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INCREASE OF THE ANTI-HIV ACTIVITY OF D4T IN HUMAN T-CELL CULTURE BY THE USE OF THE SATE PRONUCLEOTIDE APPROACH

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Abstract. The bis(*S*-acetyl-2-thioethyl) phosphotriester derivative of 2',3'-didehydro-2',3'-dideoxythymidine has been synthesized and evaluated for its inhibitory effects on the replication of HIV-1 in several cell culture systems. This pronucleotide showed potent anti-HIV activity and proved to be significantly superior to the parent nucleoside with regard to the antiviral efficiency.

In the search for effective agents against human immunodeficiency virus (HIV), a large number of nucleoside analogues have emerged as efficient drugs.[1] Their biological activity is strictly dependent on their conversion, through cellular enzymes, to the corresponding 5'-triphosphates which interact with HIV-associated reverse transcriptase. Of the three metabolic steps of nucleoside analogues, the monophosphorylation-step, which involves cellular nucleoside kinases or 5'-nucleotidases, is generally considered as being the most restrictive, and this may explain the absence or the low anti-HIV activity of some of them.[2] Moreover, the presence and activity of the intracellular enzymes necessary for the monophosphorylation of nucleoside analogues is highly dependent on the host species, the cell type, and the stage in the cell cycle.[3] The dependence on phosphorylation for activation of a nucleoside analogue may therefore be a problem in cells where the activity of phosphorylating enzymes is known to be low or even lacking.[3] Many strategies have previously been envisaged to mask or to reduce the phosphate negative charges of nucleoside 5'-monophosphate analogues with neutral substituents, thereby forming more lipophilic derivatives (pronucleotides) which would be expected to revert back to the corresponding 5'-mononucleotides once inside the cell.[4,5] In this respect, our group has recently reported the study of neutral mononucleoside phosphotriesters which incorporate the *S*-acyl-2-thioethyl (SATE) group as enzyme-labile phosphate protection.[6,7] Using 3'-azido-2',3'-dideoxythymidine (AZT) as a model, we have demonstrated that AZT bis(SATE) phosphotriester derivatives were able to liberate the parent 5'-monophosphate (AZTMP) inside the cell through a carboxylate esterase-mediated activation process.[7]

Having shown the validity of such an approach using the SATE group as a transient phosphate protection for AZTMP, we decided to extend the investigation of this group to another well-established anti-HIV drug, namely 2',3'-didehydro-2',3'-dideoxythymidine [d4T, Stavudine, Zerit^{R}] (Figure).

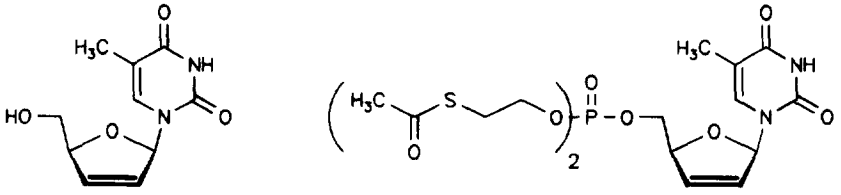

Figure. Structure of d4T and its corresponding bis(MeSATE) phosphotriester derivative

D4T has recently been approved for the treatment of advanced HIV-infected patients not responding to other approved treatments. Its selective anti-HIV activity is comparable to that of AZT *in vitro*.[8,9] Moreover, d4T has been found to be less toxic than AZT for bone marrow stem cells[9,10] and to be less inhibitory to mitochondrial DNA replication.[11] However, this dideoxynucleoside analogue is converted to its 5'-monophosphate much less efficiently than AZT in CEM and MT-4 cells.[12,13] In an effort to overcome this restriction, the bis(*S*-acetyl-2-thioethyl) phosphotriester derivative of d4T [bis(MeSATE)d4TMP, Figure] has been synthesized, and evaluated for its inhibitory effects on the replication of HIV-1 in human T4-lymphoblastoid cell lines and human primary lymphocytes.[14]

The bis(MeSATE)d4TMP has been prepared following a P(III) method using the appropriate phosphoramidite derivative.[7] The target compound was characterized by UV, high-field multinuclear NMR spectroscopy, FAB mass spectrometry, and HPLC analysis, all these data being consistent with its structure and purity.[15] The bis(MeSATE)d4TMP and its nucleoside parent have been evaluated for their inhibitory effects on the replication of HIV-1 in various cell culture systems (Table).[16]

	MT-4		CEM-SS		CEM/TK$^-$		PBM	
	$EC_{50}{}^{a}$	$CC_{50}{}^{b}$	$EC_{50}{}^{a}$	$CC_{50}{}^{b}$	$EC_{50}{}^{a}$	$CC_{50}{}^{b}$	$EC_{50}{}^{a}$	$CC_{50}{}^{b}$
bis(MeSATE)d4TMP	0.016 ± 0.009	>100 (19%)^{c}	0.006 ± 0.003	68 ± 37	0.012 ± 0.006	60 ± 22	0.007 ± 0.002	22 ± 11
d4T	0.28 ± 0.08	>100 (10%)^{c}	0.059 ± 0.016	>100 (21%)^{c}	10 ± 10	>100 (6%)^{c}	0.050 ± 0.030	42 ± 9

Table. Antiviral activity of the bis(MeSATE) phosphotriester derivative of d4T compared to its nucleoside parent in various T cells infected with HIV-1.[17]

The neutral mononucleoside phosphotriester derivative proved to be between 10 and 17 fold more effective than d4T in inhibiting HIV-1 replication in wild-type [thymidine kinase positive (TK+)] MT-4, CEM and PBM cells. Moreover, as expected, d4T proved to be weakly active against HIV-1 replication in thymidine kinase-deficient (TK^-) CEM cells with a 50% effective concentration (EC_{50}) value at 10 μM. In contrast, in CEM/TK^- cells, the corresponding bis(MeSATE) phosphotriester emerged as a potent inhibitor with an EC_{50} value at 0.012 μM which was in the same range as the EC_{50} values observed for this compound in TK+ cell lines. The slight decrease observed in the 50% cytotoxic concentration (CC_{50}) values for the bis(MeSATE)d4TMP as compared to the parent nucleoside may be related to the intracellular accumulation of the phosphorylated forms of d4T which could also interact with cellular enzymes. In this regard, the introduction of the SATE group as carboxylate esterase-labile transient phosphate protection of 5'-mononucleotide does not induce any additive toxicity (manuscript in preparation).

The use of pronucleotides, by-passing the specific first kinase step and thus giving rise to the intracellular 5'-mononucleotide delivery, can have numerous consequences on the biological activity of the parent nucleoside. Previously, we have applied this approach to circumvent the degradative metabolism pathway of 2',3'-dideoxyadenosine.[18,19] The corresponding bis(MeSATE) phosphotriester derivative emerged as a very potent anti-HIV inhibitor in cell culture, more potent than AZT in the cell lines studied. When applied to d4T, which is hampered at the first phosphorylation step, the SATE pronucleotide approach leads to an enhanced *in vitro* antiviral efficiency.

This strategy opens a wide field of studies in chemotherapy and could be of great help in the design of new anti-HIV agents. Work along these lines is currently in progress in our group.

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15. Selected physicochemical data for 2',3'-didehydro-2',3'-dideoxythymidin-5'-yl bis(*S*-acetyl-2-thioethyl) phosphate: UV (ethanol) λ_{max} 261 nm (ε 8400), 218 nm (sh, ε 12300), 205 nm (ε 14000), λ_{min} 246 nm (ε 7000), ^{1}H-NMR (Me_2SO-d_6) δ ppm 11.3 (br s, 1H, NH-3), 7.24 (d, 1H, H-6; J= 1.0 Hz); 6.82 (m, 1H, H-1'), 6.41 (m, 1H, H-3', vinyl), 6.01 (m, 1H, H-2', vinyl), 4.95 (m, 1H, H-4'), 4.16 (m, 2H, H-5',5''), 4.01 (m, 4H, CH_2O), 3.10 (t, 4H, SCH_2, J= 6.4 Hz), 2.34 and 2.33 (2s, 3H each, CH_3CO), 1.75 (d, 3H, CH_3-5, J= 0.5 Hz); ^{31}P-NMR (Me_2SO-d_6) δ ppm -0.65, mass spectrum (FAB>0, matrix: glycerol-thioglycerol, 1:1, v/v) 509 $(M+H)^+$, 383 $(S)^+$, 127 $(BH_2)^+$; (FAB<0, same matrix) 507 $(M-H)^-$, 125 $(B)^-$; HPLC retention time 34.3 min [Waters system, using a Hypersil C_{18} column (100×4.6 mm, 3μm) under isocratic conditions (ammonium acetate 0.1M pH 5.9) during 10 min, following by a linear gradient of the same eluent to 50% acetonitrile programmed over a 40 min period, with a flow rate of 1ml/min and detection at 260 nm].

16. The broad antiviral assays in cell culture were performed following previously established procedures as described in reference 7.

17. Values are means ± standard error of the mean of data obtained from at least three separate experiments. [a] EC_{50}, 50% effective concentration (in μM) or concentration required to inhibit the replication of HIV-1 by 50%. [b] CC_{50}, 50% cytotoxic concentration (in μM) or concentration required to reduce the viability of uninfected cells by 50%. [c] Percent reduction of viable cells at the indicated highest concentration tested (100 μM).

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