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RATIONAL DESIGN FOR CYTOSOLIC DELIVERY OF NUCLEOSIDE MONOPHOSPHATES : "SATE" AND "DTE" AS ENZYME-LABILE TRANSIENT PHOSPHATE PROTECTING GROUPS

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Abstract. It was demonstrated that the use of neutral 2',3'-dideoxyuridine phosphotriesters which incorporate enzyme mediated bioreversible protection such as S-acetylthioethanol (SATE) or dithiodiethanol (DTE) resulted in intracellular delivery of the parent mononucleotide. This point was corroborated by observation of an anti-HIV effect in various cell lines and decomposition data in cell extracts.

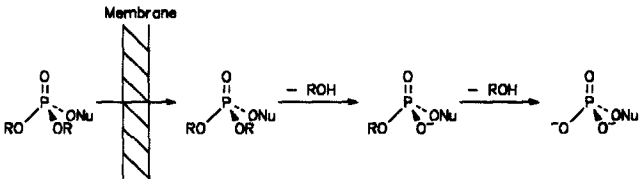
It is well established that most of the nucleoside (Nu) analogues are dependent on kinase-mediated activation to generate the bio-active triphosphate forms (NuTP).[1] Activation takes place in the cytosol after Nu uptake and involves three successive viral and/or cellular kinases, the first one being highly specific.[2-6] Nucleoside monophosphates (NuMP) themselves cannot be used as potential chemotherapeutic agents to overcome the requirement for such a first kinase as, due to their polar nature, they are not able to cross the cell membrane efficiently.[7-9] Moreover, they are readily dephosphorylated on cell surfaces and in extracellular fluids by non-specific phosphohydrolases.[10,11] Hence, the idea of temporarily masking or reducing the phosphate negative charges with neutral substituents, thereby forming more lipophilic derivatives which would be expected to revert back to the NuMP once inside the cell.

The cytosolic NuMP delivery problem has been adressed by numerous laboratories over the course of about 30 years[12-15] and the various strategies reported to date have involved dinucleoside phosphodiesters[16-18] and phosphotriesters[18-23] as well as mononucleoside phosphodiesters[24,25] or phosphotriesters.[26-30]

Considering the case of a mononucleoside phosphotriester, it must exhibit higher stability in the culture medium than inside the cell, both environments being in the same neutral pH range. However, it must undergo selective cytosolic hydrolysis. In this respect, mononucleoside phosphotriesters incorporating enzymatically bioreversible protection with acyloxymethyl groups have been introduced by Farquhar,[31,32] such transitory protecting groups being preferentially removed by intracellular carboxyesterases with concomitant elimination of formaldehyde .[32,33]

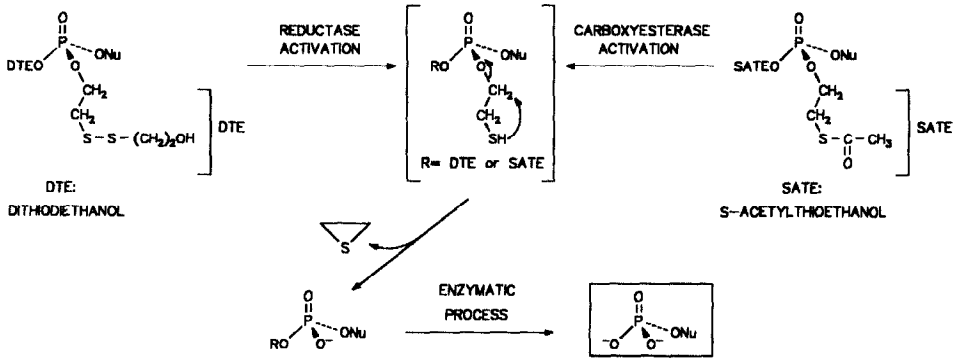
In this paper, we would like to present a rationale for NuMP delivery involving transient formation of unstable mononucleoside thioethanol phosphotriesters through the use of specific enzyme mediated bioreversible protections.

When considering a neutral mononucleoside phosphotriester, the main problem to be solved for NuMP delivery is to have - after uptake - selective cytosolic hydrolysis of the compound to the corresponding mononucleoside phosphodiester and then its conversion to NuMP (scheme I).



Scheme I

In the absence of any reported cellular phosphotriesterase activity inside cells, the only possibility of solving such a dual selectivity problem is to consider a differential in enzyme distribution between the culture medium (serum complemented) and the cell interior. Under such conditions, an unstable phosphotriester may be selectively formed inside the cells which will spontaneously decompose to the corresponding phosphodiester. In a second step, the expected NuMP may be formed through either phosphodiesterase hydrolysis or through the same enzymatic activation process as for the first step. At this point, it should be noted that due to possible hydrolysis of the starting phosphotriester in culture medium, either by nucleophilic attack on the α carbon or by enzyme action (as added FCS may not be completely inactivated), we selected - as a model - an inactive nucleoside, namely 2',3'-dideoxyuridine (ddU), the corresponding triphosphate of which is,"in vitro", a potent inhibitor of HIV reverse transcriptase.[6] In addition, we have shown in cell cultures that delivery of ddUMP by liposomes results in an anti-HIV activity.[34] Bis(POM) ddUMP, previously described,[32] fulfils the aforementioned criteria as demonstrated further by its decomposition pattern, involving the formation of an unstable phosphotriester after carboxyesterase intracellular activation.



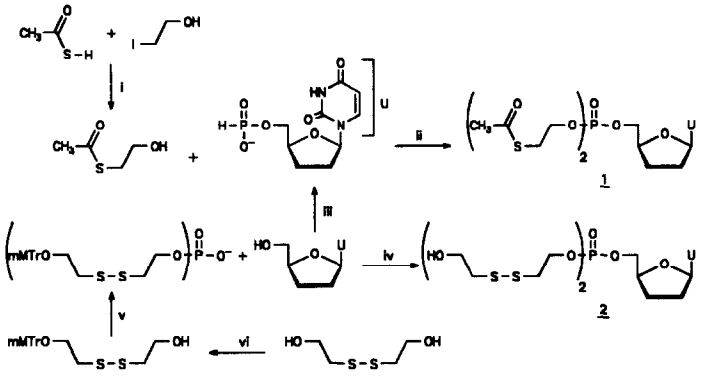
Scheme II

An alternative strategy for ddUMP delivery may be envisaged, but based on formation of a transient thioethanol phosphotriester which is known[35] to spontaneously decompose to the corresponding

phosphodiester (scheme II). Such a transformation involves rapid elimination of ethylene sulfide [Eckstein's DNA/RNA phosphorothioate sequencing method[36]] due to an internal nucleophilic attack of the thiol group on the α carbon atom which ensures the selectivity.

In order to obtain stable phosphotriester precursors, we propose to temporarily protect the nucleophilic thiol function through acylation or disulfide bridge formation, expecting preferential carboxyesterase or reductase activation respectively into cytosol.

Therefore, two ddU phosphotriesters 1 and 2 (scheme III) were synthesized with respectively SATE (S-acetylthioethanol) and DTE (dithiodiethanol) bioreversible protecting groups. Two synthetic route have been studied. The phosphotriester 1 has been obtained by reaction of the nucleoside 5'-hydrogenphosphonate[37] with 2-acetylthioethanol[38] upon pivaloyl chloride activation. The synthesis of the phosphotriester 2[30] has involved the preparation of the dithiodiethanol phosphodiester and then the condensation with the free nucleoside. The target compounds (1, 2) were characterised by UV, heteronuclear NMR, FAB mass spectrometry, and HPLC, all data being consistent with their structure and purity.[39]



Reagents and conditions
i, DBU, $C_6H_5CH_3$; ii, $(CH_3)_3CCOCl$, C_5H_5N, then $I_2/C_5H_5N/H_2O$; iii, H_3PO_3, $(CH_3)_3CCOCl$, C_5H_5N; iv, MSNT, C_5H_5N, then CH_3COOH, CH_3OH/H_2O; v, $POCl_3$, imidazole, C_5H_5N; vi, mMTrCl, $[(CH_3)_2CH]_2NC_2H_5/CH_2Cl_2$.

Scheme III

The anti-HIV activity[40] of 1 and 2 has been investigated in two human T cell lines, one of them being deficient in thymidine kinase activity (CEM TK$^-$).

	CEM-SS		CEM TK$^-$	
	EC_{50}[a]	CC_{50}[b]	EC_{50}[a]	CC_{50}[b]
bis(SATE) ddUMP (1)	$5\ 10^{-6}$	$9\ 10^{-4}$	$4\ 10^{-6}$	$7\ 10^{-5}$
bis(DTE) ddUMP (2)	$6\ 10^{-5}$	$> 10^{-4}$ (41%)[c]	$8\ 10^{-6}$	$8\ 10^{-5}$
ddU	$> 10^{-4}$ (18%)[d]	$> 10^{-4}$ (41%)[c]	$> 10^{-4}$ (0%)[d]	$> 10^{-4}$ (18%)[c]

Table I. Antiviral activity of the phosphotriester derivatives 1, 2 and their nucleoside units ddU in two cell lines infected with HIV-1.

As shown in table I,[41] these two new derivatives induce an anti-HIV effect in both CEM cell lines which may indicate cytosolic ddUMP delivery, since under the same conditions ddU and ddUMP are inactive.

Furthermore, decomposition studies[42] (Table II) in cell culture medium and in the total CEM cell extracts fully corroborated this point and unambiguously showed that the starting phosphotriesters **1** and **2** are much more readily transformed to the corresponding phosphodiesters in the cell extracts than in the culture medium. Note that even in the culture medium, the parent phosphotriesters **1** and **2** are slowly hydrolyzed, which justifies the necessity to use an inactive nucleoside such ddU as a model.[43] In addition, it was ascertained that further enzymatic decomposition of the phosphodiesters leads to the expected NuMP (data not shown).

	Culture medium	CEM cell extract
bis(SATE) ddUMP (**1**)	8.9 h	5 min <
bis(DTE) ddUMP (**2**)	29.6 h	26 min

Table II. Half-life ($t_{1/2}$) of phosphotriester transformation into the corresponding mononucleoside phosphodiester at a concentration of 5 10^{-5} M and 37°C in culture medium (RPMI + 10% heat-inactivated FCS) and in a total CEM cell extract.

In conclusion, the present results demonstrate that nucleoside phosphotriesters which incorporate enzyme mediated bioreversible protection with new groups such as SATE and DTE allow the intracellular delivery of their parent monophosphate.

Further, we would like to point out that by varying the acyl portion of the SATE group or the nature of the substitution linked to the S-S bridge, it should be possible to control the rate of generation of the bioactive NuMP, and works on this topic are currently in progress in our laboratory.

This strategy for circumventing metabolic dependency on the first nucleoside kinase opens a wide field of application in nucleoside chemotherapy. It should thus be possible to overcome the acquired resistance caused by the loss or depletion of the first kinase and/or to modify the bioavailability and the metabolic pathway of any nucleoside drug in order to increase its efficacy.

Acknowledgements

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39. Selected physicochemical data for O,O'-*bis*(S-acetyl-2-thioethyl)-O"-(2',3'-dideoxyuridin-5'-yl)phosphate (1): UV (ethanol) λ_{max} 262 nm (ε, 9 400), λ_{min} 230 nm (ε, 2 500); ^{1}H-NMR (Me_2SO-*d6*) δ ppm 11.3 (br s, 1H, NH-3), 7.64 (d, 1H, H-6; $J_{5,6}$= 8.1 Hz), 6.01 (dd, 1H, H-1'; J= 4.2 and 7.0 Hz), 5.60 (d, 1H, H-5; $J_{5,6}$= 8.1 Hz), 4.26-4.00 (m, 7H, H-4',5',5" and 2 S-CH_2-*CH_2*-O-P), 3.13 (t, 4H, 2 S-*CH_2*-CH_2-O-P; J= 6.4 Hz), 2.36 and 2.35 (2 s, 3H each, *CH_3*CO), 2.40-2.30 (m, 1H, H-2'), 2.13-1.73 (m, 3H, H-2",3',3"); ^{31}P-NMR (Me_2SO-*d6*) - 1.21 ppm; mass spectrum (FAB>0, matrix: glycerol) 497 $(M+H)^+$, 395 $(M - CH_3\text{-}CO\text{-}S\text{-}CH_2\text{-}CH_2 + 2H)^+$; HPLC retention time 13.97 min [Waters system, using an Hypersil C18 column (150×4.6 mm, 5μm particle size) in isocratic conditions (ammonium acetate 0.1 M, pH 5.9/acetonitrile, 75/25, v/v) with a flow rate of 1.0 ml/min and detection by UV at 260 nm].

Selected physicochemical data for O,O'-*bis*[S-(2-hydroxyethylsulfidyl)-2-thioethyl]-O"-(2',3'-dideoxyuridin-5'-yl)phosphate (2): UV (ethanol) λ_{max} 261 nm (ε, 9 900), λ_{min} 231 nm (ε, 3 100); ^{1}H-NMR (Me_2SO-*d6*) δ ppm 11.3 (br s, 1H, NH-3), 7.64 (d, 1H, H-6; $J_{5,6}$= 8.0 Hz), 6.00 (dd, 1H, H-1'; J= 4.1 and 7.9 Hz), 5.60 (d, 1H, H-5; $J_{5,6}$= 8.1 Hz), 4.89 (t, 2H, 2 *HO*-CH_2-CH_2-S; J= 4.9 Hz), 4.30-4.05 (m, 7H, H-4',5',5" and 2 S-CH_2-*CH_2*-O-P), 3.61 (m, 4H, 2 HO-*CH_2*-CH_2-S), 3.00 (t, 4H, 2 S-*CH_2*-CH_2-O-P; J= 6.3 Hz), 2.40-2.33 (m, 1H, H-2'), 2.10-1.90 (m, 2H, H-2",3'), 1.90-1.63 (m, 1H, H-3"); ^{31}P-NMR (Me_2SO-*d6*) - 0.88 ppm; mass spectrum (FAB>0, matrix: glycerol-thioglycerol, 1:1, v/v) 565 $(M+H)^+$, 489 $(M - S\text{-}CH_2\text{-}CH_2OH + 2H)^+$, 429 $(M - CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH_2OH + 2H)^+$.
40. The broad antiviral assays on cell culture were performed by following previously established procedures as described in reference 30.
41. [a] 50% effective molar concentration or molar concentration required to inhibit the replication of HIV by 50%; [b] 50% cytotoxic molar concentration or molar concentration required to reduced the viability of the cells by 50%; [c] percent reduction of viable cells at the indicated highest concentration tested; [d] percent inhibition of HIV replication at the indicated highest concentration tested.
42. The method for determination of kinetics of decomposition of nucleotide prodrugs has recently been described in reference 33.
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