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Synthesis, antiviral activity and enzymatic phosphorylation of 9-phosphonopentenyl derivatives of guanine

Jean-François Navé [a,*], Debra Taylor [b], Stan Tyms [b], Michael Kenny [c], Anne Eggenspiller [a], Anne Eschbach [a], Jacqueline Dulworth [c], Tara Brennan [b], François Piriou [a], Serge Halazy [a,1]

[a] *Marion Merrell Dow Research Institute, 16 rue d'Ankara, 67080 Strasbourg, France*
[b] *Medical Research Council Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London NW7 1AD, UK*
[c] *Marion Merrell Dow Inc., Park B, 10236 Marion Park Drive, Kansas City, MO 64137-1404, USA*

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Abstract

(*E*)-9-(5-Phosphonopent-4-enyl)guanine and (*E*)-9-[3-(hydroxymethyl)-5-phosphonopent-4-enyl]guanine which bear a vinyl phosphonate moiety as a mimic of the phosphate group were synthesized. Their activities against human immunodeficiency virus type-1 (HIV-1), herpes simplex virus type-1 (HSV-1) and human cytomegalovirus (HCMV) were evaluated in vitro in parallel with those of 9-(5-phosphonopentyl)guanine and 9-(5,5-difluoro-5-phosphonopentyl)guanine. Both vinyl phosphonates exhibited anti-HIV-1 and anti-HCMV activities, whereas the methyl- and difluoromethyl phosphonate analogues were inactive. The selectivity index, calculated as the ratio of the toxicity for the host cells (50% reduction in cell viability or in [methyl-^{3}H]thymidine incorporation) to the 50% inhibitory concentration for HIV-1 replication, was the highest for (*E*)-9-[3-(hydroxymethyl)-5-phosphonopent-4-enyl]guanine. The acyclonucleotide analogues were also studied as substrates of guanylate kinase, an enzyme believed to play a critical role in the conversion of acyclic phosphate and phosphonate derivatives of guanine to their antivirally active diphosphate derivatives. (*E*)-9-(5-Phosphonopent-4-enyl)guanine and (*E*)-9-[3-(hydroxymethyl)-5-phosphonopent-4-enyl]guanine were good substrates of guanylate kinase,

* Corresponding author.
[1] To whom requests for reprints should be sent. Present address: Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81106 Castres, France.

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being phosphorylated with efficiencies of 14 and 36% of that determined for GMP, respectively. These results contrast with the poor efficiency found for 9-(5-phosphonopentyl)guanine (0.3%) and the lack of phosphorylation of 9-(5,5-difluoro-5-phosphonopentyl)guanine by guanylate kinase (Navé et al. (1992) Arch. Biochem. Biophys. 295, 253–257). The role of the vinyl phosphonate group in the expression of the anti-HIV-1 activity of the phosphonopentenyl derivatives of guanine is discussed.

Keywords: 9-(Phosphonopentenyl) derivatives of guanine; Anti-HIV-1 activity; Anti-herpes virus activity; Phosphorylation; Guanylate kinase

1. Introduction

The discovery of the broad spectrum antiviral activity of the phosphonomethoxyalkyl derivative of adenine (*S*)-HPMPA (De Clercq et al., 1986) has prompted many investigators to search for new antiviral agents of the acyclonucleotide type. Initially, two series of compounds have received considerable attention. These are the 9-[2-(phosphonomethoxy)ethyl]purines and (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl] purines/pyrimidines. In the former series, 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) was found to have selective activity against retroviruses including human immunodeficiency virus (HIV) and also showed some anti-herpesvirus activity (De Clercq et al., 1987, Pauwels et al., 1988a). In the latter, (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine ((*S*)-HPMPC) exhibited selective activity against cytomegalovirus (De Clercq et al., 1987). More recently, many acyclonucleotide analogues containing the phosphonomethyl ether functionality have been described including 9-(phosphonomethoxyalkyl)purine/pyrimidine (Kim et al., 1990a,b; Balzarini et al., 1993; Yu et al., 1993), 9-(phosphonomethoxyalkoxy)purine (Duckworth et al., 1991; Perkins et al., 1992) and 9-(phosphonomethoxyalkoxyalkyl)purine/pyrimidine (Kim et al., 1991) derivatives. Several of these acyclonucleotide analogues are potent and selective inhibitors of the replication of various herpes- and retroviruses. They most likely exert their antiviral effect following sequential activation by cellular kinases to their diphosphate derivatives (nucleoside triphosphate analogues) (Votruba et al., 1987; Balzarini et al., 1991a) which act as potent inhibitors of viral DNA polymerases. Therefore, the efficiency of conversion of these acyclonucleotide analogues to their diphosphate derivatives is a critical factor for antiviral activity since it determines the level of their active metabolites in cells. There is evidence that phosphorylation of the 5′-monophosphate derivatives of the acyclic nucleoside analogues acyclovir and ganciclovir is catalyzed intracellularly by guanylate kinase (Miller and Miller, 1980; Ashton et al., 1982; Cheng et al., 1983). The resulting acyclic nucleoside diphosphate derivatives might be subsequently phosphorylated by several enzymes having broad substrate specificities (Miller and Miller, 1982). Phosphorylation by guanylate kinase has been demonstrated for several acyclonucleotide derivatives of guanine containing the phosphonomethoxyalkyl (Terry et al., 1988; Ho et al., 1991) or phosphonoalkoxyalkyl moieties (Duke et al., 1986), and it is likely that this enzyme plays a prominent role in their activation. Recently, in a study of the substrate specificity of guanylate kinase, we found that 9-(5-phosphonopentyl)guanine was a poor substrate of this enzyme (Navé et

al., 1992). In studies with adenylate kinase, a nucleoside-5′-monophosphate kinase having a catalytic mechanism similar to that of guanylate kinase, Hampton and collaborators (1976) reported that the *trans*-alkenylphosphonic acid analogue of AMP was a 3-fold more efficient substrate than its saturated phosphonate isostere. This suggested that the *trans*-alkenylphosphonic acid analogue of 9-(5-phosphonopentyl) guanine could be a substrate of guanylate kinase. If so, and provided that both further conversion to the diphosphate derivative occurs in intact cells and the diphosphate derivative is an inhibitor of viral DNA polymerase, this compound might exhibit antiviral activity. This prompted us to synthetize (*E*)-9-(5-phosphonopent-4-enyl)guanine and its 3-hydroxymethyl derivative. In this report, we describe the synthesis and in vitro antiviral activity of these compounds as well as their substrate properties for guanylate kinase. While this work was in progress, Harnden and collaborators (1993) reported the synthesis and antiviral properties of a series of phosphonoalkenyl and phosphonoalkenyloxy derivatives of purines. Yet, the phosphonopentenyl derivatives of guanine were not described in that report.

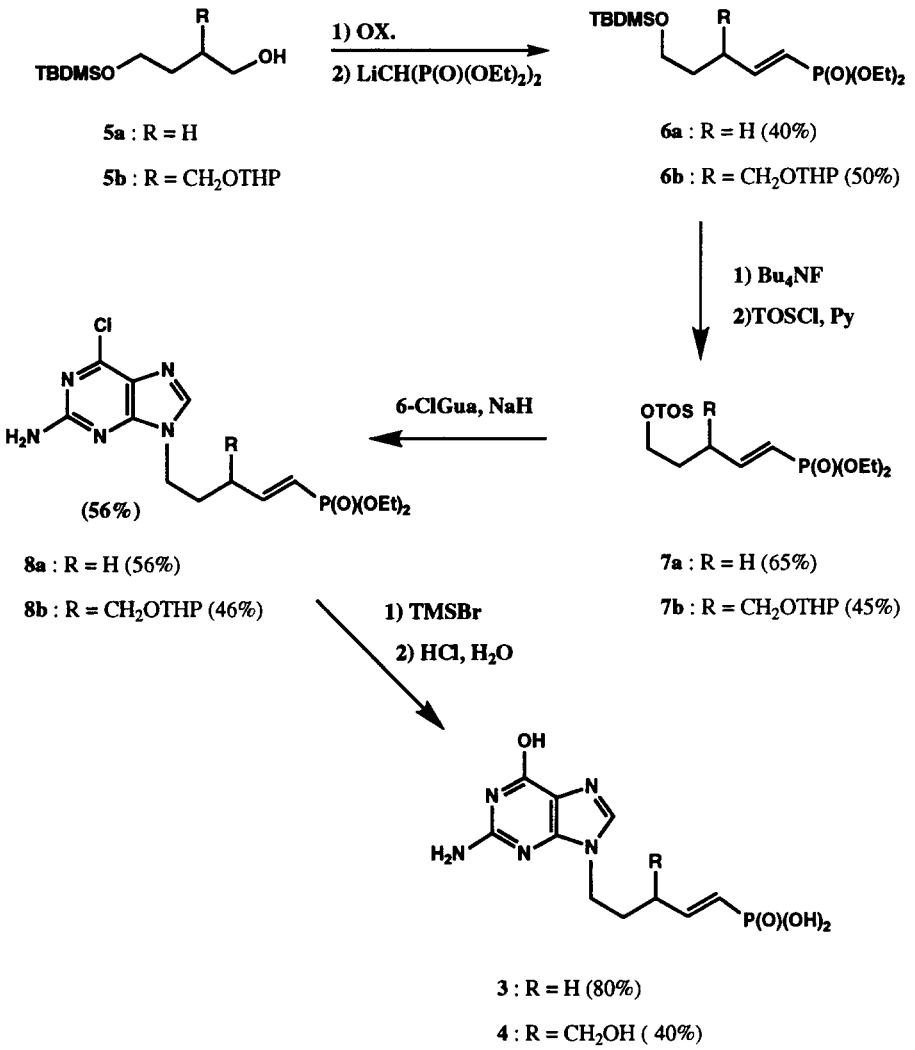
2. Materials and methods

2.1. Chemicals and enzyme

[methyl-^{3}H]Thymidine (43 Ci/mmol) was bought from Amersham. Guanylate kinase (porcine brain) was obtained from Boehringer Mannheim (Mannheim, Germany). The methylene phosphonate derivative of guanine **2** and its difluoro analogue **1** were prepared according to Halazy et al. (1991) and PMEA according to the method of Holy and Rosenberg (1987). Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide) was purchased from Viratek Inc., (Covina, CA). Acyclovir was obtained from Sigma (St. Louis, MO). For the guanylate kinase assay and the determination of the anti-HIV-1 activity, stock solutions of acyclonucleotides were prepared in 5 mM Tris-HCl (pH 7.5) and 10 mM HEPES (pH 7.6), respectively. For determination of the anti-HSV-1 and anti-HCMV activities, stock solutions (100 mM) of compounds were prepared in DMSO. These solutions were then diluted at least 250-fold in test medium.

2.2. Synthesis of (E)-9-(5-phosphonopent-4-enyl)guanine and of (E)-9-[3-(hydroxymethyl)-5-phosphonopent-4-enyl]guanine

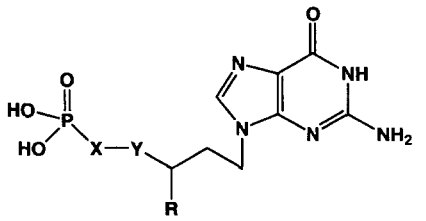
The (*E*)-vinyl phosphonate derivatives of guanine **3** and **4** were prepared, respectively, from the alcohol derivatives **5a** and **5b** according to Scheme 1. The common key step of the procedures is the stereocontrolled synthesis of the (*E*)-vinyl phosphonates **6a** and **6b**. This was achieved by condensation of the anion of tetraethylmethylene bis-phosphonate (Minami and Motoyoshiya, 1992) with the appropriate aldehydes freshly prepared by oxidation of the alcohol derivatives **5a** (by using PDC in dichloromethane) or **5b** (by using catalytic amounts of TPAP (Griffith et al., 1987) in the presence of *N*-methylmorpholine *N*-oxide in dichloromethane), respectively. The obtention of the phosphonic acid derivatives of guanine **3** and **4** from the intermediates



Scheme 1.

5a and **5b** is then straightforward (Scheme 1): the *t*-butyldimethylsilyl protecting group in the intermediates **6a** or **6b** is regioselectively removed by reaction with tetrabutylammonium fluoride in THF and the resulting alcohol is transformed into a leaving group (tosyl chloride, pyridine) suitable for displacement by the anion of 2-amino-6-chloropurine to give regioselectively the intermediates **8a** or **8b** (N9/N7 > 93% as determined by ^{1}H-NMR analysis according to Geen et al., 1990). Reaction of these intermediates with excess trimethylsilyl bromide (McKenna and Schmidhauser, 1979), followed by acid aqueous hydrolysis (1 N HCl, 80°C) allowed the isolation of the final products **3** and **4**.

[5-(2-Amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)-penten-1-yl] phosphonic acid with 0.7



Compounds	R	X—Y
1	H	$F_2C—CH_2$
2	H	$H_2C—CH_2$
3	H	HC=CH
4	CH_2OH	HC=CH

Fig. 1. Structural formulae of 9-difluorophosphonopentyl, 9-phosphonopentyl and 9-phosphonopentenyl derivatives of guanine.

HBr, 0.3 HCl and 0.5 H_2O (compound **3**) was isolated as a white solid; m.p. > 250°C (dec); ^{1}H-NMR (360 MHz, D_2O; pH 1.7): δ 2.2 (m, 2H, CH_2); 2.4 (m, 2H, CH_2); 4.15 (t, 2H, J(H-H) = 7 Hz, CH_2N), 4.7 (s, > 3H, D_2O exchangeable), 5.8 (dd, 1H, J(H-H) = 15 Hz, J(H-P) = 17 Hz, CHP), 6.5 (m, 1H, $CHCH_2$), 8.9 (s, 1H, H_8); UV(H_2O): λ_{max} 253 nm (ϵ 12775) and 270 nm (ϵ 9365); Anal. calc. ($C_{10}H_{14}N_5O_4P$, 0.7 HBr, 0.3 HCl, 0.5 H_2O): C, 31.96; H, 4.29; N, 18.64. Found: C, 31.63; H, 4.06; N, 18.32.

[5-(2-Amino-1,6-dihydro-6-oxo-9*H*-purin-9-yl)-3-hydroxymethyl-penten-1-yl] phosphonic acid disodium salt (compound **4**): The compound was purified by RP-HPLC on a 250-10 Lichrospher 100-RP-18 column (Merck) eluted with a buffer composed of 0.1 M KH_2PO_4, 5 mM tetrabutyl ammonium dihydrogen phosphate (pH 5.0), 1.6% acetonitrile. After passage through a 2.5 × 35 cm DEAE-sephadex A-25 column (Pharmacia) eluted with a linear gradient of NH_4HCO_3 (1 l, 10 mM to 1 M) and after removal of NH_4HCO_3, the compound was obtained as the pure diammonium salt. This was then converted to **4** (white solid) after passage through a 1.1 × 5.5 cm AG-50W-X8 column (Na^+ form); ^{1}H-NMR (360 MHz, D_2O): δ 1.9 (m, 1H, CH_2), 2.1 (m, 1H, CH_2), 2.35 (m, 1H, CH), 3.6 (m, 2H, CH_2O), 4.1 (m, 2H, CH_2N), 4.7 (s, > 3H, D_2O exchangeable), 5.95 (dd, 1H, CHP, J(H-H) = 17 Hz; J(H-P) = 17 Hz); 6.3 (m, 1H, CH vinyl), 7.85 (s, 1H, H_8); UV (H_2O): λ_{max} 252 nm (ϵ 10650) and 266 nm (ϵ 8210); Anal. calc. ($C_{11}H_{14}N_5O_5PNa_2$, 1.5 H_2O): C, 33.01; H, 4.28; N, 17.50. Found: C, 32.66; H, 4.55; N, 17.17.

2.3. Viruses, cells and media

The HIV-1 strain RF was obtained from the AIDS Directed Programme (ADP) Reagent Project (National Institute for Biological Standards and Controls, Potters Bar, UK). HSV-1 (strain HF) and human cytomegalovirus (HCMV; strain AD-169) were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

The human lymphoid cell lines C-8166 and MT-4 were obtained from the ADP Reagent Project and grown in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, 290 μg/ml L-glutamine, 100 U/ml penicillin and 50 μg/ml streptomycin. Human peripheral blood mononuclear cells (PBMC) were prepared from acid citrate dextrose treated buffy coat samples obtained from the North London Blood Transfusion Centre (Colindale, London). The PBMC were isolated by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and depleted of CD8-positive cells using antibody coated magnetic beads (Dynal, Oslo, Norway) according to the Manufacturer's instructions. The PBMC were cultured for 3 days in growth medium supplemented with 10 μg/ml phytohemagglutinin (PHA-M, Boehringer Mannheim) and 10 U/ml interleukin-2 (IL-2, Boehringer Mannheim) before infection with HIV-1.

African green monkey kidney cells (Vero cells; MA Bioproducts, Walkersville, MD) were grown at 37°C in Eagle's minimal essential medium with Earle's salts (EMEM; Whittaker Bioproducts Inc., Walkersville, MO) supplemented with 50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 100 μg/ml neomycin sulfate (1% PSN, GIBCO) and 8% heat-inactivated fetal calf serum (HIFCS). Human lung embryonic fibroblasts (MRC-5 cells, ATCC, Rockville, MD) were grown in EMEM supplemented with 0.1 mM non-essential amino acids (Whittaker), 1% PSN and 10% HIFCS. Vero and MRC-5 cells were maintained in EMEM supplemented with 1% PSN and 1% HIFCS.

2.4. Determination of anti-HIV-1 activity of compounds

Evaluation of the anti-HIV-1 activity of compounds in MT-4 cells was done using the MTT colorimetric assay essentially as described by Pauwels et al. (1988b). Briefly, exponentially growing cells were washed and counted. Half of the cells were infected with HIV-1 (100 $TCID_{50}$ per 5×10^4 cells) and the other half mock-infected. After a 1-h incubation at room temperature, cells were pelleted, washed once in RPMI, adjusted at 5×10^5 cells/ml and 100 μl transferred to each well of a 96-well flat-bottomed cell culture plate filled with 100 μl of growth medium containing various concentrations of the test compounds or no compound. After 6 days of incubation at 37°C, the viability of mock- and HIV-1-infected cells was determined spectrophotometrically using the MTT assay.

For evaluation of the anti-HIV-1 activity of compounds in C-8166 cells, exponentially growing cells were washed and counted. After infection with HIV-1 for 1 h at room temperature (100 $TCID_{50}$ per 1×10^5 cells), the cells were pelleted, washed 3 times in RPMI, adjusted at 10^6 cells/ml and 100-μl aliquots distributed into wells of 96-well cell culture plates filled with 100 μl of growth medium containing various concentrations of the test compounds or no compound. After 3 days of incubation at 37°C, the cell-free culture medium was assayed for levels of p24 viral core antigen using the ELISA method of Moore et al. (1990).

The anti-HIV-1 activity of compounds in PBMC was determined as follows. After isolation from buffy coat and culture for 3 days as described above, the cells were washed and infected with HIV-1 (1000 $TCID_{50}$ per 5×10^5 cells) for 1 h at room temperature. After washing twice, the cells were resuspended in growth medium containing 10 U/ml IL-2 (5×10^6 cells/ml) and 100 μl distributed into wells of

24-well cell culture plates filled with 900 μl of growth medium containing various concentrations of the test compounds or no compound. After 7 days of incubation at 37°C, the cell-free culture medium was assayed for levels of p24 viral core antigen using an ELISA (Coulter, Ltd.).

2.5. Determination of anti-HSV-1 and anti-HCMV activity of compounds

Antiviral activity against HSV-1 and HCMV was determined in Vero and MRC-5 cells, respectively. Aliquots of 0.1 ml of Vero (10^6 cells/ml) or MRC-5 (1.5×10^6 cells/ml) cells in the appropriate growth medium were transferred to 96-well flat-bottomed microtiter trays. After incubation for 24 h at 37°C, the growth medium was aspirated from the microtiter plate cultures and replaced with 100 μl maintenance medium (cell and virus controls) or compound diluted in maintenance medium at various concentrations (toxicity wells, test wells). After incubation for 3 h at 37°C, each culture received 100 μl maintenance medium (cell controls and toxicity wells) or virus (10-50 $TCID_{50}$ HSV-1 or HCMV) diluted in maintenance medium (virus controls, test wells). Microtiter trays were then incubated at 37°C and examined microscopically after 3 days (HSV-1) or 10 days (HCMV) for virus- and compound-induced cytopathic effect. Cytopathic effect was graded as 0 (no), 1 + (25%), 2 + (50%), 3 + (75%) or 4 + (100%) cell monolayer destruction.

2.6. Potentiation of the anti-HCMV effect of (E)-9-(5-phosphonopent-4-enyl)guanine by ribavirin

A checkerboard titration was employed using 0, 10, 50 or 250 μM ribavirin in combination with 0, 2.5, 5, 10, 20, 40 or 80 μM (*E*)-9-(5-phosphonopent-4-enyl)guanine. MRC-5 cell monolayers were treated with 0.1 ml appropriate compound mixture for 3 h at 37°C and 0.1 ml HCMV suspension (approximately 100 $TCID_{50}$) was then added. After incubation at 37°C for 11 days, the ability of the individual compounds or combinations thereof to reduce virus-induced cytopathic effect (compared to the no compound controls) was determined.

2.7. Determination of cytotoxicity of compounds in MT-4 cells and PBMC

Uninfected MT-4 cells were grown in the presence or absence of varying concentrations of test compound as described above. After 6 days of incubation at 37°C, cell viability was assessed using the MTT assay.

Freshly isolated human PBMC were distributed into wells (2×10^5 cells per well) of 96-well round-bottomed microtiter plates and stimulated with 2.5 μg/ml concanavalin A (Boehringer Mannheim) for 2 days at 37°C in the absence or in the presence of various concentrations of the test compounds. The cells were then labeled with [methyl-^{3}H]thymidine (1 μCi per well) for 24 h and the radioactivity incorporated was assessed by cell harvest onto glass fiber paper, followed by measurement in a β-spectrometer.

Table 1
Anti-HIV-1 activity of phosphonopentyl and phosphonopentenyl derivatives of guanine in MT-4 and C-8166 cells

Compound	EC_{50} (μM) [a,b]		CC_{50} (μM) [c]
	MT-4 cells	C-8166 cells	MT-4 cells
AZT	0.04 ± 0.01	0.04 ± 0.03	113 ± 36
ddI	13 ± 10	0.9 ± 0.4	> 1000
PMEA	20 ± 10	1.5 ± 1.0	187 ± 135
1	> 500	–	> 500
2	> 320	> 320	> 320
3	56 ± 21	12 ± 10	138 ± 63
4	48 ± 12	8 ± 6	1235 ± 442

Except for compounds **1** and **2**, data are mean values of at least 3 independent experiments ± S.D.
[a] Concentration required to inhibit HIV-1-induced cythopathic effect by 50% in MT-4 cells.
[b] Concentration required to inhibit p24 viral antigen production by 50% in C-8166 cells.
[c] Concentration required to reduce the viability of cells by 50%.

2.8. Guanylate kinase assay

The enzymatic reaction was studied with a continuous spectrophotometric assay which measures both ADP and GDP formed from ATP and GMP by coupling to the pyruvate kinase and lactate dehydrogenase reactions (Navé et al., 1992). In this assay, the phosphorylation of 1 μmol of GMP in the presence of ATP ultimately results in the oxidation of 2 μmol of NADH. In studies of stoichiometry where (*E*)-9-(5-phosphonopent-4-enyl)guanine was used as phosphate acceptor, the phosphorylation of 1 μmol of this compound by guanylate kinase resulted in the oxidation of 1 μmol of NADH (data not shown). Similarly, the phosphorylation of 1 μmol of (*E*)-9-[3-(hydroxymethyl)-5-phosphonopent-4-enyl]guanine resulted in the oxidation of 1 μmol of NADH. These stoichiometries were used for the calculation of rates of phosphorylation of these acyclonucleotides.

3. Results

3.1. Anti-HIV-1 activity of the acyclonucleotide analogues in MT-4 and C-8166 cells

Compounds **1–4** (Fig. 1) were first evaluated for their activity against HIV-1 in the human T-cell lines MT-4 and C-8166 (Table 1). The acyclonucleotide analogue PMEA and the nucleoside analogues 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI), which are selective inhibitors of HIV replication, were used as references. At a multiplicity of infection that would completely destroy MT-4 cells by day 6 postinfection, PMEA protected 50% of the cells against HIV-1-induced cytopathic effect at a concentration of 20 μM (EC_{50}). ddI and AZT were 2- and 500-fold more potent than PMEA (Table 1). This 500-fold difference between EC_{50} values of AZT and PMEA in MT-4 cells is consistent with previous results from Balzarini et al. (1989). 9-(5-Phos-

phonopentyl)guanine **2** (EC_{50} > 320 μM) and 9-(5,5-difluoro-5-phosphonopentyl) guanine **1** (EC_{50} > 500 μM) were inactive. In contrast, the vinyl phosphonate **3**, which only differs from **2** by a *trans* double bond in position α–β to the phosphorus atom, inhibited HIV-1 replication with an EC_{50} value only 3-fold higher than that of PMEA. Compound **4**, the 3-hydroxymethyl derivative of **3** was also an inhibitor of HIV-1 replication with potency similar to **3**.

Cytotoxic concentrations of compounds (CC_{50}) in MT-4 cells were determined by the MTT assay (Table 1). Compound **3** was slightly more toxic than PMEA. Compound **4** was 6-fold less toxic than PMEA. Thus, in MT-4 cells, the selectivity indexes (CC_{50}/EC_{50}) for PMEA, compounds **3** and **4** were 9.4, 2.5 and 25, respectively.

The anti-HIV-1 activity of vinyl phosphonates **3** and **4** and the lack of activity of **2** observed in MT-4 cells was confirmed in C-8166 cells (Table 1). In this cell line, the anti-HIV-1 activity was determined by measuring inhibition of p24 antigen production (Table 1). PMEA was an effective inhibitor of HIV-1 replication (EC_{50} = 1.5 μM). Compounds **3** and **4** were, respectively, 8-fold and 5-fold less potent than PMEA. At 320 μM, compound **2** had no effect on p24 antigen production. PMEA and compounds **3** and **4** exhibited little toxicity against C-8166 cells. Their CC_{50} values determined by the MTT assay were > 1000 (n = 4), 700 ± 216 (n = 3), > 1000 (n = 2) μM, respectively.

3.2. Anti-HIV-1 activity of phosphonopentenyl derivatives of guanine in peripheral blood mononuclear cells

The anti-HIV-1 activity of vinylphosphonates **3** and **4** was further investigated in human PBMC. In these cells, as observed above in MT-4 and C-8166 cells, AZT (EC_{50} = 0.002 μM) emerged as the most potent inhibitor of HIV-1 replication (Table 2). ddI (EC_{50} = 0.7 μM) and PMEA (EC_{50} = 1.0 μM), albeit less effective than AZT, exhibited potent anti-HIV-1 activity. Compounds **3** and **4** were equipotent and about 4-fold less active than PMEA.

The toxicity of compounds to PBMC was assessed by their ability to inhibit DNA synthesis in uninfected cells. Compound **3** was slightly less toxic than PMEA (Table 2), whereas compound **4** was 5-fold less toxic than PMEA. Selectivity indexes for PMEA, compounds **3** and **4** were 90, 30 and 121, respectively.

Table 2
Anti-HIV-1 activity of phosphonopentenyl derivatives of guanine in peripheral blood mononuclear cells

Compound	EC_{50} (μM) [a]	CC_{50} (μM) [b]
AZT	0.002 ± 0.002	10 ± 8
ddI	0.7 ± 0.2	680
PMEA	1.0 ± 0.5	90 ± 8
3	3.8 ± 0.5	116 ± 34
4	3.7 ± 2.0	450 ± 108

Except for ddI CC_{50} and compound 3 EC_{50} results, data are mean values of at least 3 independent experiments ± S.D.

[a] Concentration required to inhibit p24 viral antigen production by 50%.

[b] Concentration of compound required to inhibit the incorporation of [^{3}H]thymidine in uninfected cells by 50%.

Table 3
Antiherpesvirus activity of phosphonopentyl and phosphonopentenyl derivatives of guanine

Compound	EC_{50} (μM) [a]		CC_{50} (μM) [b]	
	HSV-1	HCMV	Vero cells	MRC-5 cells
Acyclovir	5 ± 2 [c]	35 ± 5 [d]	> 100	> 100
PMEA	43 ± 24 [d]	56 ± 20 [d]	292 ± 156 [c]	> 250
1	> 250	> 1000	⩾ 250	> 1000
2	> 250	> 250	> 250	> 250
3	⩾ 250	48	250	188
4	> 250	39	> 250	> 250

Except data indicated, results are mean values of two independent experiments.
[a] Concentration required to inhibit HSV-1- or HCMV-induced cythopathic effect by 50%.
[b] Concentration required to reduce cell viability by 50%.
[c] Mean of 3 independent experiments ± S.D.
[d] Mean of 2 independent experiments.

3.3. Anti-HSV-1 and anti-HCMV activity of acyclonucleotide analogues

The acyclonucleotide analogues **1–4** were tested against herpes viruses HSV-1 and HCMV. As shown in Table 3, none of these compounds was active against HSV-1, whereas acyclovir (ACV) inhibited the virus-induced cytopathic effect with an EC_{50} of 5 μM. However, both vinylphosphonates **3** and **4** were active against HCMV replication in MRC-5 cells. The EC_{50} recorded were close to those determined for PMEA and ACV (EC_{50} = 56 and 35 μM, respectively), used as references. Neither the difluorophosphonate **1** nor the phosphonate **2** were active against HCMV. Compound **3** was toxic to MRC-5 cells at a concentration only 4-fold higher than its EC_{50}. Compound **4** was not toxic at 250 μM.

3.4. Phosphorylation of acyclonucleotide analogues by guanylate kinase

At 1 mM, a concentration 31-fold higher than the K_m of GMP, using a concentration of enzyme 10-fold higher than that used for GMP, 9-(5,5-difluoro-5-phosphonopentyl)guanine **1** was not a substrate of guanylate kinase (Navé et al., 1992). In contrast, under similar conditions, its methylene phosphonate analogue **2** was phosphorylated by guanylate kinase (Table 4). However, **2** is a poor substrate since its efficiency of phosphorylation is only 0.3% of that of GMP, as estimated from the V_{max}/K_m ratios. Interestingly, compound **3**, the vinyl phosphonate analogue of **2**, was a 47-fold more efficient substrate of guanylate kinase than **2** (efficiency = 14% of that of GMP). Thus, the introduction of the *trans* double bond α–β to the phosphorus atom produces a 6.4-fold decrease in K_m and a 7.3-fold increase in V_{max}. The vinyl phosphonate **4** was also a good substrate of guanylate kinase, i.e. 2.6 times more efficient than **3**.

3.5. Potentiation of the anti-HCMV effect of (E)-9-(5-phosphonopent-4-enyl)guanine by ribavirin

Phosphorylation of the acyclonucleotide derivatives of guanine by guanylate kinase is considered as a mandatory step in the pathway leading to their diphosphate derivatives.

Table 4
Phosphorylation of phosphonoalkyl and phosphonoalkenyl derivatives of guanine by guanylate kinase

Substrate	K_m (μM)	V_{max} [a] (% of GMP)	V_{max}/K_m (% of GMP)
GMP	32	100	100
1 [b]	– [c]	– [c]	–
2 [b]	250	2.4	0.30
3 [d]	39	17.6	14
4 [d]	13	14.5	36

[a] V_{max} of GMP = 10.1 μmol/min/(mg protein).
[b] Data from Navé et al., 1992.
[c] No substrate activity at 1 mM.
[d] For compounds **3** and **4**, the kinetic parameters were determined at enzyme concentrations 4 and 5 times higher than that used for GMP, respectively.

Therefore, a decrease in the cellular level of GMP (the natural substrate of guanylate kinase) should result in an increased phosphorylation of the guanine acyclonucleotides and ultimately in a higher cellular level of their diphosphate derivatives, i.e. in a more effective inhibition of viral DNA polymerase or reverse transcriptase. The diphosphate derivatives of guanine acyclonucleotides compete with dGTP for binding to viral DNA

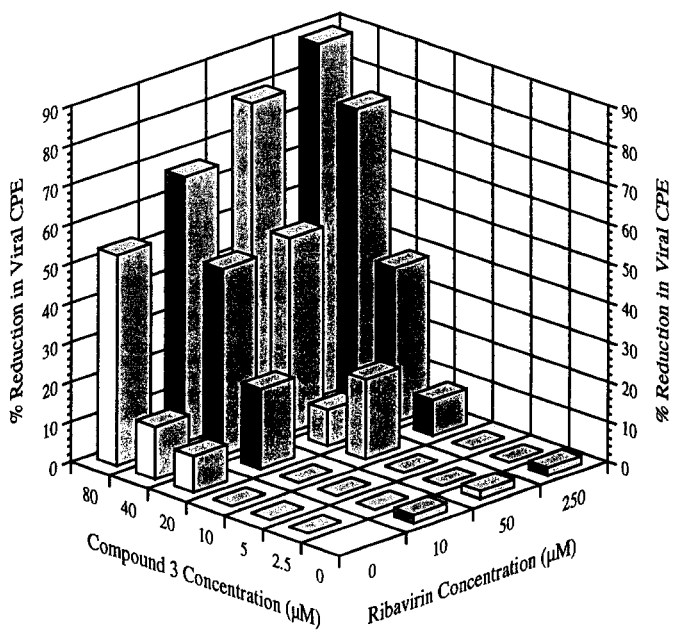


Fig. 2. Potentiation of the anti-HCMV effect of (*E*)-9-(5-phosphonopent-4-enyl)-guanine by ribavirin. For each combination of compound **3** and ribavirin, 7 wells were employed for determination of the virus-induced cytopathic effect. Results are expressed as percent reduction in virus-induced cytopathic effect when compared to no compound controls.

polymerase and/or for incorporation into the growing DNA chain (acting as chain terminators). Hence, decreasing dGTP level should lead to more efficient inhibition of viral DNA polymerase or reverse transcriptase by the diphosphate derivatives of guanine acyclonucleotides. The broad-spectrum antiviral agent ribavirin exerts various biochemical actions, prominent among which is the inhibition of inosine-5′-monophosphate (IMP) dehydrogenase by its anabolite ribavirin-5′-monophosphate (Streeter et al., 1973). Consequently, treatment of cells with ribavirin causes a marked depletion (> 70% at ribavirin concentrations higher than 40 μM) in the guanine nucleotide pools including GMP, GDP, GTP and dGTP (Streeter et al., 1973; Ahluwalia et al., 1990; Balzarini et al., 1991b).

To have a better insight into the mechanism of action of vinyl phosphonate derivatives of guanine, the effect of ribavirin on the anticytomegalovirus activity of compound **3** was investigated. As shown in Fig. 2, ribavirin alone did not reduce the cytopathic effect induced by HCMV in MRC-5 cells. In the absence of ribavirin, compound **3** reduced the virus-induced cytopathic effect by 50% at a concentration of 80 μM. In the presence of a fixed concentration of ribavirin of 10 μM, the antiviral effect of compound **3** was half-maximal at 40 μM. In the presence of 250 μM ribavirin, compound **3** reduced the virus-induced cytopathic effect by 50% at a concentration of 30 μM (Fig. 2). Hence, the addition of ribavirin led to a modest, but significant, 2- to 3-fold increase in the anti-HCMV activity of compound **3**. None of the tested combinations of ribavirin and compound **3** produced overt toxicity to uninfected MRC-5 cells (data not shown).

4. Discussion

With the exception of the recent work from Harnden et al. (1993) where synthesis and antiviral activity of several 9-phosphonobutenyl and 9-phosphonobutenyloxy derivatives of guanine are described, no information concerning acyclonucleotide analogues bearing a vinyl phosphonate group is available in literature. In this work, we describe the synthesis and the in vitro anti-HIV-1 and antiherpetic activity of two novel acyclic 9-phosphonopentenyl derivatives of guanine, namely (*E*)-9-(5-phosphonopent-4-enyl)guanine and its 3-hydroxymethyl derivative (compounds **3** and **4**; Fig. 1). Both compounds were tested against HIV-1 in parallel with PMEA, a promising acyclonucleotide analogue (Pauwels et al., 1988a; Tsai et al., 1994) undergoing clinical evaluation for the treatment of AIDS. In the lymphoid cell lines MT-4 and C-8166 and in human PBMC, the vinyl phosphonates **3** and **4** exhibited similar anti-HIV-1 activity and were, respectively, about 3, 7 and 4 times less potent than PMEA (Tables 1 and 2). The cellular toxicity of compound **3** was similar to that of PMEA (Tables 1 and 2). In MT-4 cells and PBMC, compound **4** was respectively 9- and 4-fold less toxic than its unbranched analogue **3**. Therefore, compound **4** is less potent, but more selective, than PMEA. It is interesting to compare these results with those reported by Harnden et al. (1993) for analogues of **3** and **4** which only differ by the presence of an oxygen atom in place of the methylene group linked to N-9 of guanine. In human peripheral blood lymphocytes (PBL), (*E*)-9-[(4-phosphonobut-3-enyl)oxy] guanine, the analogue of **3**

showed anti-HIV-1 activity with an IC_{50} of 0.1 μM, but was found to be very cytotoxic at 0.2 μM (inhibition of [^{3}H]thymidine incorporation) (Harnden et al., 1993). (*E*)-9-[[2-(Hydroxymethyl)-4-phosphonobut-3-enyl]oxy] guanine, the analogue of **4** showed anti-HIV-1 activity in PBL with an IC_{50} of 9 μM, but was toxic to MRC-5 cells at 19 μM, as determined by inhibition of thymidine incorporation (Harnden et al., 1993). Clearly, compounds **3** and **4** which inhibit [^{3}H]thymidine incorporation in PBMC with CC_{50} values of 116 and 450 μM (Table 2) are much less toxic than the corresponding analogues in which the acyclic chain is attached to guanine by an oxygen–nitrogen bond. The vinyl phosphonates **3** and **4** were devoid of anti-HSV-1 activity. However, both compounds exhibited anti-HCMV activity comparable to that measured for PMEA and ACV, used as references (Table 3). PMEA and ACV are known to be much less effective inhibitors of HCMV replication than (*S*)-HPMPC (De Clercq et al., 1987; Snoeck et al., 1988), an acyclonucleotide derivative of cytosine undergoing clinical evaluation for the treatment of HCMV infection in immunocompromised patients. Therefore, compounds **3** and **4** should be considered as modest anti-HCMV agents.

Compounds **1**, **2** and **3** (Fig. 1) have alkyl chains of similar length. However, they differ by the structure of their phosphate-like moiety, both sterically and electronically. In contrast to compound **3**, compounds **1** and **2** were inactive against HIV (EC_{50} > 320 μM in MT-4 cells) and HCMV (EC_{50} > 250 μM in MRC-5 cells) replication. Differences in activity of these structurally related acyclonucleotide derivatives of guanine may be explained by differences in their ability to enter the cells, to be phosphorylated by guanylate kinase, to undergo a second phosphorylation step catalyzed by cellular kinases and ultimately by differences in potency of their diphosphate derivatives as inhibitors of viral DNA polymerases. Studies of phosphorylation of these guanine acyclonucleotide analogues by guanylate kinase shed some light on their mode of action. The difluorophosphonate **1** was not phosphorylated by guanylate kinase even in the presence of a high concentration of enzyme and this could be a reason for its lack of activity. The methylene phosphonate **2** was a poor substrate, being phosphorylated with an efficiency of 0.3% of that of GMP. This might explain, at least in part, the lack of antiviral activity of **2**. However, the guanine acyclonucleotide analogue (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]guanine ((*S*)-HPMPG) was also found to be a poor substrate of guanylate kinase with an efficiency of phosphorylation of 0.6% of that of GMP (J.-F. Navé and A. Eschbach, unpublished results). Nevertheless, (*S*)-HPMPG is an effective inhibitor of HSV-1 and HCMV replication with EC_{50} values around 1 μM (Balzarini et al., 1993). Therefore, other factors (e.g. poor inhibition of viral DNA polymerase by the diphosphate derivative of **2**) may be the cause for the lack of activity of compound **2**. Interestingly, the vinyl phosphonate **3** was found to be a relatively good substrate of guanylate kinase with an efficiency of phosphorylation of 14% of that of GMP and therefore a 47-fold more efficient substrate than its saturated analogue **2**. The other vinyl phosphonate **4** also proved to be a good substrate of guanylate kinase (efficiency of phosphorylation = 36%). The ability of compounds **3** and **4** to serve as good substrates of guanylate kinase may contribute significantly to the expression of their antiviral activity. It is of note that the anti-HCMV activity of **3** could be increased approximately 2-fold by ribavirin (Fig. 2), an agent known to produce a marked depletion of the cellular GMP and dGTP pools (Streeter et al., 1973; Ahluwalia et al.,

1990; Balzarini et al., 1991b). This result shows that the level of the diphosphate derivative of **3** in competition with dGTP for binding to the active site of viral DNA polymerase is indeed critical in the expression of its antiviral activity. This result also probably reflects a more extensive cellular phosphorylation of **3** by guanylate kinase, linked to the lower intracellular concentration of GMP in the presence of ribavirin.

In conclusion, our results indicate that the vinylphosphonate group can serve as a good phosphate mimic in the 9-phosphonopentenyl derivatives of guanine described herein. The vinylphosphonate group confers to these molecules both good substrate properties for guanylate kinase and significant in vitro antiretroviral activity. (*E*)-9-[3-(Hydroxymethyl)-5-phosphonopent-4-enyl]guanine has a better selectivity index than its unbranched analogue. Synthesis of other 9-phosphonopentenyl derivatives of guanine might lead to the discovery of novel interesting antiviral agents.

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