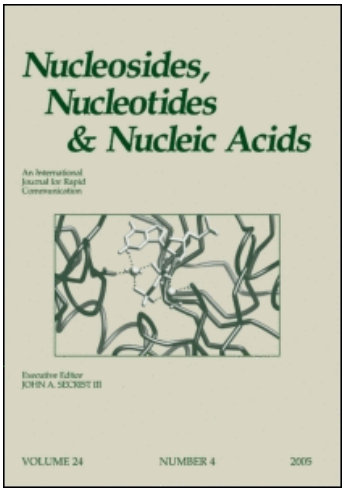

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Phosphorylation of Ganciclovir Phosphonate by Cellular GMP Kinase Determines the Stereoselectivity of Anti-Human Cytomegalovirus Activity

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Dedicated to the memory of Dr. Gertrude B. Elion

ABSTRACT

A racemic mixture of ganciclovir phosphonate was resolved by stereoselective phosphorylation using GMP kinase. The R-enantiomer of ganciclovir phosphonate was active against human cytomegalovirus but the S-enantiomer was less active. We show that enantiomeric selectivity of antiviral activity for ganciclovir phosphonate was conferred by stereoselective phosphorylations by mammalian enzymes, not by stereoslective inhibition of DNA polymerase from human cytomegalovirus.

Human cytomegalovirus (HCMV) infections are a threat to immunocompromised patients with AIDS or those undergoing immunosuppressive chemotherapy. Ganciclovir (Cytovene®, Fig. 1) is widely used to treat cytomegalovirus infections. Ganciclovir belongs to a group of nucleosides that are effective antiviral agents with a common mechanism of action. After phosphorylation to their triphosphate forms by a series of enzymatic phosphorylations, the nucleoside triphosphates inhibit viral replication by inhibition of viral polymerase or by incorporation into viral nucleic acids. Ganciclovir triphosphate inhibits DNA polymerase from HCMV[1] as a competitive inhibitor and is also incorporated as a substrate. Unlike most other nucleoside analogs, which are phosphorylated in vivo by nucleoside kinases or phosphotransferases, ganciclovir is phosphorylated by the UL97 protein of HCMV[2].

Ganciclovir phosphonate is a close structural analog (Fig. 1) of ganciclovir monophosphate that contains the biologically stable phosphonate group. Its synthesis and

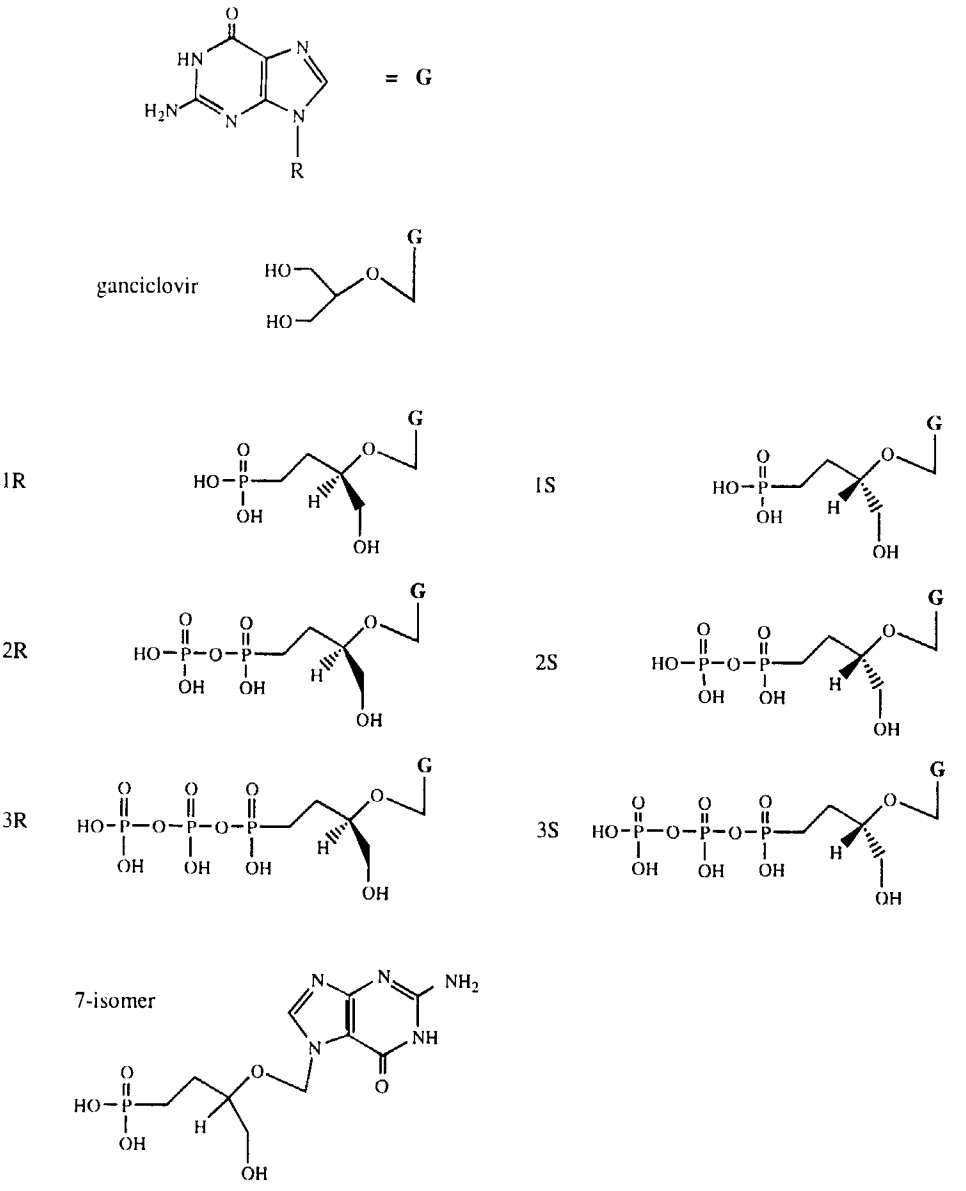

Fig. 1. Structure of R-ganciclovir phosphonate and related compounds: ganciclovir, 9-((2-hydroxy-1-(hydroxymethyl)ethoxy)methyl)guanine; R-ganciclovir phosphonate, (R)-3-((2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy)-4-hydroxybutylphosphonic acid, 1R, [also known as SR 3773[16] or DHPG-HP[32]], and its monophosphate, 2R, and diphosphate, 3R; S-ganciclovir phosphonate, (S)-3-((2-amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)methoxy)-4-hydroxybutylphosphonic acid, 1S, and its monophosphate, 2S, and diphosphate, 3S; 7-isomer, 3-((2-amino-1,6-dihydro-6-oxo-7*H*-purin-7-yl)methoxy)-4-hydroxybutylphosphonic acid.

antiviral activity against herpes virus and human and murine cytomegalovirus have been reported[3-4]. Since it already contains a phosphate isostere, it has bypassed the phosphorylation by UL97 that was needed by ganciclovir. Because ganciclovir phosphonate is structurally similar to ganciclovir monophosphate and other acyclic nucleoside analogs, GMP kinase would be expected to stereoselectively phosphorylate only one enantiomer[5-6].

In this paper we used stereoselective phosphorylation by GMP kinase as a tool to separate the enantiomers of ganciclovir phosphonate. Only one of these enantiomers had significant anti-HCMV activity. However, the triphosphate analogs of both enantiomers inhibited DNA polymerase from HCMV. The stereoselectivity of ganciclovir phosphonate as an antiviral agent was a result of the stereoselective phosphorylation of only the active enantiomer by cellular enzymes.

EXPERIMENTAL PROCEDURES

Materials— Human DNA polymerase δ was obtained from Dr. Suk Hee Lee of the St. Jude's Children's Research Hospital in Memphis TN. Nucleoside diphosphate kinase (EC 2.7.4.6) from beef liver, pyruvate kinase (EC 2.7.1.40) from rabbit muscle, creatine phosphate, and creatine kinase (EC 2.7.3.2) from rabbit muscle were from Boehringer Mannheim, Indianapolis, IN. GMP kinase (EC 2.7.4.8) from porcine brain, phosphoglycerate kinase (EC 2.7.2.3) from rabbit muscle, ATP, NAD, glyceraldehyde-3-phosphate, Pipes, Hepes, and tributylammonium pyrophosphate were purchased from Sigma Chemical Co., St. Louis, MO. GMP kinase from human erythrocytes was prepared and assayed as described previously[7]. 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone, 1,1-carbonyldiimidazole and D_2O were purchased from Aldrich, Milwaukee, WI. Coomassie protein assay reagent and bovine serum albumin standard were from Pierce Chemical Co., Rockford, IL. Ammonium bicarbonate and HPLC grade ammonium phosphate were from Fisher Scientific, Raleigh, NC.

Preparation of ganciclovir phosphonate— Racemic ganciclovir phosphonate was synthesized by the method in example 2 of reference 8. This preparation of ganciclovir phosphonate contained 10% 7-isomer (Fig. 1) which was separated from the 9-isomer as descibed below.

Enzymatic Resolution of the S-enantiomer from the R-enantiomer— Reaction mixtures contained 5.5 mM racemic ganciclovir phosphonate, 54 mM ATP (adjusted to

pH 7.5 with NaOH), 45 mM $MgCl_2$ and 0.65 units/ml GMP kinase from porcine brain (Sigma) in a final volume of 22 ml. After 5 hr at 37°C, approximately 50% of the ganciclovir phosphonate had converted to monophosphate. The reaction mixture was diluted to 75 ml with water and applied to a column (2.5 cm × 10 cm) containing DEAE Sephadex A-25 that had been equilibrated with 50 mM ammonium bicarbonate. After washing the column with 1 L of 50 mM ammonium bicarbonate, the reaction components were eluted with a 1.2 L gradient of 50- 500 mM ammonium bicarbonate. The unreacted S-ganciclovir phosphonate and 7-isomer eluted early in the gradient, followed by the monophosphate of R-ganciclovir phosphonate combined with ATP. These latter fractions were combined (150 ml) and treated with alkaline phosphatase (37 units/ml). The ATP was converted to adenosine and the monophosphate of R-ganciclovir phosphonate was converted to R-ganciclovir phosphonate after 30 min at 37°C. The adenosine and R-ganciclovir phosphonate were separated by DEAE chromatography as described above. The configuration of this ganciclovir phosphonate as the R-enantiomer (Fig. 1, 1R) was determined by comparing its optical rotation (see below) with that of authentic R-enantiomer synthesized by a chiral method[9].

UV spectrum of the R-enantiomer in 10 mM ammonium phosphate pH 5.5 had λ_{max} 253 nm, shoulder 270 nm and λ_{min} 222 nm. This spectrum was consistent with that expected for N-9-substituted guanine analogs[10]. NMR: ^{31}P NMR d (D_2O) 25.1 (s); ^1H-NMR (D_2O) δ 7.84 (s, 1H, H-8), 5.41 (m, 2H, NCH_2O), 3.52-3.36 (m, 3H, CH and $C\underline{H}_2OH$), 1.49 (m, 2H, CH_2), 1.10-1.29 (m, 2H, CH_2)

Resolution of 9-isomer from 7-isomer— Resolution of the S-enantiomer (Fig. 1, 1S) from the 7-isomer (Fig. 1) was accomplished by preparative ion-exchange HPLC on a column of Whatman Partisil 10 SAX Magnum 9 (9 mm ID × 50 cm L). The products were eluted from the column with 10 mM ammonium phosphate pH 2.4 containing 5% methanol with a flow rate of 4 ml/min. The 7-isomer eluted at 16 min followed by the 9-isomer at 31 min. Chromatography on DEAE Sephadex as described above was used to remove ammonium phosphate from the compounds.

UV spectra for the 7-isomer (Fig. 1) were: in 10 mM ammonium phosphate pH 5.5 λ_{max} 243 nm, 286 nm and λ_{min} 235 nm, 261 nm; in 10 mM ammonium phosphate pH 2.4 λ_{max} 248 nm, 275 nm and λ_{min} 232 nm, 264 nm. These spectra were consistent

with those expected for N-7-substituted guanine analogs[10]. ^{1}H-NMR (D_2O) d 8.05 (s, 1H, H-8), 5.58 (m, 2H, NCH_2O), 3.58-3.33 (m, 3H, CH and $C\underline{H}_2OH$), 1.48 (m, 2H, CH_2), 1.38-1.05 (m, 2H, CH_2).

UV spectra for the S-enantiomer of the 9-isomer (Fig. 1, 1S) were: in 10 mM ammonium phosphate pH 5.5 λ_{max} 253 nm, shoulder 267 nm and λ_{min} 221 nm; in 10 mM ammonium phosphate pH 2.4 λ_{max} 254 nm, shoulder 269 nm and λ_{min} 222 nm. These spectra were consistent with those expected for N-9-substituted guanine analogs[10]. NMR: ^{31}P NMR d (D_2O) 25.2 (s); ^{1}H-NMR (D_2O) d 7.79 (s, 1H, H-8), 5.41 (m, 2H, NCH_2O), 3.58-3.32 (m, 3H, CH and $C\underline{H}_2OH$), 1.50 (m, 2H, CH_2), 1.40-1.10 (m, 2H, CH_2).

Analytical ion exchange HPLC was performed on a Whatman Partisil 10 SAX (4.6 mm × 10 cm) column with an ammonium phosphate pH 5.5, 5% methanol gradient from 10 mM to 1M over 20 min at 2 ml/min flow. UV detection was performed with a Perkin Elmer LC480 diode array detector.

Nucleoside diphosphate kinase, creatine kinase, phosphoglycerate kinase and pyruvate kinase reactions were conducted as described previously[11]. Reactions were monitored with the HPLC analysis described above.

Synthesis of diphosphates— The R-enantiomer (Fig. 1, 2R) of ganciclovir phosphonate monophosphate (resolved using GMP kinase, see above) was converted to the diphosphate (Fig. 1, 3R) by phosphorylation with creatine kinase and creatine phosphate. Reaction components were incubated for 24 hr at 37°C in a total volume of 7 ml: monophosphate, 5.6 mM; creatine phosphate, 61 mM; magnesium acetate, 57 mM; creatine kinase (Boehringer Mannheim, rabbit muscle), 1,350 IU/ml. The R-enantiomer of ganciclovir phosphonate diphosphate (85% conversion) was purified on DEAE Sephadex as described above.

The S-enantiomer diphosphate was chemically synthesized from the phosphonate after removal of the 7-isomer analog. Phosphonate (9.6 μmol), ammonium salt, was dried *in vacuo*, redissolved in 0.5 M triethylammonium bicarbonate, and then repeatedly coevaporated with acetonitrile. 1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone was added (0.2 ml) followed by 12 mg 1,1-carbonyldiimidazole then stirred 20 hr at room temperature. Methanol (0.01 ml) was added and stirred 30 min. Tributylammonium

pyrophosphate (46 mg) was added and stirred 90 min at 44°C. The S-enantiomer (Fig. 1, 3S) of ganciclovir phosphonate diphosphate (80% conversion) was purified by DEAE Sephadex chromatography.

DNA polymerase Assays—

The K_i's for nucleoside triphosphate analogs with HCMV polymerase were determined by measuring the incorporation of [^{3}H]dGTP into activated calf thymus DNA[12]. HCMV DNA polymerase was purified from cells of the human line MRC-5 that had been infected with HCMV strain AD169[12].

DNA polymerase-catalyzed incorporation of nucleotide analogs was determined with a defined primer/template:

5'-CGCGTATATAGCTGGCATCGTAC

3'-GCGCATATATCGACCGTAGCATGCTTTGAAAAATTCATATCCAACCCACGT CCAAGTAAA

The primer was 5'-end labeled with $^{32}PO_4$ using T4 polynucleotide kinase according to Pharmacia's protocol. Primer and template were annealed by mixing equal amounts (10 μM final concentration) then heating to 70°C for 10 min followed by slow cooling to room temperature. Annealed primer/template was stored at 4°C until use. Enough radiolabelled primer/template was present to give approximately 5000 cpm/μl in the reaction mixes. Human DNA polymerase δ reaction mixes contained 40 mM Tris-Cl, pH 7.8, 5 mM $MgCl_2$, 1.0 mM dithiothreitol, 150 μg/ml bovine serum albumin, 13 μg/ml human proliferating cell nuclear antigen, and 0.2 μM (primer ends) defined primer/template in a final volume of 10 μL.

HCMV DNA polymerase assays contained 25 mM Tris-Cl, pH 8.5, 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 1.0 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 0.2 μM (primer ends) defined primer/template (same sequence as above) in a final volume of 10 μL. Ganciclovir triphosphate and the ganciclovir phosphonate diphosphate R- and S-enantiomers were 50 μM. DeoxyGTP was 5 μM and, when used, dATP, dCTP and dTTP were also 5 μM. Reaction mixes were assembled without enzyme and preincubated at 37° for 4 minutes. Reactions were initiated with enzyme and incubation was continued at 37° for 30 minutes. Reactions were terminated by addition of 10 μL 10 mM EDTA, 95% formamide. Samples (5 to 10 μL) were heated at 70° for 5 minutes and then loaded onto 15% polyacrylamide gels containing 7.0 M urea. Gels were run at 2000

volts for 2.5 hr, and then exposed to X-ray film. Gels were exposed to a phosphorimager screen (Molecular Dynamics) and bands were quantitated using the Molecular Dynamics software.

HCMV Antiviral Assays— Assays for antiviral activity were performed using a DNA hybridization assay[13] with HCMV strain AD169 and MRC-5 lung fibroblasts.

Optical rotation of enantiomers— Specific rotation values for the individual enantiomers were measured by HPLC with tandem UV and high-sensitivity optical rotation detection[14]. The optical rotation detector was a laser-based (488 nm) instrument built in-house that was described in detail by Goss et al.[15]. The enantiomers of ganciclovir phosphonate had a retention time of 4 min on SAX HPLC (Phase-Sep Chromegabond SAX, 4.6 mm ID × 10 cm L, 1 ml/min flow rate in 15 mM ammonium phosphate pH 5.5 containing 5% methanol). The specific rotations reported (in Results) were the average of three determinations, ± SE of 20%. For comparison with results for chemically synthesized R-enantiomer obtained at 589 nm, we used the $1/\lambda^2$ dependence of specific rotation on wavelength to calculate $[\alpha]_{589}^{23}$ = -4.5 deg ml g^{-1} dm^{-1} for the R-enantiomer. This agreed with the specific rotations measured for independently prepared samples of the R-enantiomer from Elmer Reist[16] ($[\alpha]_{589}^{20}$ values from -4.0 to -5.0 deg ml g^{-1} dm^{-1}) and Chamberlain et al.[9] ($[\alpha]_{589}^{20}$ = -4.5 deg ml g^{-1} dm^{-1}).

RESULTS

Resolution of Ganciclovir Phosphonate Enantiomers

Resolution of the enantiomers of ganciclovir phosphonate was achieved after stereoselective phosphorylation of the R-enantiomer by porcine brain GMP kinase. The R-enantiomer of ganciclovir phosphonate monophosphate was separated from unreacted S-enantiomer by ion-exchange chromatography. Because the phosphonate bond was stable to alkaline phosphatase, the R-ganciclovir phosphonate monophosphate could be enzymatically hydrolyzed to R-ganciclovir phosphonate with alkaline phosphatase. The S-enantiomer was separated from a small amount of 7-isomer by preparative ion exchange HPLC. Enantiomeric purity of the enzymatically resolved R- and S-enantiomers was confirmed by specific rotation measurements made using HPLC with tandem UV and high-sensitivity optical rotation detection. Specific rotations obtained for the R-enantiomer ($[\alpha]_{488}^{23}$ = -6.8 deg ml g^{-1} dm^{-1}) and the S-enantiomer ($[\alpha]_{488}^{23}$ = +7.1

deg ml g^{-1} dm^{-1}) were equal and opposite within the experimental uncertainty of about 20%. The rotations of our enzymatically-resolved material agreed with values for chemically synthesized R-enantiomer (see Experimental Procedures). The published values were used to assign configurations to the enantiomers in this study. In addition to optical rotation measurement, we assessed enantiomeric purity of the S-enantiomer by its reactivity with GMP kinase (data not shown). Under reaction conditions that would have resulted in a biphasic time course due to an initial rapid rate of phosphorylation of the R-enantiomer followed by a slower rate of phosphorylation of the S-enantiomer, only the second slower phase was observed. Extrapolation of the time course (% conversion vs. time) to zero time showed that the S-enantiomer had, at most, a 0.6% contamination by the R-enantiomer. Together, the optical rotation and kinetic results indicate high enantiomeric purity for the enzymatically-resolved enantiomers.

Anti-HCMV Activity

Anti-HCMV activity mainly resided in the R-enantiomer (Table 1). The S-enantiomer also inhibited virus growth, but with IC_{50} about 20 times higher than the R-enantiomer. As would be expected, the racemic compound had an IC_{50} value twice that of the R-enantiomer. The *in vitro* antiviral activity of the R-enantiomer was somewhat less than that of ganciclovir.

Substrate Activities with Phosphorylation Enzymes

GMP kinase from human erythrocytes catalyzed the phosphorylation of ganciclovir phosphonate. The R-enantiomer was a good substrate for GMP kinase with about one-fourth the efficiency of GMP (Table 2). R-ganciclovir phosphonate was as efficient a substrate as ganciclovir monophosphate. Additionally, the R-enantiomer was over a thousand fold more efficient as a substrate than the S-enantiomer (Table 2). To ensure that the spectrophotometric assay for phosphorylation did not measure an initial rate due to a small contamination from the R-enantiomer, the reaction progress was also followed by an HPLC assay. The formation of S-ganciclovir phosphonate monophosphate proceeded in a linear manner over 19 hr to a final 60% conversion with a rate similar to that obtained with the spectrophotometric assay.

Phosphoglycerate kinase, creatine kinase and pyruvate kinase phosphorylated the monophosphate of R-ganciclovir phosphonate, but only pyruvate kinase was able to phosphorylate the S-enantiomer. This activity was 10% the rate of the R-enantiomer

TABLE 1.

Anti-HCMV activity of ganciclovir phosphonate analogs

compound	IC_{50}, μM
racemic	5.6
R-ganciclovir phosphonate	2.8
S-ganciclovir phosphonate	57
ganciclovir	1.7

DNA hybridization assays used the AD169 strain of HCMV grown in MRC-5 lung fibroblasts[13]

TABLE 2.

Phosphorylation of ganciclovir analogs by GMP Kinase

substrate	K_m (μM)	V_{max} (relative)	V_{max}/K_m (relative)
GMP	26	100	3.8
dGMP	30	53	1.8
R-ganciclovir phosphonate	52	54	1.0
S-ganciclovir phosphonate	850	0.8	0.0009
N-7-isomer	a	< 0.1	
ganciclovir monophosphate	47[b]	44[b]	0.94

GMP kinase from human red blood cells. V_{max} for GMP was 4.8 mM/min.

[a] Not a detectable substrate at 1.2 mM, 73 μM and 7 μM.

[b] Median of six values from references 5, 32, and 19.

TABLE 3.

Phosphorylation of GDP and ganciclovir phosphonate monophosphates

enzyme	GDP	R-ganciclovir phosphonate monophosphate	S-ganciclovir phosphonate monophosphate
	relative rate	relative rate	relative rate
phosphoglycerate kinase	100	0.008	<0.00001
creatine kinase	100	0.0008	<0.0002
pyruvate kinase	100	0.00002	0.000002
nucleoside diphosphate kinase	100	<0.009	<0.009

Nucleoside diphosphates were tested at 0.1 mM, 37°C, with HPLC assay described in Experimental Procedures. All enzymes were from rabbit muscle, except nucleoside diphosphate kinase was from beef liver. Rates of GDP phosphorylation (nmol/min/mg protein) were: phosphoglycerate kinase, 440,000; creatine kinase, 14,000; pyruvate kinase, 340,000; nucleoside diphosphate kinase, 35,000.

(Table 3). Nucleoside diphosphate kinase did not phosphorylate the monophosphate of either enantiomer.

Substrate Activities with DNA Polymerases

The diphosphates of both the R- and S-enantiomers were substrates for HCMV DNA polymerase and for human DNA polymerase δ. Incorporation into a defined template/primer was observed when either enantiomer was substituted for dGTP. With HCMV DNA polymerase, the rates of incorporation were: dGTP, 1.5 fmol/hr; ganciclovir triphosphate, 1.0 fmol/hr; R-ganciclovir phosphonate diphosphate, 0.45 fmol/hr; S-ganciclovir phosphonate diphosphate, 0.33 fmol/hr. With human DNA polymerase δ, the rates of incorporation were: dGTP, 0.34 fmol/hr; ganciclovir triphosphate, 0.2 fmol/hr; R-ganciclovir phosphonate diphosphate, 0.14 fmol/hr; S-ganciclovir phosphonate diphosphate, 0.13 fmol/hr. When dATP was added to the reaction mixtures, chain elongation was observed, indicating internal incorporation and chain extension after the

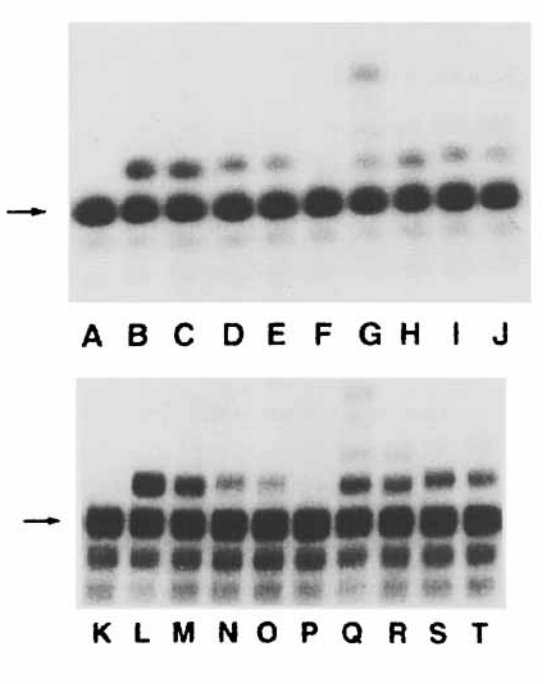

FIG. 2. Incorporation of radiolabeled dGTP into template/primer by human DNA polymerase δ (lanes A-J) or by HCMV DNA polymerase (lanes K-T). The heavy arrow indicates the band containing unextended template/primer. Bands below the arrow were a result of 3'→5' exonuclease proofreading activity of these polymerases. Nucleotide additions to the reactions were: lanes A-E, no analog, dGTP, ganciclovir triphosphate, R-ganciclovir phosphonate diphosphate, and S-ganciclovir phosphonate diphosphate, respectively; lanes F-J, the same analogs plus dATP; lanes K-O, no analog, dGTP, ganciclovir triphosphate, R-ganciclovir phosphonate diphosphate, and S-ganciclovir phosphonate diphosphate, respectively; and lanes P-T, the same analogs plus dATP, dCTP, and dTTP.

ganciclovir phosphonate nucleotide unit (Fig. 2, lanes F-J and P-T).

The diphosphates of both enantiomers were tested as inhibitors of HCMV DNA polymerase using activated calf thymus DNA as template/primer. Both enantiomers inhibited the incorporation of radiolabelled dGTP equally, with K_i values of approximately 15 μM (Table 4). The K_m for dGTP in this system was 0.22 μM.

DISCUSSION

Several nucleoside phosphonate analogs have antiviral activity, such as HPMPC, PMEC, and PMEA[17-18]. These compounds mimic nucleoside monophosphates, but have the advantages of biological stability and of having bypassed the initial phosphorylation

TABLE 4.

Inhibition of HCMV polymerase

Inhibitor	K_i, μM
R-ganciclovir phosphonate diphosphate	16
S-ganciclovir phosphonate diphosphate	13
ganciclovir triphosphate	0.43
acyclovir triphosphate	0.013

The primer/template was activated calf thymus DNA. The K_m for dGTP was 0.22μM.

by nucleoside kinases that is required for nucleosides. Bypass of nucleoside kinases is advantageous when cellular phosphorylating enzymes will not accept the nucleoside itself as substrate or when a drug resistant virus is deficient in the kinase or has altered specificity as a mechanism of resistance. The initial phosphorylation and activation step for ganciclovir phosphonate was a nucleoside monophosphate kinase, GMP kinase.

GMP kinase is essential for the anabolism of essentially all guanine-containing antiviral nucleosides and nucleoside phosphonates, such as acyclovir[7], ganciclovir[19], and HPMPG[20]. The stereoselectivity of phosphorylation by GMP kinase contributes to the stereoselectivity of antiviral activity of carbovir, ganciclovir and carbocyclic 2'-deoxyguanosine[21]. In each case, the enantiomer with the natural configuration is the preferred substrate for the enzyme.

The R-enantiomer of ganciclovir phosphonate had greater antiviral activity than the S-enantiomer (Table 1). This result with our enzymatically-resolved compounds agrees with that reported for the enantiomers that were made using a chiral chemical method[16]. The R-enantiomer was more active against HCMV than the S-enantiomer presumably because of its more efficient phosphorylation by GMP kinase and by diphosphate kinases, not because the HCMV polymerase was stereoselective. Although the S-enantiomer is a poor substrate for the kinases, this slow rate of phosphorylation may be enough to account for its *in vitro* antiviral activity. But it cannot be ruled out that the 0.6% or less of R-enantiomer (see Experimental Procedures) accounts for the antiviral activity of the S-enantiomer.

HCMV DNA polymerase was not stereoselective for the enantiomers of ganciclovir phosphonate diphosphate. Both were substrates (Fig. 2) at approximately the

same rate. Both also competed with dGTP with K_i values that were similar (Table 4). Neither enantiomer of ganciclovir phosphonate diphosphate can be considered a "potent" inhibitor of the polymerase (K_i/K_m approximately 70) compared to acyclovir triphosphate and ganciclovir triphosphate. Both enantiomers were substrates and were able to support DNA chain elongation and become internally incorporated into the lengthening DNA with chain extensions several nucleotides long after the ganciclovir phosphonate moiety (Fig. 2).

The lack of stereoselectivity of HCMV DNA polymerase should not be generalized to include other nucleoside triphosphate analogs or other DNA/RNA polymerases. This is just one instance of stereoselectivity of a polymerase with nucleoside triphosphate analogs. Human DNA polymerase α is not stereoselective with carbovir triphosphate enantiomers[22] but is stereospecific with the enantiomers of ganciclovir triphosphate[23] and cyclobutyl guanine triphosphate[24]. HIV reverse transcriptase is not stereoselective towards carbovir triphosphates[22] and cytosine-oxathiolane triphosphates[25], but is stereoselective towards thymidine-containing triphosphates[26]. Herpes simplex virus polymerase is stereoselective with enantiomers of ganciclovir triphosphate[6, 23] and cyclobutyl guanine triphosphate[24]. Hepatitis B polymerase is stereoselective but prefers the unnatural configuration of nucleoside triphosphates[27]. L-ATP is able to bind to and inhibit human DNA primase[28]. Extensive testing of a particular pair of enantiomer triphosphates has not been published for all of these polymerases. The stereoselectivity that has been observed may be attributed more to the type of molecule being tested (e.g., cyclic vs. acyclic nucleoside) rather than to the intrinsic nature of the polymerases. A recent publication has suggested that random chance, rather than evolutionary strategy, has determined the enantioselectivity of enzymes that interact with nucleosides and their phosphorylated forms[28].

Drug toxicity resulting from internal incorporation of nucleotide analogs into human DNA is considered a potential hazard for most nucleoside analogs. Acyclovir can not be incorporated due to lack of an appropriate hydroxyl group, but ganciclovir is incorporated[1, 29, 30], which presumably leads to carcinogenicity problems[31]. Both HCMV polymerase and human DNA polymerase δ incorporated ganciclovir phosphonate. The possibility of toxicity from DNA incorporation may limit the development of ganciclovir phosphonate as an anti-HCMV agent.

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