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The Effect of 4-Deoxypyridoxine Phosphate on the Activity of Thymidylate Synthase in T Lymphocytes

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INTRODUCTION

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the *de novo* synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP) and N^5,N^{10}-methylene tetrahydrofolate. TS has been studied intensively, and much is known about the structure, function and inhibition of the enzyme (1). Since high TS activitiy is found in rapidly proliferating tissues (due to increased *de novo* synthesis of dTMP), it has been a target enzyme in cancer chemotherapy (2). Although most inhibitors of TS are analogues of its substrates, they have deficiencies as potential chemotherapeutic agents. In order to gain access across cellular membranes, nucleotides must be provided as the corresponding bases or nucleosides and, therefore, must undergo appropriate metabolic activation. N^5,N^{10}-methylene tetrahydrofolate analogues often require conversion to polyglutamated forms in order to obtain potent inhibition. Specificity is also an important issue since folate analogues frequently inhibit other enzymes, which utilize folate cofactors. Thus, it would be desirable to have TS inhibitors, structurally unrelated to TS substrates. Pyridoxal-5'-phosphate is the active form of pyridoxine (vitamin B_6) and is used as an enzyme cofactor in many reactions. Among them is the one catalyzed by serine hydroxymethyltransferase (SHMT). This enzyme supplies thymidylate synthase with N^5, N^{10}-methylene tetrahydrofolate. 4-deoxypyridoxine (4-dB_6) is a B_6 analogue that causes severe B_6 deficiency upon its administritation in rats or mices and, therefore, impairs immunoresponses. It has been proposed that this impairment is due to the inhibitory effect of 4-dB_6 on the activity of SHMT (3). It has also been shown by others that B_6 analogues bind to TS active site *in vitro* and that this binding results in decrease of TS activity (4,5).

AIM

In the present study, we addressed the issue of whether 4-deoxypyridoxine binds TS *in vivo* and if such a binding results in enzyme inhibition. Our results show that 4-dB_6 addition in human T lymphocyte cultures effectively inhibits TS activity, DNA synthesis and cell proliferation.

MATERIALS AND METHODS

Human lymphocytes (10^6 cells/ml) were cultured for 72 hours in the presence or absence of 4-deoxypyridoxine (6). Cultured lymphocytes, resuspended in phosphate buffer saline, were used for all subsequent measurements. The assay for TS catalytic activity was based on published methods (7) with slight modification (8). The reaction mixture was incubated for 60 min at 37°C. Enzyme activity was expressed in pmoles of product formed/h/mg protein. 4-deoxypyridoxine was added to lymphocyte extracts at concentrations of 0.5 and 2.5 mM and TS activity was measured either immediately or 30 min later. The assay of ^{3}H-methyl-thymidine incorporation was performed using well-established methods. All data were analyzed by the Student's t-test.

RESULTS AND DISCUSSION

We investigated the effect of 4-dB$_6$ on thymidylate synthase activity of mitogen-stimulated T lymphocytes. 4-dB$_6$ was added in lymphocyte cultures at different concentrations and time intervals. Enzyme activity measurements were performed in cell extracts. It was concluded that in the presence of 4-dB$_6$, TS activity is decreased up to 30% and cell proliferation is inhibited up to 50% (Table 1).

Table 1.
Effect of 4-dB$_6$ on lymphocytes stimulation, DNA synthesis, DNA content and TS activity

Preincubation condition	Stimulated lymphocytes (%)	^{3}H-methyl-Trd incorporation (cpm/10^5 cells)	μgDNA/10^6 cells	TS activity	
				(pmoles/h/ mg prot.)	Inhibition (%)
w/o PHA	3 ± 1*	100 ± 23*	4.1 ± 0.3*	4.3 ± 0.4*	-
PHA	80 ± 4	22350 ± 1150	11.3 ± 0.9	102.1± 5.6	0
PHA+4-dB$_6$ 0.25mM	72 ± 4	21700 ± 970	8.3 ± 0.6	97.8 ± 4.8	4
PHA+4-dB$_6$ 0.5mM	58 ± 5	15190 ± 805	7.1 ± 0.5	81.9 ± 4.1	20
PHA+4-dB$_6$ 2.5mM	54 ± 2	12850 ± 911	6.6 ±0.7	76.1 ± 3.7	25
PHA+4-dB$_6$ 5.0mM	50 ± 3	11850 ± 725	5.8 ± 0.5	72.2 ± 3.9	30

[^{3}H]-thymidine incorporation experiments have shown that DNA synthesis is significantly reduced in the presence of 4-dB$_6$ (up to 49%). Our results (Table 1) clearly show that the effect of 4-dB$_6$ on nucleic acids biosynthesis or cell replication is dose dependent. n order to investigate whether 4-dB$_6$ itself or the major product of its metabolism, e.g. 4-deoxypyridoxine phosphate (4-dPNP), affects TS, we measured TS activity in lymphocyte extracts supplemented with 4-dB$_6$. In these experiments, measurement of enzyme activity started either immediately after the addition of 4-dB$_6$ or 30 min later (to ensure for adequate 4-dPNP production by cytosolic pyridoxal kinase). Our results indicated clearly that enzyme activity was significantly decreased (up to 40%) only after 4-dPNP production (Figure 1). The results obtained in this study demonstrate for the first time that deprivation of vitamin B$_6$ (by supplying the culture with its antagonist 4-dB$_6$) appreciably inhibits TS activity and DNA synthesis. We are convinced that 4-deoxypyridoxine (and possibly its phosphorylated derivative) can be tested for its pharmacological effect in cases where restraint of immune system is demanded.

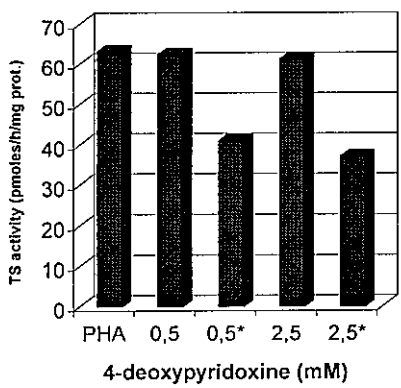

*Figure 1. Inhibition of TS activity by 4-deoxypyridoxine phosphate (4-dPNP). Control sample: PHA induced TS activity in a 72-hour culture. * Addition of 4-dB$_6$ 30 min later. Data represent the mean of 5 experiments, each one having triplicate determinations for each enzyme suspension condition.*

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