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Broad-spectrum antiviral activity of the acyclic guanosine phosphonate (*R,S*)-HPMPG

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Summary

(*R,S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)guanine [(*R,S*)-HPMPG] exhibits broad spectrum antiviral activity with an ED_{50} of less than 1 μM against herpes simplex virus (HSV) types 1 and 2, varicella zoster virus, human cytomegalovirus (HCMV) and vaccinia in plaque reduction assays. Wild type HSV-2 and its thymidine kinase deficient variant are equally sensitive to (*R,S*)-HPMPG. (*R,S*)-HPMPG is 100-fold more potent than acyclovir (ED_{50} = 0.45 μM vs. 44 μM, respectively) against HCMV in cell culture, and 10-fold more active than acyclovir in extending survival time in mice intraperitoneally infected with 70 LD_{50} HSV-1. However, (*R,S*)-HPMPG is toxic when administered repeatedly at 44 mg/kg/day in uninfected adult mice. The diphosphoryl derivative of HPMPG was enzymatically synthesized and is a competitive inhibitor of HSV-1 DNA polymerase relative to dGTP (K_I = 0.03 μM). HPMPG-PP is 70-fold less active at inhibiting HeLa DNA polymerase α than HSV-1 DNA polymerase. At concentrations between 0.3 and 1.5 μM (*R,S*)-HPMPG inhibited HSV-1 DNA replication ≥ 50% in infected cells as measured by nucleic acid hybridization. Consistent with inhibition of viral DNA synthesis, 6 to 30 μM (*R,S*)-HPMPG reduces late viral polypeptide synthesis in HSV-1 infected cells. These data indicate that (*R,S*)-HPMPG is a thymidine kinase independent broad spectrum antiviral drug which is capable of inhibiting viral DNA polymerase.

Herpesvirus; (*R,S*)-HPMPG

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Introduction

The development of acyclovir (Elion et al., 1977; Schaeffer et al., 1978) and ganciclovir (Smith et al., 1982; Field et al., 1982) as highly selective, relatively nontoxic antiherpesvirus agents has stimulated several laboratories to search for broader spectrum compounds. The specificity of acyclovir and ganciclovir is due in part to a requirement for phosphorylation by a herpesvirus encoded thymidine kinase. To circumvent this dependence, stable cyclophosphate and phosphonate nucleotide analogs have been prepared. Two such analogs, 2′-nor-cGMP (Tolman et al., 1985) and (*S*)-HPMPA (De Clercq et al., 1986) are effective against a wide array of DNA viruses, probably because these compounds are not dependent on phosphorylation by viral thymidine kinases. The capacity of cellular kinases to generate the nucleotide derivatives of (*S*)-HPMPA has been established and the resultant diphosphoryl-(*S*)-HPMPA does show selective inhibition of viral DNA synthesis (Votruba et al., 1987).

In an attempt to identify a more potent antiviral compound with broad spectrum antiviral activity, we have synthesized (*R,S*)-9-(3-hydroxy-2-phosphonomethoxypropyl)guanine [(*R,S*)-HPMPG]. In the present paper we describe the in vitro and in vivo antiviral efficacy of (*R,S*)-HPMPG along with studies concerning its mode of action. A preliminary report of these results has been presented at the Second International Conference on Antiviral Research (Terry et al., 1988).

Materials and Methods

Chemicals and enzymes

Guanylate kinase (porcine brain), nucleoside 5′-diphosphate kinase (Type 1, rabbit muscle), creatine kinase (bovine liver), phenylmethylsulfonyl fluoride, NP-40 and DNAse I activated calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, MO. Activated calf thymus DNA was extensively deproteinized with Proteinase K followed by phenol extraction before use in the DNA polymerase assays. Nucleoside triphosphates were obtained from Pharmacia (Piscataway, NJ) and phosphocreatine from Boehringer-Mannheim (Indianapolis, IN). [Methyl-^{3}H]dTTP (21 Ci/mmol), [α-^{32}P]dCTP (800 Ci/mmol) and L-[^{35}S]methionine were obtained from New England Nuclear (Boston, MA).

Acyclovir was prepared as described in Netherlands Patent 7709458 (1978) issued to The Wellcome Foundation Limited, London. (*R,S*)-HPmPG was prepared as described in Belgian Patent 898620 (1984) (A. Holý, I. Rosenberg and K. Slama) and ganciclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine was prepared as reported by Martin et al., 1983. Acyclovir triphosphate (Germershausen et al., 1983; Larsson et al., 1986) was prepared from acyclovir in 14% yield according to the general procedure of Ludwig, 1981. After chromatography on a DEAE-cellulose column using a triethylammonium bicarbonate gradient (pH 7.5), acyclovir triphosphate was isolated in the triethylammonium salt form and converted to the tetrapotassium salt by passage through a Dowex-50 (potassium form) column.

Synthesis of (R,S)-9-(3-hydroxy-2-phosphonomethoxypropyl)guanine [(R,S)-HPMPG]

2',3'-O-isopropylidene-9-(R,S)-(2,3-dihydroxypropyl)-2-amino-6-chloropurine (3). To a suspension of 2-amino-6-chloropurine (*2*) (10.17 g, 0.060 mol) in 165 ml of dimethylformamide (DMF) was added (*R,S*)-1-O-*p*-tosyl-2,3-O-isopropylideneglycerol (Ghangas and Fondy, 1971) (17.16 g, 0.060 mol) under nitrogen followed after 10 min by potassium carbonate (13.2 g, 0.096 mol). This mixture was heated at 70°C for 4 h; then an additional 5 g (0.017 mol) of (*R,S*)-1-O-*p*-tosyl-2,3-O-isopropylideneglycerol was added. The reaction was heated at 100°C for 2 h, cooled to 0°C and stirred for 16 h. After centrifugation, the solids were washed with 15 ml of DMF and the combined DMF washes were evaporated in vacuo to a residue which was slurried in 50 ml of methanol. The solids were collected by filtration, washed with chloroform:methanol (3:1, 50 ml) and dried. These solids were slurried in 170 ml of water for 20 min, filtered, washed with 30 ml of water and dried to give 5.78 g (34% yield) of *3*. ^{1}HNMR (270 MHz, DMSO-d_6): δ 8.05 (s, 1H, H-8), 6.88 (broad s, 2H, NH_2), 4.44 (m, 1H, CHO), 4.19 (dd, J=4.7, 14.2 Hz, 1H, CH_2N), 4.10 (dd, J=4.08, 14.2 Hz, 1H, CH_2N), 3.98 (dd, J=6.4, 8.5 Hz, 1H, CH_2O), 3.74 (dd, J=5.3, 8.5 Hz, 1H, CH_2O), 1.25 (s, 3H, CH_3), 1.20 (s, 3H, CH_3).

9-(R,S)-(2,3-dihydroxypropyl)guanine (4). A solution of compound *3* (5.00 g, 0.0176 mol) in 3 N HCl (117 ml) was refluxed for 2 h and cooled to 0°C. The pH was adjusted to 4 with 6 N NaOH and then to pH 7 with 1 N NaOH to give a precipitate. This suspension was concentrated to 20 ml and then applied to a 100 ml CH20P resin column. The column was washed with water and the product was eluted with acetonitrile:water (4:1) followed by water:ammonium hydroxide (19:1) to give 3.22 g (81% yield) of *4* as a solid having mp>250°C [literature: mp>260°C (Holý, 1978)]. ^{1}HNMR (270 MHz, DMSO-d_6): δ 10.52 (broad s, 1H, NH), 7.57 (s, 1H, H-8), 6.43 (broad s, 2H, NH_2), 4.95 (broad s, 1H, OH), 4.72 (broad s, 1H, OH), 4.06 (m, 1H, NCH_2), 3.81–3.76 (m, 2H, 1H of NCH_2 and CHO), 3.29 (m, H_2O + CH_2O). ^{13}CNMR (67.94 MHz, DMSO-d_6): δ 157.34 (C-6), 153.74 (C-2), 151.32 (C-4), 138.27 (C-8), 116.27 (C-5), 69.71 (C-2'), 63.43 (C-3'), 46.04 (C-1'). MS(FAB): 226 $(M+H)^+$; 224 $(M-H)^-$.

N^2-(p-anisyldiphenylmethyl)-9-(R,S)-[3-(p-anisyldiphenylmethoxy)-2-hydroxypropyl]guanine (5). To a suspension of compound *4* (2.75 g, 0.0122 mol) in 36 ml of DMF was added *p*-anisyldiphenylmethyl chloride (9.43 g, 0.030 mol), triethylamine (5.94 ml, 0.043 mol) and 4-dimethylaminopyridine (10 mg). The mixture was heated at 45°C for 7 h. After cooling, 6 ml of methanol was added and stirring was continued for 20 min. The solvent was removed in vacuo, and the residue was chromatographed on a Merck silica gel column (600 ml) using a step gradient of CH_2Cl_2 and the 7% methanol in CH_2Cl_2 to give 5.5 g of pure compound *5* and 3.3 g of impure *5*. Rechromatography of impure *5* on silica gel using a 0-3% methanol/CH_2Cl_2 gradient gave an additional 2.8 g of *5*. Crystallization of the combined products from acetonitrile gave, after drying in vacuo, 6.72 g (72% yield) of *5*. ^{1}HNMR (270 MHz, DMSO-d_6): δ 10.42 (s, 1H, NH), 7.54 (s, 1H, H-8), 6.72–7.42

(m, 28H, aromatics), 5.00 (d, J=5.8 Hz, 1H, OH), [3,74 (s, OCH_3), 3.61 (s, OCH_3), 3.5–3.8 (m, CHO), 7H], 3.37–3.29 (m, NCH_2+H_2O), 2.68 (m, 2H, CH_2O).

N^2-(p-anisyldiphenylmethyl)-9-(R,S)-[3-(p-anisyldiphenylmethoxy)-2-[(diethoxyphosphinyl)methoxy]propyl]guanine (6). Sodium hydride (1.04 g of a 60% suspension in mineral oil, 26.1 mmol) was added to a solution of compound *5* (6.72 g, 8.7 mmol) in 25 ml of DMF under nitrogen, and the mixture was stirred at room temperature for 30 min. A solution of *p*-tosyloxymethylphosphonic acid, diethyl ester (Holý, 1982) (3.6 g, 11.3 mmol) in 10 ml of DMF (previously stored over 3 Å molecular sieves for 16 h) was added and the mixture was stirred for 3 h. Additional *p*-tosyloxymethylphosphonic acid, diethyl ester (1.8 g, 5.7 mmol) was added and stirring was continued for 2 h. After storage at 0°C for 16 h, the mixture was concentrated in vacuo to give crude compound *6* as a residue which was used in the next step without purification.

9-(R,S)-[3-hydroxy-2-(diethoxyphosphinyl)methoxy]guanine (7). The residue of crude *6* (above) was dissolved in 140 ml of 80% acetic acid and heated at 65°C for 3 h. The reaction was concentrated to 15 ml and the residual acetic acid was removed by co-distillation with water (3 × 40 ml) leaving a residue. Trituration of the residue with CH_2Cl_2 (3 × 20 ml) gave solids and a combined CH_2Cl_2 extract. The CH_2Cl_2 extract was washed with 30 ml of water, and the water extract was added to the CH_2Cl_2 insoluble solids. After adjusting the pH from 4.5 to 7 with 1 N KOH, this mixture was applied to a column of CHP20P resin (400 ml). Elution with water and then a linear gradient of 0–50% acetonitrile in water gave 1.58 g (54% yield) of *7*. ^{1}HNMR (270 MHz, CD_3OD–D_2O): δ 7.98 (s, 1H, H-8), 3.83–4.49 (m, 11 H, CH_2-P-(OCH_2-C)$_2$, CH_2N, CH_2O, CHO), 1.40–1.49 (m, 6H, CH_3, CH_3).

(R,S)-9-(3-hydroxy-2-phosphonomethoxypropyl)guanine [(R,S,)-HPMPG] (1). Compound *7* (1.58 g, 4.75 mmol) was dried by co-distillation with DMF (3 × 20 ml). The residue was dissolved in 30 ml of DMF under nitrogen and cooled to 0°C. Trimethylsilyl bromide (6.27 ml, 47.5 mmol) was added and the reaction was warmed to room temperature and stirred for 18 h. The volatiles were removed in vacuo and the residue was dissolved in 30 ml of water. After adjusting the pH to 2.2 with aqueous $KHCO_3$, the solution was extracted with 20 ml of CH_2Cl_2. Further addition of CH_2Cl_2 (20 ml) to the separated aqueous layer resulted in a precipitate which was collected and washed with 5 ml of water. The precipitate was slurried in 30 ml of water, the pH was adjusted to 7.4 with 1 N KOH, and the resulting solution was filtered. Gradual adjustment of the pH of the filtrate to 2.2 with 1 N HCl gave crystals. After cooling to 0°C, the crystals were filtered, washed with water (2 × 5 ml), and dried in vacuo to give 0.951 g (63% yield) of *1* [(*R,S*)-HPMPG) having mp>250°C. Analysis: calculated for $C_9H_{14}N_5O_6P.O.37H_2O$: C, 33.17; H, 4.56; N, 21.49; P, 9.50; H_2O, 2.05. Found: C, 33.03; H, 4.52; N, 21.50; P, 9.49; H_2O, 2.05. ^{1}HNMR (400 MHz, DMSO-d_6): δ 10.56 (broad s, 1H, NH), 7.73 (s, 1H, H-8), 6.46 (s, 2H, NH_2), 5.19 (broad s, OH), 4.17 (dd, J=14.29, 4.03 Hz, 1H NCH_2), 3.98 (dd, J=14.29, 6.60 Hz, 1H, NCH_2), 3.70 (m, 1H, CHO), 3.63

(dd, J=13.55, 9.16 Hz, 1H, CH_2P), 3.58 (dd, J=13.55, 9.16 Hz, 1H, CH_2P), 3.42 (dd, J=11.72, 5.13 Hz, 1H, CH_2O), 3.38 (dd, J=11.72, 5.49 Hz, 1H, CH_2O). MS (FAB): 320 $(M+H)^+$; 318 $(M-H)^-$. UV (0.05 M phosphate + 0.85% NaCl, pH 7.2): λ_{max} 252.7 nm (12900).

Viruses, cells and media

HSV-1 strains Schooler, S, KOS and KOS(PFA^R-1) (the latter kindly provided by Dr. Y.-C. Cheng, University of North Carolina; Derse et al., 1982) as well as HSV-2 strains Curtis and 186 were prepared as extracts from infected Vero cell cultures. HCMV strain AD169 and VZV strain Ellen were prepared as extracts from infected WI-38 cell cultures. HSV-2 (186, 2'NDG^R) virus was isolated as a ganciclovir (2'NDG) resistant mutant of HSV-2 strain 186 and was at least 500-fold more resistant to acyclovir and ganciclovir than wild type virus. HSV-2 (186, 2'NDG^R) infected cells were thymidine kinase deficient and contained HSV-2 DNA polymerase which was sensitive to acyclovir triphosphate (data not shown). All virus stocks were stored at −70°C.

WI-38 (CCL75), Vero (CCL81) and HeLa S3 (CCL2.2) cells were acquired from American Type Culture Collection, Rockville, MD. Cells were grown in Eagles minimum essential medium with Earle's salts (EMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS (Gibco Laboratories, Grand Island, NY).

Plaque reduction assay

Virus was adsorbed to cell culture monolayers in 6 well culture plates (Costar, Cambridge, MA) for 1 h prior to addition of maintenance medium containing duplicate dilutions of the test compound (EMEM plus supplements, 1% carboxymethyl cellulose, 2.5% FBS, ± drug). Inhibition of plaque development was evaluated on fixed and stained monolayers after 4 days incubation at 37°C for HSV-1, HSV-2 and vaccinia; and after 6–7 days incubation at 37°C for HCMV and VZV. ED_{50} values were determined from the drug concentration which conferred at least a 50% plaque reduction compared to virus controls. HSV-1, HSV-2, HCMV and VZV were assayed on WI-38 cell monolayers; vaccinia was assayed on Vero cell monolayers.

Cell growth inhibition studies

WI-38 cells were plated at 1.2×10^5 cells per well in 12-well Costar plates containing 2 ml of growth medium. Following overnight incubation at 37°C, the cultures were refed with fresh growth medium containing acyclovir, (*R,S*)-HPMPG, (*R,S*)-HPMPA, or no drug and incubation was continued at 37°C for an additional 3 days. Quadruplicate cultures were harvested by trypsinization and counted daily for viable cells by staining with trypan blue. Untreated control cell cultures increased approximately 5-fold.

In vivo antiviral efficacy and toxicity

Randomized adult Swiss Webster (female) mice from Taconic Farms (20–24 g)

were infected intraperitoneally with HSV-1 (Schooler) at 70 times LD_{50} (approx. PFU/LD_{50}). Immediately following infection, the drug was delivered subcutaneously to 10 mice/treatment group. Half the daily dose was given each morning, the other half 7–8 h later for 5 days. Survival was measured daily for 21 days and the average survival time was compared to infected control mice.

Toxicity determinations were performed in uninfected, adult female Swiss mice (5 mice/group) treated subcutaneously twice daily for 5 days with 0.5 ml of saline or (*R,S*)-HPMPG at a daily dose of 44 mg/kg/day. Weights were recorded each day prior to treatment.

Enzymatic synthesis of the diphosphoryl derivative of (R,S)-HPMPG

The diphosphoryl derivative of HPMPG (HPMPG-PP) was prepared by incubating 3 mM (*R,S*)-HPMPG with 1 U/ml guanylate kinase and 8 U/ml creatine kinase in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 30 mM KCl, 2 mM DTT, 2.5 mM KF, 5 mM ATP, 15 mM creatine phosphate and 0.075 mg/ml BSA at 37°C for 4 h. Nucleoside 5′-diphosphate kinase (8 U/ml) was added and incubation continued overnight at 37°C. The reaction products could be resolved by HPLC indicating that 89% of the (*R,S*)-HPMPG was converted to HPMPG-PP. HPMPG-PP was isolated as a lyophilate after chromatography on a Whatman Partisil 5 ODS 3RAC column using a 1 M ammonium formate, pH 5.1, isocratic solvent. The structure and purity were confirmed by UV analysis [λ_{max} 252.3 nm (12,400)], NMR and mass spectroscopy.

Enzyme preparations

HSV-1 (Schooler) infected HeLa S3 cell extracts were prepared essentially as described by Karkas et al., 1986. Cells were infected with a multiplicity of infection of 10 PFU per cell and harvested 20 h post infection. Cell pellets were resuspended in a hypotonic solution (10 mM K_2HPO_4, pH 7, 10 mM KCl, 1 mM DTT and 1 mM PMSF) for 30 min on ice with occasional vortexing. An equal volume of 0.7 M K_2HPO_4, pH 7, 6 mM DTT, 28% glycerol, 1 mM PMSF and 0.4% NP-40 was added and cells were incubated for 30 min at 4°C with occasional vortexing. The solution was clarified by centrifugation at 125 000 × *g* for 1 h and the NP-40 concentration was adjusted to 0.4%. Aliquots were frozen in dry ice and stored at −70°C. The protein concentration was 5.0 mg/ml as determined by the Coomassie Blue protein assay (Bradford, 1976). Uninfected HeLa S3 cell extracts were prepared in an identical manner and had a protein concentration of 2.4 mg/ml.

HSV-1 DNA polymerase was purified approximately 250-fold from the crude extract described above through the ssDNA-cellulose step according to the procedure of O'Donnell et al., 1987. HSV-1 DNA polymerase was stored at −70°C in 50 mM Tris-HCl, pH 7.6, 0.15 M ammonium sulfate, 0.1 mM EDTA, 0.5 mM DTT and 50% glycerol.

DNA polymerase inhibition studies

Inhibition of HSV-1 DNA polymerase by HPMPG-PP or ACVTP was determined in: 50 mM Tris-HCl, pH 8; 5 mM $MgCl_2$; 1 mM DTT; 0.1 M ammonium sulfate; 5 μM (each) dATP, dCTP and [^{3}H]dTTP (540 cpm/pmol); 30 μg/ml activated calf thymus DNA; 0.1 mg/ml BSA; HSV-1 (Schooler) infected HeLa extracts; and varying dGTP. Incubation was for 20 min at 37°C. Incorporation of [^{3}H]dTTP into DNA was quantitated by TCA precipitation and scintillation counting. Inhibition studies were also performed substituting purified HSV-1 DNA polymerase for the HSV-1 infected cell extracts.

Inhibition constants were determined for HeLa S3 DNA polymerase α under the following conditions: 50 mM Hepes, pH 7; 5 mM $MgCl_2$; 1 mM DTT; 30 μg/ml activated calf thymus DNA; 0.1 mg/ml BSA; 5 μM (each) dATP, dCTP and [^{3}H]dTTP (540 cpm/pmol); varying dGTP; and HeLa S3 cell extracts. Incubation was for 20 min at 37°C and incorporation of radiolabel into DNA was quantitated by TCA precipitation and scintillation counting.

Inhibition of viral DNA synthesis

Quantitation of viral DNA synthesis was performed essentially as described by Gadler et al., 1984. Briefly, 96 well plates of WI-38 cells were infected with HSV-1 (KOS) at an MOI = 2 PFU/cell or mock infected. After 1 h adsorption at 37°C, the inoculum was removed and replaced with media containing varying amounts of drug; incubation was continued for 18 h post infection. At that time cells were loosened with trypsin-EDTA (100 μl/well for 10 min) and filtered through a nitrocellulose filter using a filtration manifold. Filters were processed and hybridization performed as described by Maniatis et al., 1982. The probe consisted of pNN3 plasmid DNA (obtained from Dr. M. Challberg, NIH, Bethesda, MD) containing a 5.5 kb fragment of HSV-1 (KOS) DNA derived from the region encoding the HSV-1 DNA polymerase gene and located adjacent to the origin of replication for the unique long region of the herpes genome (Challberg, 1986). This probe was radiolabeled with [α-^{32}P]dCTP to a specific activity of 1×10^7 cpm/μg and the amount of radiolabel hybridized was quantitated by cutting the spots from the filter and counting them in a liquid scintillation counter.

Inhibition of viral protein synthesis

WI-38 cells were infected with HSV-1 (KOS) at MOI = 15 PFU/cell in the presence or absence of drug in media containing limited methionine, 1% glutamine and 1% calf serum. At 1 and 7 hours post-infection, L-[^{35}S]methionine (100 μCi/ml) was added to the media and incubation was continued for 6 and 17 h, respectively. Cells were rinsed with phosphate buffered saline, harvested and lysed into 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate and 1% Triton X-100. Samples were mixed with an equal volume of 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 4% SDS and 17% glycerol. The amount of radiolabeled protein was estimated by TCA precipitation and liquid scintillation counting. Samples (2.5×10^5 cpm/well) were boiled for 3 min prior to electrophoresis on SDS-PAGE gels according to the procedure of Heine et al., 1974. Autoradio-

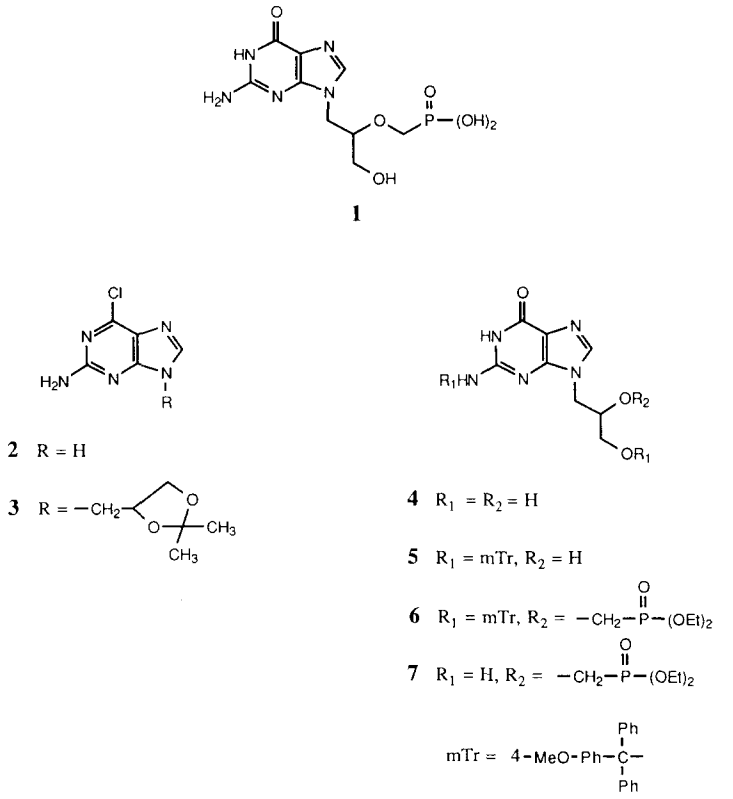

Fig. 1. Structure of (*R,S*)-HPMPG and the synthetic intermediates described in Materials and Methods.

grams of the resolved proteins were scanned with a laser densitometer (LKB Ultroscan), and the peak corresponding to the major capsid protein ICP5 was identified. Peak areas were determined with a Hewlett-Packard Integrator Model 3390A.

Results

In vitro antiviral activity and cytotoxicity

The antiviral activity of (*R,S*)-HPMPG, (*R,S*)-HPMPA, and acyclovir (ACV) were evaluated against a variety of DNA viruses as shown in Table 1. (*R,S*)-HPMPG and (*R,S*)-HPMPA inhibited plaque formation by HSV-1, HSV-2, HCMV, VZV, and vaccinia in a similar manner; the ED_{50} values for (*R,S*)-HPMPG were up to 10-fold lower than for (*R,S*)-HPMPA. Both phosphonates were effective against a wild type HSV-2 and a thymidine kinase (2'NDG^R) deficient variant. In contrast, acyclovir exhibited a marked dependence upon thymidine kinase for

TABLE 1

In vitro efficacy of (*R,S*)-HPMPG, (*R,S*)-HPMPA and acyclovir

Virus	ED_{50}, μM		
	(*R,S*)-HPMPG	(*R,S*)-HPMPA	Acyclovir
HSV-1 (Schooler)	0.9	3	0.4
HSV-1 (S)	0.2	ND	0.5
HSV-1 (KOS)	1.6	5	0.6
HSV-1 (KOS,PFA^R-1)	0.3	<1.6	3
HSV-2 (Curtis)	1	ND	0.6
HSV-2 (186)	0.8	2	0.3
HSV-2 (186, 2′NDG^R)	0.3	3	200
HCMV	0.4	0.3	40
VZV	0.2	0.6	2
Vaccinia	0.8	7	>400

ND, Not determined.

Plaque assays for HSV-1, HSV-2, HCMV and VZV were performed with WI-38 cell monolayers. Vaccinia was assayed on Vero cell monolayers. HSV-1 (KOS, PFA^R-1) is resistant to phosphonoformic acid (Derse et al., 1982) and has an altered viral DNA polymerase (Cheng et al., 1983). HSV-2 (186, 2′NDG^R) is deficient in thymidine kinase activity and is described in Materials and Methods.

antiviral activity. Additionally, (*R,S*)-HPMPG and (*R,S*)-HPMPA were about 100-fold more active against HCMV than acyclovir.

(*R,S*)-HPMPG, (*R,S*)-HPMPA, and acyclovir were also evaluated for inhibition of growth of WI-38 cells for 3 days in the presence of 3, 30 and 300 μM drug for the phosphonates and 4, 40 and 400 μM for ACV. Resultant cell yields as a percent of cells harvested from untreated control cultures are shown in Fig. 2. None of the compounds evaluated was cytotoxic as defined by an increase in the proportion of trypan blue positive cells. However, both (*R,S*)-HPMPG and (*R,S*)-HPMPA were substantially more cytostatic than acyclovir, with calculated ED_{50} cytostatic values of approximately 8 μM for (*R,S*)-HPMPG, 30 μM for (*R,S*)-HPMPA, and equal or greater than 400 μM for acyclovir. The calculated in vitro therapeutic index (ED_{50} cells/ED_{50} HSV-2) for the three compounds was estimated to be 10 for (*R,S*)-HPMPG, 15 for (*R,S*)-HPMPA and >1000 for acyclovir.

In vivo efficacy of (R,S)-HPMPG against HSV-1 infection

Young adult female Swiss mice were infected with 70 times LD_{50} and treated as described in Materials and Methods. The results are presented in Table 2. A significant increase in survival time was observed with 3.2 mg (*R,S*)-HPMPG/kg/day compared to placebo treated mice; acyclovir treatment with 25 mg/kg/day resulted in a similar increase in survival time. At 12 mg/kg/day, however, (*R,S*)-HPMPG produced apparent toxicity in the infected mice. Toxicity was more prominent at 25 and 50 mg/kg/day (data not shown) and was confirmed in a separate study with uninfected mice; mice treated for 5 days at 44 mg/kg/day lost weight resulting in early death (Fig. 3). As a result, although (*R,S*)-HPMPG exhibited a protective effect at an 8-fold lower drug concentration than acyclovir in HSV-1 infected mice,

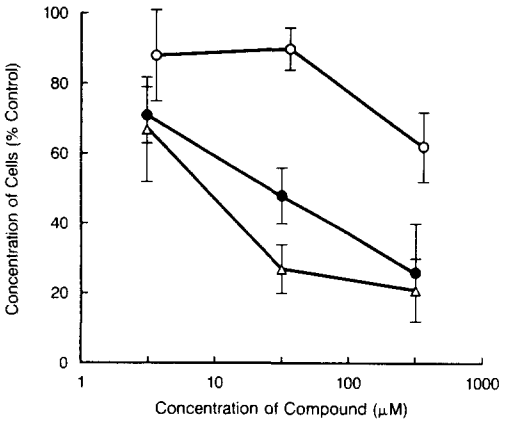

Fig. 2. Effect of (*R,S*)-HPMPG, (*R,S*)-HPMPA and acyclovir on WI-38 cell growth. Variable cell numbers were determined in quadruplicate cultures incubated with ACV, (*R,S*)-HPMPG, or (*R,S*)-HPMPA at concentrations indicated for 3 days (details in Materials and Methods). Values are means of quadruplicate determinations ± SD for each drug concentration tested relative to cell numbers in untreated cell controls as determined on day 3. Note that the compound concentration is plotted on a logarithmic scale. (○), acyclovir; (●), (*R,S*)-HPMPA; (△), (*R,S*)-HPMPG.

this protective dose was only 4- to 8-fold below the apparent toxic doses for (*R,S*)-HPMPG.

TABLE 2

Comparative efficacy of (*R,S*)-HPMPG and acyclovir against systemic HSV-1 infection

Antiviral compound	Dose mg/kg/day	Survivors total[a]	Average survival time[b]
(*R,S*)-HPMPG	12.5	1/10	14.0* ± 1.1
	6.3	4/10	12.5* ± 2.1
	3.2	4/10	10.7* ± 1.7
	1.6	3/10	9.3 ± 2.8
Acyclovir	100	3/10	14.8* ± 2.3
	50	0/10	12.4* ± 3.9
	25	3/10	10.4* ± 3.1
	12.5	0/10	9.3 ± 2.8
Infected control	–	1/13	7.7 ± 1.0

[a] Survivors were determined 21 days post infection.

[b] Survival time was determined for those mice which died up to day 21 and those mice that survived were omitted from the calculations.

* = significant difference ($P<0.05$) from infected control.

(*R,S*)-HPMPG and acyclovir were prepared in saline at pH 10.5 and given as 0.5 ml doses twice daily at a 7–8 h interval for 5 days.

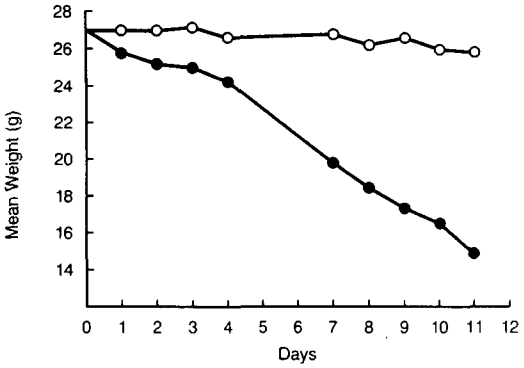

Fig. 3. Toxicity of (*R,S*)-HPMPG in female Swiss mice. Either 44 mg (*R,S*)-HPMPG/kg/day or 0.5 ml placebo was administered in two equal doses daily to uninfected mice (5 mice/group). After 5 days (day 0–4), treatment was suspended; weights were monitored daily. All placebo treated mice survived the duration of the study, while three (*R,S*)-HPMPG treated mice died (one death each day on days 9, 10, 11). (•), (*R,S*)-HPMPG, 44 mg/kg/day; (○), placebo.

Mechanism of antiviral action of (R,S)-HPMPG

(*R,S*)-HPMPG (500 μM) was not an inhibitor of either HSV-1 DNA polymerase or HeLa DNA alpha polymerase (data not shown). The diphosphoryl derivative of HPMPG (HPMPG-PP) was prepared enzymatically in order to determine if HPMPG-PP is a selective inhibitor of HSV-1 DNA polymerase. Incubation of (*R,S*)-HPMPG with guanylate kinase gave the monophosphoryl derivative, which was converted to HPMPG-PP by incubation with nucleoside 5′-diphosphate kinase using ATP as the phosphate donor.

HPMPG-PP was found to be a competitive inhibitor of HSV-1 DNA polymerase activity relative to dGTP (Fig. 4). The inhibition constant for HSV-1 DNA polymerase in cell lysates was 0.032 ± 0.015 μM for HPMPG-PP (Table 3) and compared favorably to the inhibition constant for ACVTP (0.013 ± 0.004 μM). The selectivity of HPMPG-PP for viral (vs cellular) DNA polymerase was similar to that of ACVTP; inhibition of HeLa DNA polymerase α activity in cell extracts was 70-fold less with HPMPG-PP and 100-fold less with ACVTP. Determinations of the inhibition constants were also performed with purified HSV-1 DNA polymerase and found to be 0.062 ± 0.054 for HPMPG-PP and 0.017 ± 0.005 for ACVTP (data not shown). Comparison of DNA polymerase inhibition constants determined with HSV-1 infected cell extracts and purified HSV-1 DNA polymerase indicated that the differences are not statistically significant for HPMPG-PP or ACVTP (Student's *t*-test).

The effect of (*R,S*)-HPMPG on viral replication in cell culture was investigated to determine if (*R,S*)-HPMPG inhibited viral DNA synthesis and protein synthesis. The effects on viral DNA replication were measured directly by the nucleic acid hybridization technique of Gadler et al., 1984. In the present study, (*R,S*)-HPMPG was compared to acyclovir, (*R,S*)-HPMPA and ganciclovir. The amount of viral DNA produced in infected WI-38 cells exposed to increasing amounts of

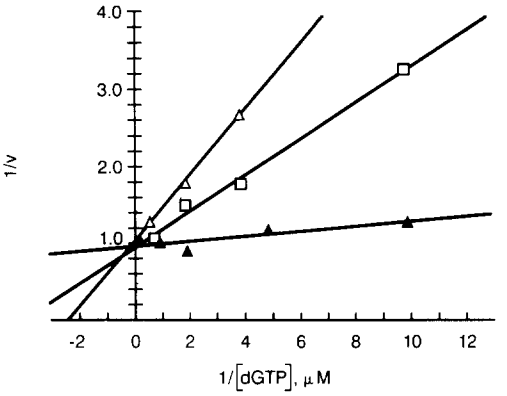

Fig. 4. Inhibition of HSV-1 DNA polymerase by HPMPG-PP. Reactions (50 μl) were performed with HSV-1 infected cell extracts (6.25 μg) at 37°C. [^{3}H]dTTP (540 cpm/pmol) incorporation into DNAse I activated calf thymus DNA was measured by TCA precipitation and scintillation counting (for details, see Materials and Methods). HPMPG-PP concentrations were: 0 μM (▲), 0.5 μM (□), and 1 μM (△).

drug was quantitated using a radiolabeled probe. This probe contains a 5.5 kb fragment of HSV-1 (KOS) DNA derived from the region encoding the HSV-1 DNA polymerase gene (Challberg, 1986). The results of this study are shown in Fig. 5. The ED_{50} for ganciclovir and acyclovir were less than 0.004 and 0.04 μM, respectively. The ED_{50} for both (*R,S*)-HPMPG and (*R,S*)-HPMPA were between 0.3 and 1.5 μM. These values are generally lower than values observed in the plaque reduction assay, but reflect the same order of efficacy. It should be noted that the two assays are not directly comparable. The hybridization assay differs from the plaque reduction assay in that it measures the effect of the drug during a short time interval (18 h) and on a uniformly infected monolayer (MOI = 2 PFU/cell). Several factors such as drug stability and the metabolism of the drug in uninfected cells may be responsible for the difference in observed ED_{50}. The experiment does demonstrate, however, that viral DNA replication is blocked in cells treated with each of the drugs.

In order to investigate the effects of the drugs on viral protein synthesis, infected cells were radiolabeled with [^{35}S]methionine. Radiolabeling intervals early

TABLE 3

Inhibition of DNA polymerase activity

Compound	K_i, μM	
	HSV-1 DNA Polymerase	HeLa α Polymerase
HPMPG-PP	0.032 ± 0.015	2.3
ACVTP	0.013 ± 0.004	1.3

Inhibition of DNA polymerase activity was determined as described in Materials and Methods.

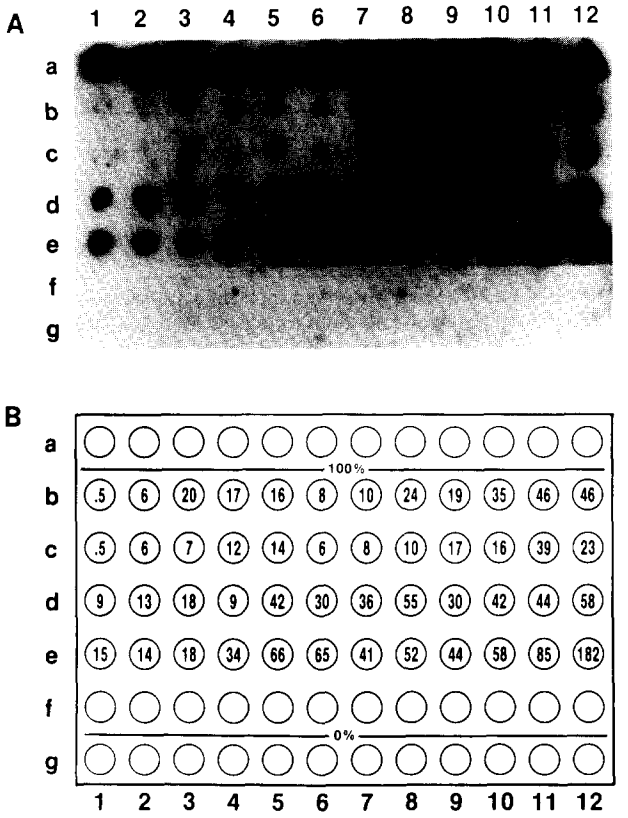

Fig. 5. Effect of antiherpes compounds on HSV-1 DNA synthesis. WI-38 cells and HSV-1 (KOS) were applied to a 96-well microtiter plate as described below. A., Results after autoradiography. B., Results after liquid scintillation counting; the percentage of HSV-1 DNA synthesized after treatment with different concentrations of compounds (compared to untreated infected cells) is indicated for each well. Six concentrations of each compound were tested in duplicate, decreasing left to right (wells numbered 1–12).

a. – e. HSV-1 infected WI-38 cells.

a. No drug.

b. Ganciclovir: 0.2, 0.08, 0.04, 0.02, 0.008, 0.004 μM.

c. Acyclovir: 2.22, 0.89, 0.44, 0.22, 0.09, 0.04 μM.

d. (*R,S*)-HPMPA: 15.9, 6.4, 3.2, 1.6, 0.64, 0.32 μM.

e. (*R,S*)-HPMPG: 6.27, 3.1, 1.6, 0.63, 0.32, 0.16 μM.

f and g. Uninfected WI-38 cells.

(1–7 h) and late (7–24 h) in infection were chosen for comparison. The accumulation of the late, or gamma class of viral proteins is known to be reduced in the presence of inhibitors of DNA synthesis (reviewed by Roizman and Batterson, 1985). Table 4 summarizes the effects of treatment with acyclovir, (*R,S*)-HPMPG and (*R,S*)-HPMPA on accumulation of the late viral capsid protein ICP5 in WI-38 cells infected with HSV-1 (KOS) at a high MOI (15 PFU/cell). This protein was chosen because it has a high molecular weight, readily resolves from the other viral

TABLE 4

Reduction in accumulation of the late viral capsid protein ICP5

Compound[a]	Reduction in ICP5 accumulation (%)[b]	
	1–7 h	7–24 h
Acyclovir	56	70
(*R,S*)-HPMPG	24	26
(*R,S*)-HPMPA	7	13

[a] Acyclovir, (*R,S*)-HPMPG and (*R,S*)-HPMPA were present at 10 μg/ml.

[b] Calculated as described in Materials and Methods from peak areas of densitometric scans. Percentage reduction is relative to peak area of ICP5 in HSV-1 infected cells without drug.

proteins on SDS-PAGE, and could easily be quantitated by densitometry. For comparisons between samples, the same quantity of radiolabeled protein was added to each lane of the gel. During both time intervals, the accumulation of ICP5 was reduced in drug treated cells compared to the control. The relative order of inhibition was acyclovir > (*R,S*)-HPMPG > (*R,S*)-HPMPA, with the most dramatic effects observed with acyclovir under the conditions studied. These results are consistent with the DNA hybridization studies and confirm that DNA synthesis is inhibited by (*R,S*)-HPMPG.

Discussion

The experiments described in this report demonstrate that (*R,S*)-HPMPG has broad spectrum antiviral activity against a variety of DNA viruses. The effective dose (ED_{50}) in vitro was less than or equal to 1 μM against HSV-1, HSV-2, HCMV, VZV and vaccinia. This efficacy compares favorably with acyclovir against HSV-1, HSV-2 and VZV infected WI-38 cells and was superior against HCMV and vaccinia. The antiviral effects of (*R,S*)-HPMPG are not dependent upon phosphorylation by viral thymidine kinase as shown by plaque reduction in a 2'NDG^R HSV-2 strain and effectiveness against HCMV. In this respect, HPMPG has a different mode of action than acyclovir. These results agree very well with a previous report on herpes virus plaque reduction in cell culture by (*R,S*)-HPMPG (De Clercq et al., 1987).

(*R,S*)-HPMPG was also effective in vivo against HSV-1 infection in mice administered a lethal dose of virus. A significant increase in survival time was observed when these mice were treated subcutaneously with as little as 3.2 mg (*R,S*)-HPMPG/kg/day. Higher doses were also efficacious, although 44 mg/kg/day of (*R,S*)-HPMPG exhibited toxicity in mice as measured by weight loss and lethargy. Therefore, there appears to be a narrow concentration range where (*R,S*)-HPMPG would be effective as an antiviral drug.

(*R,S*)-HPMPG was anticipated to ultimately interact with the viral DNA polymerase. Since (*R,S*)-HPMPG itself did not inhibit HSV-1 DNA polymerase at 500 μM, we enzymatically prepared the triphosphate equivalent of HPMPG using

guanylate kinase and nucleoside 5′-diphosphate kinase. The ease of phosphorylation of (*R,S*)-HPMPG indicates that the phosphonate linkage does not interfere with conversion to the monophosphoryl derivative and subsequent phosphorylation to the diphosphoryl derivative. The triphosphate equivalent of HPMPG (HPMPG-PP) was a competitive inhibitor of HSV-1 DNA polymerase with respect to dGTP with an inhibition constant of 0.03–0.06 μM. The selectivity of inhibition of HSV-1 DNA polymerase to HeLa DNA polymerase α was similar for HPMPG-PP and ACVTP when cell free extracts are compared (70- and 100-fold, respectively).

Cell culture studies demonstrate that viral DNA synthesis and late viral protein synthesis are reduced in (*R,S*)-HPMPG treated cultures. The effect observed with (*R,S*)-HPMPG was less dramatic than with acyclovir, but inhibition was slightly greater than that observed with (*R,S*)-HPMPA. Combined with the DNA polymerase inhibitor studies, these results imply that the antiviral activity of (*R,S*)-HPMPG results from a block in viral DNA synthesis through inhibition of the viral DNA polymerase. Furthermore, the plaque reduction studies with HSV-1 (KOS, PFA^R-1) suggest that (*R,S*)-HPMPG (and (*R,S*)-HPMPA) may interact with the viral DNA polymerase in a different manner than acyclovir. (*R,S*)-HPMPG was more active against the PFA^R-1 strain than wild type KOS whereas the converse is true for acyclovir (Table 1).

Acyclovir and similar nucleoside analogs achieve a large part of their selectivity by serving as substrates for viral thymidine kinase but not for cellular kinases. A second potential point of selectivity may be preferential inhibition of the viral DNA polymerase rather than the host polymerase. Although the selectivity of HPMPG-PP for viral vs cellular DNA polymerase was 70-fold (similar to that for ACVTP), the lack of dependence of (*R,S*)-HPMPG on viral thymidine kinase activity may account for its relatively high toxicity. Both (*R,S*)-HPMPG and (*R,S,*)-HPMPA inhibit cell growth in WI-38 cells resulting in in vitro therapeutic indices of only 10 and 15, respectively. We have also observed that (*R,S*)-HPMPG was toxic when administered to uninfected mice at doses greater than 25 mg/kg/day. Thus, although (*R,S*)-HPMPG exhibits excellent antiviral activity, it is cytotoxic in vitro and quite toxic in vivo.

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