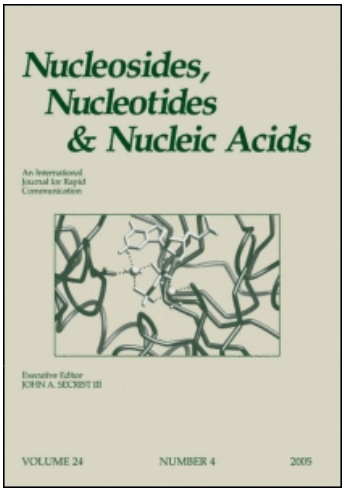

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Inhibitory Properties of Nucleotides with Difluoromethylenephosphonic Acid as a Phosphate Mimic versus Calf Spleen Purine Nucleoside Phosphorylase and Effect of These Analogues on the Viability of Human Blood Lymphocytes

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INHIBITORY PROPERTIES OF NUCLEOTIDES WITH DIFLUOROMETHYLENEPHOSPHONIC ACID AS A PHOSPHATE MIMIC VERSUS CALF SPLEEN PURINE NUCLEOSIDE PHOSPHORYLASE AND EFFECT OF THESE ANALOGUES ON THE VIABILITY OF HUMAN BLOOD LYMPHOCYTES

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□ *Several cyclic and acyclic 6-keto purine nucleotides with difluoromethylenephosphonic acid as phosphate mimic are proved to be potent inhibitors of mammalian purine nucleoside phosphorylase (PNP). Antiproliferative activity of these analogues on the growth of human blood lymphocytes was tested by MTT assay. Compared to inhibitory effects on the growth of human blood T-lymphocytes isolated from healthy donors, all analogues significantly slow down proliferation of T-lymphocytes isolated from patients with autoimmune thyroid disease—Hashimoto's thyroiditis.*

Keywords PNP; difluoromethylenephosphonic acid; difluoromethylenephosphonic acids; Hashimoto's thyroiditis

INTRODUCTION

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1.), the key enzyme of the purine salvage pathway, catalyzes the reversible phosphorolytic cleavage of the glycosidic bond of purine nucleosides. It is crucial for the integrity of the immune system since PNP deficiency in humans leads to defective T-cell response. Therefore, inhibitors of PNP may be useful in the treatment

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of some autoimmune diseases, other T-cell proliferative disorders and T-cell cancers. Since the genetic deficiency of PNP has been discovered in 1971, great efforts have been made to design potent membrane-permeable inhibitors of PNP but none has yet been approved as a drug. One of the reasons for this is that many potent inhibitors are phosphates, i.e. charged molecules and do not readily penetrate cell membranes.[1] We have synthesized several cyclic and one acyclic 6-keto purine nucleotides with difluoromethylenephosphonic acid as a phosphate mimic. Such analogues are expected to better mimic purine nucleotide phosphates and also better penetrate cell membranes.[2] Properties of two such analogues as PNP inhibitors, (±)-cis-1,1-difluoro-2-(tetrahydro-3-piranyl)ethylphosphonic acid with a (hypoxanthine-9-yl) methyl aglycone (Yokomatsu compound, (±)-cis-piranyl) and 9-(5,5-difluoro-5-phosphonopentyl)guanine (Danzin compound, DFPP-G), studied by kinetic methods, fluorescence titrations[3] and x-ray crystallography[4] have been reported. Here we describe properties as PNP inhibitors of another two compounds from the series of stereoisomers of (±)-(1,1-difluoro-2-(teterahydro-3-furanyl)ethylphosphonic acids with a (hypoxanthine-9-yl)methyl aglycone ((±)-cis-furanyl and (±)-trans-furanyl) and inhibitory effects of all four compounds on the growth of human blood lymphocytes derived from healthy donors and from patients affected by autoimmune thyroid disease—Hashimoto's thyroiditis (autoimmune lymphocytes).

MATERIALS AND METHODS

Yokomatsu compound, that is, (±)-cis-piranyl, and its two analogues (±)-cis-furanyl and (±)-trans-furanyl, as well as DFPP-G were synthesized as described.[5,6]

Properties of all analogues as inhibitors of mammalian (calf spleen) PNP were determined as described in our previous articles.[7,8] Inhibition constants were determined at 25°C and in 50 mM HEPES pH 7.0 with 7-methylguanosine as variable substrate and 1 mM phosphate as a cosubstrate. 7-Methylguanosine was chosen because phosphorolysis of this nucleoside substrate in contrast to that of natural substrates—inosine and guanosine—shows only small deviations from the Michaelis-Menten kinetics.[7]

Inhibitory effects of all analogues on the growth of human blood T-lymphocytes derived from 10 healthy donors (control) and from 10 patients affected by Hashimoto's thyroiditis (autoimmune lymphocytes) were investigated. Cells were treated with inhibitors (concentration range applied were 10^{-7} M–10^{-4} M) for 3 days and the effects on cell growth were determined by the tetrazolium (MTT) assay.[9]

TABLE 1 Inhibitory potency of compounds investigated in this study as calf spleen PNP inhibitors with intracellular (1 mM) phosphate concentration. Comparison with available literature data of inhibitory potency with human erythrocyte PNP; and antiproliferative potency of the analogues against the autoimmune lymphocytes from Hashimoto's thyroiditis as compared to the lymphocytes from healthy donors

Compound	K_i^{app} [nM] vs calf PNP at 1 mM phosphate[a]	IC_{50} [nM] vs human PNP[b] phosphate		K_i^{app} [nM] vs human PNP[b] phosphate		Inhibition[c] [%] of lymphocytes form Hashimoto healthy thyroiditis donors	
		100 mM	1 mM	100 mM	1 mM		
DFPP-G	5.0 ± 1.3 (0.5)	380	32.5	53.0	17.5	26	7
(±)-cis-piranyl	66 ± 13 (8.9)	38	8.7	26.9	3.5	30	15
(±)-trans-furanyl	52.1 ± 11.9 (5.4)	320	ND	ND	ND	42	12
(±)-cis-furanyl	43.0 ± 12.1 (5.5)	88	ND	15.2	ND	70	11

[a]With 7-methylguanosine as variable substrate. Mean from means from Dixon plot, LB plot and MM plot. In brackets standard error, ± standard deviation.

[b]With inosine as variable substrate, from Yokomatsu et al.[6]

[c]Analogue concentration 10^{-4} M.

ND = not determined.

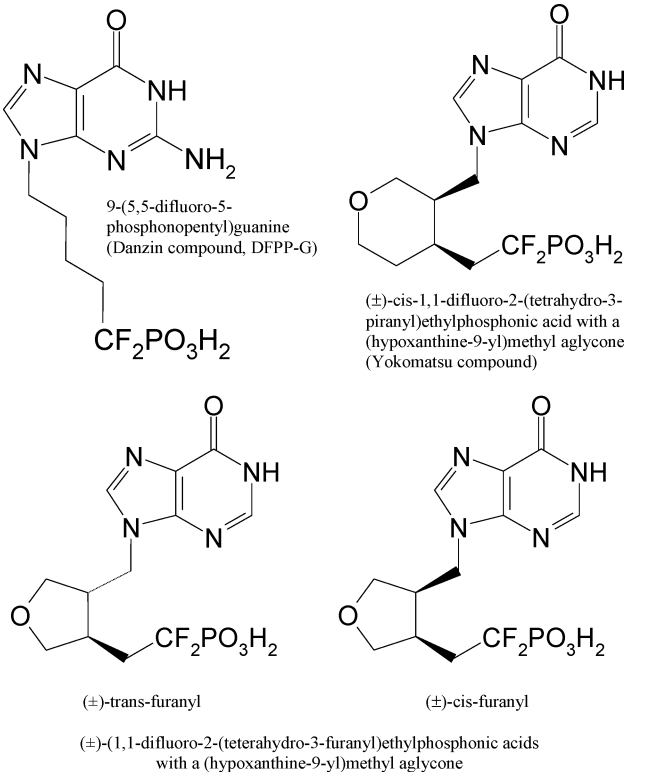

FIGURE 1 Chemical structure of compounds tested in this study as inhibitors of mammalian purine nucleoside phosphorylase and as inhibitors of proliferation of lymphocytes isolated from patients with autoimmune thyroid disease—Hashimoto's thyroiditis.

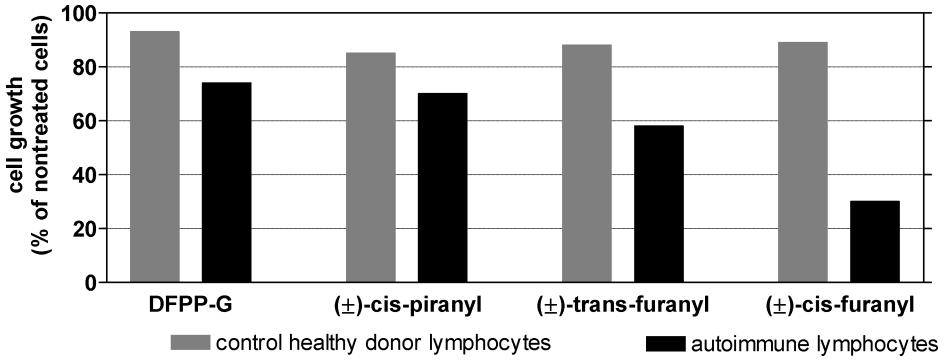

FIGURE 2 Cytotoxicity study (MTT assay) on human blood T-lymphocytes derived form healthy donor (control healthy lymphocytes; %) and blood T-lymphocytes derived from subjects affected by Hashimoto's thyroiditis (autoimmune lymphocytes; %). Cells were treated with 10^{-4} M of (±)-cis-pirany; (±)-cis-furanyl; (±)-trans-furanyl analogues, DFPP-G; see Figure 1 for their chemical structures.

RESULTS AND DISCUSSION

The chemical structures of the investigated compounds are shown in Figure 1. Their inhibitory potency with mammalian (calf spleen PNP) at orthophosphate concentration 1 mM, (i.e, corresponding to the typical concentration inside human cells) are shown in Table 1. These data are compared with inhibitory properties with human PNP.[6]

All compounds also were tested as inhibitors of proliferation of T-lymphocytes isolated from healthy subjects and patients with autoimmune thyroid disease—Hashimoto's thyroiditis. All four analogues showed increased antiproliferative potency against the autoimmune lymphocytes as compared to the controls: (±)-cis-furanyl 70% versus 11%, (±)-trans-furanyl 42% versus 12%, (±)-cis-piranyl 30% versus 15% and DFPP-G 26% versus 7% of inhibition, in the concentration of 10^{-4} M (Figure 2).

These finding testify to the potential use of cyclic and acyclic 6-keto purine nucleotides with difluoromethylenephosphonic acid mimicking phosphate as membrane permeable PNP inhibitors and immunosuppressive agents. They also show, that potential medical effect is not directly correlated with any inhibitory characteristic against mammalian PNP, although low inhibition constant (but not IC_{50}) with natural PNP substrate—inosine determined versus human erythrocyte enzyme seems to be the best indicator of biological potential of the tested compound.

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