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Phosphoramidate derivatives of d4T as inhibitors of HIV: The effect of amino acid variation

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Abstract

Phosphoramidate derivatives of the nucleoside analogue, 2′,3′-dideoxy-2′,3′-didehydro thymidine (d4T) have been prepared as potential membrane-soluble pro-drugs of the big-active free phosphate forms. In particular phenyl phosphates, linked via nitrogen to methyl-esterified amino acids, were studied. All compounds were fully characterised by a range of methods (high-field multinuclear NMR, mass spectrometry and high performance liquid chromatography (HPLC)) and were subjected to in vitro evaluation of their anti-HIV efficacy. The nature of the amino acid appeared to be extremely important for the eventual antiviral action. Of the amino acids studied, L-alanine was the most efficacious, whilst L-proline and glycine were particularly poor. However, an unnatural amino acid moiety, dimethylglycine, could substitute for alanine with little or no loss of activity. © 1997 Elsevier Science B.V.

Keywords: HIV; Nucleotide; Pro-drug; 2′,3′-Dideoxy-2′,3′-didehydro thymidine; Phosphoramidate

1. Introduction

The authors (McGuigan et al., 1990a,b, 1991a, 1992, 1993a) and others (Farrow et al., 1990; Gosselin and Imbach, 1993; Gouyette et al., 1989; Henin et al., 1991; Girardet et al., 1995; Perigaud et al., 1996) have pursued a masked phosphate approach in an attempt to improve the therapeutic potential of parent nucleoside analogues. In this approach, phosphate derivatives of the nucleoside analogue are designed to penetrate the cell membrane and liberate the bio-active nucleotides intracellularly. Masking of the phosphate

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group is necessary on account of the extremely poor membrane penetration by the polar (charged) free nucleotide.

We have noted that phosphoramidates are especially efficacious phosphate delivery systems, particularly if the amine used is an amino acid. Such amino acid-derived phosphoramidates are potent anti-HIV agents when attached to AZT (McGuigan et al., 1993b), FdT (McGuigan et al., 1991b), 2′,3′-dideoxy-2′,3′-didehydro thymidine (d4T) (McGuigan et al., 1996a,b; Balzarini et al., 1996a) or d4A (McGuigan et al., 1996c). We have recently noted that an amino acid (or close analogue) is necessary for activity, since simple amine substitution leads to a complete loss of antiviral activity (McGuigan et al., 1996d). Indeed, relatively small changes in the amino acid, such as inversion of the natural (L) stereochemistry of an alanine substituent to the (D) stereoisomer, appear to lead to a significant reduction in potency (McGuigan et al., 1996e).

We have previously surveyed several different (L) amino acids, when attached to AZT (McGuigan et al., 1993b) and noted alanine to be preferred over the others. We now report the extension of this approach to d4T and the inclusion of an expanded range of 12 amino acids. The application of this phosphate delivery strategy to d4T was anticipated to be likely to be most efficacious, on the grounds of the different phosphorylation kinetics of d4T as compared to AZT (Balzarini et al., 1989) but it would not necessarily follow that the amino acid preferences would be the same for the two nucleosides.

2. Experimental methods

2.1. General methods

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in a Bruker AVANCE DPX300 spectrometer. All ^{1}H and ^{13}C NMR chemical shifts (δ) are quoted in parts per million downfield from tetramethylsilane. All ^{31}P chemical shifts are quoted in ppm using H_3PO_4 as external reference. All NMR spectra were obtained in $CDCl_3$. Mass spectra were recorded using electrospray (ESMS), or FAB with a NOBA-matrix. A mobile phase of acetonitrile/water 1:1, with a flow rate of 4 ml min^{-1} was used with the ESMS instrument. All analytical high performance liquid chromatography (HPLC) experiments were carried out on an ACS 350/04 system provided with an ACS 351/04 gradient controller and using an ACS 750/12 detector set at 254 nm. The system used was: Ultratech 50DS (25×4.6 mm) reverse phase Column. Mobile phase: Water/acetonitrile, linear gradient conditions, 0–10 min 82% water, 10–30 min 20% water, 30–45 min 20% water. The flow was 1.0 ml min^{-1}. All retention times are quoted in minutes. Separations on silica gel were routinely performed by preparative centrifugal circular thin layer chromatography (CCTLC) on a chromatotron instrument (Kiesegel 60PF254 gipshalting, Merck, Layer thickness, 1 mm). Alternatively, flash column chromatography could be used for purification on silica; isolated yields were similar by both methods. Dichloromethane, methanol, triethylamine and pyridine were dried over calcium hydride and distilled at atmospheric pressure. Tetrahydrofuran was dried over sodium-benzophenone and distilled under nitrogen. *N*,*N*′-dimethylformamide was dried over 4 Å molecular sieves. 2′,3′-Dideoxy-2′,3′-didehydro thymidine (d4T) was supplied by Cardiff Chemicals, Cardiff, UK. All other solvents and reagents were used as supplied. All chiral amino acids were of the L-configuration.

2.1.1. General method

Phenyl *N*-methylalaninyl phosphorochoridate (McGuigan et al., 1992, 1993b) (250 mg, 0.9 mmol, 2.0 equivalents) was added to a stirred solution of d4T (100 mg, 0.45 mmol) and *N*-methyl imidazole (143.5 ml, 1.8 mmol, 4 equivs) in THF (2 ml). After 6 h, the solvent was removed under reduced pressure. The gum was dissolved in chloroform (10 ml) and washed with 1 M HCl (8 ml), sodium bicarbonate (10 ml) and water (15 ml). The organic phase was dried ($MgSO_4$) and the solvent removed in vacuo. The residue was purified by column chromatography on silica with elusion by chloroform–methanol (97:3) or by Chromatotron using dichloromethane in place of chloroform. Pooling and evaporation of the eluent gave the product as a white solid (Fig. 1).

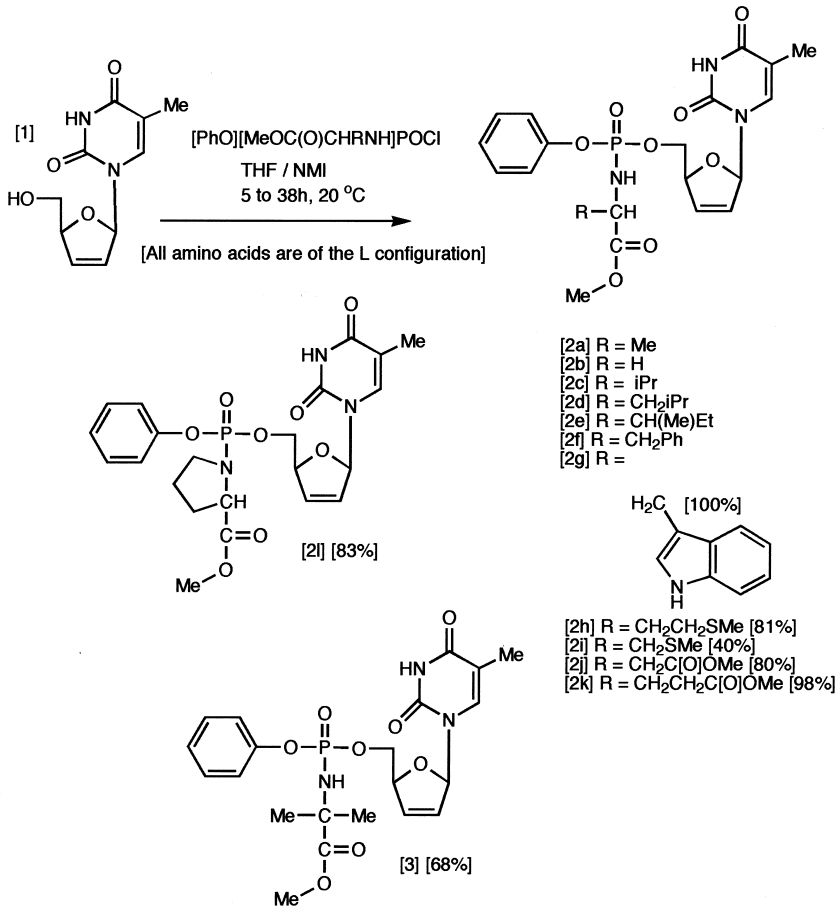

Fig. 1. The chemical structures of some anti-HIV nucleosides and nucleotides.

2′,3′-Dideoxy-2′,3′-didehydrothymidine-5′-(phenyl methoxytryptophanyl) phosphate [**2g**]. Yield = 100%. δ_P 4.15, 4.57. δ_H 1.74 (s, 3H, 5Me); 3.16 (m, 2H, CH_2 Trp); 3.60 (s, 3H, OMe); 3.75–4.05 (m, 2H, H5′); 4.10–4.33 (m, 2H, CH Trp, NH Trp); 4.84 (m, 1H, H4′); 5.79 (m, 1H, H2′); 6.15 (m, 1H, H3′); 6.86 (m, 1H, H1′); 6.91 (m, 1H, H6), 7.00–7.49 (m, 10H, Ar); 8.45 (s, 1H, NH Trp); 9.14 (s, 1H, NH). MS 580 (MH^+); 579 (M^+). HPLC RT = 43.2 min.

2′,3′-Dideoxy-2′,3′-didehydrothymidine-5′-(phenyl methoxymethioninyl) phosphate [**2h**]. Yield = 81%. δ_P 4.09, 4.86 ppm. δ_H 1.74, 1.79 (s, 3H, MeS); 1.94, 1.97 (s, 3H, 5Me); 1.80–2.40 (m, 5H, C<u>H</u>C<u>H</u>$_2$C<u>H</u>$_2$S); 3.72, 3.74 (s, 3H, OMe); 3.98–4.32 (m, 4H, H5′, CH Met, NH Met); 4.96 (m, 1 H, H4′); 5.84 (m, 1 H, H2′); 6.26 (m, 1 H, H3′); 6.96 (m, 1 H, H1′); 7.05–7.25 (m, 6H, Ar, H6); 9.58 (bs,1 H, NH). MS (FAB/NOBA) 526 (MH^+, 46); 548 (MNa^+, 21). HPLC RT = 29.9 min.

2′,3′ - Dideoxy - 2′,3′ - didehydrothymidine - 5′ - (phenyl methoxy-*S*-methylcysteinyl) phosphate [**2i**]. Yield = 40%. δ_P 4.47, 3.83. δ_H 1.85, 1.82 (s, 3H, 5Me); 1.99, 2.06 (s, 3H, SMe); 2.83 (m, 2H, CH_2 Cys); 3.71 (s, 3H, OMe); 4.1 (m, 2H, H5′); 4.4 (m, 2H, NH Cys, CH Cys); 4.41 (m, 1H, H4′); 5.8 (m, 1H, H2′); 6.4 (m, 1H, H3′); 7.04–7.35 (m, 7H, Ph, H1′, H6); 9.16 (s, 1H, NH). MS (FAB/NOBA) 511 (M^+,10); 512 (MH^+, 15); 534 (MNa^+, 100). HPLC RT = 22.5, 22.8 min.

2′,3′ - Dideoxy - 2′,3′ - didehydrothymidine - 5′ - (phenyl dimethoxyaspartyl) phosphate [**2j**]. Yield 80%. δ_P 4.78, 3.72 ppm. δ_H 1.85*,1.90* (s, 3H, Me), 2.85, 2.63 (m, 1H, CH_2 Asp), 3.05, 3.00 (m, 1H, CH_2 Asp), 3.82*, 3.75* (s, 6H, OMe), 4.21–4.52 (m, 4H, NH Asp, CH Asp, H5′), 5.04, 5.10 (m, 1H, H4′), 5.95 (m, 1H, H2′), 6.41, 6.31 (m, 1H, H3′), 7.10 (m, 1H, H1′),7.20–7.45 (m, 6H, Ph, H6), 9.22 (bs, 1 H, NH). MS (FAB/NOBA) 546 (MNa^+, 100). HPLC RT = 23.55 min.

2′,3′ - Dideoxy - 2′,3′ - didehydrothymidine - 5′ - (phenyl dimethoxyglutamyl) phosphate [**2k**]. Yield = 91%. δ_P 4.15, 4.75 ppm. δ_H 1.83, 1.86 (s, 3H, 5Me); 1.90–2.41 (m, 4H, CH_2CH_2); 3.65, 3.71 (s, 6H, OMe); 3.72 (m, 1 H, NH); 4.21–4.32 (m, 3H, H5′, CH Glu); 5.04 (m, 1H, H4′); 5.89 (m, 1H, H2′); 6.28 and 6.36 (2d, 1H, H3′); 7.03 (m, 1H, H1′); 7.18–7.36 (m, 6H, Ar, H6); 9.65, 9.66 (2s, 1 H, NH). MS (FAB/NOBA) 538 (MH^+, 100); 560 (MNa^+, 29). HPLC RT = 22.0, 22.2 min.

2′,3′ - Dideoxy - 2′,3′ - didehydrothymidine - 5′ - (phenyl methoxyprolinyl) phosphate [**2l**]. Yield = 83%. δ_P 9.04, 9.25 ppm. δ_H 1.79, 1.91 (s, 3H, 5Me); 1.80–2.10 (m, 4H, CH_2CH_2); 3.20–3.50 (m, 2H, $NC\underline{H}_2$); 3.72, 3.79 (s, 3H, OMe); 4.26–4.61 (m, 3H, H5′, NCH); 5.08 (m, 1H, H4′); 5.93 (m, 1H, H2′); 6.32, 6.46 (m, 1H, H3′); 7.07 (m, 1H, H1′); 7.20–7.50 (m, 6H, Ar, H6); 8.48 (bs, 1 H, NH). MS (FAB/NOBA) 492 (MH^+, 64); 514 (MNa^+, 28). HPLC RT = 26.95 and 27.28 min.

2′,3′ - Dideoxy - 2′,3′ - didehydrothymidine - 5′ - (phenyl methoxy dimethylglycinyl) phosphate [**3**]. Yield = 68%. δ_P 3.02, 2.33 ppm. δ_H 1.52, 1.54 (s, 6H, CMe_2); 1.91 (s, 3H, 5Me); 3.71 (s, 3H, OMe); 3.91–4.31 (m, 2H, H5′); 4.96, 4.99 (m, 1 H, H4′); 5.89 (m, 1 H, H2′); 6.28 (m, 1H, H3′); 6.99–7.31 (m, 7H, Ar, H6, H1′); 9.89 (bs, 1H, NH). MS (FAB/NOBA) 479 (M^+, 3); 480 (MH^+, 64); 481 (MH^+, ^{13}C,17); 482 (MH^+, $2 \times {}^{13}C$, 3); 502 (MNa^+, 92); 503 ($MHNa^+$, 24). HR MS found 480.1503 (MH^+, $C_{21}H_{27}N_3O_8P$ requires 480.1536). HPLC RT = 24.79 and 25.29 min.

2.2. Materials and experimental procedures

Virology human immunodeficiency virus type 1 (HIV-I/IIIB) was obtained from Dr R.C. Gallo (National institutes of Health, Bethesda, MD, now at The Institute Of Human Virology, Baltimore, MD). HIV-2/ROD was provided by Dr L. Montagnier (Pasteur Institute, Paris, France). CEM/0 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and CEM/TK^- cells were a kind gift from Drs S. Eriksson and A. Karlsson (Karolinska Institute, Stockholm, Sweden). CEM cells were infected with HIV-1 as previously described (Balzarini et al., 1993). Briefly, 4×10^5 CEM cells/ml were infected with HIV-1 or HIV-2 At 100 $CCID_{50}$ (50% cell culture infective dose) per ml of cell suspension. Then, 100 μ1 of the infected cell suspension were transferred to microtiter plate wells and mixed with 100 μl of the appropriate dilutions of the test compounds. After 4 days, giant cell formation was recorded microscopically in the hivinfected cell cultures. The 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) were defined as the compound concentrations required to reduce by 50% the number of giant cells in the virus-infected cell cultures and the number of viable cells in the mock-infected cell cultures, respectively. For MSV, C3H/3T3 cells were seeded at 20 000 cells/ml into wells of tissue culture cluster plates (48 wells/plate). Following a 24 h incubation period, cell cultures were infected with 80 focus-forming units of msv during 120 min, whereafter the culture medium was replaced by 1 ml of fresh medium containing appropriate concentrations of the test compound. After 6 days, transformation of the cells was examined microscopically.

3. Results and discussion

The synthetic strategy which we have used for the preparation of the novel phosphoramidates [**2f**–**2I**] closely follows what we have previously reported for earlier analogues [**2a**–**e**] (McGuigan et al., 1992, 1993b, 1996a). This involves the initial preparation of the appropriate phosphorochloridate from phenyl phosphorodichloridate and an amino acid methyl ester hydrochloride, followed by its reaction with the parent nucleoside analogue, Thus, reaction of phenyl methoxy-L-

Table 1
^{13}C NMR data on novel compounds [**2g**–**2l**] and [**3**]

		2g	**2h**	**2i**	**2j**	**2k**	**2l**	**3**
Base	C2	151.40*	151.41	151.35	151.14*	150.93	151.36	153.05*
	C4	164.39*	164.52	164.29	163.99*	164.03*	164.37	166.12
	C5	111.85	111.66*	111.89*	111.87*	111.33*	111.49	113.24*
	C6	136.61*	136.00*	133.80*	136.31*	135.78*	136.73	137.68*
	5-Me	12.74	12.8	12.76	12.71*	12.25*	12.83	14.27
Sugar	C1′	90.24*	89.98*	90.20*	90.19*	89.74*	89.89	91.51*
	C2′	127.92*	127.81*	127.96	127.89*	127.41*	127.46	126.88
	C3′	133.82*	133.44*	130.23*	133.78*	133.14	134.15	135.19*
	C4′	85.05	84.90*	85.14*	85.09*	84.63*	85.37*	86.57*
	C5′	67.23*	67.05 *	67.72 *	67.55*	67.20 *	66.64*	69.03 *
Amino	NH–C	55.60*	53.81*	54 25*	51.59*	53.83*	61.16*	58.88*
	C=0	173.84	173.61*	172.59*	b	d	174.68	177.69*
	OMe	52.90	53.06	53.1	c	52.52*	52.69	54.81*
	CH or Ph	109–123	—	—	—	—	—	—
	CH_2	30.45	a	39.22*	38.55*	e	f	—
	Me [1]	—	15.68	16.44	—	—	—	28.86
	Me [2]	—	—	—	—	—	—	28.70

Carbon-13 NMR data for [**2g**–**2l**] and [**3**] recorded at 75.5 MHz in $CDCl_3$. Many peaks (indicated by *) are split due to phosphorus coupling and/or the presence of diastereoisomers at the phosphate centre. Each compound also gives four sets of peaks in the region δ 120–150 corresponding to the O-Ph moiety. Additional peaks are noted in some cases as follows: a. 29.95, 33.73*; b. 172.2*, 171.58*; c. 53.34, 53.32, 52.49, 52.48; d. 172.86, 172.90, 172.97, 173.07 e. 28.92, 29.00, 29.15, 29.35; f. 25.69*, 31.55*, 46.89*.

tryptophanyl phosphorochloridate with d4T [**1**] in THF containing *N*-methylimidazole gave [**2g**] in good yield after purification by column chromatography. This was isolated as a mixture of diastereoisomers, as evidenced by the presence of two closely spaced signals in the P-31 NMR [δ_P, 4.2 and 4.6 ppm] arising from mixed stereochemistry at the phosphorus centre. The presence of these isomers was further confirmed in the ^1H NMR spectrum, where several signals showed extra multiplicity attributed to the presence of $\approx$1:1 mixture of the two (phosphate) stereoisomers. ^{13}C NMR, mass spectrometry and HPLC data also confirmed the structure, purity and isomeric nature of [**2g**]. Similarly prepared were the other analogues: [**2h**; methionine], [**2i**; cysteine], [**2j**; aspartic acid], [**2k**; glutamic acid] and [**2l**; proline]. Each of the 'simple' amino acids were carboxyl protected as their methyl esters, whilst aspartic and glutamic acid were used as their dimethyl esters, and the thiol group of cysteine was protected as the *S*-methyl ether. All spectroscopic data on [**2h**–**2l**] fully confirmed the structure and purity of these materials, which, in each case were isolated as mixtures of diastereoisomers at the phosphorus centre. All samples were pure by HPLC, and entirely free of any contaminating nucleoside. Full high field ^{13}C NMR data on [**2g**–**2l**] are given in Table 1.

The antiviral activities of compounds [**1**] and [**2g**–**l**] were evaluated in CEM cells against HIV-1 and HIV-2, data being recorded in Table 2. Data on the previous analogues {[**2a**; alanine], [**2b**; glycine], [**2c**; valine], [**2d**; leucine], [**2e**; isoleucine] and [**2f**: phenylalanine]} are included for comparison. The parent nucleoside analogue (**1**) was noted to be equiactive against HIV-1 and HIV-2. However, as previously reported, d4T was virtually inactive in a thymidine kinase-deficient cell line (CEM/TK^-) (McGuigan et al., 1996a; Girardet et al., 1995). This is attributed to the marked dependence of [**1**] on cytosolic thymidine kinase for activation. In contrast, the previously reported phenyl methoxyalaninyl derivative [**2a**] (McGuigan et al., 1996a) was noted to retain full activity in the TK^- cell line. It was $\approx$330 times more potent than d4T [**1**] in this assay.

Table 2
Anti-HIV and MSV activity and cytotoxicity of compounds [**1**], [**2a**–**2l**] and [**3**] in CEM cell cultures

Compound	Amino acid	EC_{50} (μM) CEM/O HIV-1	EC_{50} (μM) CEM/O HIV-2	EC_{50} (μM) CEM/TK$^-$ HIV-2	CC_{50} (μM) CEM/O	EC_{50} (μM) C3H/3T3 MSV	MIC (μM) C3H/3T3
1	—	0.36	0.27	25	≥100	1.62	>100
2a	Ala	0.085	0.102	0.075	>100	11.0	>100
2b	Gly	6	6	7	≥250	>100	>100
2c	Val	12.5	12.5	4	>250	94.9	>100
2d	Leu	1.1	2.23	0.4	≥250	53.0	>100
2e	Ile	5.0	12.5	2.5	>250	14.4	>100
2f	Phe	0.8	1.35	0.33	216	16.9	>100
2g	Trp	4	4	1.3	102	52.7	>100
2h	Met	0.6	0.8	0.34	≥250	56.3	>100
2i	Cys	0.95	2.0	0.33	240	≥100	>100
2j	Asp	0.55	0.65	0.33	209	31.4	>100
2k	Glu	8	5.33	1.6	≥250	88.8	>100
2l	Pro	>10	>10	>10	42.5	>100	>100
3	Me2Gly	0.29	0.5	0.06	225	22.6	>100

Data show the 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) (both in μM) for the nucleosides and nucleotides [**1**], [**2a**–**l**] and [**3**] for HIV-1 and HIV-2 in CEM and CEM/TK$^-$ cells and for MSV in C3H/3T3 cells. The MIC values represent the compound concentrations required to afford a microscopically visible alteration of morphology of the cell cultures. For full details see Section 2.

The glycine compound [**2b**] was far less active (60–70 fold) than [**2a**] in the parent cell line. This is roughly the same reduction in activity found for [**2a**] on replacing L-alanine by its D-isomer (McGuigan et al., 1996e). The simplest explanation of these data might be that the methyl group of L-alanine confers an advantage (50–100 fold) over the corresponding hydrogen atom of glycine or of D-alanine, whilst the methyl group of D-alanine confers neither advantage nor disadvantage as compared to the hydrogen of glycine. The presence of a stereospecific hydrophobic pocket at the L-amino acid sidechain site of the putative activating enzyme(s) of [**2a**–**b**] is one interpretation of these data.

With this in mind, we were interested to probe the effect of larger hydrophobic sidechains on the (L) amino acid; as in the valine analogue [**2c**]. However, it is clear that this compound is markedly less active (50–150 fold) than the alanine lead compound [**2a**]. This might indicate that the suggested hydrophobic pocket noted above may be of limited dimensions, being sufficient to accommodate a methyl group, but not an isopropyl group. Interestingly, the leucine analogue [**2d**] appeared to be five to ten-fold more active than the valine compound [**2c**], despite the larger side-chain of leucine, but the isoleucine analogue [**2e**] was again somewhat less active. These data indicate that spatial bulk of the amino acid sidechain alone is not the only determinant of the eventual activity of the compounds. Rather similar activity to [**2d**] was noted for the phenylalanine analogue [**2f**].

Moreover, the suggestion arose that a methylene unit adjacent to the chiral centre of the amino acid is required for potent antiviral action; the three most potent amino acids to date (alanine [**2a**], phenylalanine [**2f**] and leucine [**2d**]) all bear this feature.

Reported herein for the first time is the tryptophan analogue [**2g**], which retains the methylene unit in the amino acid, but has enhanced steric bulk in the side chain. It is notable (Table 2) that this analogue is somewhat less active than the lead compound [**2a**], but equiactive as the isoleucine compound [**2e**].

So far, only relatively simple aliphatic or aromatic amino acid side-chains had been studied. Since it was of interest to probe heteroatom substitution in the amino acid, we first prepared the methionine analogue [**2h**]. This compound was noted to be less active than the alanine analogue [**2a**]. However, being only 7–10 fold less active than [**2a**], the methionine analogue had an antiviral activity rather similar to the leucine [**2d**] or phenylalanine [**2f**] derivatives.

As for methionine, in the case of the cysteine compound [**2i**] it was necessary to block the thiol functionality, both to avoid spontaneous dimerisation and to facilitate the synthetic chemistry. Bearing in mind the apparent spatial constraints noted above we chose the smallest practical thiol protection, namely a methyl thioether. The protected cysteine compound [**2i**] was noted to be roughly equiactive as the methionine analogue [**2h**].

To probe the effect of oxygen-substituted amino acid side-chains we prepared the corresponding aspartic acid and glutamic acid analogues. Again, it was necessary to block the side-chains of these compounds [**2j**–**2k**]. Since the α-carboxylates were protected as methyl esters throughout this study, we considered this an appropriate protection for the extra carboxylates in these two analogues. The aspartic acid analogue [**2j**] gave an almost identical activity profile to the methionine compound [**2h**], whilst the glutamic acid derivative [**2k**] was $\sim$10-fold less active. Again, there may be some evidence here of decreasing activity with increasing bulk of the sidechain.

Finally, regarding natural amino acids it was of interest to study proline, since it is the only common natural amino acid with a blocked amino group. It is notable that [**2l**] was the least active of all the analogues studied, and also the most toxic. Indeed, [**2l**] displays no measurable antiviral selectivity, by comparison to a selectivity index of >1300 for the alanine lead structure [**2a**]. The reasons for the poor antiviral profile of [**2l**] are unclear, but may correspond to the absence of an amino acid NH for this analogue, or may be due to the extra rigidity enforced on this material by virtue of the proline ring.

Given the notable importance of the amino acid side-chain for antiviral action, we wondered if unnatural amino acids may substitute for their natural counterparts. We have previously (McGuigan et al., 1996e) noted that D-alanine is ≈20–30 fold less effective than L-alanine, in d4T phosphoramidates such as [**2a**–**l**]. However, we now suggest that this may simply correspond to the lack of the hydrophobic methyl group on the 'L'-face of the D-alanine unit, which we mentioned above to be important for antiviral action; comparing [**2a**] with [**2b**] for example. It may be that the poor activity of the D-series compounds is not related to their unusual stereochemical nature per se.

With this in mind, we prepared the novel dimethylglycine compound [**3**], using very similar chemistry to that outlined above. Compound [**3**] is an unnatural amino acid derivative containing two methyl groups on the α-carbon atom. Of major interest is the observation that the dimethylglycine analogue [**3**] is only ≈3-fold less potent than the lead alanine compound [**2a**]. Thus, the unnatural amino acid moiety of [**3**] is at least as potent as each of the (natural) amino acid units of [**2b**–**l**]. For the first time, this indicates that the phosphoramidate delivery method does not require a natural amino acid unit per se for potent antiviral action.

It is interesting to note that all of the analogues [**2a**–**2l**] and [**3**] appear to retain full activity in the thymidine kinase-deficient cell line CEM/TK$^-$. This is in very marked contrast to the situation with d4T [**1**], strongly indicating that, unlike [**1**], none of the phosphate analogues rely on thymidine kinase to express their eventual antiviral action. The simplest interpretation is that none of the compounds release free d4T [**1**] to a marked extent, but instead they all act as intracellular depots of the free monophosphate. Presumably, the considerable variation in potency for the different analogues may reflect the efficiency with which they liberate the monophosphate, but it is notable that they all appear to act by the same, thymidine kinase-independent, route. Particularly notable is the observation that the unnatural dimethylglycine system [**3**] is equiactive as the parent alanine compound [**2a**] in the thymidine kinase-deficient cell line.

The consistently higher antiviral activity of the d4T prodrugs in HlV-2-infected CEM/TK$^-$ versus CEM/O cell cultures may reflect lower endogenous dTTP levels in the CEM/TK$^-$ cells. However, it can also not be excluded that due to the lack of cytosolic TK activity in CEM/TK$^-$ cells, a lower competition may occur for the conversion of d4T-MP, released from the prodrug, to d4T-TP by intracellular anabolites of dThd. Alternatively, the CEM/TK$^-$ cells may contain slightly higher esterase activity than CEM/O cells resulting in a slightly more efficient release of d4T-MP from the prodrugs in the CEM/TK$^-$ cells than in the wild-type cells.

Also shown in Table 2, we observed inhibition of Moloney murine sarcoma virus (MSV), to varying degrees for each of the compounds [**2a**–**2l**] and [**3**]. Generally, all of the compounds were less active against MSV than HIV under the assay conditions used. However, the alanine compound [**2a**] was again the most potent. Furthermore, the most weakly active anti-HIV analogues (e.g. [**2b**], [**2c**], [**2l**]) were amongst the least active anti-MSV agents. However, the correlation is far from complete; for example, whilst the cysteine analogue [**2i**] is rather potent against HIV, it is virtually inactive against MSV. We have previously shown that murine C3H/3T3 cells (and also feline CFK cells) are rather inefficient in releasing d4T-TP from **2a** as compared to human HeLa or CD4+ T-lymphocytes (Balzarini et al., 1996b). Therefore, the intracellular release of d4T and d4T-MP from the d4T prodrug may markedly differ from one cell type to the other. These observations may explain why the aryloxyphosphoramidate d4T prodrugs are consistently less active against MSV in C3H/3T3 cells than against HIV in CEM cells. Clearly, the enzymes that eventually convert the d4T prodrug to d4T-MP are highly cell species-dependent and differ either quantitatively or qualitatively in murine and human cells. From our data, it is clear that the enzymes responsible for conversion of the d4T prodrug to d4T-MP in the murine cells must have a different amino acid substrate affinity spectrum than the enzymes from the human cells, since some compounds (i.e. **2c**, **2e** and **2k**) are only three to ten-fold less active in the C3H cells, whereas other compounds (i.e. **2a**, **2d**,

2h and **2j**) were more than 50- to 100-fold less active than d4T. The identification of the enzymes responsible for the conversion of the prodrugs to d4TMP are currently under investigation.

4. Conclusion

In conclusion, we found that phosphoramidate derivatives of d4T [**1**] are selective inhibitors of the replication of HIV-1 and HIV-2 in tissue culture. They retain full activity in thymidine kinase-deficient cells, strongly indicating a direct intracellular nucleotide release. The nature of the amino acid is very important for the eventual activity, the methoxyalaninyl analogue being the most potent studied. To some extent it appears that larger amino acid side-chains lead to a reduction in activity although this is far from absolute and indeed the (smaller) glycine analogue is rather poorly active. Finally, we note the surprising result that the unnatural dimethylglycine unit can replace alanine with little or no loss of activity. Our data strongly suggest that a natural amino acid is not required for potent antiviral action of these phosphoramidate compounds. Whether other amino acids (natural or unnatural) might be more effective than alanine, and the molecular basis of this marked selectivity are subjects of active investigation in our laboratories.

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