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Interaction of Guanine Phosphonomethoxyalkyl Derivatives with GMP Kinase Isoenzymes

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ABSTRACT. Substrate activity and inhibitory potency of guanine phosphonomethoxyalkyl derivatives towards GMP kinase isoenzymes from L1210 cells were studied. 9-[2-(Phosphonomethoxy)ethyl]guanine (PMEG) and the (*R*)- and (*S*)-enantiomers of both 9-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine (HPMPG) and 9-[2-(phosphonomethoxy)propyl]guanine (PMPG) were phosphorylated to the first step. Kinetic data showed that (*R*)-PMPG was a good substrate with a relative phosphorylation efficacy of 12% compared with the natural substrate GMP, whereas PMEG was a poor substrate with a relative phosphorylation efficacy of 1.1%. The structurally related 2,6-diaminopurine analogues 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) and (*R*)- and (*S*)-9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine (PMPDAP) were not phosphorylated by any of the GMP kinase isoenzymes tested. The inhibitory activities of the individual compounds on GMP kinase isoenzymes decreased in the following order: (*S*)-HPMPG > (*R*)-PMPG > PMEG > (*R*)-HPMPG > (*S*)-PMPG > PMEDAP = (*R*)-PMPDAP = (*S*)-PMPDAP; each compound exerted a different type of inhibition. BIOCHEM PHARMACOL **60**;12:1907–1913, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. acyclic nucleoside phosphonates; phosphorylation; GMP kinase

ANP† derivatives of both purine and pyrimidine bases are powerful antiviral agents with broad spectrum activity against DNA (adeno-, papova-, herpes-, hepadna-, irido-, pox-) viruses and/or retroviruses (i.e. HIV) [1, 2].

PMEG exerts the highest broad spectrum antiviral activity among these compounds reported to date [3–6]. In HSV-2 infected mice, its antiviral efficacy occurs at doses that are 10- to 50-fold lower than those of ACV (acyclovir) [7, 8]. PMEG also suppresses rabbit papilloma virus *in vivo* and HPV-11 infections in human skin xenografts [6]. A series of 2′-substituted derivatives of PMEG have been tested *in vitro* for anti-HIV activity [8–10]. Their anti-HIV activity depends on the size and the character of the substituent and, eventually, on the absolute configuration at the 2′-position of the PMEG molecule. In addition, these compounds generally demonstrate higher activity against HIV than HSV [8–10]. Also, the structurally related (hydroxymethyl) derivatives (*R*)- and (*S*)-HPMPG exhibit broad spectrum antiviral activity against HSV-1, HSV-2, human cytomegalovirus (HCMV), varicella zoster virus (VZV), and vaccinia virus [11].

PMEG also appears to be the most efficient cytostatic agent of all the phosphonomethoxyalkyl purines studied thus far. Its effect has been demonstrated in two types of mouse tumors *in vivo* [12], as well as in a model of spontaneous T-cell lymphoma in inbred SD/cub rats [13]. The cytostatic activity of PMEG correlates with the inhibitory potency of its diphosphate on the replicative DNA pol α and ϵ [14]. In human leukemia cell lines, PMEG reduces the proportion of the G_1 cell cycle phase and at higher concentrations induces apoptosis [15].

Once inside the cell, the ANP compounds must be activated by cellular nucleotide kinases to their active metabolites — ANPpp, i.e. nucleoside 5′-triphosphate analogues [16–19] that subsequently act as selective substrate/inhibitors of viral and cellular DNA polymerases [2, 14, 20] as well as of retroviral reverse transcriptases [21, 22]. Cellular nucleoside monophosphate kinases catalyze the first step, i.e. phosphorylation of ANPs to their monophosphates (ANPp, NDP analogues) [11, 17–19]. However, no data were available in the literature on the substrate activity of guanine ANPs towards guanylate kinases. Navé *et al.* [23] studied structurally somewhat related 9-(ω-phosphonoalkyl)- and 9-(ω-difluoro-ω-phosphonoalkyl) guanines; they did not find any phosphorylation with GMP kinase from porcine brain in either series, except for a poor reaction with 5-phosphonopentyl [23] and a 5-phosphono-4-pentenyl derivative [24].

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† *Abbreviations*: ANP, acyclic nucleoside phosphonate; ANPp, acyclic nucleoside phosphonate phosphate; ANPpp, acyclic nucleoside phosphonate diphosphate; DHPG, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; ACV, 9-[(2-hydroxyethoxy)methyl]guanine; PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; HPMPG, 9-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine; PMPG, 9-[2-(phosphonomethoxy)propyl]guanine; PMEDAP, 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine; PMPDAP, 9-[2-(phosphonomethoxy)propyl]-2,6-diaminopurine; HSV-2, herpes simplex virus type 2; and DTT, dithiothreitol.

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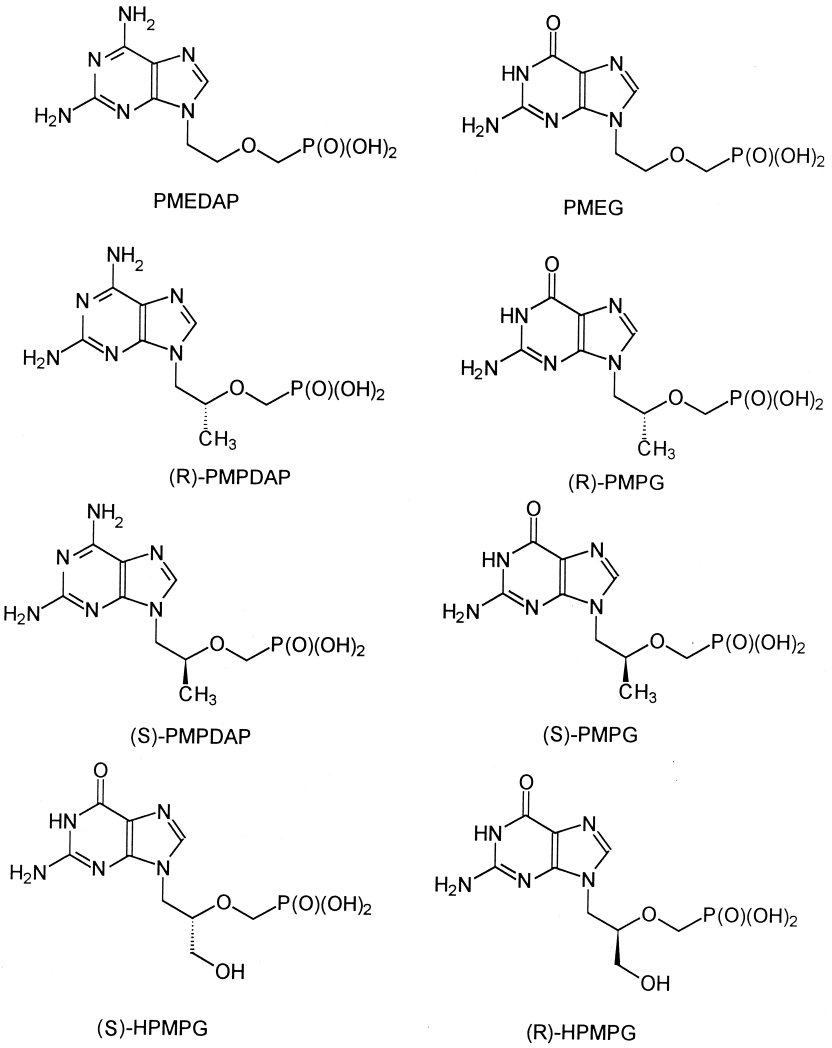

FIG. 1. Structure of purine 9-[2-(phosphonomethoxy)alkyl] derivatives.

The aim of our work was to investigate the phosphorylation of different types of biologically interesting 9-[2-(phosphonomethoxy)alkyl] compounds with GMP kinases (EC 2.7.4.8) in order to probe the structure of their GMP-binding site and the essential requirements for the phosphorylation reaction. We have chosen the recently described GMP kinase isoenzymes isolated from L1210 cells [25]. This cell line possesses high phosphorylating activity, and it is widely used for cytostatic screening. Porcine brain GMP kinase selected for comparison is the only enzyme that has been used for the phosphorylation of acyclic analogues in other laboratories [11, 23, 24]. This enzyme is available commercially and has no isoenzymes.

The study was performed with four out of five identified GMP kinase isoenzymes and involved PMEG and its congeners, (*R*)-PMPG, (*R*)-HPMPG, and (*S*)-HPMPG (Fig. 1). In addition to this set of guanine derivatives, we also investigated the structurally related, highly biologically active 2,6-diaminopurine derivatives PMEDAP [2], (*R*)-PMPDAP, and (*S*)-PMPDAP [10], which can be considered as structural analogues of GMP.

MATERIALS AND METHODS

Chemicals

[U-^{14}C]GMP (16.5 GBq mmol^{-1}) was synthesized in the Institute for Research, Production and Uses of Radioisotopes. [^{3}H]PMEG (550 GBq mmol^{-1}), [^{3}H]PMEDAP (470 GBq mmol^{-1}), [^{3}H](*R*)-HPMPG (170 GBq mmol^{-1}), [^{3}H](*S*)-HPMPG (400 GBq mmol^{-1}), [^{3}H](*R*)-PMPG (460 GBq mmol^{-1}), [^{3}H](*S*)-PMPG (530 GBq mmol^{-1}), [^{3}H](*R*)-PMPDAP (27 GBq mmol^{-1}), and [^{3}H](*S*)-PMPDAP (570 GBq mmol^{-1}) were synthesized in the Central Laboratory of Radioisotopes, Charles University. All nonlabeled acyclic analogues of nucleotides were synthesized as described by Holy *et al.* [26–28]. Polybuffer exchanger PBE 94 for chromatofocusing and Blue Sepharose were purchased from Pharmacia P-L Biochemicals. Centriprep-10 was from Amicon. All chemicals used, as well as porcine brain GMP kinase, were products of the Sigma Chemical Co. Polygram^R Cel 300 PEI/UV$_{254}$ for TLC was from Macherey–Nagel.

GMP Kinase Isoenzymes

GMP kinase isoenzymes were isolated from mouse leukemia L1210 cells using a previously described purification procedure [25]. Briefly, the frozen L1210 cells were disrupted by freeze–thawing followed by homogenization in a Dounce tissue grinder. Crude extracts were precipitated with ammonium sulfate (35–60%), and the sediments were desalted and applied onto an hydroxyapatite column. The GMP phosphorylating activity was eluted stepwise with ammonium sulfate (2–10%). The desalted active fractions were applied onto the Blue Sepharose column, and the enzyme activity was eluted with 1 mM ATP. The collected fractions containing the highest enzyme activity were concentrated using Centriprep-10 and applied onto the chromatofocusing column (polybuffer exchanger PBE 94). The five resulting peaks of GMP kinase isoenzymes (pI 5.95, 5.50, 5.08, 4.83, and 4.51) with specific activity (mU mg^{-1}) 595.4, 1 440.5, 460.9, 108.3, and 123.8, respectively, were pooled and stored at -70° for kinetic measurements.

Guanylate Kinase Assay

The standard reaction mixture (20 μL) contained 100 mM Tris–HCl, pH 7.5, 100 mM KCl, 4 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM [U-^{14}C]GMP (0.99 GBq $mmol^{-1}$), and an appropriate amount of the analogue tested. The reactions were carried out at 30° for 10 min and stopped by spotting of a 5-μL aliquot onto a polyethyleneimine-cellulose TLC plate (pretreated by soaking in distilled water and dried) that had been prespotted with 0.01 μmol of each substrate and product as carriers. The plate was developed in the solvent system 0.8 M LiCl in 0.8 M CH_3COOH. The spots were visualized under UV light (254 nm), cut out, and evaluated for radioactivity in a toluene-based scintillation fluid. One enzyme unit (U) is defined as the amount of enzyme that catalyzes the phosphorylation of 1 μmol GMP per min under the standard assay conditions.

Kinetic Experiments

Kinetic constants (K_m and V_{max}) were determined from the Lineweaver–Burk plots using various concentrations of GMP, (*S*)-HPMPG, (*R*)-PMPG (2.5–200 μM), PMEG, and (*R*)-HPMPG (10–750 μM) at a fixed concentration of ATP (1 mM saturating concentration) and an appropriate aliquot of GMP kinase isoenzyme. For GMP, 9.3 μU of isoenzyme 5.95, 10.8 μU of isoenzyme 5.50, 8.4 μU of isoenzyme 5.08, and 18.6 μU of isoenzyme 4.83 were used, while for phosphorylation of the phosphonate analogues the amounts of GMP kinase isoenzymes were approximately 5–10 times higher. The reactions were carried out at 30° for 10 min.

For the v_i/v_o assay, the reaction mixture contained 5 μM [U-^{14}C]GMP (20 GBq $mmol^{-1}$), 1 mM ATP, two concentrations of the phosphonate analogue (25 and 50 μM), and an appropriate aliquot of GMP kinase isoenzyme (isoenzyme 5.95, 9.3 μU; isoenzyme 5.50, 10.8 μU; isoenzyme 5.08, 8.4 μU; and porcine brain GMP kinase, 229 μU).

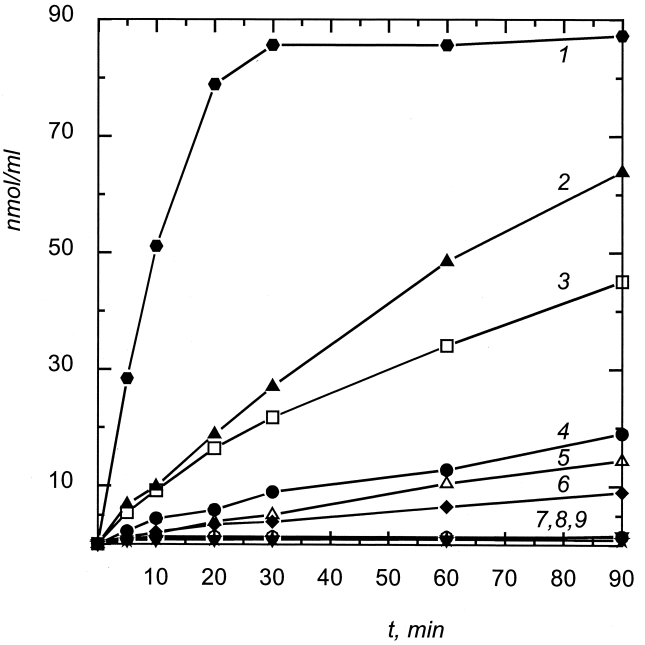

FIG. 2. Time course of the phosphorylation of phosphonomethoxyalkyl purines catalysed by GMP kinase isoenzyme 5.50. The reaction mixture contained 100 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, 1 mM DTT, 0.1 mM [^{14}C]GMP, and 64.8 μU isoenzyme 5.50, or a corresponding 0.1 mM [^{3}H]phosphonomethoxyalkyl purine and 216 μU isoenzyme 5.50. The reactions were carried out at 30°. See Materials and Methods for details. Products formed (curve number): 1, GDP; 2, (*R*)-PMPGp; 3, (*R*)-HPMPGp; 4, PMEGp; 5, (*S*)-HPMPGp; 6, (*S*)-PMPGp; 7, PMEDAPp; 8, (*R*)-PMPDAPp; and 9, (*S*)-PMPDAPp.

Four concentrations of selected phosphonate analogues, i.e. (*S*)-HPMPG (5, 10, 15, and 20 μM), (*R*)-PMPG (10, 20, 30, and 40 μM), and PMEG (25, 50, 75, and 100 μM), and four concentrations of GMP (2.5, 5, 10, and 15 μM) were used for the estimation of the inhibition constants (K_i) for the three isoenzymes mentioned above.

Data (Lineweaver–Burk, Dixon, and Hanes plots) based on results from four independent experiments were evaluated by the nonlinear regression method (Biosoft EnzFitter, 32 bit version for Windows).

RESULTS

Purine phosphonomethoxyalkyl derivatives (Fig. 1) were studied as potential substrates or inhibitors of GMP kinase isoenzymes in the kinetic experiments. To evaluate the susceptibility of phosphonate analogues to their transformation to monophosphates, the time course of their phosphorylation was compared with that of the natural substrate GMP. The same pattern of phosphorylation, which is shown in Fig. 2 for isoenzyme 5.50, was found for all GMP kinase isoenzymes from L1210 and for the porcine brain GMP kinase. Among the compounds studied, (*R*)-PMPG was the best substrate. On the contrary, we did not detect

TABLE 1. Substrate activity of guanine 9-[2-(phosphonomethoxy)alkyl] derivatives towards GMP kinase isoenzyme 5.50

Substrate	K_m (μmol/L)	V_{max} (μmol/min/mg)	V_{max}/K_m ($10^3 \cdot$ L/min/mg)	Relative efficiency of reaction* (%)
GMP	30.2 ± 2.66	2.481 ± 0.082	82	100
(*R*)-PMPG	19.1 ± 2.04	0.189 ± 0.005	9.9	12.1
(*S*)-HPMPG	11.4 ± 0.97	0.036 ± 0.0013	3.16	3.9
(*R*)-HPMPG	328 ± 24.8	0.459 ± 0.019	1.40	1.7
PMEG	209 ± 18.5	0.197 ± 0.008	0.94	1.1

Kinetic constants are averages of a least four separate experiments; K_m and V_{max} values are ± SD. See Materials and Methods for details.
*100 X $^{analogue}V_{max}/^{analogue}K_m/^{GMP}V_{max}/^{GMP}K_m$.

any phosphorylation of either PMEDAP or of the (*R*)- and (*S*)-enantiomers of PMPDAP by the GMP kinase isoenzymes tested.

The kinetic constants K_m and V_{max} were determined for PMEG, (*R*)-PMPG, and (*R*)- and (*S*)-HPMPG. As the kinetic data were approximately the same for isoenzymes 5.95, 5.50, and 5.08, only the data for isoenzyme 5.50 are presented (Table 1). Our results show that the affinity of (*R*)-PMPG (K_m values) towards all the GMP kinase isoenzymes was about equal to that of GMP. However, the comparison of its V_{max}/K_m ratios with the value for GMP showed approximately 10-fold lower substrate activity. Kinetic data for (*S*)-HPMPG demonstrated substantially higher affinity of this compound towards isoenzymes 5.95, 5.50, and 5.08, respectively, compared with GMP (Table 1). On the other hand, the V_{max} values for its enantiomer, (*R*)-HPMPG, were 10-fold higher compared with those for the (*S*)-enantiomer and 5-fold lower with respect to GMP. As a result of this dichotomy, (*S*)-HPMPG was phosphorylated in the presence of most of the GMP kinase isoenzymes tested with about twice as high catalytic efficiency as its (*R*)-counterpart (Table 1, Fig. 2).

Our data demonstrated relatively inefficient, but highly significant phosphorylation of PMEG and (*R*)-HPMPG (Table 1, Fig. 2). The affinity of these acyclic nucleotide analogues towards these isoenzymes studied was at least 10-fold lower than the affinity of GMP and the phosphorylation proceeded with 30–100 times lower efficiency (Table 1). As concerns isoenzyme 4.83, the relative efficiency of the phosphorylation reaction was the same as for the other isoenzymes. The high K_m values and very low V_{max}/K_m ratios for the isoenzyme 4.83 probably reflect the substantially lower specific activity (5–15 times).

The favorable affinity of (*S*)-HPMPG (Table 1) towards GMP kinase isoenzymes 5.95, 5.50, and 5.08 led us to investigate the potential role of acyclic nucleoside phosphonates as inhibitors of these enzymes. Preliminary experiments (v_i/v_o ratio) performed with the isoenzyme 5.50 confirmed such activity. (*S*)-HPMPG, (*R*)-PMPG, and PMEG exhibited, in addition to their substrate activity versus GMP kinases, considerable inhibitory effect on GMP phosphorylation, which decreased in the order of (*S*)-HPMPG > (*R*)-PMPG > PMEG > (*R*)-HPMPG > (*S*)-PMPG (Table 2). The 2,6-diaminopurine derivatives (*R*)-PMPDAP, (*S*)-PMPDAP, and PMEDAP, which are not substrates for GMP kinase isoenzymes, were also inactive as inhibitors (Table 2).

A detailed study of the inhibitory potency (K_i/K_m) and the type of inhibition was performed with three isoenzymes (pI 5.95, 5.50, and 5.08; Table 3) and compared with porcine brain GMP kinase. (*S*)-HPMPG was the most powerful inhibitor of GMP kinase isoenzymes from L1210 cells as well as of the porcine brain GMP kinase. While the inhibitory potency of this phosphonate analogue towards

TABLE 2. Relative inhibitory activity of 9-[2-(phosphonomethoxy)alkyl]purines towards GMP kinase isoenzyme 5.50 and porcine brain GMP kinase*

Phosphonomethoxyalkyl purine as inhibitor†	GMP kinase isozyme 5.50		Porcine brain GMP kinase	
	25 μmol/L (v_i/v_o‡)	50 μmol/L (v_i/v_o‡)	25 μmol/L (v_i/v_o§)	50 μmol/L (v_i/v_o§)
(*R*)-PMPG	0.45	0.33	0.59	0.43
(*S*)-PMPG	0.94	0.88	0.83	0.81
(*R*)-HPMPG	0.92	0.89	1.10	0.94
(*S*)-HPMPG	0.21	0.16	0.42	0.31
PMEG	0.79	0.68	0.86	0.69
PMEDAP	1.04	1.06	1.03	1.21
(*R*)-PMPDAP	1.02	1.00	0.96	1.24
(*S*)-PMPDAP	1.00	1.05	1.22	1.49

*Values (v_o, velocity of non-inhibited reaction; v_i, velocity of inhibited reaction) are averages of at least three separate experiments.
†5 μM [^{14}C]GMP as the substrate, 10 min at 30°C. See Materials and Methods for details.
‡v_o = 155.5 ± 9.33 pmol/mL/min.
§v_o = 82.6 ± 5.78 pmol/mL/min.

TABLE 3. Inhibition of GMP kinase isoenzymes by guanine 9-[2-(phosphonomethoxy)alkyl] derivatives

	(*S*)-HPMPG			(*R*)-PMPG			PMEG		
GMP kinase*	K_i (μmol/L)	$K_i/^{GMP}K_m$	Inhibition type†	K_i (μmol/L)	$K_i/^{GMP}K_m$	Inhibition type†	K_i (μmol/L)	$K_i/^{GMP}K_m$	Inhibition type†
Isoenzyme 5.95	2.87 ± 0.11	0.093	PN	23.1 ± 1.13	0.747	N	122 ± 13.2	3.95	C
Isoenzyme 5.50	2.23 ± 0.10	0.074	PC	9.4 ± 0.64	0.312	PC	103 ± 9.3	3.41	C
Isoenzyme 5.08	2.43 ± 0.17	0.088	PN	24.7 ± 1.55	0.898	N	94 ± 9.6	3.42	C
Porcine brain‡	4.61 ± 0.61	0.099	PC	61.6 ± 7.66	1.319	N	223 ± 30.0	4.78	C

Kinetic constants are averages of at least four separate experiments; K_m and V_{max} values are ± SD. See Materials and Methods for details.

*Values represent pI.

†PN, partially non-competitive; PC, partially competitive; N, non-competitive; and C, competitive.

‡$^{GMP}K_m$ = 46.7 ± 5.5 μmol/L.

individual isoenzymes (K_i values, K_i /$^{GMP}K_m$ ratios) was about the same (Table 3), the type of inhibition with respect to individual GMP kinases was different. A partially competitive inhibition was observed for porcine brain GMP kinase and isoenzyme 5.50 of GMP kinase from L1210 cells, while with isoenzymes 5.95 and 5.08 a partially non-competitive character of inhibition was noted (Fig. 3, Table 3).

An analogous inhibition of GMP phosphorylation by (*R*)-PMPG was 10-fold lower than that caused by (*S*)-HPMPG (Table 3). Its character was non-competitive for isoenzymes 5.95 and 5.08, and porcine brain GMP kinase. Finally, the parent structure PMEG exerted very weak inhibitory activity with a competitive character (Table 3).

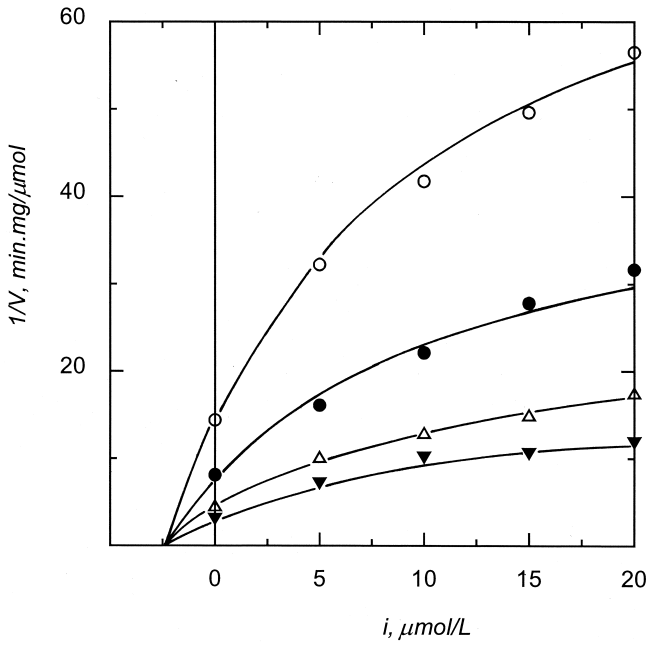

FIG. 3. Dixon plot of GMP kinase isoenzyme 5.08 inhibition by (*S*)-HPMPG. The reaction mixture contained 100 mM Tris–HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, [^{14}C]GMP [2.5 (○), 5 (●), 10 (Δ), and 15 (▼) μmol/L] and (*S*)-HPMPG (0, 5, 10, 15, and 20 μmol/L). The reaction was started by 8.4 μU isoenzyme 5.08, and proceeded for 10 min at 30°. See Materials and Methods for details.

DISCUSSION

The antiviral and cytostatic activity of acyclic nucleoside phosphonates is subdued to activation by phosphorylation that provides their diphosphates (analogues of dNTPs), which interact selectively with the cellular and/or viral DNA polymerases [14, 20–22]. The nucleoside monophosphate kinases are responsible for the key step of ANP-phosphorylation in the adenine and cytosine series [17, 18]. This process is enantiospecific, i.e. the absolute configuration at the side chain of these compounds is important for this reaction [17, 18]. Our present effort intended to provide evidence that the phosphorylation of guanine phosphonomethoxyalkyl derivatives is catalyzed by specific GMP kinases. Our data demonstrated that all GMP kinase isoenzymes from L1210 cells catalyze the phosphorylation of PMEG as well as of both enantiomers of its 2′-methyl derivative (PMPG) and/or 2′-hydroxymethyl derivative (HPMPG), respectively. These results are compatible with the biological activity of these compounds [1–4, 10, 12, 13]. Thus, both enantiomers of HPMPG, which are phosphorylated approximately to the same extent (Fig. 2, curves 3 and 5), have similar antiviral activity. It contrasts with the enantioselective action of 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl] (HPMP) derivatives of adenine and cytosine; albeit, their antivirally inactive (*R*)-counterparts are not phosphorylated by the corresponding NMP kinases [2, 17, 18].

The substantially different phosphorylation patterns of both of the enantiomers of PMPG by GMP kinase isoenzymes from L1210 cells (Fig. 2, curves 2 and 6) indicate that the GMP-binding site of these isoenzymes does not favour the binding of the (*S*)-PMPG contrary to the structurally related (*R*)-HPMPG, which bears a hydrophilic CH_2OH group instead of a CH_3 substituent. (It should be pointed out that, for formal reasons, the absolute configurations of (*R*)-HPMPG and (*S*)-PMPG are identical.)

GMP kinase is rather specific as to the nucleoside 5′-phosphate structure. The GMP-binding domain undergoes conformational changes when the enzyme binds ATP in addition to GMP. These changes are small at the domain, which binds a purine ring, but probably larger at the domain interacting with the ribose and with the

phosphate group of GMP [29]. In addition to their substrate activity, (*S*)-HPMPG, (*R*)-PMPG, and PMEG also inhibit GMP kinases from L1210 cells as well as from porcine brain. The kinetics of inhibition indicates that the binding of ANPs occurs at more than the GMP-binding site. Therefore, different types of inhibition of GMP kinases, which were observed for diverse phosphonomethoxyalkyl guanines, most probably reflect a different character of their interactions (due to the side-chain replacing ribose) with this GMP-binding site and/or with the close neighbourhood of the binding site.

The lack of phosphorylation activity of 2,6-diaminopurine derivatives confirms the stringency of the GMP-binding site for the guanine moiety [29]. These compounds (both enantiomers of PMPDAP as well as PMEDAP) are known for their potent antiviral activity [10]. Thus, some other phosphorylating system must be responsible for their phosphorylation in the cell.

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