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Certain phosphoramidate derivatives of dideoxy uridine (ddU) are active against HIV and successfully by-pass thymidine kinase

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Abstract

As part of our effort to deliver masked phosphates inside living cells we have discovered that certain phosphate triester derivatives of the inactive nucleoside analogue, dideoxy uridine (ddU) are inhibitors of HIV replication at μM levels. Moreover, we note that certain phosphoramidate derivatives retain their activity in thymidine kinase-deficient cells, which indicates that they do indeed act by intracellular release of the free nucleotide, and that they successfully by-pass the nucleoside kinase. The increased structural freedom in drug design which this allows may have implications for dealing with the emergence of resistance and may stimulate the discovery of improved therapeutic agents.

Key words: Nucleoside; Nucleotide; Anti-HIV

1. Introduction

Although nucleoside analogues, such as 3′-azidothymidine (AZT, **1**, Fig. 1) continue to dominate anti-HIV drug therapy they have a number of major limitations, such as their inherent toxicity, a dependence on kinase-mediated activation to generate the bio-active (tri)phosphate forms, and the emergence of resistance [1–2].

We [3–6] and others [7–9] have pursued a masked phosphate approach in an attempt to improve on the therapeutic potential of the parent nucleoside analogues. In this approach, inactive phosphate derivatives of the nucleoside analogue are designed to penetrate the cell membrane and liberate the bio-active nucleotides intracellularly. Masking of the phosphate group is necessary on account of the extremely poor membrane penetration by the polar (charged) free nucleotide. One mechanism by which masked phosphates may lead to enhanced selectivity of action arises from what we have termed 'kinase bypass' [3]. Thus, the complete dependence of administered (anti-HIV) nucleoside analogues on host nucleoside kinase-mediated activation places constraints upon the structures of nucleoside analogues which might be active. Nucleoside analogues which fall outside these strict constraints will be inactive, even if their 5′-triphosphates (the bio-active form) are potent and selective inhibitors of a viral target, such as reverse transcriptase (RT). Several such cases are known. Dideoxythymidine, and 3′-*O*-methylthymidine are examples of nucleoside analogues which are inactive against HIV, whilst their triphosphates are exceptionally potent inhibitors of HIV RT [10]; the inactivity of the nucleoside being attributed to poor phosphorylation by host kinases. If the masked phosphate strategy were able to deliver nucleotides intracellularly, the nucleoside kinase would be by-passed and the structural constraints such host enzymes impose would be obviated. In this way, wider structural variation of the nucleoside analogue would be permitted, and more specific (less toxic) inhibitors of viral function may arise. We have recently reported on the success of this 'kinase by-pass' strategy with several highly modified 3′-substituted nucleosides [11–12].

We now report the success of this approach with the simple nucleoside analogue dideoxy uridine (ddU, **2**). This is essentially inactive against HIV, but judicious phosphorylation leads to the introduction of a significant, selective antiviral effect. Moreover, this effect is retained in thymidine kinase-deficient cells, indicating a successful by-pass of this enzyme, and strongly supporting the suggested intracellular delivery of free nucleotides by this strategy. Whilst other researchers have recently reported the failure of the by-pass approach with phosphoramidates derived from ddu [13], we herein clearly demonstrate the success of this strategy with our previously reported aryloxy phosphoramidates [6]

2. Materials and methods

2.1. Chemistry

General synthetic procedures were similar to those we have described [5]. All nucleotides were pure by high-field multi-nuclear NMR and reverse phase High Performance Liquid Chromatography (HPLC:

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ACS system, 50 + 250 mm × 4.6 mm, Spherisorb ODS2 5 μ column, gradient elution using 5% acetonitrile in water (A), and 5% water in acetonitrile (B), with 20% B for 0–10 min, then a linear gradient to 80% B at 30 min, with a flow rate of 1 ml/min).

2.1.1. 2′,3′-dideoxy uridine (ddU, **2**).

(a) 5′-Trityl uridine. Trityl chloride (4.1 g, 14.7 mmol) was added to a solution of uridine (3 g, 12.3 mmol) in pyridine (50 ml) and the reaction mixture heated at 50°C for 24 h. The solvent was removed under high vacuum and the residue purified by chromatography on silica using 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions, followed by recrystallisation from ethanol gave the product as a white solid (5.32 g, 89%). δ_H(d_6-DMSO) 3.3(2H, dd, H5′, J = 10.8, 4.5 Hz), 4.0(1H, m, H3′), 4.15(2H, m, H2′, H4′), 5.2(1H, d, OH, J = 4.8 Hz), 5.35(1H, d, H5,J = 8.1 Hz), 5.6(1H, d, OH,J = 3.7 Hz), 5.8(1H, d, H1′, J = 3.1 Hz), 7.2–7.5(15H, m, Ar), 7.8(1H, d, H6, J = 8.1 Hz), 11.2(1H, s,NH). δ_C(d_6- DMSO) 63.24(C5′), 69.56(C3′), 73.44(C2′), 82.36(C4′), 86.45(C1′), 88.97(CPh_3), 101.47(C5); 127.18, 127.00,128.32(Ar), 140.62(C6), 143.43(Ar), 150.52(C4), 163.07(C2).

(b) 5′-Trityl-2′,3′-thiocarbonyl uridine. A solution of 5′-trityl uridine (1 g, 2.05 mmol) and thiocarbonyldiimidazole (400 mg, 2.24 mmol) in THF (20 ml) was stirred at ambient temperature for 17 h. After evaporation of the solvent under reduced pressure the residue was purified by chromatography on silica using 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product as a white solid (990 mg, 91%). δ_H ($CDCl_3$) 3.5(2H, m, H5′), 4.5(1H, m, H4′), 5.5(1H, m, H2′); 5.6–5.9(2H, m, H3′, H5), 7.2–7.6(16H, m, Ar, H6), 9.5(1H, s, NH).

(c) 2′,3′-Didehydro-2′,3′-dideoxy uridine (d4U). A solution of thiocarbonate (850 mg, 1.61 mmol) in triethylphosphite (10 ml) was heated at 150°C for 30 min. Excess triethylphosphite was evaporated under high vacuum and the residue purified by chromatography on silica using 5% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product and triethylphosphite (ratio = 60/40 by NMR). This mixture was dissolved in acetic acid and left for 17 h with stirring at ambient temperature. The solid obtained after evaporation of the solvent was purified by chromatography on silica using 8% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product (240 mg, 71%). δ_H ($CDCl_3$, CD_3OD) 3.7(2H, dd, H5′, J = 12.3, 3.0 Hz′), 4.85(1H, m, H4′), 5.6(1H, d, H5, J = 8.1 Hz), 5.8(1H, m, H2′), 6.3(1H, m, H3′), 6.9(1H, m, H1′), 7.7(1H, d,H6, J = 8.1 Hz). δ_C ($CDCl_3$, CD_3OD) 62.64(C5′), 87.42(C4′), 89.86(C1′), 101.95(C5), 125.81(C2′), 134.71(C3′), 141.29(C6), 151.01(C4), 164.25(C2).

*(d) 2′,3′-dideoxy uridine (***2***, ddU).* D4U (240 mg, 1.14 mmol) was dissolved in methanol (10 ml) and ethanol (10 ml) and 10% palladium on charcoal (200 mg) was added. This mixture was shaken under a hydrogen atmosphere for 4 h.The catalyst was removed by filtration through celite and the residue purified by chromatography on silica using 8% methanol in chloroform as eluant. Pooling and evaporation of appropriate fractions gave the product as a white solid (200 mg, 83%). δ_H ($CDCl_3$,CD_3OD) 1.9–2.1(3H, m, H2′,H3′), 2.4(1H, m, H2′), 3.8(2H, dd, H5′, J = 12.2, 2.7 Hz), 4.15(1H, m, H4′), 5.7(1H, d, H5, J = 8.1 Hz), 6.0(1H, H1′, dd, J = 6.6, 3.3 Hz) 7.9(1H, d, H6, J = 8.1 Hz); FAB MS m/e 213.0880(MH^+, 72%, $C_9H_{13}N_2O_4$ requires 213.0875), 149(32), 137(93), 129(40), 113(68), 101(100).

*2.1.2. 2′,3′-Dideoxy uridine 5′-bis[2,2,2-trichloroethyl] phosphate (***3a***).* DdU (2) (0.08 g, 0.38 mmol) and *N*-methyl imidazole (0.155 g, 1.89 mmol) were mixed in THF (10 ml) and bis(2,2,2-trichloroethyl) phosphorochloridate (0.186 g, 0.49 mmol) was added slowly at ambient temperature. After 1 h the solvent was evaporated and the residue dissolved in $CHCl_3$ (30 ml) washed with HCl (1 M; 10 ml), sodium bicarbonate solution (15 ml) and water (15 ml). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure. The crude product was purified by chromatography on silica eluting with 4% methanol in chloroform. Pooling and evaporation of appropriate fractions under reduced pressure gave the product (0.20 g, 95%). δ_H ($CDCl_3$) 2.1(3H, m, H2′, H3′), 2.45(1H, m, H2′), 4.4(2H, m, H5′), 4.55(1H, m, H4′), 4.7(4H, m, CH_2OP), 5.75(1H, d, H5, J = 8 Hz), 6.1(1H, m, H1′), 7.6(1H, d, H6, J = 8 Hz), 9.4(1H, s, NH); FAB MS m/e 552.8792(MH^+, 10%, $C_{13}H_{16}N_2O_7PCl_3$ requires 552.8826); HPLC RT = 32.6 min.

*2.1.3. 2′,3′-Dideoxy uridine 5′-(ethyl methoxyalaninyl)phosphate (***3b***).* DdU (2) (0.05 g, 0.23 mmol) and *N*-methyl imidazole (0.09 g, 1.18 mmol) were mixed in THF (10 ml) and ethyl methoxyalaninyl phosphorochloridate (0.11 g, 0.47 mmol) was added slowly at ambient temperature. After 4h the solvent was evaporated and the crude product purified entirely as described for (**3a**) above, except that a second chromatographic column was necessary, using an eluant of 15% methanol in diethyl ether, in order to obtain pure (**3b**) (0.051 g, 54%). δ_H ($CDCl_3$) 1.3(6H, m, Ala-Me, POCC), 2.0(3H, m, H2′, H3′), 2.4(1H, m, H2′), 3.70,3.71(3H, s, OMe), 3.8–4.25(7H, m, Ala-CH, Ala-NH, H4′, H5′, POC), 5.7(1H, d, H5, J = 8 Hz), 6.05(1H, m, H1′), 7.68,7.73(1H, d, H6, J = 8 Hz), 9.5(1H, bs, NH); FAB MS m/e 406.1392 (MH^+,10%, $C_{15}H_{25}N_3O_8P$ requires 406.1379), 294(100), 94(7); HPLC RT = 29.9,30.3 min.

*2.1.4. 2′,3′-dideoxy uridine 5′-(phenyl methoxyalaninyl)phosphate (***3c***).* This was prepared by a procedure which was entirely analogues to (**3a**) above, except that the reaction was stirred for 17 h, and 4% methanol in chloroform was used as chromatographic eluant. Thus, from 92 mg of ddU was isolated 178 mg (90%) of (**3c**). δ_H ($CDCl_3$) 1.32,1.33(3H, d, Ala-Me, J = 7 Hz), 1.8–2.1(3H, m, H2′,H3′), 2.3(1H, m, H2′), 3.66,3.67(3H, s, OMe), 4.0(1H, m, Ala-CH), 4.1–4.4(4H, m, H4′, H5′, Ala-NH), 5.60,5.65(1H, d, H5, J = 9 Hz), 6.0(1H, m, H1′), 7.25(5H, m, Ph), 7.60,7.61(1H, d, H6, J = 8 Hz), 10.0(1H, bs, NH); FAB MS m/e 454.1397 (MH^+,13%, $C_{19}H_{24}N_3O_8P$ requires 454.1379), 342(35), 307(14), 200(15); 154(100),136(79); HPLC RT = 34.4, 34.6 min.

2.2. Virology

2.2.1. Antiviral assays. The anti-HIV-1 activities and toxicities of compounds were assessed in two cell lines [14]. C8166 (a normal T-cell transformed by co-cultivation with leukaemia lymphocytes harbouring HTLV-1) were infected with the III-B 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 [15].

2.2.2. gp120 antigen assay. A microtiter antigen capture ELISA was developed [16] using a lectin (GNA) from Galanthus nivalis (Vector Labs., Peterborough, UK) 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, UK) 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.

3. Results and discussion

Dideoxy uridine (ddU, **2**) was prepared via three independent routes, all involving a final hydrogenation of the didehydro dideoxy compound d4U. Thus, 5′-silylation of uridine, followed by thiocarbonate formation, elimination with triethyl phosphite, and deprotection with fluoride, gave 2′,3′-dideoxy 2′,3′-didehydro uridine (d4U) [17] in an overall yield of 50%. Alternatively, a 5′-trityl protecting group could be used in this synthesis to give d4U, again via the 2′,3′-thiocarbonate, in an overall yield of 58%. Alternatively, by the procedures of Horwitz [18] 2′-deoxyuridine could be converted to its 3′,5′-dimesylate, which gave the 3′,5′-oxetane, and which could be converted to d4U on treatment with strong base. The

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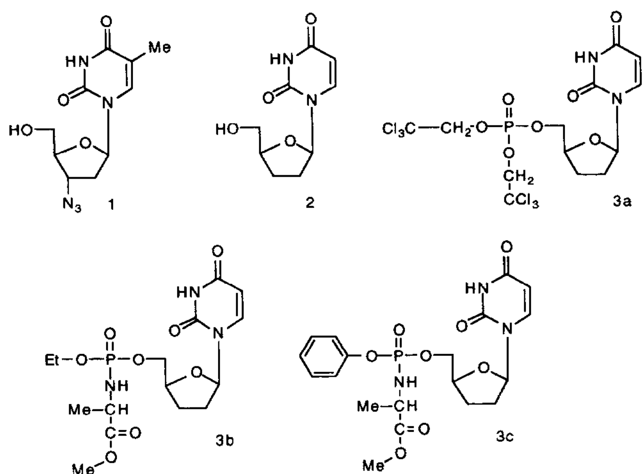

Fig. 1. The structures of potential anti-HIV nucleoside and nucleotide analogues.

overall yield by this route was 29%. Thus, in terms of yield the best route to d4U was via the 5′-trityl protected 2′,3′- thiocarbonate.

The didehydro compound d4U generated by either of these routes was hydrogenated to give ddU (**2**) in 83% yield, and the structure and purity of this compound was confirmed by ^{1}H, ^{13}C NMR, mass spectrometry and HPLC.

The nucleoside analogue was then subjected to 5′-phosphorylation with a range of phosphates. A labile phosphate blocking group is clearly a pre-requisite for the masked phosphate approach, in order to facilitate (intracellular) release of the free nucleotides. Thus, simple dialkyl phosphate derivatives of AZT are extremely resistant to phosphate hydrolysis, and they display no antiviral effect [3]. On the other hand bis(trihaloethyl) phosphate derivatives of zidovudine and of 2′,3′-dideoxycytidine (ddC) show significant lability towards hydrolysis [19], and are potent inhibitors of viral proliferation [5]. We have also noted that one of these phosphate masking groups, the bis(2,2,2-trichloroethyl) phosphate moiety is successful in the kinase by-pass activation of certain inactive 3′-modified nucleosides [11–12]. We therefore chose this phosphate as the first blocking group for ddU.

Thus, ddU was allowed to react with bis(2,2,2-trichloroethyl) phosphorochloridate in tetrahydrofuran containing *N*-methyl imidazole at room temperature to give (**3a**) in good yield. This was fully characterised by heteronuclear NMR, FAB mass spectrometry, and HPLC, all data being consistent with its structure and purity. We have also noted that certain phosphoramidate derivatives of AZT are potent and selective inhibitors of HIV [4]. Thus, ethyl methoxyalaninyl phosphorochloridate was prepared by methods we have reported [4] and was allowed to react with ddU to give the target compound (**3b**) in moderate yield. This compound was recently reported by another group, following our earlier phosphoramidate strategy [13]. However, we have found that aryloxy phosphoramidates are especially potent phosphate blocking groups for AZT, and appear to release the free nucleotides within cells, on the basis of data in thymidine kinase-deficient cells [6,20]. Thus, phenyl methoxyalaninyl phosphorochloridate was similarly prepared [20] and was allowed to react with ddU to give (**3c**) in good yield. Full carbon-13 and (where appropriate) phosphorus-31 NMR data for the nucleoside (**2**) and the phosphates (**3a-c**) are given in Table 1.

The parent nucleoside (**2**) and the corresponding masked phosphates (**3a-c**) were tested for their ability to inhibit the replication of HIV-1 in C8166 cells, and in thymidine kinase-deficient [JM] cells, data being presented in Table 2. As expected, the parent nucleoside (**2**) is active only at the highest concentrations tested, and is essentially inactive in JM cells. The bis[trichloroethyl] phosphate (**3a**) is approximately 5–10 times more active in each assay. On the other hand, the simple phosphoramidate (**3b**) is devoid of antiviral activity in this assay, as has been recently noted by other researchers in a different assay [13]. However, the aryloxy phosphoramidate (**3c**) is a potent agent, being approximately 50-times more active than the parent nucleoside analogue. This confirms the importance of data derived from assays in kinase deficient cells for the interpretation of the activities of blocked nucleotides. As we have noted [20] only

Table 1
Carbon-13 and phosphorus-31 NMR data for compounds (2) and (3a–c)

		2	3a	3b	3c
Base	C2	164.3	163.4	163.9	163.7
	C4	150.5	150.6	150.7	150.4
	C5	101.4	102.6	102.1	101.9, 102.0
	C6	140.8	139.7	140.0	139.7
Sugar	C1′	89.3	86.6	86.5, 86.3	85.9, 86.2
	C2′	24.6	25.5	25.3, 25.4	25.0, 25.2
	C3′	32.5	32.2	32.5, 32.6	32.0, 32.1
	C4′	81.8	78.6^{6}	79.4^{6}	78.8^{6}
	C5′	62.6	69.6	66.5, 66.8	66.9, 67.0
P-OR	POC	–	77.3^{a}	66.0	–
	POC<u>C</u>	–	94.7^{10}	16.2, 16.3	–
P-OAr	C1″	–	–	–	150.5
	C2″	–	–	–	119.8, 119.9^{5}
	C3″	–	–	–	129.6, 129.7
	C4″	–	–	–	125.0
P-NHR	PNC	–	–	50.0, 50.1	50.0, 50.2
	C = O	–	–	174.4, 174.3^{6}	173.8, 174.0^{8}
	OMe	–	–	52.6	52.4
	CH<u>Me</u>	–	–	21.1, 21.0^{5}	20.6, 20.7
δP		–	−3.6	7.2(d)	3.2

[a]Multiplet. All spectra were recorded in $CDCl_3$, except for (2) which were recorded in $CDCl_3$ plus CD_3OD. Data are presented as δ in ppm. All spectra were recorded using proton decoupling. In the case of carbon data phosphorus coupling constants in Hz are superscripted. Many carbon peaks for (3b–c) display diastereomeric splitting arising from mixed stereochemistry at the phosphate centre.

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Table 2
Anti-HIV1 activity of nucleoside and nucleotide analogues

Compound	C8166		JM	
	ED_{50}	CC_{50}	ED_{50}	CC_{50}
2	200	> 1,000	1,000	> 1,000
3a	40	500	80	400
3b	> 1,000	> 1,000	400	400
3c	4	400	20	500

The antiviral activity and cytotoxicity of test compounds in two different cell lines. ED_{50} represents the concentration of compound (in μM) that decreases viral antigen production in infected cells to 50% of control. CC_{50} represents the concentration of compound (in μM) which causes 50% cytotoxicity to uninfected cells.

such data will allow a clear understanding of the likely mechanism of action of blocked nucleotides, and discriminate between the release of nucleosides and nucleotides.

In conclusion, we report the antiviral activity of certain masked phosphate derivatives of the inactive nucleoside ddU. We note that aryloxy phosphoramidates are particularly efficacious, and attribute the introduction of activity to kinase by-pass. Finally we stress the importance of data in kinase-deficient cells for the clear interpretation of results on blocked phosphates.

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