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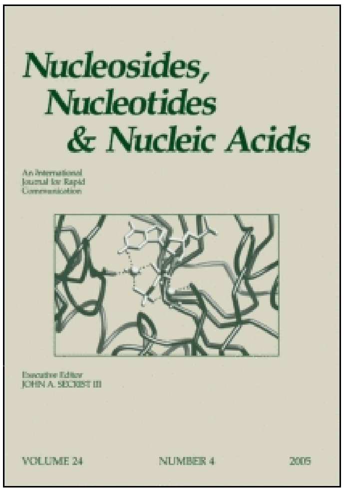

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Phosphate-Modified Nucleotides: Conversions in Human Serum

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PHOSPHATE-MODIFIED NUCLEOTIDES : CONVERSIONS IN HUMAN SERUM

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Abstract: Mechanisms and rates of hydrolytic dephosphorylation of 5'-hydrogenphosphonates, 5'-phosphorofluoridates, and 5'-phosphates of natural and 3'-substituted thymidines in human serum were studied. The stability of 5'-phosphonates of 2'-deoxy- and 2',3'-dideoxyadenosines in calf and human sera was found.

Phosphate-modified nucleotides seem to be promising prodrugs of bioactive nucleoside 5'-triphosphates. Different phosphotriesters, namely *bis*-pivaloyloxymethyl[1], *bis*-2,2,2-trihaloethyl[2], *bis*-S-substituted thioethyl[3] diesters of anti-HIV nucleotides were shown to penetrate into the cell and be hydrolyzed to the corresponding monophosphates. This was confirmed by HPLC analysis of incubation products in serum-containing medium or in cell extracts and by observation of anti-HIV effect of such compounds in thymidine kinase-deficient cells[4]. It was also reported that *bis*-S-acetylthioethyl diester of 2',3'-dideoxyadenosine 5'-phosphate was not deaminated in human T-cell culture and showed significant anti-HIV activity[5].

We studied the enzymatic dephosphorylation of several phosphate-modified pyrimidine and purine nucleotides, which were synthesized earlier in our laboratory and revealed strong inhibitory activity against HIV (Table 1).

As can be seen in Table 1, some of the compounds, namely FpAZT, HpddA, HpddA(3'-N_3), are more active than the corresponding nucleosides. This evidence could imply that during their metabolism in extracellular medium or inside cells these compounds bypass one of the anabolic steps.

Earlier the hydrolysis of 5'-hydrogenphosphonates of AZT and thymidine by *E.coli* alkaline, calf intestine, and human placental phosphatases has been examined[6]. It was shown that nucleoside 5'-hydrogenphosphonates are not hydrolyzed by any of the mentioned phosphatases after incubation for 5 h. Under similar conditions

Table 1. Anti-HIV activity and cytotoxicity of the examined nucleotide analogues

Compound	R	X	B	EC_{50},* µM	CC_{50},* µM	SI*	Cell culture	Ref.
FpAZT	F	N_3	Thy	0.1	>100	>1000	H9	7
				<0.001	578	>578000	PBL	7
HpAZT	H	N_3	Thy	0.072	2500	34700	MT-4	8
AZT				0.23	>100	>435	H9	7
				0.0047	>1000	>212766	PBL	7
				0.005	154	30800	MT-4	8
FpFLT	F	F	Thy	0.3	>100	>330	H9	7
				0.004	630	157500	PBL	7
HpFLT	H	F	Thy	0.088	>4170	>56810	MT-4	8
FLT				0.2	>100	>500	H9	7
				0.002	775	387500	PBL	7
				0.0069	190	27530	MT-4	8
HpddA	H	H	Ade	0.24	>1000	>4160	MT-4	9
HOOC-pddA	HOOC	H	Ade	>100			MT-4	6**
ddA				5.04	1490	330	MT-4	9
CH_3pdA	CH_3	OH	Ade	>100			MT-4	6**
HpdA	H	OH	Ade	--				6
HpddA-(3'-N_3)	H	N_3	Ade	0.3	104	347	MT-4	10
ddA(3'-N_3)				3.6	57	16	MT-4	10

*EC_{50} - 50% effective concentration in inhibiting HIV-1 reproduction; CC_{50} - 50% inhibitory concentration of cell growth; SI - the selectivity index (CC_{50}/EC_{50} ratio);
**- data of Dr. B.Polsky.

thymidine 5'-phosphate was hydrolyzed completely in 10-15 min. It has been reported that HpAZT was converted to pAZT in human U937 cell culture, but the

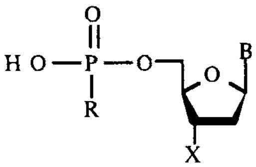

Table 2. Half dephosphorylation times for examined compounds in human serum

FpAZT	FpFLT	HpAZT	HpFLT	pAZT	pFLT	pThd
65 min	70 min	33 h	60 h	35 min	40 min	20 min

mechanism of this process is not clarified[11]. Recently the hydrolysis of HpAZT to AZT in CEM cell extract and culture medium containing 10% heat-inactivated calf fetal serum has been demonstrated[12].

We have also studied dephosphorylation of hydrogenphosphonates and phosphorofluoridates of AZT and FLT in human serum. Compounds HpAZT, HpFLT, FpAZT, and FpFLT at two concentrations (0.5 mM and 2.5 mM) were incubated with human serum at 37^{0}C. Thereafter, serum proteins were precipitated by adding methanol up to 70%. After 30 min at -20^{0}C samples were centrifuged and supernatants were evaporated in vacuo to remove methanol. Residues were dissolved in water and analyzed by RP HPLC[13]. Based on the HPLC analysis data we determined the hydrolysis rates and half times of dephosphorylation for the examined compounds (Table 2).

The data presented in Table 2 deserve the following comments. Dephosphorylation rate for the examined compounds is little affected by the 3'-substituent. The rate of dephosphorylation of 5'-hydrogenphosphonates reaches only 1-3% of that for the corresponding 5'-phosphates. After incubation of HpAZT and HpFLT we did not find any traces of pAZT and pFLT. Nucleosides were the only products of hydrolysis. On the other hand, we revealed the formation of pFLT, as well as FLT, from FpFLT, the rates of pFLT formation and its hydrolysis to FLT being of the same order of magnitude. The situation is similar for FpAZT.

Compounds HpddA, HOOCpddA, CH_3pddA, and HpdA at 2-5 mM were incubated in 10% human or calf fetal sera at 37^{0}C or 20^{0}C up to 5 days. The products of incubation were tested by HPTLC[14]. The examined compounds were stable under incubation conditions. 2'-Deoxyadenosine and its 5'-phosphate were used as controls. Under the similar conditions they were converted to 2'-deoxyinosine and then to hypoxanthine during 4 hours.

We may conclude that i) 5'-hydrogenphosphonates of 3'-substituted thymidines are dephosphorylated in human serum in 50-100 times slower than the corresponding 5'-phosphates and can serve as nucleoside prodrugs. ii) FpAZT and FpFLT are hydrolyzed to the corresponding 5'-phosphates and then to nucleosides. The first step of this process is twofold slower than the second one. The rates of cell penetration, defluorination and dephosphorylation in human serum may define which of the two metabolic courses will be taken mainly. As a result this compounds can serve both as nucleotide and nucleoside prodrugs. iii) 5'-Phosphonates of 2',3'-dideoxyadenosine and 2'-deoxyadenosine are stable in calf and human sera during several days and their anti-HIV activity has to be explained probably by other metabolic pathways.

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13. HPLC was carried out using Nucleosil 100C18 column (4x150 mm) with a linear gradient of methanol (0-35% during 25 min, 35% for 5 min) in 0.05 M KH_2PO_4 (pH 6.8). The flow rate was 0.5 ml/min.The retention time was 23.6 min (HpAZT), 20.7 min (HpFLT), 16.8 min (HpThd), 21.4 min (FpAZT), 18.6 min (FpFLT), 18.6 min (FpThd), 14.2 min (pAZT), 12.5 min (pFLT), 8.6 min (pThd), 25.7 min (AZT), 22.9 min (FLT), 17.8 min (Thd).
14. Analysis was carried out on Merck Kieselgel $60F_{254}$ HPTLC plates in the following systems (v/v): dioxane:H_2O:acetic acid, 90:10:0.2 (A); dioxane: NH_4OH, 9:1 (B); 2-propanol:ethanol:NH_4OH, 4:5:1 (C).R_f in system A, dA, 0.72; dI, 0.68; Hx, 0.57; in system B, dA, 0.35, dI, 0.27, Hx, 0.04; in system C, Hx, 0.44.