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In vitro and in vivo activities of phosphate derivatives of 9-(1,3-dihydroxy-2-propoxymethyl)-guanine against cytomegaloviruses

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Summary

The anti-cytomegalovirus activities of four phosphate derivatives of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) were evaluated against human, monkey and murine viruses. The 5′-mono-, 3′,5′-bis(mono-), and 3′,5′-cyclic monophosphate and 5′-homophosphonate forms of DHPG inhibited virus plaque formation at 1–15 μM. The cyclic phosphate and homophosphonate were more active than the other compounds against murine cytomegalovirus (MCMV) in vitro. In an in vivo MCMV infection model, DHPG homophosphonate and DHPG were equally effective at reducing mortality at ≥ 10 mg/kg. The cyclic phosphate was active at 10–20 mg/kg but toxic at ≥ 40 mg/kg. The phosphorylation of DHPG phosphate and DHPG phosphonate, as well as the inhibition of human cytomegalovirus DNA polymerase by their respective triphosphates, were also examined.

phosphate analogs of DHPG; antiviral activity; cytomegalovirus

Introduction

The deoxyguanosine analog, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) is a potent inhibitor of human cytomegalovirus in vitro [4,5,13,17,18]. Studies have shown that DHPG inhibits the replication of laboratory strains and clinical isolates of human cytomegalovirus by blocking virus DNA synthesis [8,18]. The triphosphate form of DHPG is an inhibitor of the viral-induced DNA polymerase [9]. Early viral polypeptide synthesis is also inhibited by DHPG in infected cells [8]. The remarkable activity of DHPG against these viruses prompted the synthesis of a new series of compounds which are phosphate derivatives of DHPG. The compounds are 9-(1,3-di-

hydroxy-2-propoxymethyl)guanine-monophosphate (DHPG-MP), -cyclic phosphate (DHPG-CP), also known as 2′-nor-cGMP, -bis (mono)-phosphate (DHPG-BMP), and 9-(1-hydroxy-4-phosphinyl-2-butoxymethyl) guanine (DHPG homophosphonate, DHPG-HP) (Fig. 1). DHPG-MP and DHPG-HP are racemic mixtures of what we refer to as the 3′ and 5′ phosphates (or phosphonates). The anti-herpes simplex virus activity evaluations and the synthetic procedures for these compounds will be treated in a separate communication (Prisbe, E.J., Martin, J.C., McGee, D.P.C., Barker, M.F., Smee, D.F., Duke, A.E., Matthews, T.R. and Verheyden, J.P.H., manuscript in preparation). In this report, data are presented showing anti-cytomegalovirus activities, cytotoxicity appraisals, enzymatic phosphorylation and DNA polymerase inhibition, and the comparison of efficacies of compounds in an in vivo model. The antiviral properties of 2′-nor-cGMP (DHPG-CP) have been independently described by Tolman et al. [19].

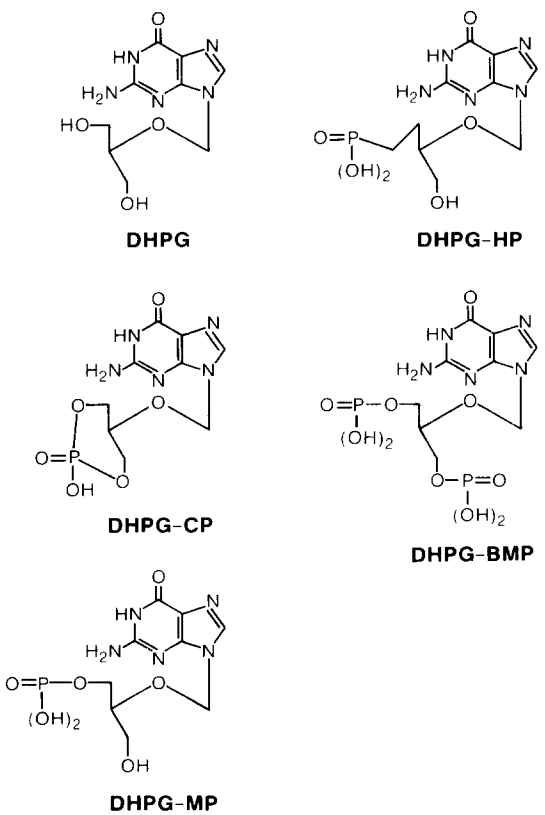

Fig. 1. Structural formulae of DHPG, DHPG-HP, DHPG-CP, DHPG-BMP, and DHPG-MP.

Materials and Methods

Chemicals

All antiviral compounds were synthesized at Syntex Research, Palo Alto, California. The triphosphate of DHPG and the diphosphate of DHPG-HP were made enzymatically by methods described previously [16]. Acyclovir triphosphate (ACV-TP) was a gift of Y.-C. Cheng, University of North Carolina, Chapel Hill, NC. Naturally occurring nucleotides (GMP, GDP, dATP, dCTP, and TTP), pyruvate kinase, human erythrocyte nucleoside diphosphokinase, and activated calf thymus DNA were purchased from Sigma Chemical Company, St. Louis, MO. [^{3}H]dGTP (12 Ci/mmol) was obtained from Amersham, Arlington Heights, IL. The antiviral compounds were dissolved in Eagle's medium (EMEM) and stored at -20°C for in vitro use, or made up fresh daily in saline for in vivo assays.

Cells

Human embryonic lung fibroblast (MRC-5) cells were purchased from Flow Laboratories, Inglewood, CA. Primary mouse embryo fibroblast (MEF) cultures were prepared from 17- to 18-day-old Swiss–Webster mouse embryos by trypsinization [12,15]. Both cell lines were maintained in EMEM containing 10% fetal bovine serum, 10 mM Hepes buffer, and 0.12% $NaHCO_3$.

Viruses

Human cytomegalovirus (HCMV) strains AD169 and Davis, vervet monkey cytomegalovirus (VCMV) strain CSG, and murine cytomegalovirus (MCMV) strain Smith were obtained from the American Type Culture Collection, Rockville, MD. Both HCMV and VCMV were grown and plaque-titered in MRC-5 cells. MCMV was propagated in weanling Swiss-Webster mice as described elsewhere [7]. MCMV titer was determined by plaquing in MEF cells.

Plaque reduction assays

Confluent monolayers of MEF and MRC-5 cells in 6-well Linbro microplates (Flow Laboratories, McLean, VA) were infected with approximately 150 plaque forming units (pfu) per well. After an adsorption period of 1.25 h the virus was aspirated, and EMEM overlay containing 0.5% sea plaque agarose (FMC Corporation. Rockland, ME), 2% fetal bovine serum, 50 μg of gentamicin per ml, and various concentrations of test compounds was applied. Three duplicate wells were used for each compound concentration and for each set of controls. After 4 days of incubation at 37°C in 5% CO_2, a second overlay was applied to HCMV plates to maintain cell viability. On days 3–6 (for MCMV and VCMV) and day 8 (for HCMV) a last overlay containing all the items mentioned above plus 0.002% (final concentration) neutral red dye was applied. Foci of virus were visible 4–24 h following neutral red overlay. Plaques were counted at 17× magnification with a Bellco plaque viewer. The evaluation of compound concentrations which reduced plaque numbers by 50% [50% inhibitory dose (ID_{50})] was done by a computer using a semilog probit analysis program [6].

Cell proliferation assays

To evaluate compound effects on cell propagation, 6-well Linbro microplates were seeded at 3×10^4 MRC-5 cells per well. 16–24 h after seeding medium was aspirated off and EMEM containing drug at various concentrations and 10% serum was added. After 3–4 days incubation (approximately 70% confluent monolayer) the wells were aspirated and cell numbers determined [17]. The drug concentration that showed 50% inhibition of cell growth (IC_{50}) was calculated by the probit method [6].

Enzyme assays

Guanylate kinase was purified from human erythrocytes based on the procedures described by Miller and Miller [10] and Boehme [3]. The activity was determined spectrophotometrically or radioisotopically as previously mentioned [1,10]. Pyruvate kinase was assayed by methods described by Miller and Miller [11]. Nucleoside diphosphokinase activity was assayed radioisotopically by quantitating the rate of conversion of [^{3}H]ATP to [^{3}H]ADP as described previously [11]. Phosphoglycerate kinase was assayed according to the methods of Miller and Miller [11], except that $NaH_2{}^{32}PO_4$ was used to produce 1,3-[^{32}P]diphosphoglycerate, and in the subsequent reaction, ^{32}P-labelled nucleoside triphosphates.

DNA polymerase extraction and purification.

MRC-5 cells seeded in roller bottles were infected at a multiplicity of infection of 1 with HCMV (AD 169) and incubated at 37°C until 100% cytopathic effect was achieved (approximately 7 days). Cells were harvested with glass beads in cold phosphate-buffered saline. Enzyme was released by sonication in 50 mM K_2HPO_4 on ice. The preparation was centrifuged at $30\,000 \times g$ for 30 min. The supernatant was added to a DE-52 (Whatman, Clifton, NJ) column which had been washed and equilibrated in 50 mM K_2HPO_4 containing 20% glycerol, 0.2 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol (DTT) and adsorbed for 30 min at 4°C. The column was then washed in 50 mM K_2HPO_4 and the enzyme was eluted with 200 mM K_2HPO_4. The above procedure was also followed to obtain the α DNA polymerase from uninfected MRC-5 cells.

DNA polymerase assay

The DNA polymerase assays were monitored by measuring the amounts of incorporation of tritium-labeled deoxyribonucleoside triphosphate into acid-precipitable product. The assay mixture contained 50 mM Tris-Cl (pH 8.0), 8 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg/ml bovine serum albumin, 625 μM dATP, dCTP and TTP. 150 mM KCl was added to the mix when the viral enzyme was used. In the presence of this concentration of salt the viral enzyme could be detected, while the cell polymerase activity was suppressed. [^{3}H]dGTP was used as the labeled substrate at concentrations of 2.0, 1.0, 0.5, 0.25 and 0.125 μM. The reaction was initiated with addition of HCMV or MRC-5 α DNA polymerase to the 100 μl reaction mixture. Samples were incubated at 37°C for 30 min and then 75 μl were spotted on glass filter paper (GF/C, Whatman). The samples were precipitated by washing the disks three times, 10 min each, in 10 mM sodium pyrophosphate solution containing 5% trichloroacetic acid, and once in

methanol. After drying, the disks were placed in 10 ml Aquasol (New England Nuclear, Boston, MA) and counted in a liquid scintillation counter.

Animal study

Groups of 16 weanling Swiss-Webster mice (Charles River Breeding Laboratories, Wilmington, MA) weighing approximately 11 g each were inoculated intraperitoneally with murine CMV. Each mouse received approximately 3×10^4 pfu of the virus to obtain an acute lethal infection. This dose was predetermined by an earlier titration experiment. All compounds were administered subcutaneously in multiple doses twice a day for 5 days, starting 6 h postinfection. Saline treated group served as the placebo. Deaths were recorded for 14 days after infection. The significance of differences in survival rates was evaluated by two-tailed Fisher Exact and Mann-Whitney tests.

Results

Antiviral and cytotoxic activities in culture

The antiviral activities of DHPG phosphate derivatives against human, vervet monkey and mouse CMVs are reported in Table 1. All four DHPG phosphate analogs had similar activities against VCMV and HCMV and were comparable to DHPG. DHPG-MP and DHPG-BMP showed less antiviral activity against MCMV compound than the other compounds, whereas DHPG-CP and DHPG-HP were more effective against MCMV than DHPG itself. All compounds inhibited cell proliferation in uninfected MRC-5 cells at levels above those showing antiviral effects (Table 1). DHPG-HP was the least and DHPG-CP the most inhibitory compound in these assays.

TABLE 1

Antiviral and anticellular activities in cell culture

Virus or cell	ID_{50} (μM)[a]				
	DHPG	DHPG-CP	DHPG-MP	DHPG-BMP	DHPG-HP
HCMV (AD169)[b]	4.5	5.9	11.7	8.0	6.2
HCMV (Davis)	4.9	6.1	6.2	6.1	5.8
VCMV (CSG)	3.6	2.4	5.2	5.8	5.3
MCMV (Smith)	6.2	1.6	15.9	28.1	1.3
	IC_{50} (μM)[a]				
MRC-5 cells	>1000	120	>1000	>1000	>1000

[a] Values are averages of three independent assays.

[b] The virus strain is given in parentheses.

TABLE 2

Phosphorylation of DHPG and DHPG homophosphonate nucleotides by cellular enzymes

Enzyme	K_m (μM)			V_{max}[a]			V_{max}/K_m (%)[b]		
	GMP	DHPG-MP	DHPG-HP	GMP	DHPG-MP	DHPG-HP	GMP	DHPG-MP	DHPG-HP
Guanylate kinase	29	40	67	107	20	24	100	14	10
	GDP	DHPG-DP	DHPG-HP-MP	GMP	DHPG-DP	DHPG-HP-MP	GMP	DHPG-DP	DHPG-HP-MP
Pyruvate kinase	940	1360	ND[c]	250	1.4	ND	100	0.4	ND
Nucleoside diphosphokinase	44	150	ND	300	0.1	ND	100	0.01	ND
Phosphoglycerate kinase	340	830	1400	870	58	22	100	2.7	0.6

[a] Units of V_{max} are μmol/min per mg protein.

[b] This value was determined by dividing the V_{max}/K_m ratio of nucleotide analog by the V_{max}/K_m ratio of the guanosine nucleotide × 100.

[c] Not detectable.

Phosphorylation of compounds by purified cellular enzymes

DHPG-MP, DHPG-HP and the diphosphate derivatives of these compounds were tested for their ability to be phosphorylated by cellular kinases. Human guanylate kinase phosphorylated DHPG-MP and DHPG-HP almost as effectively as the natural substrate GMP (Table 2). The relative rates of phosphorylation which may be expected in vitro, as estimated by the V_{max}/K_m ratios, imply that both DHPG-MP and DHPG-HP would be readily phosphorylated in infected cells. Conversion of these compounds to their triphosphate derivatives was examined using nucleoside diphosphokinase, phosphoglycerate kinase and pyruvate kinase. Investigations of these enzymes revealed that nucleoside diphosphokinase could not utilize either DHPG-DP or DHPG-HP phosphate (DHPG-HP-MP) as substrates (Table 2). Pyruvate kinase was marginally active with DHPG-DP as substrate, and inactive with DHPG-HP-MP. Nucleoside diphosphokinase and pyruvate kinase were effective in phosphorylating GDP, however. Both DHPG-DP and DHPG-HP-MP were effectively phosphorylated by phosphoglycerate kinase, but the reaction rates were less than 3% of GDP phosphorylating activity.

DNA polymerase inhibition

The effects of DHPG-TP, and DHPG-HP-DP on incorporation of [^{3}H]dGTP into DNA by partially purified HCMV DNA polymerase and MRC-5 polymerase were examined (Table 3). The triphosphate of ACV (ACV-TP), related to DHPG-TP in structure but less active in vitro against HCMV [5,17,18], was also assayed for comparison. Each compound was a competitive inhibitor of [^{3}H]dGTP incorporation into DNA based upon the Lineweaver–Burk plots (not shown). DHPG-TP was slightly more potent than DHPG-HP-DP at inhibiting the viral DNA polymerase, but both were equally active against the cell polymerase. ACV-TP was the most potent inhibitor of the viral enzyme.

Animal study

In a mouse CMV infection model in weanling mice, DHPG and DHPG-HP were able to reduce mortality rate significantly at doses as low as 10 mg/kg per day (Table 4). DHPG-CP was also active at 10 and 20 mg/kg but was toxic at 40 and 80 mg/kg. DHPG and DHPG-HP appeared to be well tolerated by the mice at all doses tested.

TABLE 3

Kinetic constants[a] for HCMV-induced DNA polymerase and cellular DNA polymerase

DNA polymerase source	K_m (μM)	K_i[a] (μM)		
	dGTP	ACV-TP	DHPG-TP	DHPG-HP-DP
HCMV (AD169 strain)	0.57	0.3	1.7	4.5
MRC-5 (α) cell	2.2	NA[b]	17.0	14.0

[a] K_m and K_i values were estimated from Lineweaver–Burk plots.
[b] Not assayed.

TABLE 4

Effect of DHPG, DHPG-CP, and DHPG-HP on a MCMC infection in weanling mice

Compounds (mg/kg)[a]	Survivors/ total	Percent survival	Mean survival time (days)[b] ± S.D.
Saline	2/16	13	5.7 ± 1.1
DHPG			
10	15/16	94[c]	10.0 ± 0.0[d]
20	16/16	100[c]	>21
40	16/16	100[c]	>21
80	16/16	100[c]	>21
DHPG-CP			
10	16/16	100[c]	>21
20	13/16	81[c]	9.6 ± 0.6[d]
40	1/16	6	7.8 ± 0.9
80	0/16	0	6.5 ± 0.5
DHPG-HP			
10	16/16	100[c]	>21
20	16/16	100[c]	>21
40	16/16	100[c]	>21
80	16/16	100[c]	>21

[a] Half-daily doses were administered at 9 am and 3 pm for 5 days beginning 6 h after inoculation.
[b] Of the mice that died.
[c] Statistically significant ($P < 0.05$) by two-tailed Fisher exact test.
[d] Statistically significant ($P < 0.05$) by two-tailed Mann–Whitney U-test.

Discussion

In vitro, all DHPG phosphate derivatives had nearly the same degree of activity against HCMV and VCMV, but they differed in efficacy against MCMV. DHPG-CP and DHPG-HP were the most potent against MCMV. DHPG-HP appears to be more selective than DHPG and DHPG-CP in that it was less inhibitory to cell proliferation. This difference could not be attributed to results observed in the MRC-5 cell α DNA polymerase assay, since the triphosphates of both DHPG and DHPG-HP were equally inhibitory.

The results of enzyme assays showed that the most likely route of phosphorylation of DHPG-MP and DHPG-HP is via guanylate kinase and phosphoglycerate kinase. The conversion of the monophosphate and phosphonate to diphosphates by guanylate kinase was efficient relative to GMP activity. Although the rates of phosphorylation of the diphosphates of both compounds were much less than that of GDP, the large excess of phosphoglycerate kinase activity present in most tissues should convert both drugs to the triphosphate form physiologically.

It is presumed from these studies that DHPG-MP and DHPG-BMP are converted to DHPG before being taken into cells. The work done with these compounds in this

report and in experiments against HSV-2 in mice (Prisbe, E.J. et al., manuscript in preparation) showed antiviral activities not different from DHPG itself, which supports this conclusion. Also, it is generally understood that most nucleotides are taken up poorly by cells, if at all [14]. DHPG-CP and DHPG-HP apparently are taken up by cells without being catabolized to DHPG. For DHPG-CP this is evident by its greater toxicity in cell culture and in mice, relative to DHPG, and because it is inhibitory to HSV thymidine kinase-deficient viruses that are resistant to DHPG (Prisbe, E.J. et al., manuscript in preparation). DHPG-HP has a carbon-linked phosphate which cannot be enzymatically hydrolyzed.

Two groups have recently reported on the inhibition of HCMV DNA polymerase activity by DHPG-TP and ACV-TP. Using HCMV (AD 169), Byron et al. [2] found K_i values nearly identical to ours. Mar and colleagues [9] indicated much lower K_i values for DHPG-TP and ACV against HCMV (Towne). Their lower K_i values may reflect different drug sensitivities of the virus strains, or perhaps were a function of preparing a more highly purified enzyme than we or Byron et al. did. The ratio of ACV-TP to DHPG-TP activity reported previously [9] was similar to that of this report, however.

In vivo, DHPG-HP was as effective as DHPG at the doses tested in preventing death due to MCMV, making this compound a good candidate for clinical evaluation against HCMV. Unlike DHPG, DHPG-HP does not demonstrate strong inhibition of herpes simplex viruses (Prisbe, E.J. et al., manuscript in preparation). The variation in sensitivity of herpes simplex viruses to DHPG-HP is a result of its poor inhibition of the HSV DNA polymerase. Although DHPG-CP was also active in vivo against MCMV, it is less likely to be considered for clinical evaluation than DHPG-HP because of its greater toxicity.

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