Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 10, pp. 1183-1186, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00195-3

PHOSPHORAMIDATE DERIVATIVES OF d4T WITH IMPROVED ANTI-HIV EFFICACY RETAIN FULL ACTIVITY IN THYMIDINE KINASE-DEFICIENT CELLS

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Abstract. New phosphate derivatives of the anti-HIV nucleoside analogue d4T were prepared as potential membrane-soluble pro-drugs of the bio-active free nucleotide. Some of the derivatives appear to have enhanced antiviral efficacy relative to the parent nucleoside analogue. Moreover, the derivatives appear to by-pass the dependence of the nucleoside on thymidine kinase-mediated activation, retaining *full* activity in thymidine kinase-deficient cells. The new analogues show particular promise for further pre-clinical development.

Recently, there has been much interest in 2',3'-dideoxynucleosides as inhibitors of HIV-1, the causative agent of AIDS.[1] The 2',3'-dideoxy-2',3'-didehydro analogue of thymidine (d4T) (**1**) has been noted to be a very potent inhibitor of HIV.[2] It exhibits a similar selective anti-HIV activity in vitro to that of the established anti-retrovirus agent 3'-azido-3'-deoxythymidine (AZT) (**3**) and in certain cells (e.g. bone marrow progenitor cells) displays lower cytotoxicity.[3] These, and other nucleoside analogues suffer from an absolute dependence on (host cell) kinase-mediated activation, a dependence which can lead to poor activity and /or the emergence of resistance.[4] In an effort to circumvent this dependence, we, 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 two particularly efficacious phosphate derivatives of d4T, designed to act as an intracellular source of the free d4T 5'-monophosphate (d4TMP). It is known that the kinetics of the three phosphorylation steps from the nucleoside analogue to the (bio-active) 5'-triphosphate differ in the case of d4T, by comparison to AZT and other 3'-modified nucleoside analogues. In particular, the rate-limiting step for AZT appears to be the conversion of mono- to di-phosphate, whereas the conversion of nucleoside to monophosphate may well be rate-limiting for d4T.[6] It could follow that the intracellular delivery of pre-formed d4TMP may be more useful than the delivery of AZTMP.

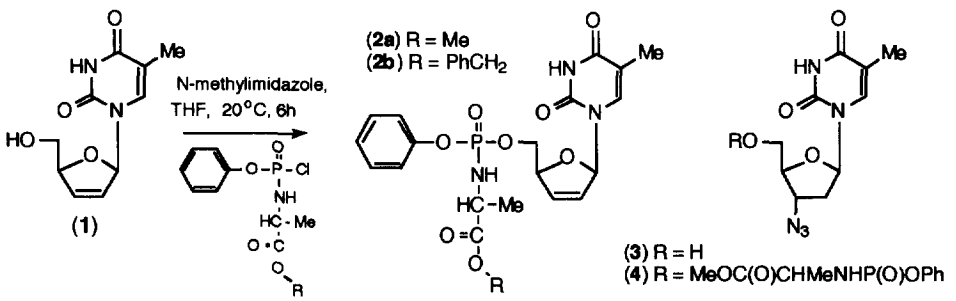
We have recently noted that simple dialkyl phosphate triesters of d4T are inactive as anti-viral agents, whereas bis(trihaloethyl) phosphates are active.[7] We have previously noted the anti-HIV activity of phosphoramidate derivatives of AZT[8] and of the 3'-fluoro analogue (FLT).[9] Thus, we were interested to apply the phosphoramidate technology to d4T.

D4T was prepared from thymidine essentially by the method of Horwitz,[10] noting the later comments

of Mansuri.[11] Then, phenyl methoxyalaninyl phosphorochloridate[12] was allowed to react with d4T using THF / N-methylimidazole, to give compound (**2a**) in good yield (88%). As anticipated,[8] this material displayed two closely spaced signals in the ^{31}P NMR (δ_P ca. 3.5),[13] corresponding to the presence of diastereoisomers, resulting from mixed stereochemistry at the phosphate centre. Similar diastereomeric splitting, and phosphorus coupling where appropriate, were also noted in the H-decoupled ^{13}C spectrum.[14] The presence of diastereoisomers was also apparent from ^{1}H NMR spectroscopy, and analytical HPLC studies on (**2a**).

Since we have also recently noted the importance of the carboxyl ester terminus for the activity of analogous phosphoramidate derivatives of AZT[15] we prepared a further d4T phosphoramidate with a benzyl ester terminus rather than the methyl; in the AZT case this was found to be the most efficacious ester group. Thus, phenyl benzylalaninyl phosphorochloridate was allowed to react with d4T as above, to give compound (**2b**) in good yield; this displayed spectroscopic and analytical data fully confirming the structure and purity, and closely resembling that of (**2a**).

Scheme



The nucleoside analogues (**1**) and (**3**), and the phosphates (**2a-b**) were tested for their ability to inhibit the replication of HIV, as previously described,[8] and the results obtained using HIV-1 or HIV-2 -infected CEM cells are displayed in the Table along with the data for the earlier AZT analogue (**4**). The clinically used nucleoside analogue AZT was included as reference material, and tests were also conducted in thymidine kinase-deficient CEM/TK$^-$ cells.

It is notable that the phosphate derivatives (**2a-b**) are approximately 2-5 fold more potent than d4T itself, (**2b**) being somewhat more potent than (**2a**). It is notable that this is significantly different to the case of AZT[8] where we noted generally reduced potency for phosphate esters such as (**4**) in the primary [TK$^+$] assay. Moreover, it is particularly striking that, whereas d4T retains only very slight activity in thymidine kinase-deficient cells, the phosphates (**2a-b**) retain virtually full activity; being ca. 300-500 fold more potent than d4T in these cells. Similarly, whilst AZT is inherently more potent than either d4T or the d4T phosphate, in thymidine kinase-competent cells, the phosphate derivative (**2b**) is ca. >1500 times more active than AZT in the kinase-deficient cell line.

Table. Anti-HIV activity for compounds [**1**], [**2a-b**], [**3**] and [**4**]. All data are in μM and represent the concentrations required to inhibit viral replication by 50% [EC_{50}][16] or the 50% cytotoxic concentration [CC_{50}][a]

Compound	Structure	EC_{50} HIV1 CEM μM	EC_{50} HIV2 CEM μM	EC_{50} HIV2 CEM-TK$^-$ μM	CC_{50} CEM μM
1	d4T	0.16±0.09	0.27±0.20	25±7.1	≥100
2a	[PhMeAla]d4T	0.085±0.025	0.102±0.023	0.075±0.007	>100
2b	[PhBzAla]d4T	0.022±0.007	0.039±0.036	0.06±0.03	52±2.5
3	AZT	0.005±0.002	0.008±0.004	>100	>100
4	[PhMeAla]AZT	0.055±0.021	0.070±0.0	12±15	172±1

a. Data represent the mean of at least 2 to 4 independent experiments.

Thus the anti-viral activity of the phosphoramidates (**2a-b**) is independent of thymidine kinase-mediated phosphorylation. The most obvious mechanism of action consistent with this observation is of intracellular delivery of the free nucleotide d4TMP by phosphoramidates (**2a-b**), and further phosphorylation to generate the active metabolite d4TTP. Further experiments are underway in our laboratories to confirm this hypothesis.

One concern regarding the phosphoramidates as potential drug candidates might be their acid instability, with a view to oral dosing. Therefore, we conducted a preliminary experiment on compound (**2a**). Storage of (**2a**) in the pH range 3-7 at 37 ^{o}C led to no change detectable by TLC after 17 hours. At pH 2 there was ca. 10% decomposition after 17 hours, and at pH 1 ca. 10% after 3 hours and ca. 40% after 17 hours. Thus, the acid lability appears to be reasonably limited for these materials. Moreover, the lipophilicity of the derivatives [**2a**, logP = 1.1; **2b**, logP = 1.4] is also rather increased, relative to d4T itself[17] indicating the possibility of enhanced membrane transport and / or brain penetration for the pro-drugs.
Each of the pro-drugs herein described (**2a-b**) was tested as a mixture of two diastereoisomers, resulting from the mixed stereochemistry at the phosphate centre; all compounds were fixed [L] stereochemistry at the amino acid. The importance of the stereochemistry at each of these chiral centres is the subject of current investigation in our laboratories.

In conclusion, the phosphate derivatives of d4T herein described show in vitro advantage over d4T itself, particularly in thymidine kinase-deficient cells. This leads to enhanced anti-viral selectivity relative to the parent drug. Such derivatives may be worthy of further study, if such *in vitro* advantage can be extrapolated to the *in vivo* situation. The precise mechanism of action of these materials remains unclear. Whilst the data in the Table clearly indicate a by-pass by (**2a-b**) of the usual dependence of (**1**) on thymidine kinase, strongly indicating intracellular nucleotide delivery, the enzymatic or chemical mechanism underlying this delivery remains to be elucidated.

Acknowledgements

We thank the AIDS Directed Programme of the MRC and the Biomedical Research Programme and Human Capital and Mobility Programme of the EC for financial support, Mr. G. O'Leary for lipophilicity measurements and Mrs. A. Absillis for excellent technical assistance.

References and Notes

1. Mitsuya, H.; Broder, S. Proc.Natl.Acad.Sci.USA **1986**, 83, 1911.
2. Balzarini, J.; Kang, G.-J.; Dalal, M.; Herdewijn, P.; De Clercq, E.; Broder, S.; Johns, D.G. Mol. Pharmacol. **1987**, 32, 162.
3. Mansuri, M.M.; Hitchcock, M.J.M.; Buroker, R.A.; Bregman, C.L.; Ghazzouli, I; Desiderio, J.V.; Starrett Jr, J.E.; Sterzycki, R.Z.; Martin, J.C. Antimicrob. Agents Chemother. **1990**, 34, 637.
4. Hao, Z.; Cooney, D.A.; Farquhar, D.; Perno, C.F.; Zhang, K.; Masood, R.; Wilson, Y.; Hartman, N.R.; Balzarini, J.; Johns, D.G. Mol. Pharmacol. **1990**, 37, 157-163; Balzarini, J.; De Clercq, E.; Herdewijn, P.; Robins, M.J. Mol. Pharmacol. **1985**, 27, 578-583.
5. McGuigan, C.; Tollerfield, S.M.; Riley, P.A. Nucleic Acids Res. **1989**, 17, 6065; Jones, B.C.N.M.; McGuigan, C.; Riley, P.A. ibid **1989**, 17, 7195; Chawla, R.R.; Freed, J.J.; Hampton, A. J. Med. Chem. **1984**, 27, 1733.
6. Balzarini, J.; Herdewijn, P.; De Clercq, E. J. Biol. Chem. **1989**, 264, 6127.
7. McGuigan, C.; Sheeka, H.M.; Mahmood, N.; Hay, A. Bioorg. Med. Chem. Lett. **1993**, 3, 1203.
8. McGuigan, C.; Pathirana, R.N.; Balzarini, J.; De Clercq, E. J. Med. Chem. **1993**, 36, 1048.
9. McGuigan, C.; Jones, B.C.N.M.; Devine, K.G.; Nicholls, S.R.; O'Connor, T.J.; Kinchington, D. Bioorg. Med. Chem. Lett. **1991**, 1, 729.
10. Horwitz, J.P.; Chua, J.; DaRooge, M.A.; Noel, M.; Klundt, I.L. J. Org. Chem. **1966**, 31, 205.
11. Mansuri, M.M.; Starrett Jr, J.E.; Ghazzouli, I; Hitchcock, M.J.M.; Sterzycki, R.Z.; Brankovan, V; Lin, T.-S.; August, E.M.; Prusoff, W.H.; Sommadossi, J.-P.; Martin, J.C. J. Med. Chem. **1989**, 32, 461.
12. McGuigan, C.; Pathirana, R.N.; Mahmood, N.; Devine, K.G.; Hay, A.J. Antiviral Res., **1992**, 17, 311.
13. Mark, V.; Dungan, C.H.; Crutchfield, M.M.; Van Wazer, J.R. *Compilation of 31P NMR data;* Grayson, M.; Griffith, E.J. Ed.; Topics in Phosphorus Chemistry, vol. 5, Wiley, New York, 1969.
14. Selected data for (**2a**): δ_P ($CDCl_3$) 3.20, 3.86; δ_H ($CDCl_3$)1.32, 1.34 (d, 3H, J=6.8Hz, CH_3 ala); 1.81, 1.84 (d, 3H, $5CH_3$); 3.69, 3.70 (s, 3H, OMe); 3.84-4.00 (m, 2H, CH-ala, NH-ala); 4.32 (m, 2H, H5'); 5.02 (m, 1H, H4'); 5.88 (m, 1H, H2'); 6.33 (m, 1H, H3'); 7.03 (m, 1H, H1'); 7.15-7.35 (m, 6H, Ar + H6); 9.22, 9.26 (bs, 1H, NH); δ_C ($CDCl_3$)12.52 ($5CH_3$); 21.02 (CH_3 ala); 50.22-50.35 (CH ala); 52.74 (OMe); 66.62-67.29 (C5'); 84.80-84.88 (C4'); 89.69-89.93 (C1'); 111.44-111.57 (C5); 120.13-120.31 (Ar ortho); 125.30 (Ar para); 127.49-127.65 (C2'); 129.87-129.93 (Ar meta); 133.19-133.50 (C3'); 135.77-136.06 (C6); 150.51 (Ar ipso); 151.16 (C2); 164.14 (C4); 174.12 (CO ala); FAB MS m/e: 466 (MH^+,7%); 340 (MH^+-base); 200 (17); 136 (47); 89 (25); 81 (C_5H_5O, 100); HPLC RT = 22.48, 22.87 min [ACS quaternary system, using a Techsphere ODS 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 1 ml/min and detection by UV at 254 nm].
15. McGuigan, C.; Pathirana, R.N.; Choi, S.S.-M.; Kinchington, D.; O'Connor, T.J. Antiviral Chem. Chemother. **1993**, 4, 97.
16. EC_{50} is the concentration of drug in μM required to reduce virus-induced cytopathicity by 50%; CC_{50} is the concentration in μM which inhibits the proliferation of uninfected cells by 50%.
17. Balzarini, J.; Cools, M.; De Clercq, E. Biochem. Biophys. Res. Commun. **1989**, 158, 413.

(*Received in Belgium* 5 *February* 1996; *accepted* 22 *April* 1996)