Antiviral Research, 17 (1992) 311–321

AVR 00534

Research Articles

Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines which are resistant to the action of AZT

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(Received 23 August 1991; accepted 19 September 1991)

Summary

Novel aryl phosphate derivatives of the anti-HIV nucleoside analogue AZT have been prepared by phosphorochloridate chemistry. These materials are designed to act as membrane-soluble pro-drugs of the bio-active free nucleotides. In vitro evaluation revealed the compounds to have a pronounced, selective antiviral activity, which, in one case, was more potent than the parent nucleoside AZT. The magnitude of the biological effect varied considerably with the nature of the phosphate-blocking group. Moreover, one of the compounds, a phosphoramidate, is particularly active in a cell line restrictive to the activity of AZT, due to poor phosphorylation therein. These data support the suggestion that the phosphate derivatives exert their biological effects via intracellular release of the nucleotide forms.

HIV; AZT; Nucleotide; Pro-drug

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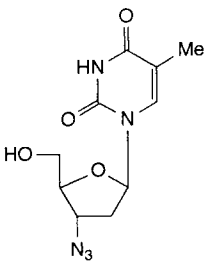
Introduction

The nucleoside analogue 3′-azido-3′-deoxythymidine (AZT, Fig. 1, 1) is now established as a useful treatment for acquired immunodeficiency syndrome (AIDS), and is presumed to be effective by virtue of its inhibition of human immunodeficiency virus (HIV1) reverse transcriptase (Mitsuya et al., 1985). As with other chemotherapeutic nucleoside analogues, AZT acts only after metabolic activation to its 5′-triphosphate form. This dependence on (kinase mediated) phosphorylation may be a limitation, especially in cellular environments low in particular nucleoside kinase activities.

We have previously outlined the potential advantages of utilising masked phosphate derivatives of anti-cancer (araC) and anti-herpetic (araA) nucleoside analogues (Jones et al., 1989; McGuigan et al., 1989). We have found that simple dialkyl phosphate derivatives of AZT, and other nucleoside analogues,



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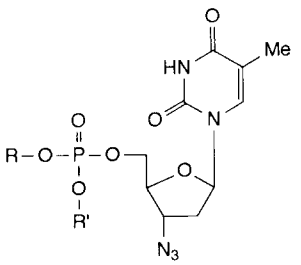

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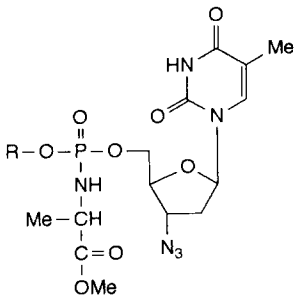
2b R=Ph, R'=Cl_3CCH_2
2c R=p-NO_2Ph, R'=p-NO_2Ph



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3b R=p-NO_2Ph
3c R=Et
3d R=Cl_3CCH_2

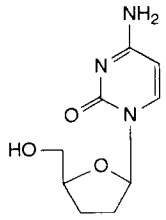

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Fig. 1. Structures of AZT (1), the nucleotide derivatives prepared (2a–c and 3a–d), and ddCyd (4).

are inactive as anti-HIV agents (McGuigan et al., 1990a), whereas substituted dialkyl phosphates are active (McGuigan et al., 1990b). In this paper we report the preparation of novel aryl phosphate derivatives of AZT, which selectively inhibit the replication of HIV. In particular, in contrast to AZT, the agents are effective in inhibiting viral replication in nucleoside kinase deficient cells.

Materials and Methods

All reactions were carried out under scrupulously dry conditions, using general procedures we have described (McGuigan et al., 1991). ^{31}P NMR spectra were recorded on a Varian XL-200 spectrometer (82 MHz) or a Jeol FX90Q (36.2 MHz) and are reported in units of δ relative to 85% phosphoric acid as external standard, positive shifts are downfield. ^{13}C NMR spectra were recorded on a Varian XL-200 spectrometer (50 MHz), a Varian VXR-400 (100 MHz) or a Bruker AM360 (90.6 MHz) δ relative to $CDCl_3$ at 77.000 ppm. Both ^{31}P and ^{13}C NMR spectra were proton noise decoupled and all signals were singlets unless otherwise stated. ^1H NMR spectra were recorded on a Varian XL-200 spectrometer (200 MHz), a Varian VXR-400 (400 MHz), or a Bruker AM360 (360 MHz) and are reported in units of δ relative to internal $CHCl_3$ at 7.240 ppm unless otherwise stated. All NMR spectra were recorded in $CDCl_3$. HPLC data were recorded as described (McGuigan et al., 1991).

3'-Azidothymidine-5'-diphenyl phosphate (Fig. 1, 2a)

3'-Azidothymidine (0.1 g, 0.38 mmol) was dissolved in THF (3 ml) and diphenyl phosphorochloridate (0.23 ml, 1.12 mmol) and *N*-methyl imidazole (0.18 ml, 2.24 mmol) were added with vigorous stirring. After 4 min at ambient temperature, methanol (1 ml) was added, and the solvents removed under vacuum. The residue was dissolved in chloroform (30 ml) and washed with saturated sodium bicarbonate solution (15 ml) and then water (2 × 15 ml). The organic phase was dried ($MgSO_4$) and evaporated under vacuum. The residue was dissolved in chloroform (10 ml) and precipitated with petroleum (bp 30–40°C; 400 ml). After cooling at 4°C overnight the solvent was decanted from the gum, which was further purified by chromatography on silica (30 g) by elution with 2% methanol in chloroform. Pooling and evaporation of appropriate fractions gave the product (0.12 g, 64%); δ_P −10.78; δ_H 8.47 (1H, sb, NH), 7.2–7.4 (11H, m, Ph, H6), 6.22 (1H, t, H1', J = 6.5 Hz), 4.55 (1H, ddd, H5'), 4.46 (1H, ddd, H5'), 4.27 (1H, m, H3'), 4.06 (1H, m, H4'), 2.41 (1H, m, H2'), 2.23 (1H, m, H2'), 1.78 (3H, d, 5-Me, J = 1.2 Hz); δ_C 163.7 (C2), 150.3 (C4), 150.1 (*ipso*-Ph, d, J = 7.1 Hz), 135.0 (C6), 129.9 (*meta*-Ph, d, J = 7.1 Hz), 125.8 (*para*-Ph), 119.9 (*ortho*-Ph, d, J = 4.7 Hz), 111.6 (C5), 84.7 (C1'), 81.9 (C4', d, J = 7.6 Hz), 67.4 (C5', d, J = 5.9 Hz), 59.9 (C3'), 37.3 (C2'), 12.3 (5-Me); HPLC retention time 29.07 min, no AZT detected.

3'-Azidothymidine-5'-(phenyl-2,2,2-trichloroethyl) phosphate (Fig. 1, 2b).

This was prepared by a method entirely analogous to that used for compound 2a above, except that stirring was continued for 48 h, the reaction was not quenched, and the chromatographic purification utilised 3% methanol in chloroform. Thus, from 0.1 g of AZT was isolated 0.11 g (52%) of compound 2b; δ_P −6.99, −7.11 (2:3); δ_H (peaks labelled * duplicated due to diastereoisomers) 9.04 (1H, sb, NH), 7.2–7.4 (6H, m, Ph, H6), 6.23* (1H, t, H1', J = 6.6 Hz), 4.71 (1H, m, CH_2OP), 4.60 (1H, m, CH_2OP), 4.50 (2H, m, H5'), 4.31 (1H, m, H3'), 4.07 (1H, m, H4'), 2.43 (1H, m, H2'), 2.30 (1H, m, H2'), 1.87* (3H, d, 5-Me, J = 1.2 Hz); δ_C 163.6 (C2), 150.2 (C4), 149.8 (*ipso*-Ph, d, J = 6.9 Hz), 135.1 (C6), 130.0 (*meta*-Ph, d, J = 4.5 Hz), 126.1 (*para*-Ph), 119.9 (*ortho*-Ph, d, J = 4.6 Hz), 111.7* (C5), 85.0 (C1'), 81.9* (C4', d, J = 8.0 Hz), 67.5 (C5', d, J = 6.1 Hz), 60.0* (C3'), 37.3* (C2'), 12.5* (5-Me); HPLC retention time 30.29, 30.47 min (2:1), no AZT detected.

Phenylmethoxyalaninyl phosphorochloridate

A solution of triethylamine (1.0 ml, 7.17 mmol) in dichloromethane (20 ml) was added dropwise with vigorous stirring to a solution of L-alanine methyl ester hydrochloride (0.5 g, 3.58 mmol) and phenyl phosphorodichloridate (0.54 ml, 3.65 mmol) in dichloromethane (20 ml) at −78°C. The reaction mixture was slowly warmed to ambient temperature with stirring over 6 h and the solvent was then removed in vacuum. The residue was treated with carbon tetrachloride (15 ml), the mixture filtered, and the filtrate evaporated in vacuum to yield the product as an oil (0.95 g, 95%); δ_P 6.22, 5.91 (1:1).

3'-Azidothymidine-5'-(phenylmethoxyalaninyl) phosphate (Fig. 1, 3a)

3'-Azidothymidine (0.15 g, 0.56 mmol) was dissolved in THF (4 ml) and phenylmethoxyalaninyl phosphorochloridate (0.52 g, 1.87 mmol) and *N*-methyl imidazole (0.26 ml, 3.36 mmol) were added with vigorous stirring. The mixture was stirred for 16 h at ambient temperature and then for 3 min at 45°C. The solvent was removed under vacuum, and the residue was dissolved in chloroform (10 ml) and washed with 1 M HCl (2 × 10 ml), saturated sodium bicarbonate solution (2 × 10 ml) and then water (3 × 10 ml). The organic phase was dried ($MgSO_4$) and evaporated under vacuum. The residue was further purified by flash column chromatography on silica by elution with 4% methanol in chloroform. Pooling and evaporation of appropriate fractions gave the product (0.18 g, 63%); δ_P 3.66, 3.34 (3:2); δ_H 8.65* (1H, s, NH), 7.30 (3H, m, Ph, H6), 7.20 (3H, m, Ph), 6.14* (1H, t, H1'), 4.35 (2H, m, H5'), 4.25 (1H, m, H4'), 4.00 (2H, m, H3', ala-CH), 3.72 (1H, m, NH), 3.70* (3H, s, OMe), 2.40 (1H, m, H2'), 2.20 (1H, m, H2'), 1.90 (3H, d, 5-Me), 1.35* (3H, d, ala-Me); δ_C 174.0* (ala-CO, m), 163.8 (C2), 150.3 (*ipso*-Ph, d, J = 2.6 Hz), 150.1 (C4), 135.3* (C6), 129.8 (*para*-Ph), 125.2 (*ortho*-Ph, d, J = 3.6 Hz), 120.0* (*meta*-Ph,

d, J = 4.8 Hz), 111.3 (C5), 84.8* (C1′), 82.2* (C4′, d, J = 7.9 Hz), 65.6* (C5′, d, J = 5.2 Hz), 60.3* (C3′), 52.6 (OMe), 50.2* (ala-CH), 37.2 (C2′), 20.8* (ala-Me, d, J approx. 6 Hz), 12.4* (5-Me); HPLC retention time 25.63, 25.82 min (1:1), no AZT detected.

Para-nitrophenyl methoxyalaninyl phosphorochloridate

This was prepared by a method entirely analogous to that used for the phenyl analogue above, except that the carbon tetrachloride treatment was omitted, and the crude reaction mixture was only evaporated to a small volume, not to dryness. Thus, from 0.92 g *p*-nitrophenyl phosphorodichloridate was isolated 0.72 g (62%) of the product; δ_P 5.97, 5.82 (3:2).

3′-Azidothymidine-5′-(p-nitrophenylmethoxyalaninyl) phosphate (Fig. 1, 3b)

This was prepared by a method entirely analogous to that used for compound 2b above, except that the chromatographic purification utilised 5% methanol in chloroform. Thus, from 0.06 g of AZT was isolated 0.06 g (47%) of compound 3b; δ_P 3.16, 2.94 (1:1); δ_H 8.50 (1H, sb, NH), 8.25 (2H, d, Ph), 7.40 (2H, d, Ph), 7.29 (1H, d, H6, J = 1.2 Hz), 6.07* (1H, t, H1′, J = 6.5 Hz), 4.4 (3H, m, H5′, H3′), 3.8–4.0 (3H, m, H4′, NH, ala-CH), 3.72* (3H, s, OMe), 2.45 (2H, m, H2′), 1.90* (3H, d, 5-Me, J = 1.0 Hz), 1.39* (3H, m, ala-Me); δ_C 173.6* (ala-CO, d, J = 7 Hz), 163.6 (C2), 155.2* (*ipso*-Ph, d, J = 5.4 Hz), 150.1* (C4), 144.7* (*para*-Ph), 136.0* (C6), 125.7 (*ortho*-Ph, d, J = 3.3 Hz), 120.7* (*meta*-Ph, d, J = 6.4 Hz), 111.4 (C5), 86.1 (C1′), 82.1* (C4′, d, J = 7.5 Hz), 66.1* (C5′, d, J = 5.2 Hz), 60.2* (C3′), 52.8* (OMe), 50.3* (ala-CH), 37.1* (C2′), 20.9* (ala-Me, d, J approx. 6 Hz), 12.4* (5-Me); HPLC retention time 26.86 min, no AZT detected.

3′-Azidothymidine-5′-bis(p-nitrophenyl) phosphate (Fig. 1, 2c)

Di-*p*-nitrophenyl hydrogen phosphate (0.61 g, 1.78 mmol) and di-*p*-tolyl carbodiimide (0.19 g, 0.86 mmol) were stirred in anhydrous dioxane (5 ml) at ambient temperature for 20 min. Anhydrous AZT (0.2 g, 0.75 mmol) was added, and the mixture stirred at 40°C for 3d. The mixture was filtered, the precipitate washed with dioxane (3 × 3 ml) and the combined filtrate and washings evaporated under reduced pressure. The resulting oil was dissolved in ethyl acetate (10 ml), extracted with water (5 × 15 ml), dried ($MgSO_4$), and evaporated in vacuum. Flash column chromatography on silica, with elution by 4% methanol in chloroform, followed by pooling and evaporation of appropriate fractions, gave the product as an oil (0.168 g, 38%); δ_P −14.0; δ_H 9.8 (1H, sb, NH), 8.27 (4H, dd, Ph), 7.40 (4H, dd, Ph), 7.17 (1H, d, H6, J = 1.2 Hz), 6.05 (1H, dd, H1′), 4.6 (2H, m, H5′), 4.4 (1H, m, H3′), 4.08 (1H, m, H4′), 2.5 (2H, m, H2′), 1.85 (3H, s, 5-Me); δ_C 163.9 (C2) 154.4 (*ipso*-Ph, d, J = 6.3 Hz), 150.3 (C4), 145.5 (*para*-Ph), 136.4 (C6), 126.0 (*ortho*-Ph, d, J = 10.5

Hz), 120.8 (*meta*-Ph, 2xd, J = 4.4 Hz), 111.7 (C5), 86.8 (C1′), 82.0 (C4′, d, J = 7.1 Hz), 68.7 (C5′, d, J = 6.4 Hz), 60.1 (C3′), 36.9 (C2′), 12.4 (5-Me); HPLC retention time 30.02 min, no AZT detected.

Materials and experimental procedures: virology

Antiviral assays

The anti-HIV-1 activities and toxicities of compounds were assessed in two cell lines (Betbeder et al., 1990). C8166 (a normal T-cell transformed by co-cultivation with leukaemia lymphocytes harbouring HTLV-1) were infected with the IIIB strain of HIV-1. Secondly, JM, a semi-mature T-cell line derived from a patient with lymphoblastic leukaemia, were infected with HIV-1 strains GB8 or IIIB. JM cells are relatively resistant to the antiviral effects of AZT and a number of its derivatives. 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 virus and incubated for 5–7 days. 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. Cell viability of infected cells and cytotoxicity to uninfected cell controls were assessed by the MTT-Formazan method (Pauwels et al., 1988).

gp120 antigen assay

A microtiter antigen capture ELISA was developed (Mahmood and Hay, 1992) using a lectin (GNA) from *Galanthus nivalis* (Vector Labs., Peterborough, U.K.) and anti-HIV antibodies in human serum. The plates were coated with lectin (0.5 μg), and after blocking with 10% calf serum, dilutions of virus supernatants in 0.25% Empigen solution (Albright and Wilson Ltd., Whitehaven, U.K.) were added to the wells and incubated at 4°C for 12–16 h. Bound antigen was detected using human anti-HIV-1 antibodies, and anti-human IgG antibodies coupled to horseradish peroxidase.

Results and Discussion

Chemistry

A synthetic strategy similar to that we have previously employed for phosphoramidates (McGuigan et al., 1990c; Curley et al., 1990) was found to be successful in the case of diaryl analogues too. Thus, diphenyl phosphorochloridate was allowed to react with AZT (Fig. 1, compound 1) in THF at ambient temperature in the presence of *N*-methylimidazole (Van Boom et al., 1975). However, by contrast to the previous studies, which required reaction

times of approx. 16 h, the aryl reaction was complete in <5 min (Jones et al., 1985). The target product (2a) was isolated in moderate yield and fully characterised by a range of spectroscopic methods. The sample was pure by HPLC, and entirely free of any contaminating AZT; this is particularly important given the high activity of AZT in the biological assay used.

We have recently noted the major effect on antiviral activity of proceeding from simple dialkyl phosphate derivatives of AZT to substituted dialkyl phosphates; indeed, this was the basis for this present study. However, we also noted antiviral activity with just one substituted alkyl group, and one simple alkyl group. Thus, it was now of interest to prepare the corresponding derivative with one simple aryl group and one substituted alkyl group. By an analogous route to that used for the parent compound (2a), phenyl-2,2,2-trichloroethyl phosphorochloridate was allowed to react with AZT, to yield the target compound (2b). The NMR and HPLC data reveal the presence of two diastereoisomers for compound 2b arising from the variable stereochemistry at the phosphate. As we have noted (McGuigan et al., 1990c; Devine et al., 1990), it may well be that the individual diastereoisomers in a particular case may differ in their biological activities; however, in the case of compound 2b the mixture of isomers was not resolved, and was tested as such.

We have noted the efficacy of certain phosphoramidate derivatives of AZT as inhibitors of HIV; it would seem that such phosphoramidate groups are analogous to substituted alkyl groups in this regard (Devine et al., 1990; Curley et al., 1990; McGuigan et al., 1990c). It was therefore of interest to combine phosphoramidate and aryl moieties in one structure. Thus, following our established synthetic route (Curley et al., 1990), the reaction of L-alanine methyl ester hydrochloride with phenyl phosphorodichloridate in the presence of triethylamine gave phenyl methoxyalaninyl phosphorochloridate in moderate yield. This was allowed to react with AZT as above, albeit for longer, to give the target compound (3a). Again, the NMR and HPLC data are entirely consistent with the structure of compound 3a, and confirm the presence of two diastereomers in the sample. Similarly prepared was the nitrophenyl analogue, 3b.

Antiviral activity

All the compounds tested inhibited the proliferation of HIV1 in C8166 cells at concentrations which were non-toxic to uninfected controls. The derivatives (2a–b) and (3a–b) were less active than AZT (1) and were more cytotoxic (Table 1; Fig. 2). It is notable that compounds 2a and 2b are very similar in their antiviral effects in this system. The former contains two phenyl groups, and the latter one phenyl and one trichloroethyl. In a simply additive sense, therefore, the phenyl and trichloroethyl groups appear to be equally efficacious in their 'activating' effect, relative to simple alkyl groups, which we have previously noted to be in-effective. Compound 3b and, to a lesser extent, 3a, are especially effective as inhibitors of virus replication. Thus, continuing the

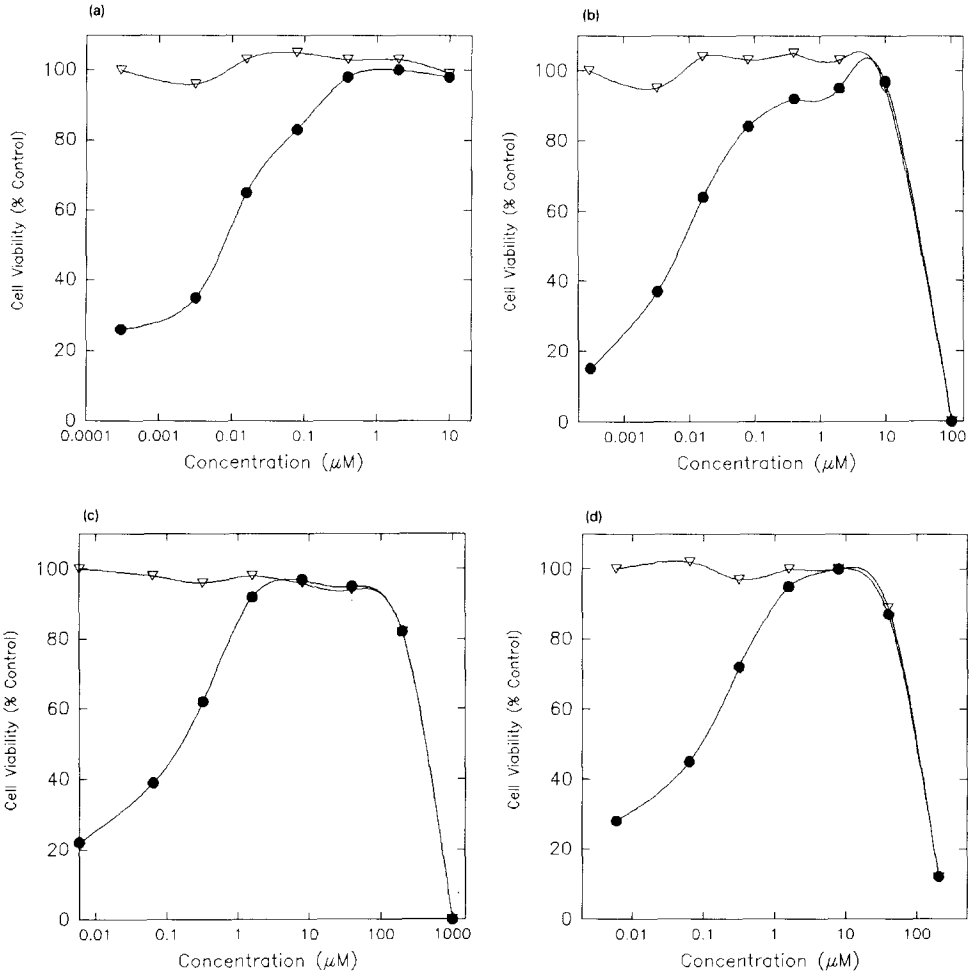

Fig. 2. Effect of compounds 1, 2c, 3a and 3b on viability of infected and uninfected C8166 cells. Five days after infection the viability of the cells was determined by the MTT-formazan method. (A) Compound 1; (B) compound 2c; (C) compound 3a; (D) compound 3b. (▽) uninfected; (●) infected.

analysis of activating effects, the nitrophenyl group appears more effective than the phenyl, and the amidate moiety in 3a is more effective than a trichloroethyl (in 2b). To extend this comparison, we tested two previous compounds (3c and d) (McGuigan et al., 1990c) in this assay system. These compounds retain the same amidate moiety as 3a and b, but carry altered alkyl groups in place of the *p*-nitrophenyl group. Compound 3c has a simple ethyl group, and 3d a trichloroethyl. The data (Table 1) showed that compound 3d is of similar activity to 2a and b, suggesting a roughly equivalent activating nature of the amidate and aryl groups. Secondly, compound 3c is an order of magnitude less

TABLE 1

Anti-HIV-1 activities and toxicities of nucleosides and nucleotides in infected C8166 and JM cells

Compound	C8166		JM	
	EC_{50}	TC_{50}	EC_{50}	TC_{50}
1	0.008	>1000	100	>1000
2a	0.3	100	>20	100
2b	0.2	70	>20	50
3a	0.08	500	0.32	500
3b	0.03	100	40	150
3c	4	300	80	200
3d	0.4	200	>20	50
2c	0.0032	40	10	20
4	0.01	>1000	0.01	0.5

EC_{50} represents the concentration of compound that decreases antigen production in infected cells to 50% of control. TC_{50} represents the concentration of compound which causes 50% cytotoxicity to uninfected cells. Data are shown for HIV-1-infected C8166 and JM cells.

active than these materials, confirming the poor activating effect of a simple alkyl group, and the marked advantage of having two 'active' groups rather than just one. In simple additive terms, the order of activating effects would be: *p*-nitrophenyl > phenyl = trichloroethyl = amidate >> alkyl. These additive effects appear to be operating reasonably well within this present study, although they must be only an approximation; we have recently noted one exception (McGuigan et al., 1991).

These structure activity relationships suggested to us a new derivative which should have even greater activity; the bis(*p*-nitrophenyl) compound 2c. The synthesis was a modification of that used for similar derivatives (Moffatt and Khorana, 1957; Chawla et al., 1984) and used phosphate, rather than phosphorochloridate chemistry. Di-*p*-nitrophenyl hydrogen phosphate was allowed to react with AZT in dioxane in the presence of di-*p*-tolyl carbodiimide. The product 2c is a particularly potent inhibitor of HIV-1 replication in C8166 cells (Table 1; Fig. 2), being approximately 3 × more potent than AZT (1). Unfortunately, however, this is at the expense of increased toxicity; in uninfected cells at concentrations as low as 40 μM. The nitrophenyl containing compounds are especially cytotoxic, whereas the amidates tend to be less toxic.

A major purpose of this study was to overcome the dependence of AZT, and other nucleoside analogues, on cellular kinase-mediated activation. In order to probe our success in this regard, and to try to understand more about the mechanism of action of these novel derivatives, their effectiveness in inhibiting HIV infection was studied in JM cells, a cell line in which HIV infection is poorly responsive to AZT due to low phosphorylation of the nucleoside (Roberts et al., 1990). The phosphate derivatives 2a–c and 3b–d are also substantially less effective in this system. However, there is one exception: compound 3a retains very significant activity against HIV infection of JM cells.

Indeed, 3a is only 4× less effective in JM cells than in C8166 cells, in marked contrast to AZT, which was 10^4× less effective. Moreover, compound 3a is relatively non-toxic to uninfected JM cells and has a selectivity index (1500) substantially greater than either AZT or 2′,3′-dideoxycytidine (ddCyd) (compound 4). In general, it would appear that the antiviral activities of the phosphate derivatives are less affected by the low activity of the thymidine kinase than are those of the free nucleosides. This is entirely consistent with the proposed mode of action of the phosphates: membrane penetration, followed by intracellular hydrolysis of the phosphate-blocking groups to liberate the nucleotide or a derivative thereof. Perhaps reduced thymidylate kinase activity, or other differences between the two cell lines, lead to the slight reduction in activity of the phosphates in JM cells, but their successful bypass of the nucleoside kinase contributes to their improved performance over AZT in this system.

In conclusion, we have reported the synthesis and anti-HIV evaluation of a series of novel aryl phosphate derivatives of AZT. The compounds show a range of activities which suggest that the phenyl, and especially *p*-nitrophenyl, groups are effective phosphate blocking groups. The data are consistent with a mode of action involving intracellular release of the bioactive free nucleotides. The bis(*p*-nitrophenyl) compound 2c is especially active in C8166 cells, and the phenyl methoxyalaninyl system (3a) retains very significant activity in JM cells, in contrast to AZT.

Acknowledgements

We thank the AIDS Directed Programme of the MRC for financial support, Mr. S. Corker (UCL) and Mr. S. Turner (Southampton) for HPLC analyses, and Miss S. Barker for assistance with the antiviral assays.

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