Phosphoramidate derivatives of stavudine as inhibitors of HIV: unnatural amino acids may substitute for alanine

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Some novel phosphoramidate derivatives of the nucleoside analogue stavudine have been prepared as membrane-soluble prodrugs of the bioactive free phosphate forms. Phenyl phosphates linked via nitrogen to methyl esterified amino acid analogues were studied, where the amino acid was an unnatural α-alkyl (or aryl) glycine or an α,α-dialkyl glycine. All compounds were characterized by a range of spectroscopic, spectrometric and analytical methods and were subjected to in vitro evaluation of their anti-human immunodeficiency virus efficacy. It is notable that certain unnatural amino acid derivatives could substitute for alanine with only a relatively small loss of activity and, moreover, that this activity did not fall-off with increasing alkyl chain length for the C2-C4 mono-alkyl series. These data are further probed by the application of our recently reported 31P-NMR-based carboxyl esterase assay, with informative results.

Keywords: HIV; nucleoside; prodrug; phosphoramidate; stavudine.

Introduction

As recently surveyed in a review by Meier (1998), a number of approaches are now emerging to the problem of the delivery of bioactive charged nucleotides into living cells. We have developed aryl phosphoramidates as an effective nucleotide delivery motif and have reported the application of this technology to potent anti-HIV drugs such as zidovudine (McGuigan et al., 1993) and stavudine (McGuigan et al., 1996a; Balzarini et al., 1996), to poorly active anti-HIV agents such as d4A (McGuigan et al., 1996b; Balzarini et al., 1997) and ddU (McGuigan et al., 1994) and to anti-herpetic agents such as netivudine (5-propynylaraU) (McGuigan et al., 1998a). Extensive structure–activity relationship (SAR) studies have revealed a clear preference for L-amino acids (McGuigan et al., 1996c) and for alanine as the preferred amino acid (McGuigan et al., 1997). In contrast, aryl substitution in general has a much lower impact on activity (McGuigan et al., 1996a; Siddiqui et al., 1999) as does carboxyl ester variation (McGuigan et al., 1998b), within certain limits. Thus, the amino acid moiety had emerged as the key determinant of intracellular phosphate delivery. With this in mind we sought to probe the application of further, unnatural amino acid analogues. We have previously noted just one such example; the achiral α-methylalanine (dimethylglycine) moiety was able to substitute for L-alanine with no loss of activity (McGuigan et al., 1997). In this manuscript we describe further novel derivatives, each of which carries an unnatural amino acid moiety, being an α-alkyl (or aryl) glycine or an α,α-sym-alkylglycine. We find that a number of these unnatural groups may substitute for alanine while retaining potent antiviral activity and selectivity. We were particularly interested to probe the application of these unnatural amino acids to the phosphoramidate-based 'protide' approach, with a view to their impact on the efficacy of nucleotide release in vitro.

Materials and Methods

General methods

All experiments involving water-sensitive compounds were conducted under dry conditions. Dichloromethane was dried by heating under reflux over calcium hydride, followed by distillation. Tetrahydrofuran was dried on sodium-benzophenone and distillation. TLC was performed using Alugram SIL G/UV254 aluminium-backed silica gel plates. Visualization of the plates was achieved using an ultrasound lamp. Chromatography refers to flash column chromatography and was carried out using Merck silica gel 60 (40–60 mM) as stationary phase.
1H, 31P and 13C NMR spectra were recorded on a Bruker Avance DPX300 spectrometer. 1H and 13C chemical shifts are quoted in p.p.m. downfield from TMS. J values refer to coupling constants and signal splitting patterns are described as singlet (s), broad singlet (bs), doublet (d), triplet (t), quadruplet (q), multiplet (m). 31P chemical shifts are quoted in p.p.m. relative to an external phosphoric acid standard. HPLC was conducted on an ACS quaternary system using ODS5 column and an eluent of water:acetonitrile (82% water during 10 min, then a linear gradient to 20% water at 30 min), with a flow rate of 1.2 ml/min and detection by UV at 265 nm.

Mass spectra were recorded in electrospray FAB mode on a Fisons VG Platform using an HPLC pump and an eluent of acetonitrile:water 1:1. All chiral amino acid analogues were of the L-configuration.

General method for phosphoramidate synthesis (1b–e)
Phenyl methoxalaninylphosphochloridate (5.34 mmol, 2 equiv., 6 ml of a solution 1 mmol/ml in THF) was added to a stirring solution of stavudine (2.67 mmol, 600 mg) and N-methylimidazole (10.7 mmol, 4 equiv., 0.85 ml) in 12 ml of dry THF. The reaction mixture was stirred for 6 h 30 min. The reaction was monitored by TLC (eluent chloroform:ethanol 98:2). Evaporation of solvents gave a white solid.

The residue was purified by column chromatography on silica gel with eluent chloroform:ethanol 98:2. The solvent was removed under reduced pressure. The product was evaluated as this mixture was not separable by chromatography on column or chromatotron and the product was evaluated as this mixture was not separable by chromatography on column or chromatotron.

Yield 62%. 31P NMR δ 4.20, 4.84. 1H NMR δ 0.83–0.90 (m, 3H, CH2CH3); 1.63–1.81 (m, 2H, CH2CH3); 1.85 and 1.89 (s, 3H, 5CH3); 3.72 and 3.73 (s, 3H, OCH3); 3.79–3.97 (m, 2H, CH, NH); 4.29–4.46 (m, 2H, H5); 5.03–5.06 (m, 1H, H4′); 5.89–5.94 (m, 1H, H2′); 6.17–6.40 (m, 1H, H3′); 7.04–7.08 (m, 1H, H1′); 7.17–7.66 (m, 6H, Ph, H6); 9.30 (bs, 1H, NH). 13C NMR δ 9.6 (CH2CH3); 12.7–12.8 (SCH2); 27.9–28.0 (CH2CH3); 52.8 (OCH3); 55.9–56.0 (NHCH); 66.9, 67.5, 67.6 (C5′); 84.9, 85.0, 85.1 (C4′); 90.0–90.2 (C1′); 111.7–111.8 (C5′); 120.4, 120.5, 120.6 (Ar ortho); 125.5–125.6 (Ar para); 127.8–127.9 (C2′); 130.1–130.2 (Ar meta); 133.5–133.7 (C3′); 136.0–136.3 (C6′); 150.7–150.8 (Ar ipso); 151.3 (C2); 164.3 (C4); 173.7–173.9 (COO). MS (FAB/NOBA) 480 (MH+, 30%), 502 (MNa+, 100%), 518 (MK+, 40%).

2,3′-Dideoxy-2′,3′-didehydrothymidine-5′- (phenylmethoxy-L-norvalinyl) phosphate (1c)
This required an extra purification on chromatotron (2% MeOH in CH2Cl2), after the initial flash column, to achieve homogeneity. Yield 44%. 31P NMR δ 4.13, 4.79. 1H NMR δ 0.88 (q, 3H, J=7.4 Hz, CH2CH3); 1.07–1.43 (m, 2H, CH2CH3); 1.46–1.77 (m, 2H, CH2CH3); 1.86, 1.90 (s, 3H, SICH3); 3.72 (s, 3H, OCH3); 3.76–3.86 (m, 1H, CHNH); 3.91–4.01 (m, 1H, NHICH); 4.25–4.46 (m, 2H, H5′); 5.03–5.06 (m, 1H, H4′); 5.89–5.94 (m, 1H, H2′); 6.17–6.39 (m, 1H, H3′); 7.05–7.07 (m, 1H, H1′); 7.17–7.36 (m, 6H, Ph, H6); 9.22 (d, 1H, J=9.21 Hz, NH). 13C NMR δ: 12.7–12.8 (SCH3); 13.9 (CH3CH2); 18.5–18.6 (CH2CH3); 32.8 (OCH3); 54.6–54.8 (NHCH); 66.9, 67.5, 67.6 (C5′); 84.9, 85.0, 85.1 (C4′); 89.9–90.2 (C1′); 111.7–111.8 (C5′); 120.4–120.6 (Ar ortho); 125.5–125.6 (Ar para); 127.8–127.9 (C2′); 130.1–130.2 (Ar meta); 133.5–133.7 (C3′); 136.0–136.3 (C6′); 150.7, 150.8, 150.9 (Ar ipso); 151.3 (C2); 164.2–164.3 (C4′); 174.0–174.2 (COO). MS (FAB/NOBA) 494 (MH+, 50%), 516 (MNa+, 100%), 532 (MK+, 65%). HPLC retention time, 24.18 min.

2′,3′-Dideoxy-2′,3′-didehydrothymidine-5′- (phenylmethoxy-L-norleucinyl) phosphate (1b)
Yield 75%. 31P NMR δ 4.05, 4.75. 1H NMR δ 0.84–0.89 (m, 3H, CH2CH3); 1.13–1.30 (m, 4H, CH2CH2CH3); 1.55–1.79 (m, 2H, CHICH3); 1.80, 1.91 (s, 3H, 5CH3); 3.69–3.77 (m, 1H, NHICH); 3.73, 3.74 (s, 3H, OCH3); 3.96–4.01 (m, 1H, NHICH); 4.30–4.47 (m, 2H, H5′); 5.04–5.08 (m, 1H, H4′); 5.91–5.95 (m, 1H, H2′); 6.30–6.41 (m, 1H, H3′); 7.04–7.18 (m, 1H, H1′); 7.21–7.40 (m, 6H, Ph, H6); 8.93 (bs, 1H, NH). 13C NMR δ 12.7–12.8 (SCH3); 14.2 (CH3CH2); 22.5–22.6 (CH2CH3); 27.3–27.4 (CH2CH3); 34.4, 34.5, 34.6 (CH2CH3); 52.8 (OCH3); 54.8–54.9 (CHICH3); 66.8, 66.9, 67.5, 67.6 (C5′); 84.9, 85.0, 85.1 (C4′); 89.9–90.2 (C1′); 111.7–111.8 (C5′); 120.3, 120.4, 120.6, 121.0 (Ar ortho); 125.5–125.6 (Ar para); 127.8–127.9 (C2′); 130.1–130.2 (Ar meta); 133.4–133.7 (C3′); 136.0–136.3 (C6′); 150.7, 150.8, 150.9 (Ar ipso); 151.4 (C2); 164.2–164.4 (C4′); 174.0, 174.1, 174.2, 174.3 (COO). MS (FAB/NOBA) 508 (MH+, 55%), 530 (MNa+, 100%).

2′,3′-Dideoxy-2′,3′-didehydrothymidine-5′- (phenylmethoxy-L-2-aminobutylbutyryl) phosphate (1d)
Uniquely for this compound the standard procedure gave some degree (approximately 15%) of racemisation at the amino acid chiral centre. The minor (racemised) by-product was not separable by chromatography on column or chromatotron and the product was evaluated as this mixture. The extra peaks are noted below for 31P NMR and
were also observed for $^{13}$C and $^1$H NMR. Yield 72% (including around 16% racemized).

$^{31}$P NMR δ 3.34, 3.96 (with 4.29, 4.61 due to racemization, ca. 15% intensity). $^1$H NMR δ 1.83, 1.84 (s, 3H, 5CH$_3$); 3.68, 3.69 (s, 3H, OCH$_3$); 4.15–4.40 (m, 2H, H$_5$); 4.60–4.85 (m, 1H, CHNH); 4.85–5.00 (m, 1H, CHNH); 4.90–5.10 (m, 1H, H$_4$'); 5.83–5.85 (m, 1H, H$_2$'); 6.16–6.31 (m, 1H, H$_3$); 6.96–7.40 (m, 12H, H$_1$; CHPH, Ph, H$_6$); 9.60 (bs, 1H, NH). $^{13}$C NMR δ 12.3–12.4 (5CH$_3$); 53.1 (OCH$_3$); 58.1 (NHCH$_3$); 66.5–67.2 (C$_5$'); 84.5–84.6 (C$_4$'); 89.6–89.7 (C$_1$); 111.3–111.4 (C$_5$); 120.0, 120.1, 120.2 (Ar ortho); 125.2 (Ar para); 127.4–127.6 (C$_2$); 127.2, 128.6, 129.9, 130.0, 137.5–137.6 (Ph); 129.7–129.8 (Ar meta); 132.9–133.3 (C$_3$'); 135.7–135.9 (C$_6$); 150.2–150.3 (Ar ipso); 151.6–151.1 (C$_2$); 164.1 (C$_4$); 171.8–171.9 (COO). MS (FAB/NOBA) 530 (MH$^+$, 55%), 550 (MNa$^+$, 100%).

2',3'-Dideoxy-2',3'-dideoxythymidine-5'-
(phenylethoxy-$\alpha$,-$\alpha$-diethylglycine) phosphate (2b)

This was prepared using an alternative synthetic route involving mesitylene nitrotriazolide (MSNT) (0.2 g, 0.66 mmol, 2.5 equiv) coupling of stavudine-5'-phenyl phosphate (0.26 mmol) with $\alpha$,-$\alpha$-diethylglycine methyl ester (0.5 mmol) in dry pyridine (5.5 ml). The reaction mixture was stirred for 45 min at ambient temperature and then for 17 h at reflux. The solvent was then removed under reduced pressure and purified by flash column chromatography on silica eluted by 36% MeOH in CHC$_3$ and then by chromatotroon with an eluent of 36% MeOH in CHC$_3$$_2$ yield 79%.

$^{31}$P NMR δ 2.03, 1.26. δ 1.05–0.85 (m, 6H, Me$_2$); 1.60–2.20 (m, 4H, CH$_2$); 1.77 (2s, 3H, 5-Me$_3$); 3.61 (s, 3H, OMe$_3$); 3.95 (bd, 1H, NH$_1$); 4.15 (m, 2H, H$_5$'); 4.90 (m, 2H, H$_4$'); 5.83 (m, 1H, H$_3$); 6.16 (m, 1H, H$_1$'); 7.87 (m, 1H, H$_7$); 7.90–7.93 (m, 6H, Ph, H$_6$); 8.20 (m, 1H, NH). $^{13}$C NMR δ 14.21 (CH$_3$-$CH_2$), 16.88 (5-Me), 22.36 (CH$_3$-$CH_2$), 34.26 (C-$CH_3$), 51.29 (OMe$_3$), 65.89 (C$_5$'), 84.26 (C$_4$'), 88.56 (C$_3$'), 110.75 (C$_5$), 119.61 (meta-Ph), 124.63 (C$_2$'), 126.26 (para-Ph), 128.39 (ortho-Ph), 133.86 (C$_6$), 135.18 (C$_3$'), 148.99 (ipso-Ph), 150.27 (C$_2$'), 163.02 (C$_4$), 172.56 (COO). MS (FAB NOBA): 508 (M$^+$, 72%), 530 (MNa$^+$, 100%).

2',3'-Dideoxy-2',3'-dideoxythymidine-5'-
(phenylethoxy-$\alpha$,-$\alpha$-diisopropylglycine) phosphate (2c)

Repeaded flash column chromatography and chromatotroon purification were needed to achieve compound homogeneity, with consequential and significant reductions in yield. Yield 2%. $^{31}$P NMR δ 1.44, 2.12. $^1$H NMR δ 0.70–1.20 (m, 11H, 4×CH$_3$, CH$_2$); 1.35 (t, 3H, O-CH$_2$CH$_2$), 1.50–2.10 (m, 4H, 2×CH$_2$); 1.85 (s, 3H, 5-Me); 3.25 (m, 3H, OCH$_3$, H$_4$'); 4.35 (m, 2H, H$_5$'); 5.10 (m, 1H, NH); 5.95 (m, 1H, H$_2$'); 6.30 (m, 1H, H$_3$'); 7.05 (m, 1H, H$_1$'); 7.20–7.50 (m, 6H, Ph, H$_6$); 8.20 (m, 1H, NH). MS (FAB NOBA): 572 (MNa$^+$, 100%).

Carboxyl esterase assay procedure
Typically 9 μmol of test compound was dissolved in a mixture of acetone (0.1 ml) and 0.5 M TRIZMA buffer pH 7.6 (1 ml made up in D$_2$O) and exposed to 10 mg of pig liver esterase (Sigma; activity 15 units/mg). The mixture was maintained at 37°C and evaluated directly by $^{31}$P NMR at intervals (1–48 h). Where possible, reactions were followed to completion by NMR; however, kinetic data were confined to the early portion of the reaction and were collected in all cases by 24 h.

Results
Following synthetic methods that were reported previously (McGuigan et al., 1993, 1996b, 1997) we prepared novel aryl phosphorochloridates by the reaction of phenyl dichlorophosphoryl with the appropriate $\alpha$-$\alpha$-alkyl glycines (ethyl, propyl and butyl) or $\alpha$-phenylglycine as appropriate. These phosphorochloridates were allowed to react with stavudine in THF containing N-methylimidazole to give target phosphoramidates (1b-1e) in moderate yield (Figure 1). As previously noted for natural amino acid-derived compounds, these materials each displayed two closely spaced $^{31}$P NMR signals, corresponding to two diastereomers resulting from mixed phosphate stereochemistry. Indeed, the absence of further splitting in any of the $^{31}$P

![Figure 1. Structures of some antiviral nucleotides](image-url)
Table 1. Anti-HIV activity and cytotoxicity of nucleoside and nucleotide analogues

<table>
<thead>
<tr>
<th>Structure</th>
<th>EC50 HIV-1 CEM (µM)</th>
<th>EC50 HIV-2 CEM (µM)</th>
<th>EC50 HIV-2 CEM/TK (µM)</th>
<th>CC50 CEM (µM)</th>
</tr>
</thead>
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<tr>
<td>1a</td>
<td>0.075</td>
<td>0.075</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>1b</td>
<td>0.6</td>
<td>0.8</td>
<td>0.47</td>
<td>≥250</td>
</tr>
<tr>
<td>1c</td>
<td>1.1</td>
<td>0.4</td>
<td>≥250</td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>0.50</td>
<td>0.69</td>
<td>0.22</td>
<td>168</td>
</tr>
<tr>
<td>1e</td>
<td>15.3</td>
<td>15.3</td>
<td>4.5</td>
<td>≥250</td>
</tr>
<tr>
<td>1f</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>≥250</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.6</td>
<td>225</td>
</tr>
<tr>
<td>2b</td>
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<td>28.3</td>
<td>50</td>
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</tr>
<tr>
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<td>50</td>
<td>&gt;50</td>
<td>98.6</td>
</tr>
<tr>
<td>Stavudine</td>
<td>0.65</td>
<td>0.77</td>
<td>33</td>
<td>174</td>
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</table>

spectra for 1b–1d indicated no racemisation at the amino acid chiral centre in these cases and the retention of the ‘natural’ L-stereochemistry. In contrast, compound 1e displayed a second, minor pair of signals in the 31P NMR corresponding to a small degree (<15%) of racemisation. This was presumably catalysed by the basic conditions of both the phosphorochloridate preparation and the final preparation of 1e and arose uniquely in the case of this analogue as a result of the significantly enhanced asymmetric C-H acidity. Such racemisation had previously been noted by us for any of a range of natural amino acids (McGuigan et al., 1997) or elsewhere in this study. Compounds 1b–1e were further characterized by 1H and 13C NMR and again, 1b–1d were confirmed to consist of two (phosphate) diastereomers, whilst 1e was noted to additionally contain the minor (carbon) epimer.

We were particularly interested in the possibility of substituting achiral α,α-sym-dialkylglycines for (chiral) alanine in the phosphoramidate approach, with a view to removing any possibility of racemisation at the amino acid centre in vivo. Although replacement of alanine by glycine achieves this, we have previously noted (McGuigan et al., 1997) a very significant reduction in potency on this substitution; we hoped that the α,α-sym-dialkyl systems would retain the potency of the parent L-alanine analogue. Indeed, given our previous experience, that α,α-dimethylglycine could substitute for alanine in the phenyl phosphoramidate prodrg of stavudine with no loss of activity (McGuigan et al., 1997), we were interested to pursue further, novel α,α-sym-dialkyl systems.

The α,α-dimethyl glycine system (2a) was previously prepared using our established phosphorochloridate method as described above and we initially embarked on the dipropyl analogue (2c). However, the reaction proceeded rather poorly in this case and extensive chromatographic purification was required. As a result, the isolated yield of pure 2c was only 2%, the lowest yield ever recorded by us for any phosphoramidate. However, spectroscopic data fully supported the structure and purity of this material.

Given the poor yield of 2c, an alternative synthetic route was pursued for the ethyl analogue (2b). Thus, stavudine 5′-monophosphoryl phosphate was prepared by established methods (Jastorff & Hettler, 1969) and coupled to α,α-dimethylglycine methyl ester using MSNT. This reaction proceeded more cleanly than the phosphorochloridate-based method for 2b above and the yield was enhanced.

The nucleotides (1a–e and 2a–c) were evaluated for their ability to inhibit the replication of HIV-1 and HIV-2 in CEM cells (Table 1). Data for the parent nucleoside analogue, stavudine, and data for the previously prepared glycine compounds (1f) are included for comparison.

Discussion

It is evident from the antiviral data shown in Table 1 for the mono-alkyl series (1a–e), that extension of the side-chain beyond the methyl group of natural L-alanine (1a) leads to a reduction in antiviral potency of approximately 10-fold. It is interesting to note that this reduced antiviral potency is constant for alkyl chains from ethyl to n-butyl, showing no further reduction in activity as the chain is lengthened within this range. It could be reasoned that the ethyl side chain of 1b for example, might have made this homologue more closely resemble the methyl group of the alanine compound (1a) and that activity may have decreased gradually through the homologous series. The observation that this is not the case, at least through the series C2–C4, may indicate that still longer chains may be acceptable in this region. However, the phenyl substituted system 1e was poorly active; being approximately 200-fold less potent than 1a and approximately 25-fold less potent than 1b–d. The reason for this decrease in potency may be connected with either the steric bulk of the phenyl substituent or its electronic nature. However, it is instructive to compare the activity of 1e to that of the previously reported valine analogue (side-chain: isopropyl) (McGuigan et al., 1997), which displayed an almost identical lack of antiviral activity. This would argue for steric factors being most important in determining the (poor) antiviral potency of 1e and supporting our earlier conclusion (McGuigan et al., 1997) that α-branching of the amino acid side-chain is detrimental to activity. Indeed, this conclusion is further supported by comparison of data for 1e to that of the previously noted phenylalanine analogue (side chain PhCH2) (McGuigan et al., 1997). The latter compound was around 10-times less active than the alanine compound 1a and is thus 20-fold more potent than the phenyl glycine compound 1e.

Finally, for the mono-alkyl systems (1a–e) it is demonstrated that full activity is retained in the thymidine kinase-deficient cell line CEM/TK. This was also observed for the alanine analogue 1a, but is in contrast with parent
stavudine which lost its potency in the CEM/TK– assay. Thus, within error, each of the analogues 1a–e retained full activity in the absence of thymidine kinase. This supports the view that the primary mechanism of action of these compounds is TK– independent, consistent with the suggestion that these compounds act via intracellular delivery of the 5′-monophosphate, stavudine-MP. Therefore, any variations in potency down the series 1a–e may simply reflect variations in the efficiency of cellular penetration and intracellular hydrolysis of the prodrug to the free monophosphate. Assuming membrane permeation for these (unnatural) blocked nucleotides is principally via passive diffusion and thus lipophilicity driven, it might be likely that membrane permeation may increase down the series 1a–c. This being the case, the antiviral data would tend to suggest a reduced prodrug hydrolysis for 1b–d, and particularly for 1e. Since the precise hydrolysis mechanism is multi-step and remains to be fully elucidated, it is not clear which step the largest alkyl (aryl) substituents are impedesting.

In an attempt to identify the rate-determining step in the hydrolysis mechanism of the novel alkyl compounds 1b–d, we applied our recently described carboxyl esterase assay (McGuigan et al., 1998c). Compounds 1b–d were exposed to carboxyl esterase from porcine liver and the generation of their corresponding hydrolysed intermediates 3b–d was followed by 31P NMR spectroscopy at regular intervals. Our previously described alanine (1a) and glycine (1f) analogues were also included in the assay for comparison. The hydrolysis of glycine analogue (1f) was the most rapid, with a half-life of 16 h and produced 63% of 3f after 24 h. The alanine analogue (1a) was processed around 18-times more slowly, with only 5.4% of 3a generated after 24 h. It is notable that this efficient esterase-mediated hydrolysis of 1f over 1a is in contrast to the considerably higher antiviral potency of the latter compound (McGuigan et al., 1997). This indicates that the predicted efficiency of esterase-mediated conversion of parent phosphoramidates to the appropriate charged intermediates such as 3a–f is only one factor in determining antiviral potency. Other factors, notably the efficient onward hydrolysis of compounds 3a–f to free nucleoside 5′-monophosphate, may be equally important in this regard. Indeed, these data might indicate that 3f may be very poorly processed in vitro to stavudine-MP, in comparison to 3a.

Concerning the rate of esterase-mediated hydrolysis, the novel compounds 1b–d were processed most slowly of all. In each case, approximately 1.7–2.3% of the corresponding hydrolysis product (3b–d) was noted after 24 h. In this case, the data seem to correlate with the in vitro biological activity indicating that the presence of an alkyl group in the amino acid moiety produces an obvious decrease in the rate of enzymatic hydrolysis that could ultimately affect the delivery of the 5′-monophosphate, stavudine-MP. Whether compounds 3b–d are also poorly processed to the monophosphate remains unclear. However, the relatively comparable antiviral potency of 1b–d with 1a might appear to indicate otherwise.

We have previously noted that the α,α-dimethylglycine compound 2a is a potent antiviral agent, thus representing the first successful phosphoramidate prodrug with an unnatural amino acid moiety (McGuigan et al., 1997). It was therefore of interest, given that unnatural chain-extended mono-alkyl systems are rather potent antiviral agents, to similarly extend the alkyl chains of the dialkyl analogues.

As shown in Table 1, compounds 2a–c were evaluated against HIV-1 and HIV-2 in CEM cells and against HIV-2 in a CEM/TK– assay. In marked contrast to the mono-alkyl system however, the high potency of the parent (di)methyl system (2a) was not retained as the alkyl chains were extended in this series (2b–c). Indeed, activity was virtually lost for the diethyl- and dipropyl homologues, which were approximately 100-fold less potent than 2a. These higher homologues were somewhat more cytotoxic than 2a and any apparent antiviral activity may simply be due to cytotoxicity for 2b–c. As discussed above for the mono-alkyl series, the reduced potency of 2b–c is likely to reflect impeded nucleotide hydrolysis of these compounds; the exact nature of this impediment remains the subject of ongoing studies in our laboratories.

In conclusion, we have noted that chain extension of the alkyl side chain of alanine leads to an approximately 10-fold reduction in antiviral potency of phosphoramidate prodrugs of stavudine. Activity did not decrease further with chain extension in the range C2–C4 and in each case potency was retained in TK– cells. These data further support the view that a natural amino acid side chain is not necessary for potent antiviral activity of these compounds. However, side chain substitution with a phenyl group or chain elongation of α,ε- dialkyl systems does lead to a significant reduction in antiviral potency.

The combined antiviral profiles of 1a–d and 2a–e may indicate that asymmetric dialkyl systems with a methyl group on the 'D-face' and a longer, n-alkyl group on the 'L-face' may display interesting biological activities.

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