

# Antiproliferative Activity of Purine Nucleoside Phosphorylase Multisubstrate Analogue Inhibitors Containing Difluoromethylene Phosphonic Acid against Leukaemia and Lymphoma Cells

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**Potent inhibitors of purine nucleoside phosphorylase (PNP) are expected to act as selective agents against T-cell tumours. Five compounds with guanine, three with hypoxanthine, and five with 9-deazaguanine, all connected by a linker with difluoromethylene phosphonic acid, were studied on their inhibitory potential against human and calf PNPs. Antiproliferative activity of these analogues against lymphocytes as well as lymphoma and leukaemia cells has been also investigated. All tested compounds act as multisubstrate analogue inhibitors of PNP with the apparent inhibition constants in the range 5–100 nM, and also show a slight antiproliferative activity. Analogues with 9-deazaguanine aglycone have better anti-leukaemic and anti-lymphoma activities compared to the guanine and hypoxanthine analogues, and applied in the concentration of 100 μM, caused a statistically significant decrease in the cell viability in all human leukaemia and lymphoma cells used. Despite the high PNP inhibitory potential of tested analogues, no differences were observed between the effects on the growth of tumour cells sensible to the inhibition of PNP, such as human adult T-cell leukaemia and lymphoma cells, and other investigated cells. Obtained poor effects on cell proliferation could be explained probably by a poor ability of tested compounds to penetrate cell membranes.**

**Key words:** 9-deazaguanine analogues, antitumour activity, guanine analogues, hypoxanthine analogues, PNP inhibitors

**Abbreviations:** m<sup>7</sup>Guo, 7-methylguanosine; HEPES, (N[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); K<sub>i</sub><sup>app</sup>, apparent inhibition constant; PNP, purine nucleoside phosphorylase; MTT assay, the methyltetrazolium assay; Ly, human lymphocytes; K562, chronic myeloid leukaemia in blasts crisis; JURKAT and MOLT, T-cell leukaemia; RAJL, Burkett's lymphoma; HL-60, acute myeloid leukaemia; CCRF-CEM, acute T-cell lymphoblastic leukaemia; HuT78, T-cell lymphoma; DFPP-G, 9-(5', 5'-difluoro-5'-phosphonopentyl)guanine, **1**; γ-met-DFPP-G, 9-(2'-methyl-5',5'-difluoro-5'-phosphonopentyl)guanine, **2**; γ-prop-DFPP-G, 9-(2'-propyl-5',5'-difluoro-5'-phosphonopentyl)guanine, **3**; γ-benz-DFPP-G, 9-(2'-benzyl-5',5'-difluoro-5'-phosphonopentyl)guanine, **4**; γ-isobut-DFPP-G, 9-(2'-isobutyl-5',5'-difluoro-5'-phosphonopentyl)guanine, **5**; (±)-cis-piranyl, (±)-cis-1,1-difluoro-2-(tetrahydro-3-piranyl)ethylphosphonic acid with (hypoxanthine-9-yl)methyl aglycone, **6**; (±)-trans-furanyl, (±)-trans-(1,1-difluoro-2-(tetrahydro-3-furanyl)ethylphosphonic acid with (hypoxanthine-9-yl)methyl aglycone, **7**; (±)-cis-furanyl, (±)-cis-(1,1-difluoro-2-(tetrahydro-3-furanyl)ethylphosphonic acid with (hypoxanthine-9-yl)methyl aglycone, **8**; DFPP-DG, 9-(5',5'-difluoro-5-phosphonopentyl)-9-deazaguanine, **9**; nor-DFPP-DG, 9-(5',5'-difluoro-5'-phosphonobutyl)-9-deazaguanine, **10**; homo-DFPP-DG, 9-(5',5'-difluoro-5'-phosphonoheptyl)-9-deazaguanine, **11**; 6C-DFPP-DG, 9-(5',5'-difluoro-5'-phosphonoheptyl)-9-deazaguanine, **12**; 7C-DFPP-DG, 9-(5',5'-difluoro-5'-phosphonoheptyl)-9-deazaguanine, **13**; 9-DG, 9-deazaguanine, **14**.

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Purine nucleoside phosphorylase (PNP, E.C.2.4.2.1) is the key enzyme of the purine salvage pathway of mammalian cells, for which inosine, guanosine and 2'-deoxyguanosine derived mainly from ribonucleotide and deoxyribonucleotide hydrolysis, respectively, are its main substrates (1,2).

As T-cells rely heavily on PNP activity to maintain their functions and are particularly sensitive to PNP deficiency, which is attributed to a relatively high level of kinase and low level of nucleotidase activity compared to other cells (3), PNP has been recognised as a potential target for novel drugs designed for treatment of type IV autoimmune disorders, organ transplant rejection, T-cell lymphoma, and T-cell leukaemia (4–9). In addition, PNP inhibitors can also be used to avoid cleavage of anticancer and antiviral drugs because many of these drugs mimic natural purine nucleosides and can thereby be cleaved by PNP before accomplishing their therapeutic role (10–13).

The best PNP inhibitors reported to date belong to two classes. These are either transition-state analogues, such as forodesine

(immucillin), which bear features of the proposed PNP transition state (7), or multisubstrate analogue inhibitors interacting with both the nucleoside- and phosphate-binding sites of the enzyme (1). Immucillins show a  $pK_a$  for imminoribitol (the pentose analogue) protonation at neutral pH ( $pK = 6.9$ ) and easily enter cells (7). By contrast, multisubstrate analogue inhibitors are anions, or even a mixture of mono- and di-anions at neutral pH, and as charged molecules, do not readily penetrate cell membranes. They also have short plasma lifetimes, because of susceptibility to phosphatases. Hence, they are not promising candidates for effective *in vivo* drugs. This was the reason why the synthesis of some mimics, analogues of guanine, hypoxanthine, and 9-deazaguanine, connected by a linker to difluoromethylene phosphonic acid, was undertaken (14–21). In this study, five compounds with guanine moiety, three with hypoxanthine moiety, and six compounds with 9-deazaguanine moiety were tested on their PNP inhibitory potential on calf spleen and human erythrocyte PNPs *in vitro*. In addition, their cytotoxic activity against human lymphocytes and a panel of leukaemia and lymphoma cells was examined.

## Materials and Methods

### Chemicals

The synthesis of the inhibitors, analogues of 9-(5',5'-difluoro-5'-phosphonopentyl)-guanine and 9-(5',5'-difluoro-5'-phosphonopentyl)-9-deazaguanine, was described elsewhere (19–21). Calf spleen PNP (about 30 units/mg) was obtained from Sigma and used without further purification, except that sulphate and phosphate present in the commercial preparation were carefully removed as previously described (22).

Concentration of the substrate (7-methylguanosine) and all inhibitors was determined spectrophotometrically. For  $m^7\text{Guo}$  at pH 7.0 (i.e. for the mixture of cationic and zwitterionic forms of  $m^7\text{Guo}$ ), the extinction coefficient (260 nm) is  $8\,500\text{ M}^{-1}/\text{cm}^{-1}$  (23), while for all inhibitors with guanine base, the extinction coefficient for guanosine was used in calculations, (253 nm) =  $13\,650\text{ M}^{-1}/\text{cm}^{-1}$  (24). Extinction coefficients for other inhibitors were used from our published data (19–21).

### Preparation of tested compounds

To prepare 10-mM stock solution, nor-DFPP-DG (**10**), DFPP-DG (**9**) and homo-DFPP-DG (**11**) were dissolved in dimethylsulphoxide (Sigma Chem. Co., St. Louis, MO, USA) and heated for 5 min at 80 °C. 6C-DFPP-DG (**12**) was dissolved in dimethylsulphoxide; DFPP-G (**1**), ( $\pm$ )-cis-piranyl (**6**), ( $\pm$ )-cis-furanyl (**8**), and ( $\pm$ )-trans-furanyl (**7**) were dissolved in a highly pure water;  $\gamma$ -met-DFPP-G (**2**) and  $\gamma$ -isobut-DFPP-G (**5**) in methanol; and  $\gamma$ -benz-DFPP-G (**4**) and  $\gamma$ -prop-DFPP-G (**3**) were dissolved in 10% dimethylsulphoxide.

### Inhibition studies

Inhibition constants were determined at 25 °C and in 50 mM Hepes/NaOH pH 7.0 buffer with 7-methylguanosine as a variable substrate and 1 mM orthophosphate as a co-substrate. 7-Methylguanosine ( $m^7\text{Guo}$ ) was chosen because phosphorolysis of this

nucleoside substrate shows only small deviations from the Michaelis–Menten kinetics (22). For some inhibitors, competitions versus phosphate was also studied, and in those cases phosphate was a variable substrate and concentration of  $m^7\text{Guo}$  was kept constant (350  $\mu\text{M}$ ). Direct spectrophotometric assay was used to measure the reaction velocity. Observation was made at 260 nm, with  $\Delta\epsilon = 4\,600\text{ M}^{-1}/\text{cm}^{-1}$  (23).

Standard initial rate procedures were employed in kinetic studies. The initial velocities were measured directly from the computer controlling the spectrophotometer. Linear regression program was used for the determination of the slopes, with their standard errors, of absorbance versus time. At least four initial substrate concentrations and typically four or five different inhibitor concentrations (including no inhibitor added) were analysed. Therefore, the typical data set consisted of initial velocity values measured for 20 or 25 combinations of  $c_0$  and  $I$ . The measurements were taken at least twice for each pair ( $c_0$ ,  $I$ ).

Inhibition constants were determined by fitting kinetic equations for competitive type inhibition model to the whole data sets using the weighted least-squares non-linear regression implemented in the program Leonora (25).

### Cell culture

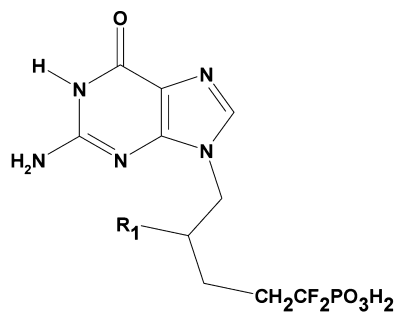
Cell lines: chronic myeloid leukaemia in blasts crisis (K562), T-cell leukaemia (JURKAT and MOLT), Burkett's lymphoma (RAJI), and acute myeloid leukaemia (HL-60) were obtained from ATCC, USA. Acute T-cell lymphoblastic leukaemia (CCRF-CEM) and T-cell lymphoma (HuT78) were purchased from ECACC, Health Protection Agency, Salsbury, UK. Human lymphocytes are isolated from heparinised blood of 10 healthy donors (Ly) by a standard method of density-gradient centrifugation over Ficoll-Hypaque reagents according to manufacturer's recommendations.

Cells were grown in RPMI-1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% FBS, streptomycin (100  $\mu\text{g}/\text{mL}$ ), and penicillin G (100 U/mL). Cells were cultured in a humidified (95% air, 5%  $\text{CO}_2$ )  $\text{CO}_2$  incubator (Shell Lab, Sheldon Manufacturing, Cornelius, OR, USA) at 37 °C. Cell viability was assessed by the trypan blue dye exclusion method.

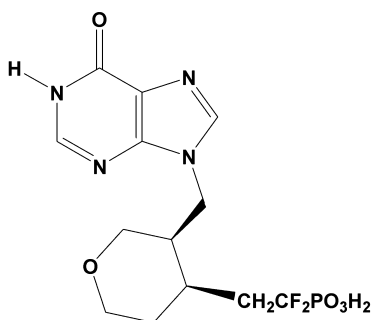
### Cytotoxicity test

Cytotoxic effects on the tumour cell growth were determined using the methyltetrazolium (MTT) assay (26). At day zero of the experiment, tumour cells,  $1 \times 10^5$  cells/mL, were plated onto 96-microwell plates (Costar, Cambridge, MA, USA) and allowed to attach overnight in a  $\text{CO}_2$  incubator (Shell Lab, Sheldon Manufacturing). After 24 h, the medium was replaced with a fresh medium containing various well-defined concentrations of investigated compounds. All working dilutions (0.1–100  $\mu\text{M}$ ) were prepared immediately before each experiment in a phosphate-buffered saline (Sigma Chem. Co.).

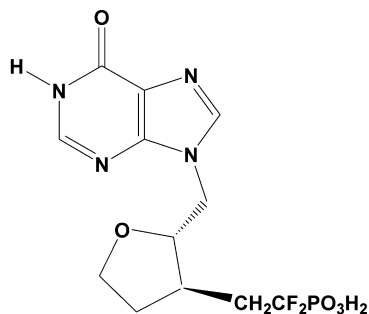
Control cells were grown under the same experimental conditions without addition of tested compounds. The analogous



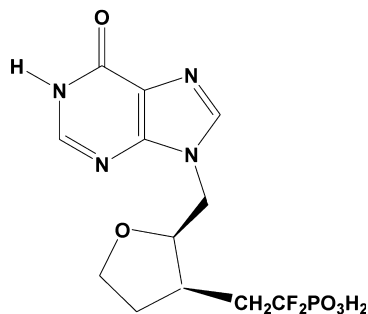
- |   |                |                         |
|---|----------------|-------------------------|
| 1 | $R_1 = H$      | DFPP-G                  |
| 2 | $R_1 = Me$     | $\gamma$ -met-DFPP-G    |
| 3 | $R_1 = Prop$   | $\gamma$ -prop-DFPP-G   |
| 4 | $R_1 = Benz$   | $\gamma$ -benz-DFPP-G   |
| 5 | $R_1 = isoBut$ | $\gamma$ -isobut-DFPP-G |



6 (±)-cis-piranyl

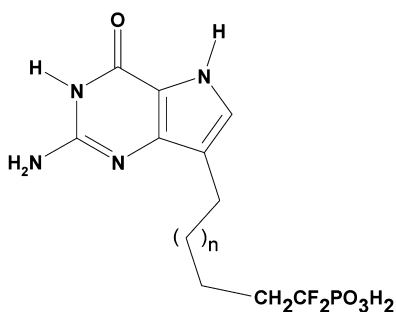


7 (±)-trans-furanyl



8 (±)-cis-furanyl

- |    |         |              |
|----|---------|--------------|
| 10 | $n = 0$ | nor-DFPP-DG  |
| 9  | $n = 1$ | DFPP-DG      |
| 11 | $n = 2$ | homo-DFPP-DG |
| 12 | $n = 3$ | 6C-DFPP-DG   |
| 13 | $n = 4$ | 7C-DFPP-DG   |



**Scheme 1:** Chemical structures of analogues of 9-deazaguanine, guanine and hypoxanthine connected by a linker to difluoromethylene phosphonic acid.

concentrations of the solvents, dimethylsulphoxide and methanol were tested for eventual cytotoxic activity. Dimethylsulphoxide and methanol in applied concentrations did not inhibit the growth of treated cells. After 72 h of incubation, the medium was removed, and 40  $\mu$ L of MTT (5 mg/mL of phosphate buffered saline) was added. To each well, 10% dimethylsulphoxide with 0.01 mol/L HCl was added to dissolve water-insoluble MTT-formazane crystals. Absorbance was measured at 570 nm on Elisa plate reader (Stat fax 2100, Pharmacia Biotech, Uppsala, Sweden). All experiments were performed at least three times, with three wells each.

A percentage of viable cells was determined by the following equation:

$$\% \text{ viable cells} = (A_{\text{COMPOUND}} - A_{\text{BLANK}} / A_{\text{CONTROL}} - A_{\text{BLANK}}) \times 100$$

BLANK = medium containing cytostatic and MTT.

### Statistics

The Kolmogorov–Smirnov test, a normality distribution test, was applied. The differences between groups were assessed by a non-parametric Kruskal–Wallis test ( $p < 0.05$ ). Statistical analyses were performed with STATISTICA™ software (version 7.0). Data are presented as mean values  $\pm$  SD of three separate experiments.

### Results and Discussion

With the ageing of the world's population, the westernization of diet, and the increasing environmental pollution associated with the global economy, cancer has emerged as the top threat to human life worldwide. Maximizing therapeutic response by increasing selectivity is a major goal in the development of anticancer therapy (27).

**Table 1:** Inhibitory properties of 9-deazaguanine, (DG, **14**) and analogues (**1-13**), with indicated base connected by a linker to difluoromethylene phosphonic acid, versus calf spleen and human erythrocyte PNPs<sup>a</sup>

Number	Compound	Base	K <sub>i</sub> <sup>app</sup> [nM] human PNP	K <sub>i</sub> <sup>app</sup> [nM] calf PNP
<b>1</b>	DFPP-G	Guanine	10.8 ± 0.7	6.9 ± 0.7 <sup>b</sup>
<b>2</b>	γ-met-DFPP-G	Guanine	12.1 ± 2.6	7.2 ± 0.6
<b>3</b>	γ-prop-DFPP-G	Guanine	7.6 ± 2.2	7.5 ± 0.8
<b>4</b>	γ-benz-DFPP-G	Guanine	15.4 ± 2.8	10.9 ± 1.0
<b>5</b>	γ-isobut-DFPP-G	Guanine	7.2 ± 2.5	11.2 ± 1.0
<b>6</b>	(±)-cis-piranyl	Hypoxanthine	96 ± 11	66 ± 13 <sup>b</sup>
<b>7</b>	(±)-trans-furanyl	Hypoxanthine	68 ± 6	52 ± 12 <sup>b</sup>
<b>8</b>	(±)-cis-furanyl	Hypoxanthine	63 ± 5	43 ± 13 <sup>b</sup>
<b>9</b>	DFPP-DG	9-deazaguanine	8.1 ± 0.6 <sup>c</sup>	4.4 ± 0.6 <sup>c</sup>
<b>10</b>	nor-DFPP-DG	9-deazaguanine	47 ± 5	19 ± 5
<b>11</b>	homo-DFPP-DG	9-deazaguanine	5.3 ± 0.4 <sup>c</sup>	5.7 ± 0.6 <sup>c</sup>
<b>12</b>	6C-DFPP-DG	9-deazaguanine	13 ± 1	21 ± 2
<b>13</b>	7C-DFPP-DG	9-deazaguanine	5.6 ± 0.8	6.1 ± 0.9
<b>14</b>	DG	9-deazaguanine	–	160 <sup>d</sup>

PNP, purine nucleoside phosphorylase.

<sup>a</sup>Reactions were carried out in 50 mM hepes/NaOH buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate, in the presence of 1 mM phosphate. Standard initial velocity procedure was used. <sup>b</sup>From Glavas-Obrovac *et al.*, 2007 (18); <sup>c</sup>From Hikishima *et al.*, 2007 (19). <sup>d</sup>From Stepniak *et al.*, 2007 (33), dissociation constant, K<sub>d</sub>, from fluorimetric titrations.

The unique sensitivity of human immature T-cells to deficiency of purine nucleoside phosphorylase (PNP) has been a leading base for the design of several novel classes of PNP inhibitors that may be useful for the treatment of T-cell-mediated tumour diseases, such as cutaneous T-cell lymphoma and acute lymphoblastic leukaemia, as well as other T-cell disorders (28).

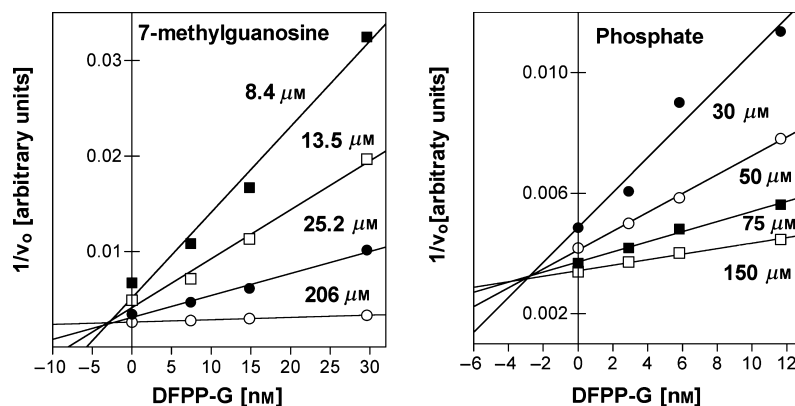
In search of novel compounds acting as multisubstrate analogue PNP inhibitors, we have synthesized several new compounds, analogues of guanine, hypoxanthine, and 9-deazaguanine (14–17, 19–21). As shown in Scheme 1, novel analogues have a terminal

phosphate replaced by a difluoromethylene phosphonate, which confer metabolic stability. Moreover, some phosphonates appear to be capable of slowly traversing the cell membrane, conceivably via an endocytosis-like process (1).

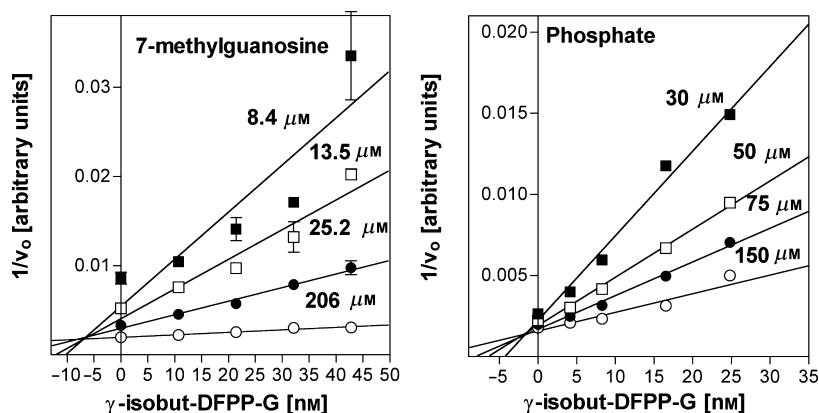
Five compounds with guanine moiety, three with hypoxanthine moiety and six compounds with 9-deazaguanine moiety, all connected by a linker with difluoromethylene phosphonic acid, were studied on their inhibitory potential against human and calf PNPs. Inhibition data for trimeric calf spleen PNP are displayed in Table 1. Data were determined at 25 °C, as majority of existing inhibition data for similar compounds including transition-state analogues, immucilins, and trimeric PNPs were obtained at this temperature (29,30). For calf spleen PNP, all compounds showed apparent inhibition constant, K<sub>i</sub><sup>app</sup>, in the nM range. For comparative purposes, some previously published data for human erythrocyte PNP were included as well (Table 1), showing that similar values were obtained for both enzymes. Furthermore, inhibition was competitive for both substrates of the phosphorolytic reaction (nucleoside and phosphate), as shown in Figures 1-3. Data for DFPP-G (**1**) versus calf spleen PNP (Figure 1), γ-isobut-DFPP-G (**5**) versus calf spleen PNP (Figure 2), and 7C-DFPP-DG (**13**) versus human erythrocyte PNP (Figure 3) were displayed in the form of the Dixon plot to clearly visualize the inhibition type. Taken together, obtained data confirm that investigated analogues act as multisubstrate analogue inhibitors, and hence, compete with both substrates, nucleoside and phosphate, for the binding sites of the enzyme.

All analogues inhibited mammalian PNPs with inhibition constants ranging from 5 to 100 nM. Nevertheless, inhibition constants shown in Table 1 should be regarded only as approximate values. Indeed, true constants are expected to be even lower, as DFPP-DG (**9**) and analogues act as slow-binding inhibitors, with no equilibrium reached during the time course of the classical initial velocity experiment (K. Breer, L. Magnowska, A. Bzowska, unpublished data).

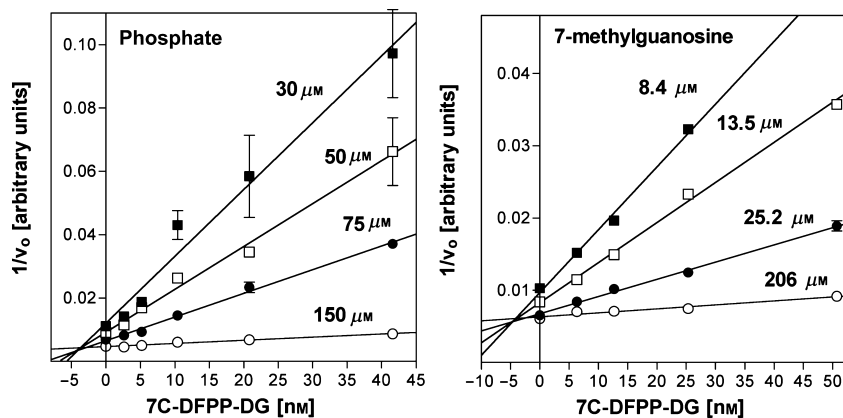
Having confirmed PNP inhibitory potential of novel analogues, we tested their antiproliferative activity against human lymphocytes as



**Figure 1:** Inhibition of calf spleen PNP by DFPP-G (**1**) shown in the form of Dixon plot. Reactions were carried out in 50 mM Hepes buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate in the presence of 1 mM phosphate (left panel) or with phosphate as a variable substrate with 350 μM of 7-methylguanosine (right panel). Kinetic data were presented in the form of the Dixon plot only to visualize the type of inhibition. Data ( $v_0$ ,  $c_0$  and  $[I]$ ) were analysed, and kinetic constants were obtained with the use of the weighted least-squares non-linear regression using the program Leonora, Cornish-Bowden, 1995 (25).



**Figure 2:** Inhibitions of calf spleen PNP by  $\gamma$ -isobut-DFPP-G (**5**) shown in the form of Dixon plot. Reactions were carried out in 50 mM Hepes buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate in the presence of 1 mM phosphate (left panel) or with phosphate as a variable substrate with 350  $\mu$ M of 7-methylguanosine (right panel).



**Figure 3:** Inhibitions of human erythrocyte PNP by 7C-DFPP-DG (**13**) shown in the form of Dixon plot. Reactions were carried out in 50 mM Hepes buffer pH 7.0, at 25 °C, with 7-methylguanosine as a variable substrate in the presence of 1 mM phosphate (left panel) or with phosphate as a variable substrate with 350  $\mu$ M of 7-methylguanosine (right panel).

well as lymphoma and leukaemia cells of the B- and T-cell origin. For those purposes, we selected a panel of seven lymphoma and leukaemia cell lines, four of them having the T-cell lineage given their specific biochemical, immunological, and clinical features that separate them from the non-T-cell malignancies (31). In addition to the multisubstrate analogue inhibitors, the base moiety 9-deazaguanine (9-DG, **14**), which inhibits calf spleen PNP with  $IC_{50} = 2.3 \mu$ M (32) and binds to the enzyme with a dissociation constant  $K_d = 160 \pm 10$  nM (33), was included in the study as well. Here, 9-DG (**14**) had no influence on lymphocytes and tumour cells' growth compared to control, non-exposed cells (Figure 4B). In contrast, for novel compounds, a slight inhibition (up to 20%) of human lymphocytes from healthy donors (Ly) was observed after 3 days of exposure, depending on the concentration applied.

The tumour cells' growth was influenced by all studied analogues; however, the effect varied by cell type and the dose applied. At lower concentrations, little or no effect on cell viability was observed up to 100  $\mu$ M (data not shown). By contrast, (Figure 4B), at 100  $\mu$ M, 9-deazaguanine analogues, nor-DFPP-DG (**10**), DFPP-DG (**9**), homo-DFPP-DG (**11**), and 6C-DFPP-DG (**12**) produced a significant reduction in the cell viability of all human leukaemia and lymphoma cell lines used ( $p < 0.05$ ). Furthermore, when compared to other 9-DG analogues with better  $K_i$  values (DFPP-DG, **9** and homo-DFPP-DG, **11**, Table 1), nor-DFPP-DG (**10**) was one of the most potent inhibitors of the cell growth at 100  $\mu$ M (Figure 4B). Thus,

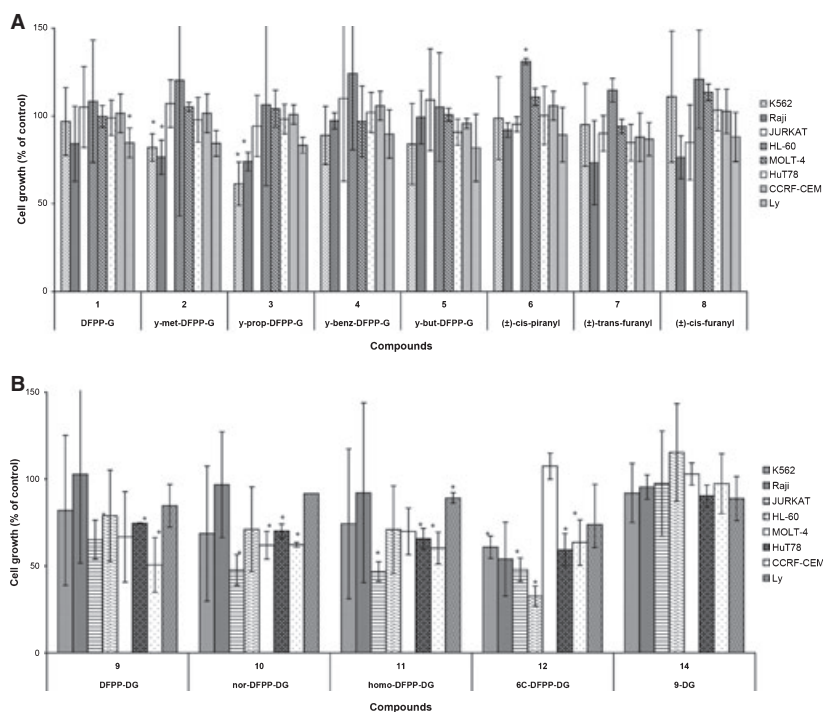
despite inferior inhibitory potency of nor-DFPP-DG (**10**) against human PNP ( $K_i = 47$  nM) compared to other 9-DG analogues, and hence, weaker antiproliferative effect expected, no differences in antiproliferative capacities were observed between nor-DFPP-DG (**10**) and other difluoromethylene phosphonate-linked compounds.

Over a concentration range of 0.1–100  $\mu$ M, guanine and hypoxanthine multisubstrate analogue inhibitors produced no significant effects upon cell viability and morphology of T-cell leukaemia (MOLT-4, Jurkat), acute T-cell lymphoblastic leukaemia (CCRF-CEM), T-cell lymphoma, and HL-60-acute myeloid leukaemia cells (data not shown). In contrast, chronic myeloid leukaemia in blasts crisis (K562) and Burkett's lymphoma (RAJI) cells were sensitive to guanine analogues  $\gamma$ -met-DFPP-G (**1**) and  $\gamma$ -prop-DFPP-G (**3**) applied in 100  $\mu$ M concentration (Figure 4A).

Despite the potent PNP inhibitory potential of investigated compounds, we did not observe any growth differences between tumour cells sensitive to PNP inhibition (e.g. human adult T-cell leukaemia and lymphoma cells) and other treated leukaemia or lymphoma cells of B- or non-T/non-B-cell lineages (32, 34, 35).

The observed antiproliferative effect of novel compounds on the tumour cell growth could be because of their PNP inhibitory capacity or just as a part of multistep processes, but this still remains to be investigated.





**Figure 4:** Growth inhibitory effects against leukaemia and lymphoma cells and normal lymphocytes. (A) Effects of analogues with guanidine and hypoxanthine aglycone. (B) Effects of 9-deazaguanine and analogues with 9-deazaguanine moiety. Cell lines: human lymphocytes (Ly), chronic myeloid leukaemia in blasts crisis (K562), T-cell leukaemia (JURKAT and MOLT), T-cell lymphoma (HuT78), Burkett's lymphoma (RAJI), acute T-cell lymphoblastic leukaemia (CCRF-CEM), and acute myeloid leukaemia (HL-60) Exponentially growing cells were treated with  $10^{-4}$  M concentration of investigated compounds during a 72-hour period. Cytotoxicity was analysed with methyltetrazolium (MTT) survival assay. Data are shown as mean values from three independent experiments  $\pm$  SD. Statistically significant change ( $p < 0.05$ ) is presented by\*.

## Conclusion and Future Directions

In summary, the studied analogues of guanine, hypoxanthine, and 9-deazaguanine containing difluoromethylene phosphonic acid act as potent multisubstrate analogue PNP inhibitors with nM inhibition constants. Based on these observations, *in vitro* screening of their anti-leukaemic and anti-lymphoma activities provided evidence that 9-deazaguanine compounds have better inhibitory potential compared to the guanine and hypoxanthine-derived analogues, but only at the highest concentration applied. Nevertheless, considering their good PNP inhibitory potential, stronger antiproliferative activity against haematological malignancies, especially of the T-cell origin, has been expected. It is likely that the poor effects on lymphoma and leukaemia cell proliferation could be explained by a poor ability of investigated compounds to permeate plasma membranes and reach PNP. For that reason, future studies will be directed towards the synthesis of cell-penetrating DFPP-DG pro-drugs. Yet another possibility is to exploit one of the recently developed drug-delivery systems (e.g. 36, 37). Finally, upon *in vitro* selection of the most potent candidates, the use of fluorescence dye to mark DFPP-DG analogues and other candidate compounds may help to elucidate their intracellular fates.

## Acknowledgments

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