ELSEVIER

Antiviral Research 43 (1999) 37–53

Antiviral Research

www.elsevier.com/locate/antiviral

Synthesis and antiviral activity of phosphoralaninate derivatives of methylenecyclopropane analogues of nucleosides

Yao-Ling Qiu [a], Roger G. Ptak [b], Julie M. Breitenbach [b], Ju-Sheng Lin [c], Yung-Chi Cheng [c], John C. Drach [b], Earl R. Kern [d], Jiri Zemlicka [a,*]

[a] *Department of Chemistry, Experimental and Clinical Chemotherapy Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201-1379, USA*

[b] *Department of Biologic and Materials Science, School of Dentistry, University of Michigan, Ann Arbor, MI 48019-1078, USA*

[c] *Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510-8066, USA*

[d] *Department of Pediatrics, The University of Alabama at Birmingham, Birmingham, AL 35294, USA*

Received 17 November 1998; accepted 18 March 1999

Abstract

Phenylmethylphosphoro-L-alaninate prodrugs of antiviral *Z*-methylenecyclopropane nucleoside analogues and their inactive *E*-isomers were synthesized and evaluated for their antiviral activity against HCMV, HSV-1, HSV-2, HHV-6, EBV, VZV, HIV-1 and HBV. The adenine *Z*-analogue was a potent inhibitor of all these viruses but it displayed cellular toxicity. The guanine *Z*-derivative was active against HCMV, HBV, EBV and VZV and it was not cytotoxic. The 2,6-diaminopurine analogue was the most potent against HIV-1 and HBV and somewhat less against HHV-6, HCMV, EBV and VZV in a non-cytotoxic concentration range. The 2-amino-6-cyclopropylamino and 2-amino-6-methoxypurine prodrugs were also more active than parent analogues against several viruses but with a less favorable cytotoxicity profile. In the *E*-series of analogues, adenine derivative was active against HIV-1, HBV and EBV, and it was non-cytotoxic. The guanine analogue exhibited a significant effect only against HBV. The 2,6-diaminopurine *E*-analogue was inactive with the exception of a single EBV assay. The 2-amino-6-methoxypurine *Z*-methylenecyclopropane nucleoside analogue was an effective inhibitor of HCMV, MCMV and EBV. The 2,6-diaminopurine *Z*-prodrug seems to be the best candidate for further development.

Keywords: Antivirals; EBV; HBV; HCMV; HHV-6; HIV-1; HSV-1; HSV-2; MCMV; Methylenecyclopropane analogues; Phenyl phosphoralaninates; Prodrugs; VZV

* Corresponding author. Tel.: +1-313-833-0715, ext. 2452; fax: +1-313-832-7294.
E-mail address: zemlicka@kci.wayne.edu (J. Zemlicka)

0166-3542/99/$ - see front matter
PII: S0166-3542(99)00029-7

1. Introduction

Recently, we have described a new series of nucleoside analogues based on a methylenecyclopropane structure (Fig. 1, formula 1) which exhibit a broad-spectrum activity against herpesviruses and hepatitis B virus (Qiu et al., 1998a,b; Hartline et al., 1998). Several of the purine analogues are active in vivo in a mouse model of CMV infection (Rybak et al., 1998). Our previous results with unsaturated nucleoside analogues lacking the ribofuranose moiety indicated that introduction of a phosphoralaninate residue can (i) potentiate antiretroviral activity of active analogues and (ii) activate inactive compounds (Winter et al., 1996, 1997). These lipophilic pronucleotides are capable of releasing the activated (phosphorylated) analogues inside the cells. A distinct advantage of this type of lipophilic phosphoramidates is that in contrast to other lipophilic phosphate esters (Perigaud et al., 1996) they do not require action of phosphodiesterase(s) in the process of intracellular activation. This is particularly important for analogues which do not incorporate structural features (e.g. cyclic ribofuranose-like moiety) deemed important for function of these enzymes. It is therefore of interest to examine similar phosphate derivatives derived from methylenecyclopropane nucleoside analogues. An additional facet of importance is seen in the fact that to the best of our knowledge an evaluation of lipophilic phosphoralaninate derivatives of a series of nucleoside analogues in several different antiviral assays has not been described.

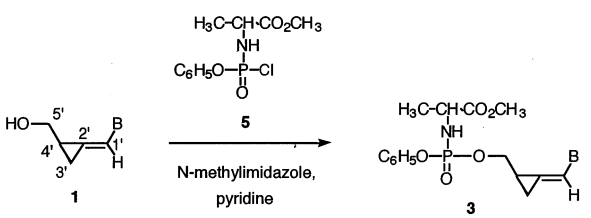

Fig. 1. Synthesis of phosphoralaninate diesters of *Z*-methylenecyclopropane analogues **3a**–**3e**. Series a, B = Ada; series b, B = Gua; series c, B = 2,6-diaminopurine; series d, B = 2-amino-6-cyclopropylaminopurine; series e, B = 2-amino-6-methoxypurine; series f, B = 2-amino-6-chloropurine.

Several aspects of lipophilic phosphoramidate prodrugs of methylenecyclopropane analogues are of particular importance: (i) potentiation of antiviral activity of biologically active methylenecyclopropane derivatives; (ii) broadening the scope of antiviral activity; (iii) possibility of activation of inactive *E*-methylenecyclopropane analogues; and (iv) relationship of antiviral activity and cytotoxicity. Regarding the last point, it is recognized that an increase of intracellular concentration of analogues of nucleoside triphosphates can lead to inhibition of mammalian enzymes, e.g. DNA polymerase and, thus, to cytotoxic effects. In such a case the advantage of circumvention of the first phosphorylating step by a phosphoramidate prodrug may be partially or completely lost.

It also should be noted that lipophilic phosphoramino acid derivatives of nucleoside analogues with an intact furanose ring were investigated primarily as anti-HIV agents (McGuigan et al., 1996). Only rarely a potency higher than the parent highly active analogue was achieved and importance of these agents rests in their efficacy against viral infections of kinase-deficient cells (Meier, 1998). Activity of phosphoramino acid analogues against viruses other than retroviruses (Balzarini et al., 1996a) or HBV (Balzarini et al., 1998) has not been reported to the best of our knowledge. However, a lack of potentiating effect of phosphoralaninate function against herpesviruses was very recently noted in case of antiherpetic agent 5-(*E*-2-bromovinyl)-2′-deoxyuridine (BVDU, McGuigan et al., 1998).

The previous work in our laboratory has shown that phosphoralaninate approach can be applied to allenic analogues which lack the furanose moiety (Winter et al., 1996, 1997). We have now widened this concept to methylenecyclopropane nucleoside analogues. From the latter group, purine derivatives **1a**–**1f** (Fig. 1) exhibit potent antiviral effects with broad selectivity (Qiu et al., 1998a,b) and effectivity in vivo (Rybak et al., 1998, 1999). In this communication, we describe the synthesis and evaluation of antiviral activity of a total of eight phosphoralaninate prodrugs **3a**–**3e** and **4a**–**4c**. A new methylenecyclopropane analogue with a broad-spectrum antiviral activity, O^6-methylsynguanol **1e**, is also reported. Ana-

logues **3a**, **3b**, **3d** and **3e** have been derived from potent inhibitors of several herpesviruses—synadenol **1a**, synguanol **1b** (Qiu et al., 1998a), 2-amino-6-cyclopropylamino analogue **1d** (Qiu et al., 1998b) as well as 2-amino-6-methoxypurine derivative **1e** first reported herein. Phosphoralaninate analogue **3c** derived from a less effective 2,6-diaminopurine methylenecyclopropane **1c** (Qiu et al., 1998b) was also included in our study. In addition, phosphoramidates **4a**–**4c** of the *E*-isomers (Qiu et al., 1998a,b) **2a**–**2c** were synthesized to investigate whether introduction of a phosphoralaninate function can activate these inactive parent methylenecyclopropane analogues. A preliminary account of this work was reported (Zemlicka et al., 1998).

2. Materials and methods: chemistry

2.1. General methods

See Qiu et al., 1998a,b.

2.2. Methylchlorophenylphosphoryl $P \rightarrow N$-L-alaninate (5)

A described procedure (McGuigan et al., 1992) was modified as follows. A solution of triethylamine (2.79 ml, 20 mmol) in CH_2Cl_2 (60 ml) was added dropwise with stirring during 2 h into a suspension of methyl *L*-alaninate hydrochloride (1.40 g, 10 mmol) and phenyl dichlorophosphate (1.56 ml, 10 mmol) in CH_2Cl_2 (80 ml) at -70°C. The solvent was evaporated, ether (30 ml) was added to the residue, the insoluble portion was filtered off and washed with ether (2×5 ml). The solvent was evaporated to give **5** as a colorless oil (2.80 g, 100%). The reagent was used as 0.184 M stock solution in tetrahydrofuran.

2.3. (Z)-9-[(2-(Hydroxymethyl)cyclopropylidene) methyl]adenine (methylphenylphosphoryl) $P \rightarrow N$-L-alaninate (3a)

A suspension of synadenol **1a** (200 mg, 0.92 mmol) in pyridine (30 ml) was sonicated for 5 min, and a solution of phosphochloridate **5** in THF (0.184 M, 25 ml, 4.6 mmol) was added dropwise at room temperature. After addition of *N*-methylimidazole (0.73 ml, 9.2 mmol) the mixture was stirred for 3.5 h. The solvents were evaporated and the residue was dried in vacuo overnight. It was then chromatographed on a silica gel column using CH_2Cl_2–MeOH (95:5 → 9:1) to give the crude product, which was rechromatographed in CH_2Cl_2–MeOH (95:5). Phosphodiester alaninate **3a** was obtained as a white amorphous powder after washing with ether and drying in vacuo. Yield 270 mg (64%). UV (EtOH) λ_{max} 277 nm (shoulder, ε 9300), 262 (ε 13500), 227 (ε 28600), 213 (ε 28400); IR (KBr) 3500 (shoulder), 3360, 3280 (shoulder), 3200 and 3140 cm^{-1} (shoulder, NH and NH_2), 1750 (C=O, ester), 1665, 1605, and 1580 (olefin, adenine and aromatic ring), 1265 and 1245 (P=O), 1155 (C–O), 1040, 1010, and 940 (phosphoramidate and cyclopropane); ^{1}H NMR (CD_3SOCD_3) δ 1.11–1.20 (m, 3H, CH_3 of Ala), 1.34–1.43 (m, 1H) and 1.56–1.67 (m, 1H, $H_{3'}$), 2.30–2.44 (m, 1H, $H_{4'}$), 3.51, 3.52 and 3.55 (3s, 3H, OCH_3), 3.62–3.88 (m, 1H, CH of Ala), 3.90–4.23 (m, 2H, $H_{5'}$), 5.91–6.05 (m, 1H, NH of Ala), 7.06–7.17 (m, 3H) and 7.24–7.40 (m, 2H, C_6H_5), 7.35 (s, 2H, NH_2), 7.41–7.46 (m, 1H, $H_{1'}$), 8.16 and 8.17 (2s, 1H, H_2 of adenine), 8.45, 8.46 and 8.48 (3s, 1H, H_8 of adenine);^{13}C NMR 7.59 ($C_{3'}$), 17.23, 17.29, and 17.33 ($C_{4'}$), 68.13 and 68.17 ($C_{5'}$), 111.92 ($C_{1'}$), 113.76 and 113.94 ($C_{2'}$); alanine: 19.96, 20.05, and 20.12 (CH_3), 50.08, 50.12 and 50.21 (CH), 52.25 and 52.29 (OCH_3), 173.56 and 173.85 (C=O); C_6H_5: 120.56, 120.61 and 120.65 (C_{meta}), 124.89 (C_{ortho}) 129.96 (C_{para}), 151.05 (C_{ipso}); adenine: 118.87 (C_5), 137.97 (C_8), 148.63 (C_4), 153.51 (C_2), 156.60 (C_6). ^{31}P NMR 3.46, 3.64, 3.73 and 3.90; FAB-MS (thioglycerol) 567 (M + thioglycerol + H, 41.0), 459 (M + H, 11.9), 308 (17.5), 281 (14.2), 234 (21.1), 200 (M − OP(=O)(OPh)NHCHMeCO$_2$Me, 100.0), 136 (adenine + H, 60.5). Anal. Calcd. for $C_{20}H_{23}N_6O_5P \cdot 0.3\ H_2O$: C, 51.79; H, 5.13; N, 18.12. Found: C, 51.90; H, 5.20; N, 18.14.

2.4. (E)-9-[(2-(Hydroxymethyl)cyclopropylidene)-methyl]adenine (methylphenylphosphoryl) $P \rightarrow N$-L-alaninate (4a)

The procedure described above for **3a** was followed on the same scale with the *E*-isomer **2a** as

a starting material. After removal of solvents, the residue was dissolved in MeOH (1 ml) and partitioned between ethyl acetate (250 ml) and water (100 ml). The aqueous phase was extracted with ethyl acetate (60 ml). The combined organic phase was washed with water (4 × 80 ml), brine (80 ml), dried over Na_2SO_4 and evaporated to a syrup. Chromatography on silica gel using CH_2Cl_2–MeOH (95:5→92:8) gave the pure product **4a** as a white foam (328 mg, 74%) after washing with ether (5 ml) and drying in vacuo. UV λ_{max} (EtOH) 277 nm (shoulder, ε 8900), 262 (ε 12800), 225 (ε 28000), 214 (shoulder, ε 27200); IR (KBr) 3330 (br) and 3180 cm^{-1} (br, NH and NH_2), 1750 (C=O, ester), 1645, 1600, and 1580 (olefin, adenine and aromatic ring), 1250 (P=O), 1156 (C–O), 1025 and 937 (phosphoramidate and cyclopropane). ^1H NMR (CD_3SOCD_3) δ 1.20 (apparent t, 3H, $J = 3.5$ Hz, CH_3 of Ala), 1.52–1.65 (m, 1H) and 1.78–1.90 (m, 1H, $H_{3'}$), 2.18 (m, 1H, $H_{4'}$), 3.54, 3.54, 3.56 and 3.58 (4s, 3H, OCH_3), 3.77–3.93 (m, 1H, CH of Ala), 3.93–4.10 (m, 2H, $H_{5'}$), 6.01 (apparent dt, 1H, $^3J = 10.2$ Hz, $^2J_P =$ 12.75 Hz, NH of Ala), 7.09–7.23 (m, 3H) and 7.29–7.45 (m, 2H, C_6H_5), 7.38 (s, 2H, NH_2), 7.50–7.56 (m, 1H, $H_{1'}$), 8.18 (s, 1H, H_2 of adenine), 8.478 and 8.484 (2s, 1H, H_8 of adenine); ^{13}C NMR 10.25 and 10.31 ($C_{3'}$), 15.32 ($C_{4'}$), 68.50 ($C_{5'}$), 111.85 ($C_{1'}$), 114.36 ($C_{2'}$); alanine: 20.02 and 20.10 (CH_3), 50.10 and 50.24 (CH), 52.25 (OCH_3), 174.11 and 174.18 (C=O); C_6H_5: 120.62 (C_{meta}), 124.89 (C_{ortho}), 123.00 (C_{para}), 151.18 and 151.26 (C_{ipso}); adenine: 118.84 (C_5), 137.65 (C_8), 148.71 (C_4), 153.54 (C_2), 156.49 (C_6). ^{31}P NMR 3.55, 3.91 and 3.97; EI-MS 459 (M + H, 1.5), 458 (M, 6.7), 307 (1.1), 264 (4.1), 200 (M − OP(=O)(OPh)NH-CHMeCO$_2$Me, 100.0), 135 (adenine, 40.8). HR-MS Calcd. for $C_{20}H_{23}N_6O_5P$: 458.14675. Found: 458.1477. Anal. Calcd. for $C_{20}H_{23}N_6O_5P \cdot 0.3\ H_2O$: C, 51.79; H, 5.13; N, 18.12. Found: C, 51.77; H, 5.26; N, 17.86.

2.5. (E)-9-[(2-(Hydroxymethyl)cyclopropylidene)-methyl]guanine (methylphenylphosphoryl) P→N-L-alaninate (4b)

A suspension of analogue **2b** (163 mg, 0.70 mmol) in dry pyridine (20 ml) was sonicated for 5 min. A solution of phosphorochloridate **5** in THF (0.184 M, 19.4 ml, 3.50 mmol) was then added during 5 min with stirring at room temperature. After additional 5 min., *N*-methylimidazole (0.56 ml, 7.0 mmol) was added to the resultant orange suspension and the mixture was stirred for 18 h. TLC showed the presence of a faster moving component (UV λ_{max} 294 nm in EtOH) as the predominant product. Solvents were evaporated in vacuo at room temperature to give a reddish syrup which was dissolved in acetic acid (80%, 25 ml). This solution was stirred for 22 h at room temperature. TLC showed a complete disappearance of the faster moving component and formation of product **4b**. Volatile components were removed by evaporation in vacuo (oil pump) at room temperature. The residue was chromatographed on a silica gel (55 g) column using CH_2Cl_2–MeOH (95:5→9:1) as eluent to give product **4b** as a gel-like material. Addition of ether (10 ml) resulted in formation of a white solid (368 mg, 92% yield based on UV spectrophotometry at 270 nm). A suspension of this product in MeOH (30 ml) and Na_2HPO_4 (0.1 M, pH 7.5, 10 ml) was stirred for 24 h at room temperature. The solvents were evaporated and the residue was washed with CH_2Cl_2–MeOH (9:1, 50 ml). The insoluble portion was filtered off and it was washed with the same solvent. The organic phase was evaporated and the residue was chromatographed on a silica gel (20 g) column using CH_2Cl_2–MeOH (9:1) as eluent to give product **4b** as a colorless solid (254 mg, 76.5%). UV λ_{max} (EtOH) 270 nm (ε 12600), 229 (ε 33100); IR (KBr) 3330 and 3150 cm^{-1} (NH and NH_2), 1750–1580 (ester, olefin, guanine and aromatic ring), 1250 and 1215 (P=O), 1155 (C–O), 1020 and 937 (phosphoramidate and cyclopropane); ^1H NMR (CD_3SOCD_3) δ 1.14–1.24 (m, 3H, CH_3 of Ala), 1.42–1.57 (m, 1H) and 1.72–1.85 (m, 1H, $H_{3'}$), 2.05–2.21 (m, 1H, $H_{4'}$), 3.53, 3.547, 3.554 and 3.56 (4s, 3H, OCH_3), 3.74–3.88 (m, 1H, CH of Ala), 3.88–4.06 (m, 1H) and 4.09 –4.22 (m, 1H, $H_{5'}$), 5.92–6.07 (m, 1H, NH of Ala), 6.56 (s, 2H, NH_2), 7.08–7.22 (m, 3H) and 7.22–7.38 (m, 3H, $H_{1'}$ and C_6H_5), 8.04 (s, 1H, H_8 of guanine), 10.71

(s, 1H, NH of guanine); ^{13}C NMR 10.05 and 10.10 ($C_{3'}$), 15.11, 15.16 and 15.23 ($C_{4'}$), 68.50, 68.56 and 68.65 ($C_{5'}$), 111.75 ($C_{1'}$), 113.93 and 114.02 ($C_{2'}$); alanine: 19.96, 20.03, and 20.11 (CH_3), 50.09 and 50.25 (CH), 52.28 (OCH_3), 174.09 and 174.16 (C=O); C_6H_5: 120.55, 120.61 and 120.67 (C_{meta}), 124.93 (C_{ortho}), 130.02 (C_{para}), 151.15 and 151.25 (C_{ipso}); guanine: 116.73 (C_5), 134.18 (C_8), 150.33 (C_4), 154.41 (C_2), 157.13 (C_6); ^{31}P NMR 3.47, 3.54, 3.82 and 3.96; FAB-MS (thioglycerol) 605 (25.5), 583 (M + thioglycerol + H, 9.6), 519 (8.5), 497 (36.0), 475 (M + H, 24.1), 324 (9.4), 304 (13.4), 281 (5.7), 250 (17.3), 238 (29.6), 216 (M − OP(=O)(OPh)NHCHMeCO$_2$Me, 100.0), 200 (82.4), 174 (61.4), 152 (guanine + H, 64.4). Anal. Calcd. for $C_{20}H_{23}N_6O_6P$: C, 50.62; H, 4.89; N, 17.72. Found: C, 50.45; H, 5.12; N, 17.87.

2.6. (Z)-9-[(2-(Hydroxymethyl)cyclopropylidene)-methyl]guanine (methylphenylphosphoryl) P → N-L-alaninate (3b)

The reaction was performed as described above for the *E*-isomer **4b** with synguanol **1b**. The faster moving compound was hydrolyzed in 80% acetic acid for 70 h at room temperature to give **3b**. Column chromatography gave a white gum (330 mg, 99%) which was stirred in methanol (30 ml) and Na_2HPO_4 (0.1 M, pH 7.5, 20 ml) for 20 min. The precipitate was dissolved by addition of water (10 ml) and the stirring was continued for 8.5 h. The solution was then allowed to stand at 0°C for 16 h to give product **3b** (70 mg, 21.1%) which was collected by filtration. A further work-up followed the procedure described for compound **4b** to afford product **3b** (195 mg, 58.9%). UV λ_{max} (EtOH) 270 nm (ε 11200), 229 (ε 27600); IR (KBr) 3330 and 3150 cm^{-1} (NH and NH_2), 1750–1580 (ester, olefin, guanine and aromatic ring), 1250 and 1215 (P=O), 1155 (C–O), 1020 and 937 (phosphoramidate and cyclopropane); ^{1}H NMR (CD_3SOCD_3) δ 1.12–1.20 (m, 3H, CH_3 of Ala), 1.28–1.40 (m, 1H) and 1.51–1.63 (m, 1H, $H_{3'}$), 2.25–2.41 (m, 1H, $H_{4'}$), 3.53 and 3.55 (2s, 3H, OCH_3), 3.66–3.89 (m, 1H, CH of Ala), 3.89–4.09 (m, 1H) and 4.09–4.22 (m, 1H, $H_{5'}$), 5.90–6.07 (m, 1H, NH of Ala), 6.56 (s, 2H, NH_2), 7.08–7.21 (m, 4H) and 7.26–7.36 (m, 2H, $H_{1'}$ and C_6H_5), 8.02, 8.03 and 8.04 (3s, 1H, H_8 of guanine), 10.71 (s, 1H, NH of guanine); ^{13}C NMR 7.34 and 7.54 ($C_{3'}$), 17.24 ($C_{4'}$), 68.04 and 68.10 ($C_{5'}$), 111.92 ($C_{1'}$), 113.28 and 113.46 ($C_{2'}$); alanine: 19.97, 20.06 and 20.16 (CH_3), 50.07, 50.14 and 50.21 (CH), 52.26 (OCH_3), 174.19 (C=O); C_6H_5: 120.61 and 120.65 (C_{meta}), 124.93 (C_{ortho}), 139.97 (C_{para}), 151.05 and 151.13 (C_{ipso}); guanine: 116.81 (C_5), 134.47 and 134.57 (C_8), 150.28 (C_4), 154.41 (C_2), 157.13 (C_6); ^{31}P NMR 3.56, 3.82 and 4.02; FAB-MS (thioglycerol) 605 (29.9), 583 (M + thioglycerol + H, 41.2), 519 (8.5), 497 (54.2), 475 (M + H, 37.1), 324 (17.0), 281 (24.7), 250 (48.7), 216 (M − OP(=O)(OPh)NHCHMeCO$_2$Me, 100.0), 200 (84.8), 174 (48.7), 152 (guanine + H, 96.1). Anal. Calcd. for $C_{20}H_{23}N_6O_6P{\cdot}0.5\ H_2O$: C, 49.69; H, 5.00; N, 17.38. Found: C, 49.93; H, 5.09; N, 17.05.

2.7. (Z)-2,6-Diamino-9-[(2-(hydroxymethyl)cyclopropylidene)methyl]purine (methylphenylphosphoryl) P → N-L-alaninate (3c)

Pyridine (2 × 10 ml) was evaporated from the starting analogue **1c** (140 mg, 0.6 mmol) which was then suspended in the same solvent (20 ml). Phosphorochloridate **5** in THF (0.184 M, 16.65 ml, 3 mmol) was added followed, after 5 min, by *N*-methylimidazole (0.48 ml, 6 mmol) at room temperature with stirring. After 3 h, the precipitated gum was redissolved by addition of pyridine (20 ml) and the stirring was continued for 16 h. The TLC showed no starting material **1c** and the presence of *N*-phosphorylated compound(s) moving on TLC faster than the desired product **3c**. The volatile components were evaporated in vacuo and the residue was dissolved in 80% acetic acid (35 ml). The solution was stirred for 88 h at room temperature. TLC showed a complete disappearance of a faster moving component. After evaporation, the residue was chromatographed on a silica gel column using CH_2Cl_2–MeOH (95:5) to give a colorless syrup. The latter product was dissolved in MeOH (20 ml) and 0.1 M Na_2HPO_4 (pH 7.5, 20 ml) and the solution was stirred for 16

h at room temperature. The solvents were evaporated and the residue was washed with CH_2Cl_2–MeOH (9:1, 60 ml). The insoluble portion was filtered off and it was washed with the same solvent. The organic phase was evaporated and the residue was chromatographed on a silica gel (20 g) column using CH_2Cl_2–MeOH (9:1) as eluent to give the product **3c** as a hygroscopic gum. The latter was dissolved in methanol (1 ml), water (10 ml) was added and the resultant suspension was lyophilized to afford compound **3c** (170 mg, 60% yield) as a white solid. UV λ_{max} (EtOH) 280 nm (ε 11800), 229 (ε 35500); IR (KBr) 3350 and 3200 cm^{-1} (NH and NH_2), 1745 (C=O, ester), 1670–1580 (olefin, purine and aromatic ring), 1220 (P=O), 1160 (C–O), 1020 and 940 (phosphoramidate and cyclopropane); ^1H NMR (CD_3SOCD_3) δ 1.12–1.22 (m, 3H, CH_3 of Ala), 1.27–1.38 (m, 1H) and 1.50–1.62 (m, 1H, $H_{3'}$), 2.22–2.40 (m, 1H, $H_{4'}$), 3.52 and 3.55 (2s, 3H, OCH_3), 3.68–3.88 (m, 1H, CH of Ala), 3.88–4.05 (m, 1H) and 4.10–4.30 (m, 1H, $H_{5'}$), 5.91 (s, 2H, 2-NH_2), 5.85–6.06 (m, 1H, NH of Ala), 6.79 (s, 2H, 6-NH_2), 7.08–7.20 (m, 3H) and 7.20–7.36 (m, 3H, olefin and C_6H_5), 8.037, 8.040, 8.05 and 8.06 (4s, 1H, H_8 of purine); ^{13}C NMR 7.43, 7.50 and 7.61 ($C_{3'}$), 17.32, 17.41, and 17.80 ($C_{4'}$), 68.25 and 68.41 ($C_{5'}$), 111.77 ($C_{1'}$), 113.26 ($C_{2'}$); alanine: 19.97, 20.06 and 20.12 (CH_3), 49.94, 50.07, 50.16 and 50.25 (CH), 52.28 (OCH_3), 174.10 and 174.17 (C=O); C_6H_5: 120.66 (C_{meta}), 124.93 C_{ortho}), 130.00 (C_{para}), 151.04 and 151.14 (C_{ipso}); purine: 112.16 (C_5), 134.48 and 134.56 (C_8), 150.56 and 150.93 (C_4), 156.60 (C_2), 161.07 (C_6); ^{31}P NMR 3.62, 3.85, 3.90 and 4.08; FAB-MS (thioglycerol) 582 (M + thioglycerol + H, 48.6), 474 (M + H, 11.6), 323 (19.4), 281 (15.7), 249 (27.5), 215 (M − OP(=O)(OPh)NHCHMeCO$_2$Me, 43.7), 200 (36.2), 151 (2,6-diaminopurine + H, 100.0). Anal. Calcd. for $C_{20}H_{24}N_7O_5P$: C, 50.72; H, 5.11; N, 20.72. Found: C, 50.95; H, 5.06; N, 20.49.

*2.8. (E)-2,6-Diamino-9-[(2-(hydroxymethyl)-cyclopropylidene)methyl]purine (methylphenyl-phosphoryl) P → N-L-alaninate (**4c**)*

The experiment followed the procedure described above on a 0.43 mmol scale of **2c**. The mixture was stirred for 5 h at room temperature. TLC showed the presence of product **4c** and a faster moving component (UV λ_{max} 292 nm in EtOH) in approximately 1:1 ratio. The volatile components were evaporated in vacuo and the residue was dissolved in 80% acetic acid (30 ml). The solution was stirred for 166 h at room temperature when TLC showed a complete hydrolysis of the faster moving product. After evaporation, the residue was chromatographed on a silica gel column using CH_2Cl_2–MeOH (95:5) to give a colorless syrup. The latter was dissolved in a mixture of MeOH (20 ml) and Na_2HPO_4 (0.1 M, pH 7.5, 20 ml) and the solution was stirred for 24 h at room temperature. The solvents were evaporated and the residue was washed with CH_2Cl_2–MeOH (9:1, 60 ml). The insoluble portion was filtered off and it was washed with the same solvents. The organic phase was evaporated and the residue was chromatographed on a silica gel (20 g) column using CH_2Cl_2–MeOH (9:1) as eluent to afford product **4c** as an amorphous white powder (120 mg, 59%). UV λ_{nm} (EtOH) 281nm (ε 12700), 229 (ε 37700); IR (KBr) 3350 and 3200 cm^{-1} (NH and NH_2), 1745 (C=O, ester), 1670–80 (olefin, purine and aromatic ring), 1220 (P=O), 1160 (C–O), 1025 and 940 (phosphoramidate and cyclopropane); ^1H NMR (CD_3SOCD_3) δ 1.15–1.25 (m, 3H, CH_3 of Ala), 1.43–1.58 (m, 1H) and 1.73–1.86 (m, 1H, $H_{3'}$), 2.10–2.22 (m, 1H, $C_{4'}$), 3.54, 3.55, 3.56 and 3.57 (4s, 3H, OCH_3), 3.76–3.91 (m, 1H, CH of Ala), 3.91–4.07 (m, 2H, $H_{5'}$), 5.92 (s, 2H, 2-NH_2), 5.84–6.07 (m, 1H, NH of Ala), 6.83 (s, 2H, 6–NH_2), 7.09–7.27 (m, 3H) and 7.27–7.40 (m, 3H, $H_{1'}$ and C_6H_5), 8.06 and 8.07 (4s, 1H, H_8 of purine); ^{13}C NMR 10.11 and 10.16 ($C_{3'}$), 15.10 and 15.17 ($C_{4'}$), 68.63, 68.70 and 68.78 ($C_{5'}$), 111.94 ($C_{1'}$), 113.18 ($C_{2'}$); alanine: 20.09 (CH_3), 50.10 and 50.25 (CH), 52.27 (OCH_3), 174.12, 174.21 and 174.27 (C=O); C_6H_5: 120.61 and 120.65 (C_{meta}), 124.92 (C_{ortho}), 129.03 (C_{para}), 151.01 and 151.14 (C_{ipso}); purine: 112.34 (C_5), 134.04 (C_8), 151.22 (C_4), 156.59 (C_2), 161.04 (C_6); ^{31}P NMR 3.51, 3.54, 3.86 and 3.96; FAB-MS (thioglycerol) 582 (M + thioglycerol + H, 20.3), 474 (M + H, 79.4), 323 (7.6), 249 (5.8), 215 (M–OP(=O)(OPh)NHCHMeCO$_2$Me, 100.0), 200 (14.7), 151 (2,6-diaminopurine + H, 23.4). Anal. Calcd. for $C_{20}H_{24}N_7O_5P \cdot 0.2H_2O$: C, 50.36; H,

5.16; N, 20.55. Found: C, 50.50; H, 5.32; N, 20.47.

2.9. (Z)-2-Amino-6-cyclopropylamino-9-[(2-(hydroxymethyl)cyclopropylidene)methyl]-purine (methylphenylphosphoryl) P→N-L-alaninate (3d)

The procedure described above for synadenol (**1a**) was followed on 0.6 mmol scale of **1d** to give product **3d** as a foam after chromatography on silica gel using CH_2Cl_2–MeOH (98:2→95:5). It was dissolved in MeOH (1 ml), water (8 ml) was added and the solution was lyophilized to give a white powder (223 mg, 72.4% yield). UV λ_{max} (EtOH) 286 nm (ε 14800), 223 (ε 36000); IR (KBr) 3500, 3340, and 3210 cm^{-1} (NH and NH_2), 1750 (C=O, ester), 1630–1580 (olefin, purine and aromatic ring), 1270 and 1210 (P=O), 1160 (C–O), 1025 and 940 (phosphoramidate and cyclopropane); ^1H NMR ($CDCl_3$) δ 0.59 (br s, 2H) and 0.81 (q, 2H, $^3J = 6.6$ Hz, two CH_2 of 6-cyclopropylamino), 1.25–1.39 (m, 4H) and 1.52–1.62 (m, 1H, $H_{3'}$ and CH_3 of Ala), 2.18–2.34 (m, 1H, $H_{4'}$), 2.98 (br s, 1H, CH of 6-cyclopropylamino group), 3.627, 3.634, 3.64 and 3.66 (4s, 3H, OCH_3), 3.86–4.30 (m, 4H, CH and NH of Ala, $H_{5'}$), 5.24 (br s, 2H, NH_2), 6.25 (br s, 1H, 6-NH), 7.02–7.32 (m, 6H, $H_{1'}$ and C_6H_5), 7.90 and 7.94 (2s, 1H, H_8 of purine); ^{13}C NMR 7.35 and 7.87 ($C_{3'}$ and two CH_2 of 6-cyclopropylamino group), 17.23, 17.35, 17.46 and 17.54 ($C_{4'}$), 23.77 (CH of 6-cyclopropylamino group), 69.14 and 69.19 ($C_{5'}$), 112.27 ($C_{1'}$), 114.04 ($C_{2'}$); alanine: 20.86 and 20.91 (CH_3), 50.01 and 50.13 (CH), 52.44 (OCH_3), 174.05, 174.10 and 174.14 (C=O); C_6H_5: 120.10 and 120.16 (C_{meta}), 124.81 and 124.86 (C_{ortho}), 129.57 (C_{para}), 150.56 and 150.64 (C_{ipso}); purine: 112.11 (C_5), 114.04 (C_8), 134.78 and 134.90 (C_4), 156.09 (C_2), 160.23 (C_6); ^{31}P NMR 2.68, 2.73, 2.84 and 2.87; FAB-MS (thioglycerol) 622 (M + thioglycerol + H, 62.6), 514 (M + H, 25.9), 363 (34.5), 289 (19.3), 255 (M − OP(=O)(OPh)NHCHMeCO$_2$Me, 58.2), 191 (2-amino-6-cyclopropylaminopurine + H, 100.0). Anal. Calcd. for $C_{23}H_{28}N_7O_5P$: C, 53.78; H, 5.50; N, 19.10. Found: C, 53.56; H, 5.40; N, 18.87.

2.10. (Z)-2-amino-6-methoxy-9-[(2-hydroxymethyl)cyclopropylidene)methyl]purine (1e)

A solution of compound **1f** (Qiu et al., 1998a, 50 mg, 0.17 mmol) and K_2CO_3 (84 mg, 0.61 mmol) in MeOH–H_2O (9:1, 5 ml) was stirred at room temperature for 60 h. Volatile components were removed by evaporation. The residue was chromatographed on silica gel using CH_2Cl_2–MeOH (95:5→9:1), giving the desired product as a white solid (40 mg, 95%), mp 196–199°C. UV (EtOH) λ_{max} 280 nm (ε 11600), 224 (ε 31000); IR (KBr) 3400, 3310, 3210, and 3100 cm^{-1} (NH_2 and OH), 1645 and 1585 (olefin and purine ring), 1050 (cyclopropane); ^1H NMR (CD_3SOCD_3): 1.16 (ddd, 1H, $^2J = 8.1$ Hz, $^3J_{trans} = 5.1$ Hz, $^4J = 1.8$ Hz) and 1.44 (td, 1H, $^2J = {}^3J_{cis} = 8.55$ Hz, $^4J = 1.5$ Hz, $H_{3'}$), 2.07 (dqd, 1H, $^3J_{cis} = 9.0$ Hz, $^3J_{trans} = {}^3J = 6.3$ Hz, $^4J = 2.1$ Hz, $H_{4'}$), 3.33 (ddd, overlapped with H_2O at 3.30 ppm, 1H, $^2J = 11.1$ Hz, $^3J = 7.2$ Hz, $^3J = 6.3$ Hz) and 3.69 (dt, 1H, $^2J = 10.5$ Hz, $^3J = 5.1$ Hz, $H_{5'}$), 3.41 (s, 3H, OCH_3), 5.08 (dd, 1H, $^3J = 6.0$ Hz, $^3J = 4.5$ Hz, OH), 6.52 (s, 2H, NH_2), 7.19 (d, 1 H, $^4J = 1.8$ Hz, $H_{1'}$), 8.47 (s, 1H, H_8). ^{13}C NMR 6.59 ($C_{3'}$), 19.57 ($C_{4'}$), 63.68 ($C_{5'}$), 63.33 (OCH_3), 110.54 ($C_{1'}$), 115.09 ($C_{2'}$), 113.87 (C_5), 136.94 (C_8), 152.94 (C_4), 160.55 (C_2) and 161.13 (C_6). EI-MS 247 (M, 65.4), 230 (M − H_2O + H, 63.3), 217 (M − CH_2OH + H, 30.2), 198 (M − H_2O–OCH_3, 11.8), 178 (10.9), 166 (100.0). HRMS Calcd. for $C_{11}H_{13}N_5O_2$ (M): 247.1069; Found: 247.1074. Anal. Calcd. for $C_{10}H_{12}N_6O$: C, 53.42; H, 5.30; N, 28.33. Found: C, 53.28; H, 5.19; N, 28.18.

2.11. (Z)-Amino-6-methoxy-9-[(2-(hydroxymethyl)cyclopropylidene)methyl]purine (methylphenylphosphoryl) P→N-L-alaninate (3e)

The procedure described above for synadenol analogue **3a** was followed on 0.6 mmol scale of compound **1e**. A syrupy product obtained after chromatography on silica gel using CH_2Cl_2–MeOH (98:2→96:4) was dissolved in *tert*.-butyl alcohol (3 ml). Water (6 ml) was added and the solution was lyophilized to give compound **3e** as a white powder (230 mg, 78.5% yield). UV λ_{max} (EtOH) 279 nm (ε 11700), 223 (ε 30100), 204

(ε 26900); IR (KBr) 3500, 3350, and 3220 cm^{-1} (NH and NH_2), 1750 (C=O, ester), 1630–1580 (olefin, purine and aromatic ring), 1270 and 1215 (P=O), 1155 (C–O), 1020 and 937 (phosphoramidate and cyclopropane); ^1H NMR ($CDCl_3$) δ 1.25–1.37 (m, 4H) and 1.52–1.62 (m, 1H, $H_{3'}$ and CH_3 of Ala), 2.20–2.35 (m, 1H, $H_{4'}$), 3.61, 3.62, 3.64 and 3.65 (4s, 3H, OCH_3 of Ala), 4.027 and 4.033 (2s, 3H, 6-OCH_3), 3.85–4.33 (m, 4H, CH and NH of Ala, $H_{5'}$), 5.00–5.50 (brs, 2H, NH_2), 7.05–7.32 (m, 6H, $H_{1'}$ and C_6H_5), 7.99, 8.03 and 8.04 (3s, 1H, H_8 of purine); ^{13}C NMR 7.62, 7.73, and 8.01 ($C_{3'}$), 17.64, 17.75, 17.82 and 17.86 ($C_{4'}$), 53.91 (6-OCH_3), 69.30 ($C_{5'}$), 112.17, 112.32, 112.49, 112.63 and 112.72 ($C_{1'}$ and $C_{2'}$); alanine: 20.78 (CH_3), 50.01, 50.14 and 50.22 (CH), 52.42 (OCH_3), 173.89 and 173.98 (C=O); C_6H_5: 120.09, 120.14 and 120.19 (C_{meta}), 124.82 and 124.86 (C_{ortho}), 129.56 (C_{para}), 150.53 and 150.60 (C_{ipso}); purine: 115.18 (C_5), 136.69, 136.87, 136.93 and 137.04 (C_8), 152.43 (C_4), 159.78 (C_2), 161.53 (C_6); ^{31}P NMR 2.70, 2.89 and 3.04; FAB-MS (thioglycerol) 597 (M + thioglycerol + H, 59.6), 489 (M + H, 37.7), 338 (21.8), 264 (31.0), 230 (M − OP(=O)(OPh)NHCHMeCO_2Me, 100.0), 200 (56.1), 166 (2-amino-6-methoxypurine + H, 97.9). Anal. Calcd. for $C_{21}H_{25}N_6O_6P \cdot 0.3$ tBuOH: C, 52.21; H, 5.53; N, 16.46. Found: C, 52.57; H, 5.66; N, 16.72.

2.12. Antiviral assays

The antiviral assays were described in detail in the previous communications (Qiu et al., 1998a,b). Briefly, the following assays were performed: HCMV plaque reduction assay in HFF cell culture with Towne and AD 169 strains of virus. In selected cases, yield reduction assay was also performed with Towne strain of HCMV.

The HSV-1 was assayed by ELISA with BSC-1 cells, HSV-1, HSV-2, VZV and MCMV in HFF, Vero or MEF (MCMV) by plaque reduction. The HHV-6 strain GS was assayed by ELISA in HSB-2 cell culture, EBV by viral capsid antigen immunofluorescence assay (VCA-IFA) in Daudi cells and by DNA hybridization in H-1 cells. The HBV assays were performed with transfected human hepatitis HepG-2 2.2.15 cell line. The anti-HIV-1 activity was determined with strain III_B in CEM-SS culture by supernatant reverse transcriptase assay. The results are given in Tables 1–5.

2.13. Cytotoxicity assays

Cytotoxicity studies were carried out in uninfected cell lines that were used either to grow stocks of the respective viruses or were used to propagate these viruses in the drug evaluation experiments (Qiu et al., 1998a). Thus, cytotoxicity was measured in the following cell lines using methods given in footnotes to the tables: stationary and proliferating human foreskin fibroblasts (HFF), stationary murine embryonic fibroblasts (MEF), stationary and proliferating Daudi cells, proliferating CEM, CEM-SS and KB cells. More details regarding the cell lines and assays used with each are provided in the footnotes to the tables. The cytotoxicity results are summarized in Tables 1, 2, 4 and 5.

3. Results

3.1. Chemical synthesis

Procedures elaborated previously for the preparation of lipophilic phosphoralaninates of allenic and other unsaturated nucleoside analogues (Winter et al., 1996, 1997) were not suitable for phosphorylation of analogues **1a**–**1e** and **2a**–**2c** with phosphorochloridate **5**. Because of a limited solubility of analogues, pyridine (Franchetti et al., 1996) was employed as a solvent. Phosphorylation of adenine derivatives **1a** and **2a** gave the corresponding phosphoralaninates **3a** and **4a** in 64 and 74% yield, respectively (Figs. 1 and 2) without a significant *N*-phosphorylation. Analogue **3a** was obtained by repeated column chromatography. In case of **4a** this necessity was obviated by inclusion of a simple extraction step and single chromatographic separation was sufficient. The reaction was also smooth in case of analogues **3d** and **3e** (72 and 78.5% yield). In case of guanine and 2,6-diaminopurine analogues **1b**, **1c**, **2b** and **2c** formation of more lipophilic products of phosphorylation of the heterocyclic moieties was ob-

Table 1
Inhibition of human and mouse cytomegalovirus (HCMV and MCMV) replication by phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues[a]

Cmpd.	Base	Isomer	Prodrug	Antiviral activity (EC_{50}, μM)[b]			Cytotoxicity (IC_{50}, μM)[c]		
				HCMV/HFF Plaque Towne	AD 169	MCMV/MEF Plaque	HFF Stationary	Dividing	MEF
1a	Ade	*Z*	−	2.1[d] (1.3)[d]	2.3	2.1	>460	166	>92
3a	Ade	*Z*	+	0.14 (0.21)	NT	NT	4.3	42	NT
1b	Gua	*Z*	−	2.1[d] (1.8)[d]	1.2	0.3	>429	279	>429
3b	Gua	*Z*	+	4.2	25	3.3	>206	>206	>206
1c	DAP	*Z*	−	24[d] (18)	16	0.6	266	440	293
3c	DAP	*Z*	+	4	7.8	3.2	121	>211	211
1d	ACAP	*Z*	−	0.4 (1.0)	2.4	0.4	327	257	220
3d	ACAP	*Z*	+	0.25	6.2	0.94	126	2.7	101
1e	AMP	*Z*	−	3.1	5.3	0.40	>404	243	261
3e	AMP	*Z*	+	0.16	4.5	0.24	34	2.4	126
2a	Ade	*E*	−	>100 (>100)	NT	NT	>460	>460	NT
4a	Ade	*E*	+	21	NT	NT	141	>216	NT
2b	Gua	*E*	−	>100	NT	NT	>413	>413	NT
4b	Gua	*E*	+	>100	NT	NT	>211	>211	NT
2c	DAP	*E*	−	>100	NT	NT	>431	266	NT
4c	DAP	*E*	+	>100	NT	NT	>211	>211	NT
Ganciclovir				7.4 ± 6.5[e]	0.78	3.8 ± 1.8[e]	>392	157	NT

[a] Abbreviations of bases: DAP, 2,6-diaminopurine; ACAP, 2-amino-6-cyclopropylaminopurine; AMP, 2-amino-6-methoxypurine. NT, not tested.

[b] For description of antiviral assays, see Qiu et al. (1998a,b). Dose–response curves were constructed using from four to seven different drug concentrations, each in duplicate. Results from plaque assays are reported as EC_{50} values; results for yield reduction experiments (in parentheses) given as EC_{90} values. Towne and AD169 are laboratory strains of HCMV.

[c] Cytotoxicity was determined in stationary and rapidly proliferating human foreskin fibroblasts (HFF) by neutral red dye uptake and cell enumeration, respectively. Visual cytotoxicity was scored on murine embryonic fibroblasts (MEF) and HFF cells at the time of plaque enumeration. Data for HFF visual cytotoxicity are not shown but they were in agreement with neutral red dye uptake. Exceptions: **3d** (IC_{50} 10 μM) and **3e** (IC_{50} 3.7 μM).

[d] Averaged results from duplicate or triplicate experiments.

[e] Averages ± S.D. derived from more than four separate experiments in which ganciclovir was used as a positive control.

served. The O^6-phosphorylated products were noticed previously during phosphorylation of unsaturated analogues of inosine with reagent **5** (Winter et al., 1996, 1997). These compounds were not isolated but they were converted in situ to the desired phosphoralaninates **3b**, **3c** and **4b**, **4c** by hydrolysis with 80% acetic acid. A work-up with pH 7.5 buffer was necessary to release free bases **3b**, **3c**, **4b** and **4c** from the corresponding salts formed during the work-up in acidic solution. The yields of analogues **3b** and **4b** were 80 and 77%, respectively, whereas those of **3c** and **4c** amounted to 60 and 59%.

All these analogues were obtained as amorphous solids without definite melting point. They are significantly more stable than the corresponding allene derivatives (Winter et al., 1996) and no special precautions for handling of these materials are necessary. Interestingly, prodrugs **3a**-**3e** and **4a**–**4c** are, with the exception of *E*-analogue **4a**, less soluble in organic solvents such as ethyl acetate or CH_2Cl_2 than the respective acyclic unsaturated analogues (Winter et al., 1996, 1997). The NMR spectra of analogues **3a**–**3e** and **4a**–**4c** reflect their diastereoisomeric composition (four diastereoisomers are possible). Thus, the NMR spectra of **3d**, **4b**, and **4c** showed the expected four peaks for CH_3O and ^{31}P. Analogues **4a** and **3e** had also four CH_3O (alaninate) signals but only three in the ^{31}P NMR, whereas **3a** and **3c** exhibited

only two or three CH_3O peaks but a full number (four) of ^{31}P signals. Only compound **3b** did not display the four peaks for either CH_3O or ^{31}P signal. All these signals form close clusters and overlaps cannot be ruled out.

3.2. Activity against HCMV and MCMV

The antiviral potency of analogue **1e** and phosphoralaninates **3a**–**3e** and **4a**–**4c** was examined in the following assays: Human cytomegalovirus (HCMV), herpes simplex virus 1 and 2 (HSV-1 and HSV-2), human herpes virus 6 (HHV-6), Epstein-Barr virus (EBV), varicella zoster virus (VZV), hepatitis B virus (HBV) and human immunodeficiency virus (HIV-1). The results are summarized in Tables 1–5.

We have previously disclosed that analogues **1a**–**1d** are anti-HCMV agents with synadenol (**1a**), synguanol (**1b**) and cyclopropylamino derivative **1d** being especially effective (Qiu et al., 1998a,b). We now report that 2-amino-6-methoxypurine analogue **1e** is also a potent agent against HCMV and MCMV (Table 1) with no cytotoxicity. The phosphoralaninate derivative **3a** displayed an enhanced anti-HCMV activity in plaque and yield reduction assays relative to parent synadenol **1a**. The antiviral effect was accompanied by an increased cytotoxicity in host (HFF) cells. By contrast, the anti-HCMV activity of synguanol phosphoramidate **3b** was lower than that of synguanol (**1b**) in both HCMV/HFF and MCMV/MEF assays with no host cell cytotoxicity of **3b** in uninfected HFF and MEF cells. The inhibitory effect of phosphoralaninate **3c** was im-

Table 2
Inhibition of herpes simplex virus type 1 (HSV-1) replication and cell growth of KB cells by phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues

Cmpd.	Base	Isomer	Prodrug	Antiviral activity (EC_{50}, μM)[a] HSV-1			Cytotoxicity (IC_{50}, μM)[b] KB
				BSC-1 ELISA	HFF Plaque	Vero Plaque	
1a	Ade	*Z*	−	26[c]	43.3	28	78[c]
3a	Ade	*Z*	+	2.5	NT	>1[d]	0.5
1b	Gua	*Z*	−	100[c]	NT	85	>100
3b	Gua	*Z*	+	>100	22.1	>100	>100
1c	DAP	Z	−	>100	>100	>100	>100
3c	DAP	*Z*	+	50	5.5	30	40
1d	ACAP	*Z*	−	15	NT	31	>100
3d	ACAP	*Z*	+	5	0.8	0.9	65
1e	AMP	*Z*	−	33	NT	>50	>100
3e	AMP	*Z*	+	2[c]	1.1	0.6	80
2a	Ade	*E*	−	>100	>460	>100	>100
4a	Ade	*E*	+	65	NT	>10[e]	50
2b	Gua	*E*	−	>100	NT	>100	>100
4b	Gua	*E*	+	>100	NT	>100	>100
2c	DAP	*E*	−	>100	NT	>100	>100
4c	DAP	*E*	+	>100	NT	>100	>100
Acyclovir				3.5 ± 2.1[f]	3.5 ± 1.5[f]	9	NT

[a] For abbreviations see Table 1. For description of antiviral assays, see Qiu et al. (1998a,b). Dose–response curves were constructed using from four to seven different drug concentrations. All compounds assayed by enzyme-linked immunosorbent assay (ELISA) were run in quadruplicate wells, other assays in duplicate.

[b] Inhibition of KB cell growth was determined as described in Qiu et al. (1998a) in quadruplicate assays.

[c] Averaged results from duplicate or triplicate experiments.

[d] Cytotoxic at 10 μM.

[e] Cytotoxic at 100 μM.

[f] Averages ± S.D. derived from more than four separate experiments in which acyclovir was used as a positive control.

Table 3
Inhibition of herpes simplex virus type 2 (HSV-2) and human herpes virus 6 (HHV-6) replication cells by phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues[a]

Antiviral activity (EC_{50}, μM)[b]						
Compd.	Base	Isomer	Prodrug	HSV-2 HFF Plaque[c]	Vero Plaque[c]	HHV-6 HSB-2 ELISA
1a	Ade	*Z*	−	37.8	59	14
3a	Ade	*Z*	+	NT	>1[d]	NT
1b	Gua	*Z*	−	NT	>100	42.5
3b	Gua	*Z*	+	39.2	>100	90
1c	DAP	*Z*	−	NT	>100	>100
3c	DAP	*Z*	+	12.7	15	20
1d	ACAP	*Z*	−	NT	41	NT
3d	ACAP	*Z*	+	0.8	1.8	NT
1e	AMP	*Z*	−	NT	>50	NT
3e	AMP	*Z*	+	0.88	0.6	NT
2a	Ade	*E*	−	>460	>100	90
4a	Ade	*E*	+	NT	>10[e]	NT
2b	Gua	*E*	−	NT	>100	>100
4b	Gua	*E*	+	NT	>100	>100
2c	DAP	*E*	−	NT	>100	>100
4c	DAP	*E*	+	NT	>100	55
Control				5.1 ± 0.5[f]	25[f]	31 ± 14[g]

[a] For abbreviations see Table 1.
[b] See footnote (a) in Table 2. For cytotoxicity in HFF, KB and CEM cells see Tables 1, 2 and 5.
[c] Plaque reduction assay.
[d] Toxic at 10 μM.
[e] Toxic at 100 μM.
[f] Acyclovir.
[g] Foscarnet.

proved over that of the parent 2,6-diaminopurine analogue **1c** only in HCMV plaque reduction assay. Surprisingly, compound **3c** was less effective than **1c** against MCMV. The overall anti-CMV effect of **3c** was almost identical with that of synguanol phosphoralaninate **3b** and both analogues were non-cytotoxic. The activity of the cyclopropylamino analogue **3d** against both viruses was not significantly improved over that of the parent compound **1d**. Prodrug **3d** was somewhat more toxic in HFF cells as determined by visual cytotoxicity but it was non-cytotoxic by neutral red uptake and in MEF cells. Analogue **3e** had an improved antiviral activity over that of the parent compound **1e**. A cytotoxicity effect was observed in HFF cells but not in MEF culture.

In contrast to the prodrugs of the *Z*-isomers, the *E*-phosphoralaninates **4a**–**4c** were devoid of potency against HCMV. The adenine derivative **4a** was an exception with a moderate effect in non-cytotoxic concentration range as determined by HCMV plaque reduction assay. The parent analogue **2a** was inactive.

3.3. Activity against herpesviruses

A potent activity of phosphoralaninate analogues **3a**, **3d** and **3e** relative to the parent compounds **1a**, **1d** and **1e** was observed in cell cultures infected with HSV-1 (Table 2) but this effect was not well separated from cytotoxicity determined in HFF or CEM cells. Some improvement of

antiviral activity of analogue **3b** in cytopathic effect inhibition over that of synguanol (**1b**) was noted with no observable cytotoxicity (data not shown). Moderate efficacy and cytotoxicity in CEM cells was observed with 2,6-diaminopurine phosphoralaninate **3c**.

A similar pattern of antiviral action was observed in HSV-2 assays (Table 3). Again, analogues **3a**, **3d** and **3e** were the most effective. The 2,6-diaminopurine derivative **3c** was less potent, **3b** and *E*-phosphoralaninates **4a**–**4c** were devoid of anti-HSV-2 activity.

From the group of phosphoralaninates tested against HHV-6 (Table 3), the 2,6-diaminopurine analogue **3c** was the most active followed somewhat surprisingly by the *E*-isomer **4c**. The parent analogues **1c** and **2c** were inactive. Surprisingly, a decrease of anti-HHV-6 efficacy was noted in case of phosphoralaninate **3b** relative to synguanol (**1b**). It should be noted that activity of **3c** falls in the range of that observed for reference compound, drug foscarnet.

The 2-amino-6-methoxypurine analogue **3e** was the most effective against VZV in HFF cells (Table 4) in a non-cytotoxic concentration range (Table 1). It was followed by 2-amino-6-cyclopropylaminopurine derivative **3d** which was less cytotoxic than **3e**. The parent analogues **1e** and **1d** were much less potent. The 2,6-diaminopurine analogue **3c** had EC_{50} 1.0 μM without apparent

Table 4
Inhibition of hepatitis B virus (HBV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV) replication human by phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues[a]

Compd.	Base	Isomer	Prodrug	Antiviral activity (EC_{50}, μM)[b]				Cytotoxicity (IC_{50}, μM)[c]		
				HBV 2.2.15[d]	EBV H-1	Daudi	VZV HFF[e]	2.2.15[d]	H-1	Daudi[f]
1a	Ade	*Z*	−	2	0.2	3.2	2.5	NT	>50	368 (NT)
3a	Ade	*Z*	+	0.01	0.1	1.0	7.6	0.3	NT	>108 (0.73)
1b	Gua	Z	−	10	0.3	5.6	61.3	NT	>50	>214 (13)
3b	Gua	*Z*	+	2	>10	0.31	8.1	NT	>50	>103 (62)
1c	DAP	*Z*	−	10	1.5	6.9	93	NT	>50	>215 (>215)
3c	DAP	*Z*	+	0.08	5.5	3.8	1.0	45[g]	NT	26 (10.6)
1d	ACAP	*Z*	−	2	4	11.8	13.2	NT	NT	>184 (28)
3d	ACAP	*Z*	+	2	10	2.3 (1.7)[h]	0.64	NT	23	18.8 (>97.3)
1e	AMP	*Z*	−	>10	1.6	17.6 (1.2)[h]	23.9	NT	>50	>202 (162)
3e	AMP	*Z*	+	2	0.4	0.75 (0.5)[h]	0.12	NT	4.6	4.35 (3.7)
2a	Ade	*E*	−	>10	3	71.4	90.2	NT	>50	>230 (NT)
4a	Ade	*E*	+	1.2	4.0	3.2	111	4.5	NT	65 (4.3)
2b	Gua	*E*	−	>10	>50	>206	263	NT	>50	>206 (>206)
4b	Gua	*E*	+	>10	8	>105	>211	NT	>50	>105 (>105)
2c	DAP	*E*	−	>10	>50	58.1	NT	NT	>50	>215 (164)
4c	DAP	*E*	+	10	>10	4.0	>211	NT[f]	>50	>106 (63.4)
Control				1.4[i]	5[j]	4.9[k]	9.3[k]	NT	75[j]	>222[k] (>222)[k]

[a] For abbreviations see Table 1.
[b] See footnote (a), Table 2.
[c] For cytotoxicity in KB and CEM cells see Tables 1 and 2.
[d] Transfected human hepatitis cell line HepG-2 (2.2.15). Averaged data from duplicate experiments.
[e] Plaque reduction assay.
[f] Stationary cells, values for proliferating cells are in parentheses.
[g] The IC_{50} of inhibition of mitochondrial DNA synthesis in CEM cells was >50 μM.
[h] The IC_{50} of inhibition of EBV DNA synthesis in Daudi cells.
[i] Zalcitabine (ddC).
[j] Ganciclovir.
[k] Acyclovir.

Table 5
Inhibition of replication of human immunodeficiency virus (HIV-1) by phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues[a]

Compd.	Base	Isomer	Prodrug	Antiviral activity (EC_{50}, μM)[b]		Cytotoxicity (IC_{50}, μM)
				HIV_{IIIB}[c]/CEM-SS		CEM-SS[d]
				High moi	Low moi	
1a	Ade	*Z*	−	20	0.8	>100
3a	Ade	*Z*	+	0.05	<0.14	1.1
1b	Gua	*Z*	−	>100	1.1[e]	>100
3b	Gua	*Z*	+	>100	NT	>100
1c	DAP	*Z*	−	>100	16	>100
3c	DAP	*Z*	+	0.2	0.26[e]	37.7
1d	ACAP	*Z*	−	NT	>100	>100
3d	ACAP	*Z*	+	NT	2.9	32 (9)
1e	AMP	*Z*	−	NT	>100	>100
3e	AMP	*Z*	+	NT	1.3	21 (2.2)
2a	Ade	*E*	−	>100	14	>100
4a	Ade	*E*	+	1.3	1.6	66 (40)
2b	Gua	*E*	−	>100	1.9	>100
4b	Gua	*E*	+	>100	NT	>100
2c	DAP	*E*	−	>100	>100	>100
4c	DAP	*E*	+	>100	>100	>50
AZT				0.017	0.003	NT

[a] For abbreviations see Table 1.
[b] See footnote (a) in Table 2.
[c] HIV strain IIIB assayed by supernatant reverse transcriptase (RT) activity. The data were obtained at high (~1 pfu/cell) and/or low (<0.1 pfu/cell) multiplicity of infection (moi). All assays were performed in triplicate.
[d] The IC_{50} values obtained with CEM cells were in good agreement with these values. More significant differences are in parentheses.
[e] Average of duplicate experiments.

cytotoxicity. Parent compound **1c** was virtually inactive. Analogues **3a** and **3b** were almost equipotent but **3a** was less effective than synadenol (**1a**) and it was cytotoxic (Table 1). The guanine derivative **3b** is non-cytotoxic and much more active than synguanol (**1b**). The *E*-analogues **4a**, **4b**, **4c** and the respective parent compounds **2a**, **2b**, **2c** were inactive against VZV.

3.4. Activity against HBV

Inhibition of HBV replication in transfected human liver HepG-2 (2.2.15) cell line indicated that phosphoralaninate **3a** was the most active agent (Table 4). This effect was accompanied by cytotoxicity. The parent synadenol (**1a**) was less potent but it lacked cytotoxicity in CEM cells. Guanine analogue **3b** was less effective than **3a** but it was significantly more potent than synguanol (**1b**) and devoid of cytotoxicity in CEM cells (Table 2). The most interesting activity profile was shown by 2,6-diaminopurine analogue **3c** with an impressive EC_{50} of 0.08 μM in a non-cytotoxic concentration range (IC_{50} 45 μM, SI 563). Prodrug **3c** was more than 100 times more potent that parent compound **1c**. The long-term (mitochondrial) toxicity of this analogue was

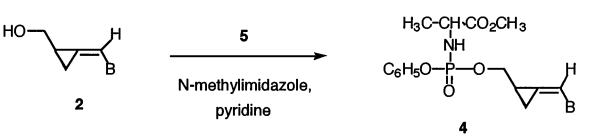

Fig. 2. Synthesis of phosphoralaninate diesters of *E*-methylenecyclopropane analogues **4a**–**4c**. Series a, B = Ada; series b, B = Gua; series c, B = 2,6-diaminopurine.

also favorable ($IC_{50} > 50$ μM). Phosphoralaninate **3d** was as effective against HBV as the parent compound **1d** but it displayed a higher cytotoxicity in CEM cells. An improvement of potency of analogue **3e** over that of **1e** was also noted but, again, cytotoxicity in CEM cells was apparent. From the *E*-series of analogues, phosphoralaninate **4a** was the most potent in a non-cytotoxic concentration range. Analogues **4b** and **4c** were less effective. The parent compounds **2a**–**2c** were inactive.

3.5. Activity against EBV

The *Z*-series of phosphoralaninates was also most effective against EBV (Table 4). The activity of phosphoralaninate **3a** against EBV in both H-1 and Daudi cells corresponded to that of parent synadenol (**1a**). In case of analogue **3b** the potency relative to synguanol (**1b**) was reversed in both assays. A significant cytotoxicity of **3a** was observed in HFF and CEM cell cultures but not in stationary Daudi cells. Analogue **3b** was generally non-cytotoxic. The 2,6-diaminopurine derivative **3c** was also active with some cytotoxicity in CEM and Daudi cells. The anti-EBV effects of **3c** were comparable to those of the parent analogue **1c**. The activity of phosphoralaninate **3d** corresponded roughly to that of the parent analogue **1d** in both H-1 and Daudi cells but the pattern of potency was reversed. Compound **3d** was also a strong inhibitor of EBV DNA synthesis. Analogue **3e** was more active than **1e** in both H-1 and Daudi cells but at the expense of higher cytotoxicity. Both analogues inhibited EBV DNA synthesis to approximately equal extent.

From the *E*-series of analogues, phosphoralaninate **4a** was the most effective but it was almost equipotent with the parent *E*-analogue **2a** against EBV in H-1 cells. Nevertheless, it was significantly more active in Daudi cells than **2a**. By contrast, phosphoralaninate **4b** was quite a potent and non-cytotoxic inhibitor of EBV replication in H-1 culture but it was inactive in Daudi cells. Compound **4c** inhibited EBV in Daudi culture with a lesser effect in H-1 cells. Both parent analogues **2b** and **2c** were devoid of a significant anti-EBV effect.

3.6. Activity against HIV-1

As found earlier (Qiu et al., 1998a,b), methylenecyclopropane nucleoside analogues **1a**–**1d** exhibit little efficacy against HIV-1 in CEM-SS cell culture at higher virus input. Thus, the most active analogue **1a** had EC_{50} 20 μM, whereas the rest of the group was essentially inactive. Transformation to the respective phosphoralaninates led in several instances to a significant increase of anti-HIV potency (Table 5). Thus, adenine analogue **3a** when assayed by reverse transcriptase activity in CEM-SS cells infected with HIV-1 had an EC_{50} of 0.05 μM accompanied by cytotoxicity (IC_{50} 1.1 μM) but guanine derivative **3b** was inactive. This finding is more in line with the results obtained with HSV-1 and HSV-2 assays (Tables 2 and 3) than the respective HCMV assay (Table 1), where **3b** was a potent inhibitor. As in the case of HBV assay (Table 4), the most interesting finding was obtained with 2,6-diaminopurine analogue **3c** (EC_{50} 0.2 μM, IC_{50} 37.7 μM, SI 189). Compounds **3d** and **3e** tested at low moi of HIV-1 were very potent at non-cytotoxic concentrations, whereas the parent analogues **1d** and **1e** were inactive. Interestingly, *E*-analogue **4a** had a significant anti-HIV effect in a non-cytotoxic concentration range, whereas the parent analogue **2a** was devoid of activity. By contrast, *E*-analogues **4b** and **4c** were inactive.

Inhibition of growth of KB cells, a human cell line derived from epidermoid oral carcinoma, was used as a complementary measure of cytotoxicity (Table 2). Prodrug of synadenol **3a** was the most inhibitory from the tested phosphoralaninates, whereas the rest of the group were either moderately effective or inactive. Compounds **3a** and **4a** were inactive as antitumor agents against murine leukemia L1210, mouse solid tumors C-38, M-17/Adr and human solid tumor H-116.

4. Discussion

Most of the described *Z*-phosphoralaninate diesters exhibited an improvement in the antiviral spectrum and potency over the parent analogues. Thus, activity of adenine phosphoralaninate **3a**

was increased over synadenol (**1a**) in almost all antiviral assays performed. A similar trend was noted with phosphoralaninates **3d** and **3e** as compared with compounds **1d** and **1e**. Nevertheless, the increased antiviral potency of **3a**, **3d** and **3e** was accompanied by a varying degree of cytotoxicity not seen in the parent analogues. By contrast, phosphoralaninate **3b** lacked the cytotoxicity but the anti-HCMV activity remained at about the same level as that of synguanol (**1b**). The anti-HBV potency increased about five times.

The 2,6-diaminopurine phosphoralaninate **3c** differs from the aforementioned analogues in one significant aspect. An increase of the antiviral activity relative to the parent compound **1c** observed in several viral assays was not accompanied by a substantial elevation of cytotoxicity. This pattern is most apparent in inhibition of HBV, HIV-1, HHV-6 and VZV. Phosphoralaninate **3c** is also effective against HCMV, MCMV and EBV but the activity is roughly comparable with that of 2,6-diaminopurine analogue **1c**. By contrast, the antiviral effect of **3c** against HSV-1 and HSV-2 although significantly increased relative to the parent (inactive) compound **1c** is only moderate.

Inactive *E*-isomers also acquired an antiviral potential after introduction of the phosphoralaninate residue (compounds **4a**–**4c**) although their antiviral spectrum is more narrow and potency weaker. It should be noted that the respective parent analogues **2a**–**2c** are devoid of antiviral activity with the exception of the *E*-isomer of synadenol **2a** which showed effect in EBV/H-1 culture (Qiu et al., 1998a). Thus, adenine analogue **4a** is an effective agent against HBV, EBV and HIV-1 with a strong cytotoxicity apparent only in 2.2.15 and dividing Daudi cells. Analogues **4b** and **4c** are active against HBV and EBV/Daudi cell system but ineffective in other assays.

Noteworthy is a significant anti-HIV activity (EC_{50} 1.3 μM) of *E*-phosphoralaninate **4a** accompanied by a moderate cytotoxicity (IC_{50} 66 μM, SI 51). The selectivity index of this analogue is then more favorable than that of the *Z*-derivative **3a** (SI 22). There are indications (Qiu et al., 1998a) that the parent compound **2a** may be a more distant structural analogue of nucleosides than the corresponding *Z*-isomer **1a**. This could then explain an apparent lower reactivity toward cellular enzymes leading to a decreased cytotoxicity. All these results indicate that transformation to phosphoralaninate 'pronucleotides' can confer antiviral activity on inactive *E*-methylenecyclopropane nucleoside analogues.

It is clear that the phosphoralaninate group of **3b** has, with the exception of HBV, EBV/Daudi and VZV, little favorable effect on antiviral parameters of a potent anti-CMV agent synguanol **1b**. Analogue **3b** is also devoid of significant cytotoxicity. Presumably, intracellular phosphorylation of **1b** is very efficient and, therefore, a circumvention of the first phosphorylation step provides no advantage. A similar effect was described (McGuigan et al., 1993) in case of AZT.

Comparison of anti-HIV profiles of **3a** and **3c** is also of interest. Both prodrugs had a similar level of efficacy but compound **3a** comprising a natural DNA base (adenine) was significantly more cytotoxic than **3c** which is derived from an unnatural base analogue (2,6-diaminopurine). Nevertheless, this favorable effect is diminished in purine analogues **3d** and **3e** comprising heterocyclic bases structurally more distant from adenine. In nucleoside series, 2,6-diaminopurine derivatives are usually converted to guanine analogues (Mitsuya et al., 1991) by the action of adenosine deaminase and they must be therefore considered prodrugs. Nevertheless, parent compound **1c** is neither a substrate for adenosine deaminase nor non-specific AMP deaminase from *Aspergillus* sp. (Qiu et al., 1998b). Very different antiviral profiles seem also to rule out that **3c** or a phosphorylated species generated from it (Fig. 3) is a prodrug of **3b**.

There are studies showing that the 2,6-diaminopurine base is a better mimic of adenine than guanine (Rackwitz and Scheit, 1977; Stoeckler et al., 1997). A lower cytotoxicity of **3c** relative to **3a** may then indicate that phosphate metabolite derived from a modified nucleobase is less likely to be processed by cellular enzymes than similar derivative containing a natural nucleobase. A possible extension of this observation to more conventional nucleoside analogues could be of interest.

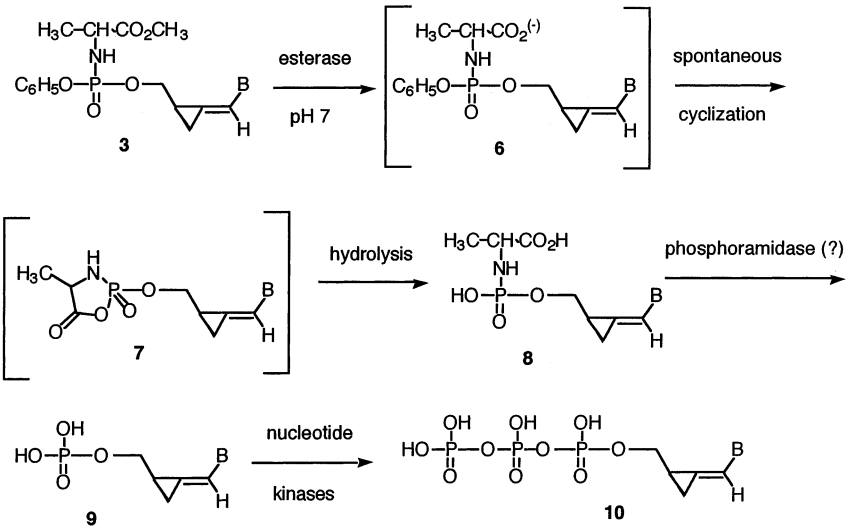

Fig. 3. Proposed mechanism of activation of phosphoralaninate diesters of methylenecyclopropane analogues **3a**–**3e**.

The present findings also strengthen the indirect evidence (Drach et al., 1997) that phosphorylated intermediates are important in the mechanism of action of both *Z*- and *E*-methylenecyclopropane analogues of nucleosides.

From all the analogues studied, compound **3c** is the most promising antiviral agent. Further investigation of this prodrug as a potential chemotherapeutic agent against HIV and HBV infections appears to be warranted. It has a broad range of activity including all the tested viruses with the exception of HSV-1 and HSV-2, and a favorable selectivity index. It is also worthwhile of noting that analogues **3a** and **3c** are effective (Yoshimura et al., 1998; Uchida et al., 1999) against clones of HIV-1 resistant to several drugs against AIDS.

It is possible that mechanism of action of phosphoralaninates **3** and **4** can be related to intracellular transformations of similar prodrugs (Balzarini et al., 1996b; Winter et al., 1996) derived from other nucleoside analogues (Fig. 3). After penetration of the cell membrane the alaninate ester moiety of methylenecyclopropane analogue **3** is hydrolyzed by an intracellular esterase to give acid **6**. In the next step, a nucleophilic attack of the ionized carboxyl group leads to expulsion of the phenoxide moiety and cyclization. The resultant cyclic anhydride **7** undergoes a hydrolysis to give phosphoralanine derivative **8**. The latter is then hydrolyzed, possibly by intracellular phosphoramidase, to give monophosphate **9** which is then brought to the triphosphate level (compound **10**) by nucleotide kinases. Our present and previous results (Winter et al., 1996, 1997) indicate that the putative phosphoramidase can tolerate structures distant from carbohydrate (ribose) moiety found in most of the nucleoside analogues.

Acknowledgements

We thank Drs M.B. Ksebati and M.B. Kempff, Central Instrumentation Facility, Department of Chemistry, Wayne State University (Director, Dr Robin H. Hood) for NMR and mass spectra. The antitumor assays were performed by Dr T.H. Corbett, Department of Internal Medicine, Wayne State University School of Medicine. The work described herein was supported in part by US Public Health Service Research Grants RO1-CA32779 (J.Z.) from the National Cancer Institute, U19-AI31718, RO1-AI36872 (J.C.D.), NO1-AI35177 (E.R.K.) and RO1-AI33655, RO1-AI38204 (Y.-C.C.) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

References

Balzarini, J., Egberink, H., Hartmann, K., Karlsson, A., Perno, C.-F., Cahard, D., Naesens, L., Thormar, E., De Clercq, E., McGuigan, C., 1996a. Mechanism of anti-HIV action of masked alaninyl 2′,3′-dideoxy-2′,3′-didehydrothymidine 5′-monophosphate derivatives. Antiviral Res. 30, 18.

Balzarini, J., Karlsson, A., Aquaro, S., Perno, C.-F., Cahard, D., Naesens, L., De Clercq, E., McGuigan, C., 1996b. Mechanism of anti-HIV action of masked alanyl d4T-MP derivatives. Proc. Natl. Acad. Sci. USA 93, 7295–7299.

Balzarini, J., Wedgwood, O., Kruining, J., Pelemans, H., Heijtink, R., De Clercq, E., McGuigan, C., 1998. Anti-HBV and anti-HBV activity and resistance profile of 2′,3′-dideoxy-3′-thiacytidine (3TC) and its arylphosphoramidate derivative CF 1109. Biochem. Biophys. Res. Commun. 225, 363–369.

Drach, J.C., Fan, B.Y., Ptak, R.G., Breitenbach, J.M., Borysko, K.Z., Qiu, Y.-L., Zemlicka, J., 1997. Selective activity of 2-hydroxymethylcyclopropylidenemethyl purines against human cytomegalovirus. Antiviral Res. 34, 83.

Franchetti, P., Cappelacci, L., Grifantini, M., Messini, L., Sheikha, G.A., Loi, A.G., Tramontano, E., De Montis, A., Spiga, M.G., La Colla, P., 1996. Synthesis and evaluation of the anti-HIV activity of aza and deaza analogues of isoddA and their phosphates as prodrugs. J. Med. Chem. 37, 3534–3541.

Hartline, C.B., Zemlicka, J., Qiu, Y.-L., Bidanset, D.J., Harden, E., Sommadossi, J.-P., Kern, E.R., 1998. In vitro activity of methylenecyclopropane analogues of nucleosides against cytomegalovirus infections. Antiviral Res. 37, 82.

McGuigan, C., Pathirana, R.N., Balzarini, J., De Clercq, E., 1993. Intracellular delivery of bioactive AZT nucleotides by aryl phosphate derivatives of AZT. J. Med. Chem. 36, 1048–1052.

McGuigan, C., Pathirana, R.N., Mahmood, N., Devine, K.G., Hay, A.J., 1992. Aryl phosphate derivatives of AZT retain activity against HIV-1 in cell lines which are resistant to the action of AZT. Antiviral Res. 17, 311–321.

McGuigan, C., Wedgwood, O.M., De Clercq, E., Balzarini, J., 1996. Phosphoramidate derivatives of 2′,3′-didehydro-2′,3′-dideoxyadenosine (d4A) have markedly improved anti-HIV potency and selectivity. Bioorg. Med. Chem. Lett. 6, 2359–2362 and references cited therein.

McGuigan, C., Wedgwood, O.M., Yarnold, C.J., Hiouni, A., Sutton, P.W., Harris, S.A., De Clercq, E., Balzarini, J., 1998. The phosphoramidate approach to the intracellular delivery of free nucleotides can enhance potency and antiviral selectivity of a variety of nucleosides. Antiviral Res. 37, 48.

Meier, C., 1998. Pro-nucleotides-recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. Synlett 233–242.

Mitsuya, H., Yarchoan, R., Kageyama, S., Broder, S., 1991. Targeted therapy of human immunodeficiency virus-related disease. FASEB J. 5, 2369–2381.

Perigaud, C., Girardet, J.-L., Gosselin, G., Imbach, J.-L., 1996. Comments on nucleotide delivery forms. In: Advances in Antiviral Design, vol. 2. JAI Press, Greenwich, CT, pp. 147–172.

Qiu, Y.-L., Ksebati, M.B., Ptak, R.G., Fan, B.Y., Breitenbach, J.M., Drach, J.C., Lin, J.-S., Cheng, Y.-C., Kern, E.R., Drach, J.C., Zemlicka, J., 1998a. (*Z*)- and (*E*)-2-(Hydroxymethylcyclopropylidene)methyladenine and -guanine—new nucleoside analogues with a broad-spectrum antiviral activity. J. Med. Chem. 41, 10–23.

Qiu, Y.-L., Ptak, R.G., Breitenbach, J.M., Drach, J.C., Lin, J.-S., Cheng, Y.-C., Kern, E.R., Zemlicka, J., 1998b. (*Z*)- and (*E*)-2(Hydroxymethylcyclopropylidene)-methylpurines and pyrimidines as antiviral agents. Antiviral Chem. Chemother. 9, 341–352.

Rackwitz, H.R., Scheit, K.H., 1977. The stereochemical basis of template function. Eur. J. Biochem. 72, 191–200.

Rybak, R.J., Zemlicka, J., Qiu, Y.-L., Hartline, C.B., Kern, E.R.. 1999. Effective treatment of murine cytomegalovirus infections with methylenecyclopropane analogues of nucleotides. Antiviral Res., in press.

Rybak, R.J., Zemlicka, J., Qiu, Y.-L., Kern, E.R., 1998. Effective treatment of murine cytomegalovirus infections with methylenecyclopropane analogues of nucleosides. Antiviral Res. 37, 82.

Stoeckler, J.D., Poirot, A.F., Smith, R.M., Parks, R.E. Jr., Ealick, S.E., Takabayashi, K., Erion, M.D., 1997. Purine nucleoside phosphorylase. 3. Reversal of purine base specificity by site-directed mutagenesis. Biochemistry 36, 11749–11756.

Uchida, H., Kodama, E., Yoshimura, K., Maeda, Y., Kosalaraksa, P., Maroun, V., Qiu, Y.-L., Zemlicka, J., Mitsuya, H., 1999. In vitro anti-human immunodeficiency virus activities of *Z*- and *E*-methylenecyclopropane nucleoside analogues and their phosphoro-L-alaninate diesters. Antimicro. Agents and Chemother. 43, 1487–1490.

Winter, H., Maeda, Y., Mitsuya, H., Zemlicka, J., 1996. Phosphodiester amidates of allenic nucleoside analogues: anti-HIV activity and possible mechanism of action. J. Med. Chem. 39, 3300–3306.

Winter, H., Maeda, Y., Uchida, H., Mitsuya, H., Zemlicka, J., 1997. Phosphodiester amidates of unsaturated nucleoside analogues: synthesis and anti-HIV activity. J. Med. Chem. 40, 2191–2195.

Yoshimura, K., Uchida, H., Kodama, E., Maeda, Y., Feldman, R., Qiu, Y.-L., Zemlicka, J., Mitsuya, H., 1998. In vitro anti-HIV-1 activity and viral drug resistance profiles against phosphoralaninate diesters of *Z*- and *E*-methylenecyclopropane analogues. In: 5th Conference on Retroviruses and Opportunistic Infections, Chicago, IL, 1–5 February 1998, p. 197, abstract 627.

Zemlicka, J., Qiu, Y.-L., Lin, J.-S., Cheng, Y.-C., Ptak, R.G., Breitenbach, J.M., Drach, J.C., Kern, R.E., 1998. Phosphoralaninate prodrugs of methylenecyclopropane analogues of nucleosides as antiviral agents. Antiviral Res. 37, 6.