucleoside analog into DNA.

MATERIALS AND METHODS

Chemicals and Enzymes—DHPG, DHPGMP, and [3H]DHPG (16 Ci/mmol) were donated by Syntex (U.S.A.), Inc., Palo Alto, CA. Other nucleosides, nucleotides, and dithiothreitol were purchased from Sigma. Proteinase K and micrococcal nuclease were obtained from Boehringer Mannheim GmbH. Spleen phosphodiesterase was from Worthington and Escherichia coli alkaline phosphatase was from Sigma.

Cells and Virus—Cells were grown at 37°C in RPMI 1640 medium (GIBCO) containing 100 µg/ml of Kanamycin supplemented with 5% horse serum and 5% fetal calf serum for HeLa S1 and Vero cells, respectively. HSV-1 (strain KOS) was maintained as described previously.

Preparation of HSV-1 Thymidine Kinase and Human Erythrocyte Lysate—A highly purified enzyme was prepared from HSV-1-infected HeLa BU (TK-) cells using affinity column as described previously (5). Twenty-five ml of fresh heparinized blood was diluted with phosphate-buffered saline (0.14 M NaCl, 4 mM KC1, 0.94 mM Na2HPO4, 0.15 M KH2PO4). Erythrocytes were pelleted by centrifugation and 4 volumes of cold water were added to lyse the cells. After centrifugation at 12,000 × g for 20 min, supernatant was collected and solid ammonium sulfate was added to 70% saturation. Precipitate was removed by centrifugation and 4 volumes of cold water were added to lyse the cells. After centrifugation at 12,000 × g for 20 min, supernatant was collected and solid ammonium sulfate was added to 70% saturation. Precipitate was removed by centrifugation and 4 volumes of cold water were added to lyse the cells. After centrifugation at 12,000 × g for 20 min, supernatant was collected and solid ammonium sulfate was added to 70% saturation. Precipitate was removed by centrifugation and 4 volumes of cold water were added to lyse the cells. After centrifugation at 12,000 × g for 20 min, supernatant was collected and solid ammonium sulfate was added to 70% saturation. Precipitate was removed by centrifugation and 4 volumes of cold water were added to lyse the cells.

Preparation of Acid-soluble and -insoluble Fractions—HeLa S1 cells in 25-cm² flasks were infected with 5 plaque-forming units/cell of HSV-1. Various concentrations of [3H]DHPG were added at 0 h postinfection and the cells were harvested at 6 h and washed three times with phosphate-buffered saline. The cell pellets (5 × 10⁷ cells) were extracted with 50 µl of 1.5 M perchloric acid at 0°C for 30 min. The extract was neutralized with 1 N potassium hydroxide (77 µl) and 23 µl of 0.5 M potassium phosphate buffer (pH 7.4) was added. Precipitate was removed by centrifugation and the supernatant was used as the acid-soluble fraction. The precipitate from the perchloric acid extraction was washed twice with 1.5 M perchloric acid and designated as the acid-insoluble fraction.

Alkaline Hydrolysis of Acid-insoluble Fractions—The acid-insoluble fraction was dissolved in 100 µl of 1 M NaOH and incubated for 2 h at 37°C. Four hundred µl of 5% trichloroacetic acid was added to the solution. The supernatant and an additional 200-µl wash of trichloroacetic acid were neutralized with KOH solution and counted in a liquid scintillation counter as the alkali-labile fraction. The pellet was redissolved in dimethyl sulfoxide and counted as the alkali-stable material.

HPLC Analysis—An anion exchange column, Partisil 10 SAX/25 (Whatman), was used in a solvent system with a gradient of potassium phosphate buffer (pH 6.6) from 0.03 to 0.15 M. The solvent system for a reverse phase column, C18 (ALTECH), was acetonitrile, 0.03 M acetic acid (1:5000, v/v).

Sodium Iodide and Cesium Sulfate Density Gradient Centrifugation—HSV-1-infected cells were washed with phosphate-buffered saline and lysed with 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1 M NaCl, 0.5% Sarkosyl, 5.5% sodium dodecyl sulfate (250 to 300 µl). After 30 min at room temperature, proteinase K was added to the lysis (6 mg/ml) and the mixture was incubated overnight at 37°C.

The mixture (300 µl) was directly layered on sodium iodide solution (4.7 ml) which contained 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 0.5 mg/ml of NaH2SO4, and 1.53 to 1.54 g/cm³ of sodium iodide. Centrifugation was performed at 45,000 rpm for 48 h at 20°C using a Beckman SW 50.1 rotor.

For cesium sulfate density gradient centrifugation, the mixture was extracted with 1/5 volume of phenol and the aqueous phase was further extracted with ether. One-tenth volume of 3 M sodium acetate buffer (pH 5.9) and 2.5 volume of ethanol were added to the aqueous phase.

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‡The abbreviations used are: DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; DHPGMP, DHPGDP, DHPGTP, 9-(1,3-dihydroxypropoxymethyl)guanine mono-, di-, and triphosphate, respectively; HSV-1, herpes simplex virus type 1; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; HPLC, high performance liquid chromatography.

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RESULTS

Uptake of DHPG in Virus-infected and Mock-infected HeLa Cells—HeLa cells were infected with HSV-1 and various amounts of \([^{3}H]DHPG\) were added at 0 h post-virus adsorption. The cells were harvested at 6 h post-virus adsorption. The uptake of DHPG into an acid-soluble and -insoluble fraction of these cells was analyzed. The results are presented in Table I. The more DHPG added, the more uptake into both fractions was observed, with the majority of \([^{3}H]DHPG\) uptake taking place in the acid-soluble fraction. When 5 \(\mu M\) DHPG was added to mock-infected HeLa cells, the uptake of DHPG into the acid-soluble fraction was similar to that in infected cells exposed to 0.5 \(\mu M\) DHPG. The uptake of DHPG into the acid-insoluble fraction was about the same as that of infected cells exposed to between 1.0 and 2.0 \(\mu M\) DHPG. More than three-quarters of \([^{3}H]DHPG\) in the acid-insoluble fraction could be rendered acid-soluble following treatment with 1 N KOH.

HPLC Analysis of DHPG Metabolites in Acid-soluble Fraction—The acid-soluble fraction of virus-infected and mock-infected HeLa cells after the treatment with \([^{3}H]DHPG\) was analyzed by HPLC using a Partisil 10 SAX column system. The results are shown in Fig. 1. In addition to the radioactivity associated with the DHPG marker fraction (Fraction 5), three other metabolites of DHPG were found in either virus-infected or mock-infected cells. One of the metabolites (Fraction 20) was co-eluted with a DHPGMP marker. The amount of each metabolite formed in infected cells was dependent on the extracellular concentration of DHPG. In comparison with the virus-infected cells, very small amounts of metabolites could be found in the mock-infected cells and most of the radioactivity was in the DHPG fraction. When the extract from HSV-infected cells was treated with alkaline phosphatase for 4 h and then analyzed with the same HPLC system, the radioactivity associated with each of these metabolites was decreased with a corresponding increase of radioactivity associated with the DHPG fraction (Fig. 1D). When alkaline phosphatase-treated sample was subjected to the reverse phase C\(_{3}\) HPLC column system, the majority of the radioactivity was found to be associated with DHPGMP.

In order to identify these three metabolites further, each compound was isolated by HPLC and subjected to DEAE-Sephadex column chromatography in urea-containing buffer (Fig. 2). This allows the separation of nucleotides based on the number of ionic charges on phosphate groups. The metabolites associated with fractions 19 and 20 (Compound 1), 25 and 26 (Compound 2), and 52 to 55 (Compound 3) in Fig. 1 eluted slightly faster than GMP, GDP, and GTP, respectively. These results indicate that Compounds 1, 2, and 3 were mono-, di-, and triphosphate derivatives of DHPG.

In Vitro Formation of Metabolites—Highly purified HSV-1 thymidine kinase was incubated with \([^{3}H]DHPG\) in the presence of ATP. The major metabolite formed co-eluted with DHPGMP on HPLC/Partisil 10 SAX system as shown in Fig. 3. \([^{3}H]DHPGMP\) was isolated and then incubated with human erythrocyte lysate in the presence of ATP. As shown

\(^{1}\) Y.-C. Cheng, S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow, unpublished observations.

\(^{2}\) Incorporation of DHPG into DNA—HSV-1-infected cells were treated with 2 \(\mu M\) \([^{3}H]DHPG\) for 8 h and the cell lysate was subjected to sodium iodide density gradient centrifugation. Although this method allows separation of virus DNA (density \(=\) 1.52) from host cell DNA (density \(=\) 1.50), they were not distinguishable for \([^{3}H]DHPG\)-labeled DNA, which distributed into the fractions between the two densities (Fig. 5A).
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Fig. 2. DEAE-Sephadex column chromatography of [3H]DHPG metabolites. An aliquot of Compound 1 (A), 2 (B), or 3 (C) (as designated in the text), together with 100 nmol each of GMP, GDP, and GTP, were applied on a DEAE-Sephadex column (0.3 x 25 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 7 M urea. Elution was performed with a NaCl gradient (30 ml) from 0 to 150 mM in the same buffer as above. One-ml fractions were collected. After absorbance at 260 nm was measured to locate the elution position of GMP, GDP, and GTP (arrows), each fraction was mixed with 10 ml of ACS scintillant (Amersham) and counted in a liquid scintillation counter to determine the elution of the [3H]DHPG metabolites.

Fig. 3. In vitro phosphorylation of [3H]DHPG with HSV-1 thymidine kinase. [3H]DHPG (0.26 pCi, 7.3 nmol) was incubated at 37 °C in a mixture (100 μl) containing 4.0 mg/ml of bovine serum albumin, 12.0 mg/ml of creatine phosphate, 0.4 mg/ml of creatine phosphokinase, 67 mM Tris-HCl buffer (pH 7.5), 3 mM sodium fluoride, 0.6 mM dithiothreitol, 9 mM ATP-Mg²⁺, and 4 units of HSV-1 thymidine kinase. Acid-soluble fractions at 0 h (A) and 2 h (B) of incubation were analyzed by HPLC using Partisil 10 SAX column as described under "Materials and Methods." Elutions of standard DHPG and DHPGMP were indicated by arrows.

It is quite possible that [3H]DHPG-labeled DNA has different density from normal DNA, which makes the virus and host DNA indistinguishable by density.

When these fractions were treated with alkali, a portion of radioactivity became acid-soluble and the radioactivity in the alkal-stable form resulted in two peaks with densities 1.50 and 1.52, respectively (Fig. 5B).

Fig. 4. In vitro formation of di- and triphosphate derivatives from [3H]DHPGMP with human erythrocyte lysate. [3H]DHPGMP was prepared with HSV-1 thymidine kinase and purified by HPLC as described in the legend to Fig. 3. Aliquots of [3H]DHPGMP were incubated with 10 units of HSV-1 thymidine kinase for 5 h (A) or 80 μl of human erythrocyte lysate for 5 h (B) and 17 h (C) in the reaction mixtures described in the legend to Fig. 3. Reaction was terminated by the addition of perchloric acid and acid-soluble fraction was analyzed by HPLC using Partisil 10 SAX column as described under "Materials and Methods."

Fig. 5. Sodium iodide density gradient centrifugation by [3H]DHPG-labeled nucleic acid. HeLa S3 cells in a 25-cm² flask were infected with HSV-1 and incubated in medium (2 ml) containing [3H]DHPG (53 μCi, 9.3 nmol) for 8 h. Extraction of nucleic acid and centrifugation were as described under "Materials and Methods." Seventy-five μl of each fraction were collected. Twenty-five μl of each fraction were absorbed into fiber glass discs (Whatman GF/A) and the discs were washed three times with ice-cold 5% trichloroacetic acid and once with ethanol. When dry, the discs were counted in ACS scintillant (A). Five μl of 5 N NaOH was added to another 25 μl of each fraction. The mixtures were incubated at 37 °C for 2 h, absorbed into discs, and washed as above (B).
of radioactivity was associated with DHPGMP and other material, which was possibly dinucleotide resulting from incomplete digestion. These results indicate that DHPG was internally incorporated into DNA and therefore does not act as pure chain terminator for DNA synthesis.

**DISCUSSION**

Like several other selective anti-HSV nucleoside analogs (6-15), DHPG could be preferentially phosphorylated in HSV-infected cells. The primary metabolic scheme is shown in Fig. 7. In spite of two symmetrical —CH2OH groups at carbon 2 of the propoxyl group of DHPG, the phosphorylation of DHPG could take place at one of those two —OH groups by virus-induced thymidine kinase. Two stereoisomers at carbon 2 could be anticipated. It is probable that only one stereoisomer of DHPGMP which could be utilized for the further metabolism was formed since all the DHPGMP was converted to DHPGDP and DHPGTP by the erythrocyte lysate. However, the possibility that the enzyme(s) responsible for further phosphorylation could not distinguish the two stereoisomers requires further experimentation. It seems likely that the isomer formed should have a steric conformation similar to dGMP, but regardless of the outcome of this issue, the host cells apparently have an efficient system to carry out further phosphorylation as indicated in Fig. 1E. There was no accumulation of DHPGMP in mock-infected cells, although DHPG could be found in the acid-insoluble fraction. This implies that a cell enzyme could perform the initial phosphorylation of DHPG but in a relatively inefficient manner since other human herpesviruses are less susceptible to DHPG than HSV and these viruses do not induce virus-specific thymidine kinase (1-3). The host enzymes responsible for the phosphorylation of DHPGMP to DHPGDP could be GMP kinases since GMP could inhibit this process. A similar suggestion was also made recently (16). Although there are several GMP kinase isozymes (17), it is not clear whether there is a preference for a particular GMP kinase isozyme in this process.

The amount of accumulated DHPGTP in infected cells treated with DHPG at concentrations of 0.5, 1, 2, and 5 μM was 0.5, 1.2, 2.8, and 8.4 pmol/10⁶ cells, respectively. The amount of DHPG incorporated into the acid-insoluble fraction was found to be less than 5-propyl-dUrd (13). This suggested the possibility that DHPG is acting as a chain terminator such as acyclovir or possibly a pseudochain terminator such as arabinosyl adenine (19). A detailed study of the mode of DHPG (18) on virus DNA replication is in progress. Since a portion of DHPG incorporated into the acid-insoluble fraction is alkali-labile, the possibility is raised that either DHPG could be incorporated into RNA which is alkali-labile or into DNA with an alkali-labile linkage. Using the Cs₂SO₄ isopycnic centrifugation technique, all the radioactivity of [³H]DHPG in the nucleic acid from the infected cells was found to be with DNA. DHPG was also demonstrated to be incorporated into the internucleotide chain of DNA. The unpublished results of this laboratory using an in vitro DNA synthesis system catalyzed by HSV DNA polymerase further confirms the incorporation of DHPG into the internucleotide chain of DNA. The alkaline lability of DHPG in the internucleotide DNA chain or at the terminal of DNA is currently under investigation.

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