ELSEVIER

Antiviral Research 24 (1994) 69–77

Antiviral Research

Synthesis and anti-HIV activity of some novel diaryl phosphate derivatives of AZT

Christopher McGuigan[a,*], Michael Davies[a], Ranjith Pathirana[a], Naheed Mahmood[b], Alan J. Hay[c]

[a]*Department of Chemistry, University of Southampton, Highfield, Southampton, SO9 5NH, UK*
[b]*Medical Research Council Collaborative Centre, 1–3 Burtonhole Lane, Mill Hill, London, NW7 1AD, UK*
[c]*National Institute for Medical Research, The Ridgeway, London, NW7 1AA, UK*

(Received 20 January 1994; accepted 21 February 1994)

Abstract

Novel diaryl phosphate triester derivatives of the anti-HIV nucleoside analogue AZT have been prepared by phosphorochloridate chemistry. These materials were designed to act as membrane-soluble pro-drugs of the bio-active free nucleotides. In particular, novel para-substituted diaryl phosphate derivatives were prepared. In vitro evaluation revealed the compounds to have a pronounced and selective antiviral effect, the magnitude of which varied considerably with the nature of the aryl substituent. In particular, strongly electron-withdrawing aryl substituents correlate with high anti-HIV potency in C8166 cells. Along with AZT, the compounds are poorly effective in JM cells, which appear to lack thymidine kinase, indicating the phosphates to act as pro-drugs of the nucleoside rather than of the free phosphate.

Key words: HIV; AZT; Nucleotide; Pro-drug

1. Introduction

In an attempt to improve on the therapeutic potential of current anti-HIV agents such as AZT (**1**), there have been reports on a variety of masked phosphate derivatives, designed to act as membrane-soluble pro-drugs of the bio-active phosphate forms (Farrow et al., 1990; Freed et al., 1989; Henin 1991; McGuigan et al., 1990a;

*Corresponding author. Fax: +44 0703 593781.

SSDI 0166-3542(94)00023-2

McGuigan et al., 1990b; McGuigan et al., 1991; and McGuigan et al., 1992). We have noted that aryloxy phosphoramidates, such as compound (**2**) are particularly promising derivatives, since, unlike the parent nucleoside (**1**), they appear to retain a potent anti-viral effect in cells deficient in thymidine kinase. They may thus represent true intracellular phosphate delivery forms (McGuigan et al., 1992; McGuigan et al., 1993). Symmetrical diaryl phosphate triesters of AZT are also potent and selective inhibitors of the replication of HIV in vitro. The bis(para-nitrophenyl) phosphate triester of AZT (**3a**) was previously reported by us to be especially active (McGuigan et al., 1992). Recently, we have noted the ortho- and meta-analogues (**4**, **5**) to be very potent and non-toxic inhibitors of HIV (McGuigan et al., 1994). These compounds were prepared on the premise that the nitrophenyl groups would act as bio-reversible blocking groups for the charge on the free phosphate, thus enabling intracellular delivery of free AZT-5′-monophosphate. Indeed, the bis(nitrophenyl)phosphate triesters of AZT have comparable activity to the parent nucleoside in the in vitro whole cell assay in C8166 cells and in CEM cells. However, in JM cells and in CEM/TK$^-$, lacking the ability to express thymidine kinase, these compounds displayed similarly poor activity to the parent nucleoside; in contrast to the enhanced activity of aryloxy phosphoramidates, such as (**2**) (McGuigan et al., 1992; McGuigan et al., 1993). Thus it is apparent that, whereas the phosphoramidates are able to act as nucleotide delivery forms, the diaryl phosphates are only able

Table 1
Carbon-13 and phosphorus-31-NMR data for (**3a–f**) recorded in $CDCl_3$

		3a	3b	3c	3d	3e	3f
Base	C2	163.9	164.0	163.9	163.9	163.9	164.0
	C4	150.3	150.4	150.5	150.4	150.4	150.6
	C5	111.7	111.5	111.7	111.8	111.7	111.6
	C6	136.4	136.2	136.2	135.8	135.5	135.2
	Me	12.4	12.4	12.5	12.5	12.5	12.4
Sugar	C1′	86.8	86.2	85.1	86.0	85.5	84.8
	C2′	36.9	36.9	37.4	37.3	37.4	37.3
	C3′	60.1	60.0	60.0	60.1	60.0	60.0
	C4′	82.0[7]	81.8[8]	82.0[7]	82.0	82.0	82.0
	C5′	68.7[6]	68.4[6]	66.0[7]	68.2	67.9[6]	67.4
OAr	ipso	154.4[6]	153.1	147.8[8]	152.5[6]	150.0[7]	157.2
	ortho	126.0[11]	121.1[3]	120.5[5]	120.5	122.2[4]	120.9[5]
	meta	120.8[4]	134.4[6]	128.2[9]	128.3	139.1[5]	114.8[7]
	para	145.5	110.0	135.3	127.6	89.9	143.7[7]
substituent		–	117.8	16.4	123.7[a]	–	55.6
δ_P		−13.8	−13.0	−12.7	−13.3	−12.4	−11.0

Data are presented as δ in ppm, with superscripted phosphorus coupling constants in Hz where noted.
Notes: a: q, $J_F = 273$.

to act as pro-drugs of the free nucleoside. Further evidence for this was gained from a study of the behaviour of AZT bis(para-nitropheny)phosphate (**3a**) in human serum. AZT para-nitrophenyl phosphate diester was the initial major product, after 1 day, followed by the slow appearance of AZT (**1**); the free AZT-5′-phosphate was not detectable at any stage (McGuigan et al., 1994).

As a follow up to the AZT-bis-nitrophenyl phosphate triesters, further para-substituted diaryl phosphate triesters of AZT were prepared, in the hope that these would retain the potent activity of the nitrophenyl series, but would also display activity in kinase-deficient cells.

2. Results

As there was little variation in anti-viral activity observed between the ortho-, meta- and para-nitrophenyl analogues (EC_{50}: 0.005, 0.004, and 0.003 μM, respectively; see McGuigan et al., 1994), it was decided that the present series would consist only of para-substituted analogues and the para substituent would be varied considerably, in terms of electronic and steric properties, in order to probe the existence of a Quantitative Structure Activity Relationship (Topliss, 1972). The target compounds (**3a–f**) were prepared by methods previously noted for the diphenyl phosphate (**3g**) (McGuigan et al., 1992). Firstly, the appropriate diaryl phosphorochloridate was prepared by reaction of 2 equivalents of the phenol with phosphoryl chloride in the presence of triethylamine. The crude products were judged to be sufficiently pure by ^{31}P-NMR (δ_P approximately -5 ppm, see Mark et al., 1969) to be used without further purification. These were allowed to react with AZT (**1**) in anhydrous pyridine/tetrahydrofuran. Isolation and purification by column chromatography gave the target compounds (**3a–f**) in good yield (74–92%). These materials were fully characterised by ^{3}P- and ^{13}C-NMR (Table 1), proton-NMR, mass spectrometry and HPLC. All data were fully consistent with the structure and purity of the target compounds. It is important to note the absence of contaminating AZT (**1**) from the phosphate esters, as judged by HPLC, given the activity of AZT in the biological assay system.

3. Discussion

The target compounds (**3b–f**) were tested for the inhibition of HIV1 replication in vitro using procedures which have been described (Betbeder et al., 1990; Mahmood and Hay, 1992). AZT (**1**) and the previously reported diphenyl- and bis(para-nitrophenyl)phosphates (**3g,3a**) were also included in the assay. As shown in Table 2, each of the compounds displayed potent and selective inhibition of viral replication. Some of the compounds (**3a**, **3b**) appear to be 2–3-fold more active than the parent nucleoside. Although such a small difference may not be significant, it is notable that this distinction was seen throughout the repeated assays conducted with these lead samples.

The antiviral effect of the diaryl phosphates varied over three orders of magnitude, depending on the nature of the aryl substituent. It is notable that the activity appears to vary inversely with respect to the pK_a of the appropriate phenol, there being an apparent relationship between the logarithm of the anti-viral potency and the pKa of the phenol (Table 3, Graph 1). To some extent this might be explained by the suggestion that phenol acidity may correlate with leaving group ability, and thus to P-O-aryl lability, and hence to release of AZT phosphate(s) and biological activity. It is certainly notable that the most active compounds in the series (**3a** and **3b**) were also the most difficult to isolate and purify on account of their chemical lability.

As noted above, despite being extremely potent in C8166 T-cells, the bis(para-nitrophenyl) compound (**3a**) is poorly active in JM cells (McGuigan et al., 1992), which appear to lack the ability to phosphorylate AZT (Roberts et al., 1990). This indicates that (**3a**) acts by nucleoside release, rather than as a nucleotide pro-drug. It was of interest to examine the effectiveness of the alternative diaryl phosphates in the kinase-deficient cell line, and data for this assay are recorded in Table 2. It is notable that, as with AZT, all of the diaryl phosphates (**3a–g**) are less potent in JM cells than in C8166 cells, indicating that they all, to some extent, function as AZT pro-drugs. The degree of reduction in activity varies very considerably with the nature of the aryl phosphate substituent, with some of the less active compounds retaining activity in JM cells, and all compounds being more active than AZT in JM cells.

Although the poor activity of all of the diaryl phosphates in thymidine-kinase deficient cells strongly indicates that they do not act as intracellular phosphate delivery forms, their potential to act as depot forms of the free nucleoside may confer some advantage in vivo; this possibility is under current investigation in our laboratory.

4. Experimental methods

All reactions were performed under an atmosphere of nitrogen, unless stated

Table 2
Anti-HIV-1 activity and cytotoxicity for nucleoside and nucleotide derivatives in two cell lines

Compound	Z	C8166		JM	
		EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
3a	NO_2	0.0032	40	10	20
3b	CN	0.0032	500	50	400
3c	MeS	0.4	500	15	200
3d	CF_3	0.0064	500	45	100
3e	I	0.16	400	10	400
3f	OMe	1.6	100	45	100
3g	H	0.32	100	40	100
1	–	0.008	>1000	100	>1000

otherwise, at temperatures which were those of the external bath. Proton and carbon nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were recorded on Bruker AM 360 (360 MHz, 90 MHz), Bruker AC 300 (300 MHz, 75 MHz), JEOL JNM GX270 (270 MHz, 67.5 MHz) spectrometers with residual non-deuterated solvent as internal standard. Phosphorus (^{31}P-NMR) spectra were recorded on a JEOL FX90 (36.4 MHz) spectrometer, with chemical shifts quoted in parts per million relative to an external phosphoric acid standard. Coupling constants (J) are given in Hz and signal splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) or broad (br) or combinations thereof. Mass spectra were recorded on a VG Analytical 70 SE Mass spectrometer. The mode of ionisation was fast atom bombardment (FAB). Chromatography refers to flash column chromatography and was carried out using Merck silica-gel 60H (40–60μ, 230–400 mesh) as stationary phase. Thin-layer chromatography (TLC) was performed using Alugram SIL G/UV$_{254}$ aluminium backed silica-gel plates. Visualisation was achieved by ultra-violet lamp or by treatment with 5% ethanolic solution of dodeca-molybdophosphoric acid followed by heating. HPLC was carried out using an ACS quaternary system, with an ODS5 column and an eluant of water/acetonitrile, with 82% water 0–10 min, then a linear gradient to 20% water at 30 min, with a flow rate of 2 ml/min and detection by UV at 265 nm. Detection limits for AZT were approx. 0.01%. Tetrahydrofuran (THF) was dried over sodium/benzophenone and distilled under an atmosphere of nitrogen. Dichloromethane, triethylamine and pyridine were dried over calcium hydride and distilled. Brine refers to saturated aqueous sodium chloride solution, ether refers to diethyl ether.

4.1. The preparation of diaryl phosphorochloridates

To a stirred solution of freshly distilled phosphoryl chloride (0.25 mmol/ml) in dichloromethane, stirred at −80°C, was added dropwise, a solution of triethylamine (2.0 eq) (0.50 mmol/ml) and the appropriate phenol (2.0 eq) in dichloromethane. The resultant solution was allowed to warm to ambient temperature over a period of at least 12 h (depending on the alcohol, progress followed by ^{31}P-NMR). The

Table 3
Analysis of biological activity of AZT diaryl phosphate derivatives (**3a–g**) in C8166 cells in terms of the acidity of the corresponding phenol

Compound	EC_{50} (μM)	$1/EC_{50}$*	Log ($1/EC_{50}$)	pK_a (phenol)
3a	0.003	313	2.5	7.15
3b	0.003	313	2.5	7.95
3c	0.4	2.5	0.4	9.81
3d	0.006	156	2.2	8.87[a]
3e	0.16	6.3	0.8	9.38
3f	1.6	0.63	−0.2	10.0
3g	0.32	3.1	0.5	10.0

*$1/EC_{50}$ might be regarded as a measure of antiviral 'potency' in this assay.

[a]The PK_a value for the para-trifluoromethyl phenol has been extrapolated using its reported PK_a in DMSO (Ellington and Arnett, 1988).

reaction mixture was concentrated in vacuo and the residues dissolved in ether. The resulting white precipitate was removed by filtration under nitrogen and the filtrate was concentrated in vacuo. The crude products were purified by high vacuum distillation or recrystallisation. Purity was determined by ^{31}P-NMR, with chemical shifts noted for the phosphorochloridate reagent precursors as follows: 3b, −5; 3c, −5; 3d, −6; 3e, −5; 3f −2.

4.2. The coupling reaction between AZT and a diaryl phosphorochloridate

To a solution of AZT (**1**) in pyridine (0.075 mmol/ml), stirred at ambient temperature, was added dropwise by syringe, a solution of diaryl phosphorochloridate (2.0–3.0 equivalents) in THF. Concentration in vacuo followed by flash column chromatography afforded the nucleoside 5′-phosphate triester.

4.3. 3′-Azido-3′-deoxythymidine-5′-(bis-4-cyanophenyl)phosphate triester (3b)

δ_H ($CDCl_3$) 1.79 (3H, s, 5-Me), 2.48 (2H, m, 2′C$\underline{H}_2$), 4.06 (1H, m, 4′C$\underline{H}$), 4.35 (1H, m, 3′C$\underline{H}$), 4.53 (2H, m, 5′CH_2), 6.04 (1H, t, J 7.6, 1′C$\underline{H}$), 7.17 (1H, s, 6C$\underline{H}$), 7.24–7.40 (4H, m, Ar$\underline{H}$), 7.58–7.78 (4H, m, Ar$\underline{H}$), 10.14 (1H, s, N$\underline{H}$); *m/z* (FAB) 550(MH^+, 22%), 127(65), 81(100), Found MH^+ 550.126, $C_{24}H_{21}N_7O_7P$ requires 550.1240.

4.4. 3′-Azido-3′-deoxythymidine-5′-(bis-4-methylthiophenyl)phosphate triester (3c)

δ_H ($CDCl_3$) 1.91 (3H, s, 5-Me), 2.23–2.57 (8H, m, 2′C$\underline{H}_2$, SC$\underline{H}_3$), 4.10 (1H, m, 4′C$\underline{H}$), 4.35 (1H, m, 3′C$\underline{H}$), 4.55 (2H, m, 5′CH_2), 6.25 (1H, t, J 8.1, 1′C$\underline{H}$), 7.10–7.30 (8H, m, Ar$\underline{H}$), 7.35 (1H, s, 6C$\underline{H}$), 9.80 (1H, s, N$\underline{H}$); *m/z* (FAB) 592 (MH^+,14%),

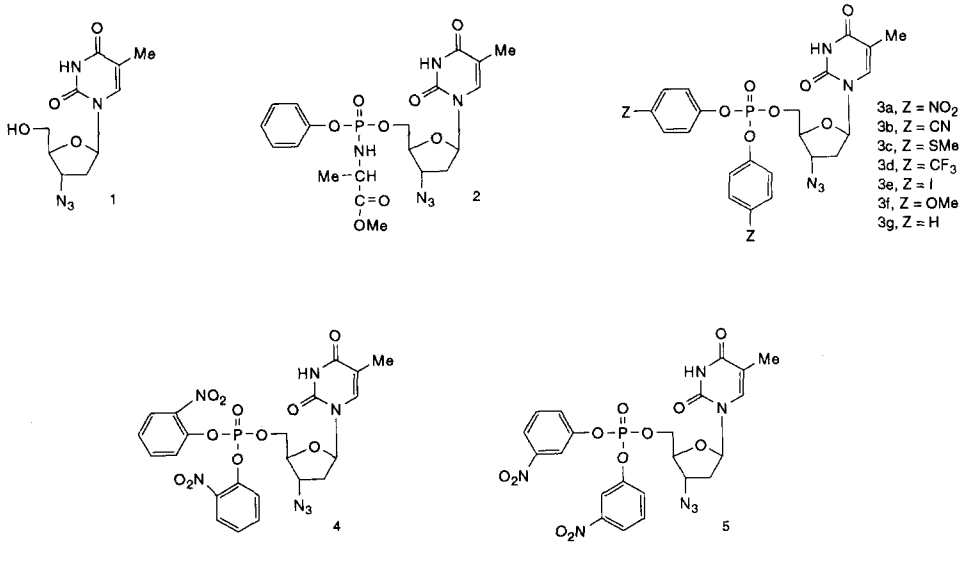

Fig. 1. Structures of nucleosides and nucleotides.

312(64), 81(100), Found MH^+ 592.1077, $C_{24}H_{27}N_5O_7S_2P$ requires 592.1089.

4.5. 3'-Azido-3'-deoxythymidine-5'-(bis-4-trifluoromethylphenyl)phosphate triester (3d)

δ_H ($CDCl_3$) 1.83 (3H, s, 5-Me), 2.40 (2H, m, 2'C$\underline{H}_2$), 4.08 (1H, m, 4'C$\underline{H}$), 4.35 (1H, m, 3'C$\underline{H}$), 4.60 (2H, m, 5'C$\underline{H}_2$), 6.14 (1H, t, J 8.1, 1'C$\underline{H}$), 7.22 (1H, s, 6C$\underline{H}$), 7.29–7.40 (4H, m, Ar$\underline{H}$), 7.56–7.72 (4H, m, Ar$\underline{H}$), 9.80 (1H, s, N$\underline{H}$); *m/z* (FAB) 636(MH^+, 8%), 387(6), 127(37), 81(100), Found MH^+ 636.1097, $C_{24}H_{21}F_6N_5O_7P$ requires 636.1082.

4.6. 3'-Azido-3'-deoxythymidine-5'-(bis-4-iodophenyl)phosphate triester (3e)

δ_H ($CDCl_3$) 1.72 (3H, s, 5-Me), 2.32 (2H, m, 2'C$\underline{H}_2$), 3.97 (1H, m, 4'C$\underline{H}$), 4.24 (1H, m, 3'C$\underline{H}$), 4.42 (2H, m, 5'CH_2), 6.09 (1H, t, J 8.1, 1'C$\underline{H}$), 6.84–6.93 (4H, m, Ar$\underline{H}$), 7.17 (1H, s, 6C$\underline{H}$), 7.50–7.63 (4H, m, Ar$\underline{H}$), 9.74 (1H, s, N$\underline{H}$); *m/z* (FAB) 752(MH^+, 27%), 127(22), 81(100), Found MH^+ 751.9247, $C_{22}H_{21}I_2N_5O_7P$ requires 751.9268.

4.7. 3'-Azido-3'-deoxythymidine-5'-(bis-4-methoxyphenyl)phosphate triester (3f)

δ_H ($CDCl_3$) 1.75 (3H, s, 5-Me), 2.24 (1H, m, 2'C$\underline{H}$), 2.39 (1H, m, 2'C$\underline{H}$), 3.73 (3H, s, OMe), 3.75 (3H, s, OMe), 4.05 (1H, m, 4'C$\underline{H}$), 4.30 (1H, m, 3'C$\underline{H}$), 4.47 (2H, m, 5'CH_2), 6.20 (1H, t, J 8.1, 1'C$\underline{H}$), 6.75–6.88 (4H, m, Ar$\underline{H}$), 7.04–7.15 (4H, m, Ar$\underline{H}$), 7.28 (1H, s, 6C$\underline{H}$), 9.94 (1H, s, N$\underline{H}$); *m/z* (FAB) 560(MH^+, 2%), 277(7), 185(75), 93(100), Found MH^+ 560.1526, $C_{24}H_{27}N_5O_9P$ requires 560.1546.

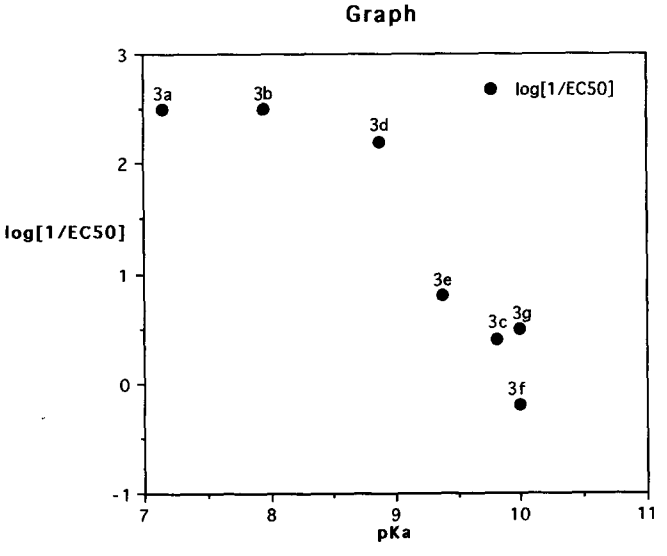

Fig. 2. A plot of the logarithm of the inverse of EC_{50} (μM) of nucleoside derivatives (**3a–g**) measured against HIV-1 in C8166 cells, vs. the pK_a of the corresponding phenol (see Table 3).

4.8. Antiviral Assays

The anti-HIV-1 activities and toxicities of compounds were assessed in two cell lines:

C8166 cells. Cells were grown in RPMI 1640 with 10% calf serum. 4×10^4 cells per microtiter plate well were mixed with 5-fold dilutions of compound prior to addition of 10 $CCID_{50}$ units of the III-B strain of HIV-1 and incubated for 5–7 days (Betbeder et al., 1990). Formation of syncytia was examined from 2 days post-infection. Culture fluid was collected at 5–7 days and gp120 antigen production measured by ELISA (Mahmood and Hay, 1992). The EC_{50} is that concentration of drug (in μM) required to reduce gp120 production by 50%. Cell viability of infected and uninfected cells were assessed by the MTT-Formazan method (Pauwels et al., 1988).

JM cells. JM cells, which are relatively resistant to the antiviral effects of AZT and a number of its derivatives, were infected with HIV-1 strains and the antiviral and toxic effects of compounds assessed as for C8166 cells. Both GB8 or IIIB strains of HIV1 were used, with no detectable differences in the end-points noted.

Each assay was carried out in duplicate, on at least two separate occasions, and data quoted are the average of each separate assay.

Acknowledgements

We thank the AIDS Directed Programme of the MRC for financial support, Dr. G.J. Langley for Mass spectra and Mr. S. Turner for HPLC analyses.

References

Betbeder, D., Hutchinson, D.W., Richards, A.O'L., Mahmood, N. and Kinchington, D. (1990) The enzymatic synthesis and anti-HIV-1 activity of 9-β-D-2',3'-dideoxynucleosides of N(6)-substituted purines. Antiviral Chem. Chemother. 1, 241–247.

Ellington, J.C., Jr. and Arnett, E.M. (1988) Kinetics and thermodynamics of phenolate silylation and alkylation. J. Am. Chem Soc. 110, 7778–7785.

Farrow, S.N., Jones, A.S., Kumar, A., Walker, R.T., Balzarini, J. and De Clercq, E. (1990) Synthesis and biological properties of novel phosphotriesters: A new approach to the introduction of biologically active nucleotides into cells. J. Med. Chem. 33, 1400–1406.

Freed, J., Farquhar, D. and Hampton, A. (1989) Evidence for acyloxymethyl esters of pyrimidine 5'-deoxyribonucleotides as extracellular sources of active 5'-deoxyribonucleotides in cultured cells. Biochem. Pharmacol. 38, 3193–3198.

Henin, Y., Gouyette, C., Schwartz, O., Debouzy, J.-C., Neumann, J.-M. and Huynh-Dinh, T. (1991) Lipophilic glycosyl phosphotriester derivatives of AZT: Synthesis, NMR transmembrane transport study and antiviral activity. J. Med. Chem. 34, 1830–1837.

Mahmood, N. and Hay, A.J. (1992) An ELISA utilising immobilised snowdrop lectin GNA for the detection of envelope glycoproteins of HIV and SIV. J. Immunol. Meth. 151, 9–13.

Mark, V., Dungan, C.H., Crutchfield, M.M. and Van Wazer, J.R. (1969) Compilation of 31P NMR data. In: M. Grayson and E.J. Griffith (Eds), Topics in Phosphorus Chemistry, Vol. 5, Wiley, New York.

McGuigan, C., O'Connor, T.J., Nicholls, S.R., Nickson, C. and Kinchington, D. (1990a) Synthesis and anti-HIV activity of some novel substituted dialkyl phosphate derivatives of AZT and ddCyd.

Antiviral Chem. Chemother. 1, 355–360.

McGuigan, C., Devine, K.G., O'Connor, T.J., Galpin, S.A., Jeffries, D.J. and Kinchington, D. (1990b) Synthesis and evaluation of some novel phosphoramidate derivatives of 3′-azido-3′-deoxythymidine. Antiviral Chem. Chemother. 1, 107–113.

McGuigan, C., Devine, K.G., O'Connor, T.J. and Kinchington, D. (1991) Synthesis and anti-HIV activity of some haloalkyl phosphoramidate derivatives of 3′-azido-3′-deoxythymidine (AZT): potent activity of the trichloroethyl methoxyalaninyl compound. Antiviral Res. 15, 255–263.

McGuigan, C., Pathirana, R.N., Mahmood, N., Devine, K.G. and Hay, A.J. (1992) Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines which are resistant to the action of AZT. Antiviral Res. 16, 311–321.

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

McGuigan, C., Pathirana, R.N., Davies, M.P.H., Balzarini, J. and De Clercq, E. (1994) Diaryl phosphate derivatives act as pro-drugs of AZT with reduced cytotoxicity compared to the parent nucleoside. BioOrg. Med. Chem. Lett. 4, 427–430.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmeyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Meth. 20, 309–321.

Roberts, N.A., Martin, J.A., Kinchington, D., Broadhurst, A.V., Craig, J.C., Duncan, I.B., Galpin, S.A., Handa, B.K., Kay, J., Krohn, A., Lambert, R.W., Merrett, J.H., Hills, J.S., Parkes, K.E.B., Redshaw, S., Ritchie, A.J., Taylor, D.L., Thomas, G.T. and Machin, P.J. (1990) Rational design of peptide-based HIV proteinase inhibitors. Science 248, 358–361.

Topliss, J.G. (1972) Utilization of operational schemes for analog synthesis in drug design. J. Med. Chem. 15, 1006–1011.