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DIARYL PHOSPHATE DERIVATIVES ACT AS PRO-DRUGS OF AZT WITH REDUCED CYTOTOXICITY COMPARED TO THE PARENT NUCLEOSIDE

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Abstract Diaryl phosphate derivatives of the anti-HIV nucleoside analogue AZT were prepared as potential pro-drugs of the bio-active free nucleotide. The compounds are potent inhibitors of HIV replication in several cell lines, and show reduced cytotoxicity in vitro, by comparison to the parent nucleoside. However, in contrast to previously reported aryloxy phosphoramidate derivatives of AZT the diaryl phosphates are poorly active in HIV-infected thymidine kinase-deficient CEM cells.

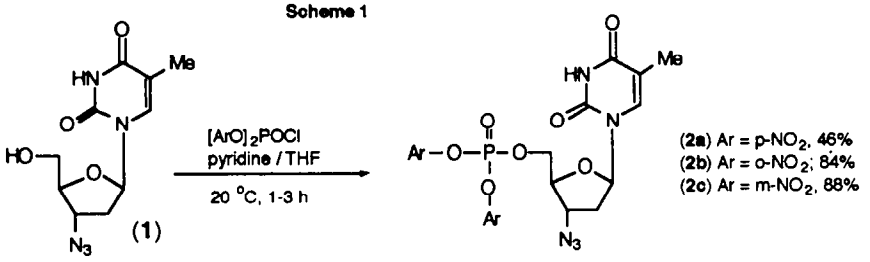
Recently, there has been much interest in 2',3'-dideoxynucleosides as inhibitors of HIV, the causative agent of AIDS.[1] The dideoxy nucleoside analogue AZT (**1**) is firmly established as a treatment for AIDS.[2] AZT has an absolute dependence on (host cell) thymidine kinase-mediated activation.[3] In an effort to circumvent this kinase dependence, we,[4] and others[5] have suggested the use of masked phosphate pro-drugs of the bio-active nucleotide forms of several chemotherapeutic nucleoside analogues. We now report the preparation and biological evaluation of several diaryl phosphate derivatives of AZT, designed to act as an intracellular source of the free 5'-monophosphate AZTMP. In particular, we report herein that certain derivatives appear to be less cytotoxic than the parent nucleoside analogue, while retaining full antiviral efficacy. If confirmed in vivo, the enhancement of the antiviral selectivity of AZT may be of clinical significance.

We have previously reported the synthesis and biological activity of the p-nitrophenyl target compound (**2a**).[6] We now report for the first time an alternative synthesis of this material, and the evaluation of the test compounds in a thymidine kinase-competent and -deficient assay system.

The synthetic strategy followed that we have previously developed for the dialkyl analogues of the present aryl systems.[7] p–Nitrophenol reacted with phosphoryl chloride in diethyl ether to give the diaryl phosphorochloridate (51%, δ_P -8). This was allowed to react with AZT (**1**) in pyridine and tetrahydrofuran to give the target compound (**2a**) in moderate yield (Scheme 1). As previously noted,[6] this material displayed an up-field signal in the P-31 NMR (δ_P ca. -15). Carbon- and proton-NMR data were also entirely consistent with the structure and purity of this material. Similarly, o-nitrophenol reacted with phosphoryl chloride to give the intermediate diaryl phosphorochloridate (72%, δ_P -6). This was allowed to react with AZT (**1**) to give the ortho- compound (**2b**) in good yield.[7]

Similarly prepared was the meta- compound (**2c**) (88%, δp ca. -15).

Scheme 1



The parent nucleoside AZT (**1**), and the phosphate derivatives (**2a-c**) were evaluated for their ability to inhibit the replication of HIV-1 and HIV-2 in human lymphocyte MT-4 and CEM cells. The results are displayed in Table 1.[8] It is notable that all of the compounds are potent inhibitors of viral replication, being comparable to the parent nucleoside (**1**). There is some variation in activity with the position of the nitro groups on the aryl rings; the para compound (**2a**) is slightly less active than the other isomers in CEM cells, and the meta isomer (**2c**) is the most active, in both CEM and MT4 cells. There is generally no difference in the effectiveness of any given compound against either HIV-1 or HIV-2.

Table 1. EC_{50} values for AZT and diaryl phosphate derivatives (nM)[8]

	CEM/0 cells		MT4 cells	
	HIV-1	HIV-2	HIV-1	HIV-2
Compound				
1	3±0	3.5±0.7	2.5±0.2	2.6±0.1
2a	9	15	13	10
2b	5.3±2.0	6.2±0.3	12.1±3.4	14±1.0
2c	3.8±0.4	3.8±0.4	2.5±0.2	3.1±0.4

We have previously noted that certain aryl phosphoramidate derivatives of AZT retain their antiviral activity in thymidine kinase-deficient cells, which lack the ability to phosphorylate (and thus activate) AZT.[6,9]

This has been interpreted that such compounds are able to act as intracellular delivery forms for the free nucleotides, as had been intended in their design. It was therefore of interest to examine the current diaryl phosphates in thymidine kinase-deficient cells. Thus, CEM/TK$^-$ cells were used, a cell line deficient in cytosolic thymidine kinase, the data being shown in Table 2. It is apparent that, as with AZT, the phosphates are extremely poor inhibitors of viral replication in the thymidine kinase-deficient cell line, in marked contrast to the earlier reported aryl phosphoramidate analogues.[9]

This strongly suggests that the diaryl phosphates act as delivery forms for the free nucleoside, rather than the nucleotide, as had been intended. Since it was considered that advantage might emerge from delivery of the nucleotide, the diaryl phosphates might be regarded as less promising than their phosphoramidate analogues.[9]

Table 2. EC_{50} and CC_{50} values for AZT and diaryl phosphate derivatives (nM)[8]

Compound	EC_{50} CEM/TK⁻ cells HIV-2	CC_{50} MT4 cells
1	>100,000	6,000
2a	50,000	50,000
2b	>100,000	42,000
2c	85,000	36,000

However, further examination of the compounds revealed a promising advantage over the parent nucleoside, in particular with regard to their cytotoxicity towards uninfected cells (Table 2). The para compound (**2a**) in particular appears to be at least one order of magnitude less cytotoxic than AZT. Given the known clinical toxicity of the parent nucleoside analogue,[10] such an advantage could be of major importance, if this phenomena were to be confirmed in vivo. It is interesting to note that the cytotoxicity noted above for [2a] is almost identical [40μM] to that previously noted[6] in C8166 T-cells, whilst the toxicity of AZT is markedly increased in MT4 cells [6μM, c.f. >1000μM in C8166].

The mechanism of action of the diaryl phosphates remains unclear; although it was surprising that compounds bearing such good leaving groups on the phosphate should act as delivery forms for the nucleoside rather than for the nucleotide. One possibility is that the high lability of the nitrophenyl groups leads to rapid hydrolysis in vitro to release the nucleotide extracellularly. Due to the poor membrane penetration by such a polar species as AZTMP,[11] the products would only act following further extracellular cleavage to the nucleoside (AZT), followed by passive membrane penetration, and intracellular re-phosphorylation. Such a mode of action would be entirely consistent with the observed poor antiviral activity in the thymidine kinase-deficient cell line. In order to probe this, we examined the stability of the p-nitrophenyl compound (**2a**) in several media. The compound showed complete hydrolysis after 24 hours at 25 ^{o}C in human plasma as judged by thin layer chromatography. By contrast, there was no detectable decomposition in water, and only partial decomposition in pH 7.6 buffer, even after 1 week. Further analysis of the plasma sample by HPLC revealed a complete absence of the free nucleoside AZT and the presence of one major, polar product. This was confirmed to be AZT nitrophenyl phosphate diester by proton NMR. Judging from its HPLC retention time, this material is much more polar than either the triester (**2a**) or AZT, further supporting the suggestion of poor membrane penetration by such a compound. In plasma, there was further slow hydrolysis, over a period of 2 weeks, liberating AZT as the major product.

In conclusion, we have demonstrated that bis(nitrophenyl) phosphate esters of AZT are potent and selective inhibitors of HIV in several cell lines. The materials do not retain their antiviral activity in thymidine kinase-deficient cells, which implies that they are not able to deliver the nucleotides intracellularly, but rather act as nucleoside delivery forms. Lastly, it is noted that the phosphate

triesters are markedly less toxic than the parent nucleoside analogue towards uninfected cells. Consequently, their therapeutic index in vitro was increased by approximately one order of magnitude, by comparison to the parent nucleoside. The possibility that this advantage might translate to the clinic suggests that such compounds are worthy of further study.

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7. Data for (2b): $\delta_P(CDCl_3)$ -15.2; $\delta_C(CDCl_3)$ 164.0(C2), 157.0(Ph-para), 150.5(C4), 142.7(d, Ph-ipso, J=5Hz), 141.2(2xd, Ph-ortho), 135.4(C6), 135.1(2xd, Ph), 126.3(2xd, Ph), 122.9(d, Ph, J=2Hz), 111.8(C5), 84.9(C1'), 81.7(m, C4', J=8Hz), 68.9(d, C5', J=7Hz), 60.0(C3'), 37.2(C2'),12.4(5-Me); $\delta_H(CDCl_3)$ 9.6(1H, sb, NH), 7-8(9H, m, H6, Ph), 6.2(1H, t, H1'), 4.7(2H, m, H5'), 4.4(1H, m, H3'), 4.1(1H, m, H4'), 2.4(2H, m, H2'), 1.8(3H, s, 5-Me); m/e (FAB) 590(MH^+, 7%), 307(15), 154(100), 136(75), 81(C_5H_5O, 35); HPLC retention time 30.1 min [ACS quaternary system, using 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]

8. EC_{50} (50% effective concentration) is the concentration of compound that inhibits virus-induced cytopathicity in HIV-infected cells by 50%, and CC_{50} (50% cytotoxic concentration) is the concentration of compound which causes a 50% reduction in cell viability. For full details see (9).

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11. See for example: Liebman, K.C.; Heidelberger, C. J. Biol. Chem **1955**, 216, 823.

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