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Nucleoside analogues previously found to be inactive against HIV may be activated by simple chemical phosphorylation

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Nucleoside analogues previously found to be inactive against the human immunodeficiency virus (HIV) may be activated by simple chemical derivatisation. As part of our effort to deliver masked phosphates inside living cells we have discovered that certain phosphate triester derivatives of inactive nucleoside analogues become inhibitors of HIV replication. This discovery underlies the importance of the masked phosphate approach, and has significant implications for the future design of chemotherapeutic nucleoside analogues. If highly modified nucleoside analogues may be active without the intervention of nucleoside kinase enzymes, major advantage may accrue in terms of low toxicity and enhanced selectivity. Moreover, the increased structural freedom may have implications for dealing with the emergence of resistance. The concept herein described as 'kinase bypass' may thus stimulate the discovery of a new generation of antiviral agents.

Nucleoside; Nucleotide; Anti-HIV

1. INTRODUCTION

Although nucleoside analogues, such as zidovudine (see Fig. 1, **1a**) 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]. There remains the need for potent and selective agents acting by different mechanisms and on novel targets during the process of viral replication. We herein report a recent discovery we have made regarding the antiviral activity of certain nucleotide derivatives, which may facilitate the development of new antiviral compounds.

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. As we have noted [3] such masked pro-drugs may have improved antiviral selectivity by two quite separate means.

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The most straightforward mechanism of enhanced selectivity is selective phosphate cleavage; could the phosphate blocking group be designed to allow selective hydrolysis and nucleotide release in the viral-infected cell? We have pursued this idea, with some success, by the synthesis of some phosphoramidate derivatives, with the phosphate blocked with amino acids [4]. The second 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. For example 3′-*O*-methylthymidine (**1b**), an analogue of zidovudine, is inactive against HIV, whilst its triphosphate is an exceptionally potent inhibitor of HIV RT [10]; the inactivity of the nucleoside being attributed to poor phosphorylation by host kinases. Dideoxythymidine itself is another example of a 3′-modified nucleoside which has low anti-viral activity on account of its poor phosphorylation. 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 nucle-

oside analogue would be permitted, and more specific (less toxic) inhibitors of viral function may arise.

We now report the success of this strategy. In particular, we have been able to demonstrate that judicious phosphorylation of certain inactive nucleoside analogues leads to the introduction of a significant, selective antiviral effect. Moreover, such non-AZT derivatives are fully active against several AZT-resistant strains of virus; this could be another major advantage of the by-pass strategy.

2. MATERIALS AND METHODS

2.1. *Chemistry*

General synthetic procedures were similar to those we have described [5]. Full details are given for compound (**3b**); subsequent compounds were similarly prepared and characterised. All materials were pure by high-field multi-nuclear NMR and reverse phase High Performance Liquid Chromatography (HPLC). 3′-*O*-mesylthymidine 5′-bis(2,2,2-trichloroethyl)phosphate (**3b**). A solution of methane sulphonyl chloride (0.048 g, 0.42 mmol) in dry dichloromethane (5 ml) was added to a mixture of thymidine 5′-bis(2,2,2-trichloroethyl)phosphate (**3a**) (0.19 g, 0.32 mmol) and triethylamine (0.045 g, 0.45 mmol) in dichloromethane (20 ml) with stirring at −20°C. The mixture was allowed to warm to ambient temperature, with stirring for 30 min, and was washed with saturated sodium bicarbonate solution (4 × 50 ml) and saturated brine (2 × 50 ml). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure, to yield the product as a white solid (0.11 g, 52%) m.p. 72–74°C. δ_P ($CDCl_3$) −4.8; δ_C ($CDCl_3$) 165.3 (C2), 151.2 (C4), 137.2 (C6), 111.3 (C5), 95.1 (d, CCl_3, J = 11.2 Hz), 86.3 (C1′), 82.7 (d, C4′, J = 7.6 Hz), 79.3 (C3′), 77.7(d, CH_2OP, J = 4.3 Hz), 68.2 (d, C5′, J = 6.0 Hz), 37.5 ($MeSO_2$), 37.1 (C2′), 11.8 (5-Me); δ_H ($CDCl_3$) 8.9 (1H, bs, NH), 7.3 (1H, s, H6), 6.3 (1H, dd, H1′), 5.4 (1H, m, H3′), 4.7 (4H, m, CH_2OP), 4.4 (3H, m, H4′, H5′), 3.1 (3H, s, $MeSO_2$), 2.6 (1H, m, H2′), 2.4 (1H, m, H2′), 1.9 (3H, s, 5-Me); FAB MS m/e 667 (MH_2^+, 3 × ^{37}Cl, 3%), 666 (2), 665 (7), 664 (3), 663 (8), 662 (2), 661 (MH^+, 4), 303 (8), 207 (6), 127 (11), 81 (C5H5O$^+$, 100); Found C 27.11%, H 2.92, N 3.79, P 4.29, $C_{15}H_{19}Cl_6N_2O_{10}PS \cdot [H_2O]_{0.25}$ requires C 26.99%, H 2.87, N 4.19, P 4.64; Analytical HPLC retention time 30.54 min [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].

2.2. *Virology*

Compounds were assayed for anti-HIV activity as described [11]. High titre virus stocks of the human immunodeficiency virus HIV-1_{RF} were grown in H9 cells with RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum and penicillin (100 IU /ml) and streptomycin (100 μg/ml). Cell debris was removed by low speed centrifugation, and the supernatant stored at −70°C until required. In a typical assay C8166 T-lymphoblastoid CD4+ cells were incubated with 10 TCID50 HIV-1 at 37°C for 90 min and then washed three times with phosphate-buffered saline (PBS). Cell aliquots (2×10^5) were resuspended in 1.5 ml growth medium in 6 ml tubes, and compounds at half log dilutions [200 to 0.1 μM] were added immediately. The nucleotides were sparingly soluble in aqueous solution, and 20 mM stock solutions of each compound were made up in DMSO. The final DMSO concentration in the tissue culture medium was 1%. The cells were incubated at 37°C in 5% CO_2. At 72 h post-infection 200 μl of supernatant was taken from each culture and assayed for HIV using an antigen capture ELISA [11] which recognises all of the core proteins equally (Coulter Electronics Ltd., Luton, UK). The ELISA plates were read with a spectrophotometer. The following controls were used: supernatants taken from infected and uninfected cells, infected cells treated with AZT (Roche Products UK, Ltd.) and ddC (Roche Products UK, Ltd.). The activities of AZT and ddC in infected cells gave IC_{50} values of 10–20 nM and 200 nM respectively. Compounds were tested in duplicate at each concentration and experiments were carried out on at least 2 different occasions. The same protocols were used to evaluate HIV-1_{RF} and the AZT-sensitive and resistant strains of HIV-1 in the MT2 cells. The 018/A and 105/A sensitive strains were isolated before treatment with AZT. The 018/C resistant strain was isolated 6 months after treatment [12]. The 105-F strain was isolated after 136 weeks of treatment and contained 4 mutations in the RT coding region [13]. To test for compound toxicity aliquots of 2 x 10^5 uninfected cells were cultured with the compounds at the same log dilutions for 72 h. The cells were then washed with PBSA and resuspended in 200 μl of growth medium containing ^{14}C protein hydrolysate. After 12 h the cells were harvested and the ^{14}C incorporation measured. Uninfected, untreated cells were used as controls. The compounds (**1a–4c**) showed a range of antiviral activities (IC_{50} values: Table I), whilst most were non-toxic (CC_{50} values) in this system at the highest concentration tested (100 or 200 μM).

3. RESULTS AND DISCUSSION

A labile phosphate 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 (**2a–c**) of zidovudine are extremely resistant to phosphate hydrolysis, and they display no antiviral effect [3]. On the other hand

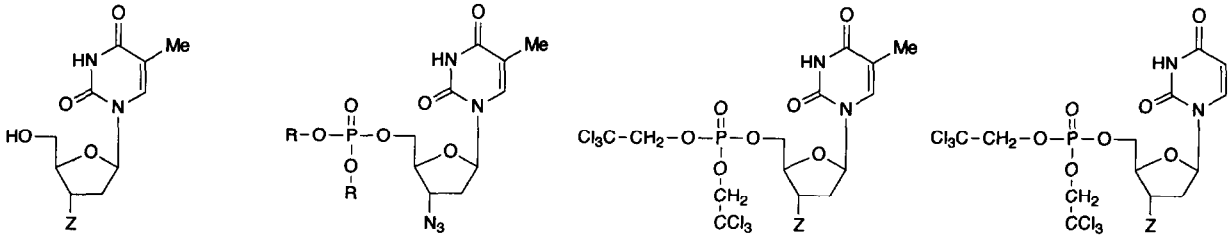

Fig. 1. The structures of potential anti-HIV nucleoside and nucleotide analogues.

bis(trihaloethyl) phosphate derivatives of zidovudine (**2d,e**) and of 2′,3′-dideoxycytidine (ddC) show significant lability towards hydrolysis [14], and are potent inhibitors of viral proliferation [5]. We now note that one of these phosphate masking groups, the 2,2,2-trichloroethyl moiety is successful in the kinase by-pass activation of certain inactive 3′-modified nucleosides.

The first 3′-modification studied was the methanesulphonyl (mesyl) group. It has been shown [10] that 3′-mesyl nucleosides have little or no anti-HIV activity, and it was of interest to probe the potential introduction of activity by suitable phosphorylation and consequential kinase by-pass. One potential interest of the mesyl group is its leaving-group ability, which could lead to irreversible modification (suicide) if such a nucleotide (or a metabolite thereof) were to bind to the active site of a (viral) enzyme. Thus, thymidine was allowed to react with bis(2,2,2-trichloroethyl) phosphorochloridate in pyridine at low temperature to give (**3a**) as we have described [5]. This was selectively mesylated at the 3′-position by treatment with mesyl chloride in dichloromethane to give target compound (**3b**) in moderate yield. This was fully characterised by heteronuclear NMR, FAB mass spectrometry, and HPLC, all data being consistent with its structure and purity. The next 3′-modification studied was the acetyl group. A particular interest here is the possibility of esterase-induced cleavage of the acetyl function [15] providing a potential de-toxification route, since it would liberate thymidine nucleotides. Much of the selectivity of the anti-cancer nucleoside analogues FU and FUDR is now attributed to a more rapid metabolic breakdown in normal cells [16]. Thus, 3′-acetyl thymidine was prepared by standard methods [17] and was allowed to react with bis(trichloroethyl) phosphorochloridate to give target compound (**3c**) in good yield.

As discussed above, the metabolic instability of the mesyl and acetyl groups could be of therapeutic interest. However, it was of value to study also stable 3′-modifications, the ether moiety being suitable. Thus, 3′-*O*-ethyl thymidine (**1e**) was prepared from thymidine in three steps by the alkylation of 5′-trityl thymidine [18]. The nucleoside analogue (**1e**) was then phosphorylated as usual to give the masked nucleotide (**3d**).

Each of the parent nucleosides (**1a**, **1c–e**) and the corresponding masked phosphates (**3b–d**) were tested for their ability to inhibit the replication of HIV-1 in C8166 T-cells, data being presented in the table. It is apparent that whilst the parent nucleosides (**1c–e**) are devoid of antiviral activity at the highest concentrations tested (100–200μM), the phosphate derivatives (**3b–d**) exert a significant anti-HIV effect at concentrations as low as 8μm. In a subsequent assay, compounds (**3b–d**) were noted to be of similar potency in a second cell line (MT2). There remains a significant antiviral effect in every case, with activity noted at non-cytotoxic concentrations. Surprisingly, there is little variation in activity with variation in the 3′-substituent, although this is a limited study; further 3′-modifications are being studied in our laboratories at the moment.

Analogues of 2′-deoxyuridine are ordinarily poorly active against HIV, this being either due to poor phosphorylation, or poor interaction of the 5′-triphosphate with viral RT, or a combination of both. It was of interest to examine the activity of masked phosphate derivatives of inactive deoxyuridine nucleoside analogues [19]. Thus, the 5′-bis(trichloroethyl) phosphate of 2′-deoxyuridine (**4a**) was prepared as described above, and was mesylated and acetylated to give (**4b**) and (**4c**), respectively. As noted in the table, these compounds are of very limited antiviral activity, in each case being significantly less active than the corresponding

Table I

Anti-HIV-1 and cytotoxic effects of nucleoside and nucleotide analogues

Cell:	C8166		MT2		MT2			MT2		
Virus:	RF		RF		Res018	Sens018		Res105F	Sens105A	
	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}	IC_{50}	Ratio	IC_{50}	IC_{50}	Ratio
Sample										
1a	0.02	500	0.04	>200	0.9	0.002	450	0.2	0.003	67
1c	>100	>100	–	–	–	–	–	–	–	–
1d	>100	>100	–	–	–	–	–	–	–	–
1e	100	>100	–	–	–	–	–	–	–	–
3b	8	>100	45	>200	110	100	1.1	80	105	0.8
3c	35	>100	30	200	55	80	0.7	50	45	1.1
3d	28	150	30	200	24	24	1	40	52	0.8
4b	>100	>100	200	>200	>200	110	>1.8	90	100	0.9
4c	>100	>100	>200	>200	>200	100	>2	100	100	1

The antiviral activity and cytotoxicity of test compounds. IC_{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 (protein hydrolysate assay).

thymidine analogue. This would suggest that subsequent phosphorylation steps from the mono- to triphosphate are inefficient in this case, or alternatively that the triphosphate is not a potent inhibitor of RT. Moreover, these data would suggest the base moiety to be important for activity, and would make it unlikely that antiviral action arises simply from the bis(trichloroethyl) phosphate group.

Each of the compounds (**1a**), (**3b–d**), and (**4b–c**) were tested for their ability to inhibit viral proliferation using a range of AZT-sensitive and AZT-resistant isolates. Whereas the IC_{50} values for AZT were increased 66–450-fold for the two resistant isolates, the activity of the phosphate derivatives (**3b–d**) was not affected by the transition. There is very clearly no cross-resistance between AZT and the kinase-bypass compounds. This is entirely as expected, since the scope of AZT resistance appears to be restricted to nucleosides carrying azide groups at the sugar 3′-position. Indeed, one major advantage of the by-pass approach may be the opportunity to utilise a wide range of different structures, in order to reduce the emergence of resistance. Although the structural variation we herein describe is limited to modifications at the 3′-position of the sugar, it appears likely that the by-pass approach may be applicable to modifications elsewhere in the sugar, or base regions.

In conclusion, we report the antiviral activity of certain masked phosphate derivatives of inactive 3′-modified nucleosides, and attribute this introduction of activity to kinase by-pass. It is quite likely that the use of alternative nucleosides and/or phosphate blocking groups may yield yet more potent anti-HIV agents, and it has not escaped our attention that this discovery may have implications in other aspects of chemotherapy. Lastly, we note that the increased structural freedom which arises from the by-pass approach may have implications for dealing with the emergence of resistant strains of viruses.

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