Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2949–2954

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

LACTATE CANNOT SUBSTITUTE FOR ALANINE IN D4T-BASED ANTI-HIV NUCLEOTIDE PRODRUGS - DESPITE EFFICIENT ESTERASE-MEDIATED HYDROLYSIS.

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Received 18 August 1998; accepted 22 September 1998

Abstract. As part of our on-going effort to deliver masked phosphates of antiviral nucleosides inside living cells we have previously discovered that amino acid-derived phosphoramidates are particularly effective. Here we report that lactate analogues, with a simple change of bridging nitrogen for oxygen, are virtually inactive as antiviral agents and apparently do not achieve intracellular nucleoside phosphate delivery.

Despite the recent introduction of HIV protease and non-nucleoside reverse transcriptase inhibitors, nucleoside analogues continue to dominate both HIV mono- and combination chemotherapy, and they show continued clinical utility against herpesviruses such as herpes simplex virus (HSV). The didehydro dideoxy nucleosides such as d4T (**1**, Figure) show particular efficacy against retroviruses, most notably HIV.[1] However, as with all nucleoside analogues, d4T has a complete, and often deleterious, dependence on intracellular metabolism to the bio-active triphosphate form. As with most nucleoside analogues, the thymidine kinase - mediated first phosphorylation step of d4T to d4T 5'-monophosphate (d4TMP) appears to be rate-limiting in a variety of cell types.[2] Given these kinetics, we[3] and others[4-5] have been keen to apply emerging phosphate pro-drug approaches to probe the feasibility to deliver d4TMP inside cells, thus by-passing the (rate-limiting) dependence of administered d4T upon thymidine kinase.

Indeed, applying a phosphoramidate based approach that we had earlier developed for AZT[6] we have reported the synthesis and evaluation of amino acid derived d4TMP pro-drugs such as **2**.[3,7] We carried out extensive Structure Activity Relationship (SAR)[8] and mechanism of action[9] studies on these materials and revealed them to display a very potent and selective antiviral action and to achieve clear and efficient intracellular phosphate delivery.[10]

In these and subsequent studies, several major observations emerged. Firstly, the generation of a partial hydrolysis product, the free amino acyl phosphoramidate (e.g. **3**), as the major intracellular metabolite,

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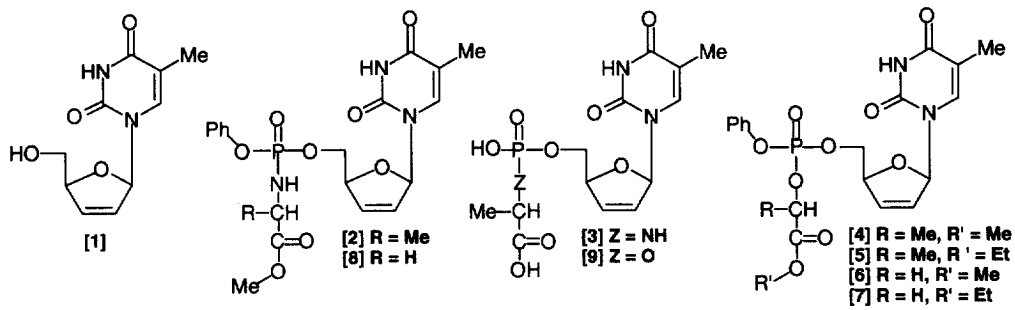
0960-894X/98/$ - see front matter
PII: S0960-894X(98)00530-7

appeared to correlate with antiviral potency.[9,10] Secondly, SAR studies revealed alanine to be particularly effective as the phosphoramidate moiety[8,11] with even rather 'similar' amino acids such as glycine and valine being considerably less potent. Finally, we recently noted[12] that the ability of carboxyl esterase to liberate the amino acyl phosphoramidate intermediates (e.g. **3**) may be a necessary condition for pro-drug action and antiviral potency. In fact, we have described a simple P-31 NMR assay to allow this test to be conducted[12] and in initial trials have found a direct correspondence between the efficiency of esterase-mediated activation and in vitro antiviral potency. Given this background we were keen to pursue close analogues of the lead pro-drugs such as **2**. In this article we report for the first time the synthesis and evaluation of lactate-based analogues **4** and **5** in which the bridging N-H is replaced by an oxygen atom. We assumed that these compounds would also be likely to be activated by carboxyl esterase to give charged phosphate intermediates such as **9** which might then serve as d4TMP prodrugs. Furthermore, we report the application of the P-31 NMR-based carboxyl esterase assay to these materials, with informative results.

Figure. Structures of some nucleosides and nucleotides
[Alanine and lactate side-chains are all L stereochemistry].



The synthetic methods we have previously developed for phosphoramidate derivatives[8] were herein successfully applied by the preparation of oxygen-based lactate analogues (e.g. **4**). Thus, reaction of phenyl dichlorophosphate with methyl-L-lactate lead to the production of the intermediate phosphorochloridate reagent, which was allowed to react with d4T (**1**) in THF containing N-methylimidazole. Isolation and purification by standard methods[8] gave the target lactate ester (**4**), in high yield.[13] As previously noted for the amino-acyl phosphoramidate analogues,[8] this material also existed as a pair of diastereoisomers, resulting from mixed stereochemistry at the phosphate centre. This was evidenced by the duplication of signals in the P-31 NMR spectrum, where two peaks were noted at ca. -5.7 and -6.2 ppm. This chemical shift is some 13 ppm further upfield than noted for the corresponding phosphoramidates, as may have been anticipated on the basis of similar compounds in the literature.[14] Peaks were also duplicated in the H-1 and C-13 NMR spectra of this compound, data otherwise being entirely consistent with the structure and purity of **4**. The mass spectrum and HPLC chromatogram of the compound also supported this structure, although interestingly the latter only revealed one peak, presumably due to the coincidence of the retention times of the two diastereoisomers.

To probe initial SARs in this lactate series, we similarly prepared a small number of closely related analogues. Thus, prepared by similar chemistry were the ethyl L-lactyl analogue **5**, and the methyl- **6**[15] and ethyl glycolate **7** compounds. It was of interest to prepare the glycolate, given their close analogy to glycine-derived phosphoramidates and the striking differences in anti-HIV activity found in this latter series for alanine (c.f. **4**) versus glycine (c.f. **6**).[11] All of the analogues (**5-7**) were tested for structure and purity as described above for **4**, and all revealed by NMR the presence of diastereoisomeric mixtures.

The parent nucleoside (**1**) and the lactate and glycolate derived pro-drugs (**4-7**) were tested for their ability to inhibit the replication of HIV-1 and HIV-2 in both thymidine kinase-competent and -deficient cell lines,[16] the data being presented in the Table. For comparison, data previously obtained on the phosphoramidate analogues (**2**) and (**8**) are included.

Table.

Anti-HIV activity and cytostatic activity of d4T and nucleotide analogues. All data are in μM.

Compound	EC_{50}	EC_{50}	EC_{50}	CC_{50}
	HIV-1	HIV-2	HIV-2	CEM
	CEM/0	CEM/0	CEM/TK$^-$	
1 (d4T)	0.25	1.2	>100	>100
4	40	50	>250	≥250
5	28	23	160	>250
6	27.5	50	>250	>250
7	12.5	12.5	150	≥250
2	0.075	0.075	0.075	>100
8	6	6	7	≥250

The major, and striking feature of these data is the virtual inactivity of the oxygen-linked analogues (**4-7**). It is particularly notable for the lactate analogue (**4**) where there is simply a replacement of the bridging NH in (**2**) by an oxygen atom, and yet about 500-fold reduction in potency, such that **4** is virtually devoid of a selective antiviral effect. This result also applies to the analogues **5-7** where the ester terminus and side-chain are varied. Thus, a clear conclusion is that O cannot substitute for NH as the phosphate bridging atom without sacrificing antiviral activity.

To probe this informative result we undertook to apply our recently described P-31 NMR based carboxyl esterase assay[12] to the lactate analogues (**4-5**). Given our previous observations with phosphoramidate analogues of **2** we anticipated that the poor antiviral action of **4-5** might correlate with impaired carboxyl esterase-mediated hydrolysis. In fact, the outcome was very diverse from this expectation. Thus, under conditions where the parent phosphoramidate (**2**) proceeded to the key intermediate (**3**) with a half-life of

about 300 hours,[17] we noted far more rapid hydrolysis of **4-5** to give the analogous (common) intermediate, **9**. The estimated half-life of **4** by P-31 NMR was 33 hours, thus 9 times faster than the analogous hydrolysis of **2** under identical conditions. For **5** the half-life was estimated to be 37 hours, thus not being significantly different from that of **4**; the change of methyl ester (**4**) to ethyl ester (**5**) appeared to have no impact on the rate of hydrolysis under these conditions. It is also notable that (**4**) and (**5**) displayed virtually identical biological profiles (Table).

No detectable hydrolysis of **4-5** to **9** took place in the absence of enzyme over the time scale of the experiment, indicating the process to be entirely esterase-mediated. Furthermore, several pieces of evidence support **9** as the structure of the hydrolysis product. Firstly, it is notable that both **4** and **5** lead to the same product. Secondly, it is clear from P-31 NMR that the chirality at the phosphate is lost; whilst both **4** and **5** give two closely spaced singlets by P-31 NMR, the product of the enzyme hydrolysis gives only one peak. This strongly supports the loss of the phenyl (phosphate) moiety, being also observed in the (much slower) esterase-mediated hydrolysis of **2**. Lastly, chemical hydrolysis of **4** gave one major product whose NMR and mass spectrometric characteristics correspond entirely to that expected for **9**.

Thus, it is clear that there is a major enhancement in the efficacy of esterase - mediated hydrolysis of **4** and **5** to **9** by comparison to the analogous hydrolysis of **2** to **3**. However, this kinetic data makes the notably poor antiviral profile of **4-5** (Table) even more striking.

We would previously[12] have regarded esterase hydrolysis to charged phosphate intermediates to be a predictive test for antiviral action. However, this must now be qualified; it may be that this is a sufficient requirement for phosphoramidates such as **2**, but it is clearly not so for lactates such as **4**. It may well be that such hydrolysis efficiency is a *necessary,* but not a *sufficient* parameter for activity.[18]

Thus, the precise reasons for the poor antiviral profile of **4-7** remain unclear. The simplest explanation consistent with the data is that **9** is generated in vitro, but, unlike **3**, is not further processed to liberate d4TMP - which we believe to be a necessary condition for antiviral potency. Instead, the further decreased activity of **4-7** in CEM/TK$^-$ cells as compared to CEM/0 cells point to a slow release of d4T (rather than d4TMP) from the intermediate (**9**). The mechanism of the intracellular conversion of **3** to d4TMP remains a matter of speculation,[19] but it may be that the putative 'phosphoramidase-type' enzyme involved may not accept **9** as a substrate, and that no efficient alternative enzyme systems exist. However, at this time we cannot exclude other possible explanations of the data. These include poor processing of **4** and **5** to **9** under the precise conditions of the in vitro assay, or poor intracellular transport of **4** and **5** or early metabolites (such as **9**) thereof. Further studies are underway in our laboratories to explore these possibilities.

Acknowledgements.

We thank the UK Medical Research Council and the Biomedical Research Programme and the Human Capital and Mobility Programme of the European Commission for financial support. We also thank the CNRS (France) and the Royal Society (UK) for support. Finally we thank Mrs A. Absillis for technical assistance and Ms H. Murphy for secretarial support.

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13. Procedure and selected data for (**4**): 2',3'-dideoxy-2',3'-didehydrothymidine-5'-phenyl (methyl lactyl) phosphate. Phenyl (methyl lactyl) phosphorochoridate (0.9 mmol, 2.0 equivs) in a 1M THF solution was added to a stirred solution of d4T [**1**] (100 mg, 0.45 mmol) and N-methylimidazole (143.5 ml, 1.8 mmol, 4 equivs) in THF (2 ml). After 6 hours, the solvent was removed under reduced pressure. The gum was dissolved in chloroform (10 ml), and washed with 1M HCl (8ml), sodium bicarbonate (10 ml) and water (15 ml). The organic phase was dried, and the solvent removed in vacuo. The residue was purified by column chromatography on silica gel with elution by chloroform-methanol (97:3). Pooling and evaporation of the eluent gave the product as a white solid. Yield 83%. δ_P -5.73, -6.15; δ_H 1.41, 1.51 (d, 3H, J=6.8Hz, lactyl Me); 1.76, 1.81 (s, 3H, 5 Me); 3.64, 3.72 (s, 3H, OMe); 4.34, 4.47 (m, 2H, H5'); 4.93 (m, 2H, H4', CH lactyl);

5.84 (m, 1H, H2'); 6.27 (m, 1H, H3'); 7.00 (m, 1H, H1'); 7.09-7.31 (m, 6H, Ph, H6); 9.14, 9.23 (br s, 1H, NH); δ_C 12.12-12.15 (5 Me); 18.71-19.11 (lactyl Me); 52.52-52.62 (OMe); 68.09-68.29 (C5'); 72.73-72.85 (CH lactyl); 84.18-84.54 (C4'); 89.39-89.43 (C1'); 111.29-111.50 (C5); 119.88-120.19 (Ar ortho); 125.49-125.68 (Ar para); 127.35-127.71 (C2'); 129.71 (Ar meta); 132.72-133.05 (C3'); 135.71-135.96 (C6); 149.73-150.11 (Ar ipso); 150.92-150.95 (C2); 163.84-163.90 (C4); 169.98-170.74 (CO lactyl); m/e FAB (NOBA matrix) $C_{20}H_{23}O_9N_2P$: 467 (MH^+, 11%); 489 (MNa^+, 100); HPLC retention time 27.72 min. HPLC conditions were as follows: ACS system 50 x 250 mm x 4.6 mm spherisorb ODS 2 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 1ml/min.

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15. Selected data for (**6**): 2',3'-dideoxy-2',3'-didehydrothymidine-5'-phenyl (methyl glycolyl) phosphate. Yield = 80%. δ_P -4.51, -5.05; δ_H 1.84, 1.86 (s, 3H, 5 Me); 3.79, 3.81 (s, 3H, OMe); 4.48-4.83 (m, 4H, H5', CH_2 glycolyl); 5.07 (m, 1H, H4'); 5.93 (m, 1H, H2'); 6.39 (m, 1H, H3'); 7.05 (m, 1H, H1'); 7.19-7.40 (m, 6H, Ph, H6); 9.46, 9.50 (br s, 1H, NH); δ_C 12.05-12.07 (5 Me); 52.41-52.45 (OMe); 63.76-63.93 (CH_2); 68.20-68.38 (C5'); 84.21-84.38 (C4'); 89.35-89.41 (C1'); 111.28 (C5); 119.82-120.00 (Ar ortho); 125.61-125.68 (Ar para); 127.42-127.49 (C2'); 129.70-129.79 (Ar meta); 132.84-132.89 (C3'); 135.81-135.88 (C6); 149.70-149.91 (Ar ipso); 150.97 (C2); 163.92-163.95 (C4); 167.69-167.91 (CO glycolyl); m/e (NOBA matrix) $C_{19}H_{21}O_9N_2P$ 453 (MH^+, 10%), 475 (MNa^+, 100).

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17. Pig liver esterase assay. Test compounds [**4-5**] (ca. 9 μMol) were dissolved in a mixture of acetone (0.1 ml) and 0.5 M pH 7.6 TRIZMA buffer (1 ml; made up in D_2O) and exposed to 10 mg of pig liver esterase (activity 15 units /mg). The mixture was maintained at 37 °C for evaluation directly by P-31 NMR at different time intervals (1 - 75 h). Where possible, reactions were followed to completion by NMR; however, kinetic data were confined to the early portion of the reaction, and were collected in all cases by 24 h. After 75 h methanol (10 ml) was added to precipitate the enzyme. The mixture was filtered and the solvent removed under reduced pressure. The crude product was examined by TLC, H-1 and P-31 NMR and mass spectrometry. For example, hydrolysis of **5**: δ_P (D_2O) 0.30; MS (ES^-) m/e 374 (**9**, M^+-2, 100%).

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