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THE CONCENTRATION OF 5-PHOSPHORIBOSYL 1-PYROPHOSPHATE IN MONOLAYER TUMOR CELLS AND THE EFFECT OF VARIOUS PYRIMIDINE ANTIMETABOLITES

G. J. PETERS*, E. LAURENSSE, A. LEYVA and H. M. PINEDO

Section of Experimental Chemotherapy, Netherlands Cancer Institute, Amsterdam and Department of Oncology, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. [*Tel.* (020) 548 2162]

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Abstract—1. 5-Phosphoribosyl 1-pyrophosphate (PRPP) was determined in several murine and human cancer cell lines grown in monolayer, and harvested by trypsinization.

2. For all cell lines a large variation in the PRPP concentration (5–1300 pmol/10^6 cells) was found.

3. A 1-hr incubation in Dulbecco's medium reduced the variation in PRPP concentration. After this incubation the highest concentration was found in the murine B16 melanoma cell line (about 200 pmol/10^6 cells).

4. The human melanoma cell lines IGR3 and M5 and the human colon carcinoma cell line WiDr contained about 100 pmol/10^6 cells.

5. After this preincubation of 1-hr these cell suspensions were used to study the effect of several antimetabolites on PRPP concentration. A 2-hr incubation with 1mM *N*-(phosphonacetyl)-L-aspartate (PALA) increased the PRPP concentration only in M5 cells, whereas methotrexate caused an increase in all cell lines.

6. When 5-fluorouracil (5FU) was added, no significant decrease was found in any cell line. Addition of 5FU after a 2-hr preincubation with PALA resulted in a lower concentration in B16, M5 and WiDr cells.

7. The prodrug, 5-fluoro-5'deoxyuridine altered the PRPP concentration only in WiDr cells when it was added after PALA.

8. The activity of the 5FU metabolizing enzyme orotate phosphoribosyl transferase was comparable in B16, M5 and WiDr cells, but much lower in IGR3 cells.

INTRODUCTION

PRPP is a high-energy sugar phosphate which is essential for the synthesis of purine, pyrimidine and pyridine nucleotides. The concentration of PRPP is regulated by various factors including inorganic phosphate (Raivio *et al.*, 1981a) as an allosteric activator, the substrates ATP and ribose-5-phosphate and by feedback inhibition by various nucleotides (Becker *et al.*, 1979). Extensive utilization of PRPP by phosphoribosyl-transferases, as in the conversion of hypoxanthine to IMP, can lead to fluctuations in PRPP concentrations (Gordon *et al.*, 1979; Torrelio *et al.*, 1982). The activity of the hexose monophosphate shunt also regulates PRPP availability (Raivio *et al.*, 1981b).

Several frequently used antimetabolites require PRPP for their conversion to active forms. The fluoropyrimidine, 5FU, can be phosphorylated by the action of OPRT (Reyes and Hall, 1969). Purine phosphoribosyl-tranferases are essential for the conversion of purine antimetabolites such as 6-mercaptopurine and thioguanine to their active forms. A drug like cordycepin depletes PRPP pools (Tyrstedt and Sartorelli, 1969) in addition through inhibition of PRPP synthetase by cordycepin-monophosphate (Peters *et al.*, 1981) while other drugs such as methotrexate increase PRPP levels (Buesa-Perez *et al.*, 1980). Combining a drug that increases the PRPP concentration with a PRPP utilizing drug provides a biochemical basis for combination cancer chemotherapy.

Methotrexate can enhance the antitumor activity of 5FU (Tisman and Wu, 1980). PALA, an inhibitor of pyrimidine *de novo* biosynthesis which shows antitumor activity in several experimental sold tumors in mice, also increases PRPP levels (Major *et al.*, 1982). Most of these results were obtained with leukemic cell lines, but 5FU is widely used in the treatment of solid tumors. When available as a cell line these tumors only grow in monolayers. The few reports on PRPP concentration in monolayer cells are mostly limited to normal fibroblasts and do not relate to tumor cell lines. In fibroblasts, PRPP concentrations appears to fluctuate with the condition of the cells and the exhaustion of the medium (Gay and Amos, 1983; Torrelio *et al.*, 1982). In this paper we describe the measurement of PRPP concentration in tumor cell lines. The effect of PRPP concentration by several antimetabolites was studied after a preincubation of the cells as a suspension and the results were

* Present and correspondence address: Laboratory of Biochemical Pharmacology, Department of Oncology, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.

Abbreviations: PRPP — 5-phosphoribosyl 1-pyrophosphate; 5FU — 5-fluorouracil; FUMP — 5-fluorouridine 5'monophosphate; PALA — *N*-(phosphonacetyl)-L-aspartate; OPRT — orotate phosphoribosyl transferase (EC 2.4 2.10); ODC — orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23); HBSS — Hanks balanced salt solution.

related with the activity of OPRT, which is responsible for the direct conversion of 5FU to nucleotides.

MATERIALS AND METHODS

Materials

[Carboxyl-^{14}C]orotic acid, [carboxyl-^{14}C]orotidine-5'monophosphate and Omnifluor were obtained from New England Nuclear Corporation, Dreieichenhain, F.R.G. Hydroxide of Hyamine 10 X was from Packard, Groningen, The Netherlands; Dulbecco's modified Minimal Essential Medium and fetal bovine serum were from GIBCO, Grand Island, New York, U.S.A. PRPP and a preparation from brewer's yeast containing OPRT and ODC were from Boehringer, Mannheim, F.R.G. PALA was from the NCI, Division of Cancer Treatment, Bethesda, Maryland, U.S.A.; 5FU from Sigma, St Louis, Missouri, U.S.A. 5-fluoro-5'deoxyuridine was a gift from Hoffmann–La Roche, Basel, Switzerland; and methotrexate was from Lederle Laboratories, Pearl River, New Jersey, U.S.A. All other chemicals were of the highest quality commercially available.

Culture and harvesting of cells

Cells were grown in 75-cm^2 Falcon flasks in 25 mM Hepes-buffered Dulbecco's medium supplemented with 15% dialyzed, heat-inactivated fetal bovine serum. Origins of the murine melanoma cell line B16 and the human melanoma cell lines IGR3 and M5 were described previously (Leyva *et al.*, 1981; 1983), the human colon carcinoma cell line was obtained from the American Type Culture Collection (ATCC CCL 18). Cells were passaged every 2–3 days. Cells for metabolic studies were obtained from cultures in logarithmic growth phase. The monolayers were washed with HBSS without Ca^{2+} and Mg^{2+}, and subsequently treated for 2–5 min with 1 ml trypsin solution (1 mg/ml in HBSS containing 5 mM EDTA and 4.2 mM $NaHCO_3$) at room temperature. Unless otherwise indicated, the cells were suspended in Dulbecco's medium, supplemented with 7.5% dialyzed, heat-inactivated fetal bovine serum, counted with a hemacytometer, spun down at 200 *g* and suspended in appropriate incubation medium or assay buffer for PRPP determination. The buffer for PRPP and enzyme assays consisted of 50 mM Tris (pH 7.4) containing 1 mM EDTA.

Determination of PRPP concentration

Extracts for PRPP determination were prepared either immediately after harvesting the cells or from cells rapidly frozen as a pellet at −70°C. In the first case the cells were lysed in the Tris–EDTA buffer by sonication (2 cycles of 5 sec at 50 W output, Branson sonifier). When the cells had been frozen, the pellet was thawed by resuspending the cells in an appropriate volume of the Tris–EDTA buffer; no further lysis was performed. PRPP concentrations in cell pellets stored for several weeks at −70°C were similar to the concentration of PRPP in fresh cells. The PRPP concentration was determined according to the procedure described previously for erythrocytes and lymphocytes (Tax and Veerkamp, 1977; Peters and Veerkamp; 1979). The method is based on measurement of the $^{14}CO_2$ release from [carboxyl-^{14}C]orotic acid, catalyzed by the OPRT–ODC enzyme complex. The amount of $^{14}CO_2$ (trapped by hyamine) was proportional to the PRPP present in the cell extract (0.2–5×10^6 cells). Recovery of PRPP standards added to monolayer cells before the assay was always higher than 90%. Since a heating step is included in the procedure, intracellular enzymes will be denaturated and will not affect the assay. The yeast OPRT–ODC enzymes were not influenced by the drugs. Furthermore their concentration would be too low since only cell pellets were used. PRPP concentration is given in pmol per 10^6 cells or per mg total cell protein. Protein was determined by a dye-binding assay (Sedmak and Grosberg, 1977).

Drug incubations

The effect of drugs was studied in cells harvested 2 days after plating, in Hepes-buffered Dulbecco's medium with 7.5% fetal bovine serum. These cells were incubated in this medium for 1 hr at 37°C in a shaking water-bath as a 2 ml cell suspension ($0.2–1\times10^6$ cells). After addition of drugs in a concentrated solution, incubation was continued for an additional 2 or 4 hr. Also several control tubes without drugs were incubated during this time period. The incubation was stopped by spinning down the cells at 400 *g* for 2 min. The cell pellet was frozen at −70°C until PRPP assays were performed. Statistical significance of data was determined using the Student's *t*-test for paired data.

Enzyme assays

The activities of OPRT and ODC were estimated in cells cultured and harvested in Dulbecco's medium plus serum. After suspension of the cells in Tris–EDTA buffer ($0.5–2\times10^6$ cells/ml) extracts were prepared by sonication (3 cycles of 5 sec with intervals of 5 sec at maximal output, Branson sonifier). Extracts were immediately used for measurement of OPRT and ODC according to methods described earlier (Peters and Veerkamp, 1979). Linearity with the amount of protein and time was assured.

RESULTS

Figure 1 shows the large variation in the PRPP concentration in each of the cell lines studied. This variation was found with both buffers that were used during harvesting of the cells. The isotonic Tris buffer appeared to be the most suitable isolation buffer for erythrocytes and various lymphoid cells (Tax and Veerkamp, 1979; Peters and Veerkamp, 1979; Peters *et al.*, 1982b), since this buffer contains no nutritional compounds that stimulate PRPP synthesis or consume PRPP. Using Tris–saline we also found a varia-

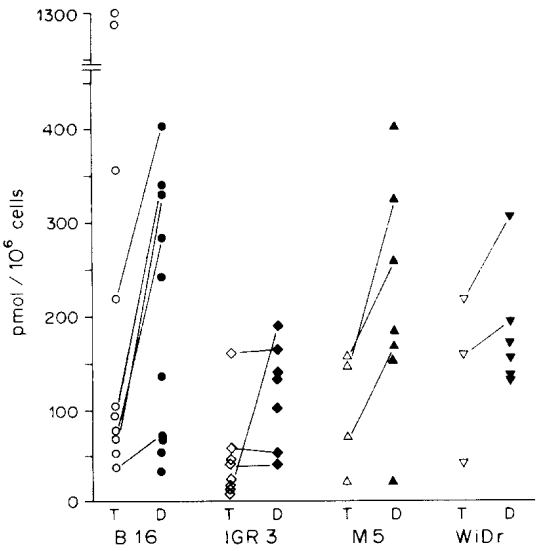

Fig. 1. Effect of isolation buffer on PRPP concentrations. Cells were harvested in isotonic Tris (T) buffer (50 mM Tris, 100 mM saline, pH 7.4) or Dulbecco's medium (D) and PRPP concentration was measured immediately. Lines connect PRPP concentrations measured in cells from the same culture, but harvested either in Tris–buffer or Dulbecco's medium.

tion in concentration related with the day of harvesting after plating the cells. However, both an increase and a decrease during culture was found.

Culture period and harvesting appear to affect the PRPP concentration of the cells. However, for drug modulation studies it is essential to have a cell population that consists of cells in a comparable condition. Therefore, the cells harvested either in isotonic Tris buffer or in Dulbecco's medium, were suspended in Dulbecco's medium supplemented with serum and incubated for 1 hr in a shaking water-bath. The effect of this incubation is shown in Fig. 2. With all cell lines studied the variation in PRPP concentration decreased, especially when the cells had been harvested in Dulbecco's medium. PRPP concentrations appear to equalize. Longer incubation periods up to 5 hr did not affect significantly PRPP concentrations. Nucleotide concentrations and the energy charge (ATP/ADP ratio higher than 2.5) did not change during the incubation (data now shown). Table 1 shows the concentration of PRPP in cells harvested in Dulbecco's medium at the second day after plating the cells and incubated for 1 hr in Dulbecco's medium. The concentration was highest in B16 cells.

Cells preincubated as a suspension for 1 hr, were used to investigate the effect of drugs on PRPP concentrations. In preliminary studies B16 melanoma cells were incubated with 5FU and with PALA, a potent inhibitor of aspartate transcarbamylase. Although 0.1 mM PALA completely blocks the growth of B16 cells (Leyva *et al.*, 1981), no significant effect on the PRPP concentrations was caused by 0.1 and 1 mM PALA (Table 2). B16 cells have a very low uridine phosphorylase activity (Leyva *et al.*, 1983). Therefore, in these cells 5FU can only be converted to FUMP by OPRT, an enzyme that consumes PRPP. However, incubation of B16 cells with 5FU did not decrease PRPP concentrations (Table 2). The combination PALA and 5FU also did not affect PRPP concentrations.

For comparative purposes, effects of drugs in the other cell lines were expressed relative to the PRPP concentration in the control incubations (Table 3). PALA caused an increase in PRPP concentration only in M5 cells after an exposure of 2 hr, but not after 4 hr (Table 3). Methotrexate enhanced PRPP levels in all cell lines. Consumption of PRPP for 5FU anabolism to nucleotides did not significantly

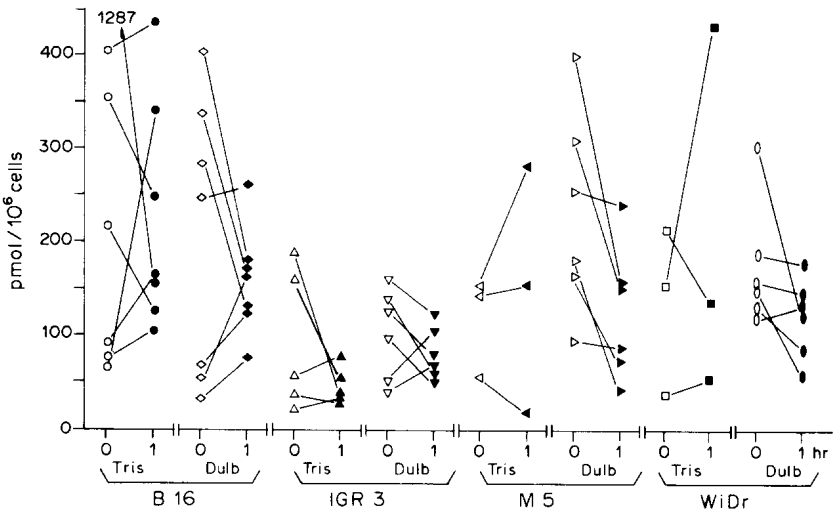

Fig. 2. Effect of a 1-hr incubation in Dulbecco's medium on the PRPP concentration. Cells were harvested in isotonic Tris–buffer (Tris) or Dulbecco's medium (Dulb), and PRPP concentration was measured immediately in one part of the cells (0 hr). After centrifugation the other part of the cells was suspended in Dulbecco's medium and incubated for 1 hr at 37°C in a shaking water-bath. PRPP concentration was measured after centrifugation of the cells as described in "Materials and Methods". Lines connect the 0 and 1 hr values for the same sample.

Table 1. Concentration of PRPP in various tumor cell lines incubated for 1 and 3 hr in Dulbecco's medium

Cell line	PRPP concentration 1 hr	3 hr
B16	200 ± 20 (11)	253 ± 40 (8)
IGR3	87 ± 10 (7)	58 ± 7 (3)
M5	110 ± 19 (7)	112 ± 18 (5)
WiDr	131 ± 19 (6)	185 ± 30 (6)

Cells were harvested after 2 days in Dulbecco's medium and incubated during 1 or 3 hr after suspension in fresh Dulbecco's medium. Concentrations (in pmol/10^6 cells) are given as means ± SEM for the number of different samples indicated within parentheses. Protein contents were about 160, 127, 152 and 249 μg/10^6 cells for B16, IGR3, M5 and WiDr, respectively.

Table 2. Effect of PALA and 5FU on the PRPP concentration in B16 cells

Drug	PRPP concentration
0.1 mM PALA	364 ± 38
1 mM PALA	346 ± 25
25 μM 5FU	252 ± 52
50 μM 5FU	242 ± 29
0.1 mM PALA + 25 μM 5FU	308 ± 50

Concentrations (in pmol/10^6 cells) are means ± SEM for 3–4 separate experiments, unless otherwise indicated within parentheses. Values for controles are given in Table 1. Concentrations were measured at the indicated time. Drugs were added after 1 hr and PRPP concentrations were assayed after 3 hr.

Table 3. Effect of several drugs on the PRPP concentration in various tumor cell lines

Drug	Relative concentration in			
	B16	IGR3	M5	WiDr
No addition	100	100	100	100
1 mM PALA	121 ± 11	102 ± 11	183 ± 34*	130 ± 32
1 mM PALA (4)	119 ± 20	98 ± 11	132 ± 21	115 ± 17
50 μM FU	90 ± 5*	100 ± 10	113 ± 24	86 ± 9*
1 μM MTX	231 ± 67*	231 ± 56*	158 ± 27**	183 ± 14***

Values (in % of the concentration after 3 hr incubation without additions) are means ± SEM of 3–5 separate experiments. Drugs were present for 2 hr, PALA (4) for an additional 2 hr. The levels of significancy for differences between incubations with and without drug were at the level: * $0.02 < P < 0.05$; ** $0.01 < P < 0.02$; *** $P < 0.01$.

decrease PRPP levels, although inhibition of cell growth at 50 μM 5FU was at least 50% with all cell lines (Peters *et al.*, submitted for publication).

Preincubation of cultured cells with PALA increases the incorporation of 5FU into RNA (Major *et al.*, 1982; Peters *et al.*, 1984). This enhanced anabolism could possibly be catalyzed by OPRT. Therefore, we also measured PRPP concentration after sequential addition of PALA and 5FU (Table 4). In B16, M5 and WiDr cells the PRPP concentration decreased significantly. The fluoropyrimidine analog, 5-fluoro-5′-deoxyuridine, decreased PRPP levels only in WiDr cells. This analog is active only after its cleavage to 5FU which is catalyzed by uridine phosphorylase. This enzyme that is also involved in subsequent metabolism of 5FU, has a very high activity in IGR3 cells, an intermediate activity in M5 and WiDr cells and is almost absent from B16 cells (Leyva *et al.*, 1983).

Table 4. Effect of fluoropyrimidines on the PRPP concentration after PALA treatment

Cell line	Relative concentration in the presence of	
	5FU	5′FUdR
B16	82 ± 7*	103 ± 15
IGR3	85 ± 10	108 ± 2
M5	68 ± 5**	98 ± 20
WiDr	74 ± 4**	82 ± 3*

Values (in % of the concentration of PRPP after 4 hr incubation with 1 mM PALA) are means ± SEM of 3–4 separate experiments. PALA was present in all incubations at 1 mM for 4 hr; 50 μM 5FU and 100 μM 5′FUdR were present during the last 2 hr of the incubation. Significantly different from the PRPP concentration in cells treated only with PALA at the level: * $0.01 < P < 0.2$; ** $P < 0.01$. 5′FUdR is 5-fluoro-5′-deoxyuridine.

We also measured the activities of OPRT and ODC in these cell lines. OPRT is also capable of anabolizing 5FU and catalyzes the direct conversion to FUMP. In IGR3 cells the activities of OPRT and the next enzyme in pyrimidine nucleotide synthesis, ODC, were lower than in the other cell lines (Table 5). The ODC/OPRT ratios, calculated from the separate experiments, were comparable for all cell lines studied.

Table 5. Activities of OPRT and ODC in various tumor cell lines

Cell line	OPRT	ODC	ODC/OPRT
B16	6.7 ± 0.4	27.8 ± 1.7	5.4 ± 0.6
IGR3	2.2 ± 0.3	10.8 ± 1.6	6.1 ± 1.1
M5	6.5 ± 1.8	20.6 ± 1.8	3.6 ± 0.6
WiDr	6.9 ± 1.0	24.4 ± 2.9	3.5 ± 0.6

Activities (in nmol/hr per 10^6 cells) are given as means ± SEM of 3–4 separate experiments. ODC/OPRT ratio was calculated from the separate experiments.

DISCUSSION

Cancer cell lines are useful tools to study the mode of action of chemotherapeutic agents. Leukemic cell lines that grow in suspension are easy to handle, whereas cell lines derived from solid tumors are primarily grown in monolayers. For most biochemical studies these cells must be detached from the plastic surface. Previous studies on the modulation of PRPP by methotrexate (Buesa-Perez *et al.*, 1980) and purine nucleosides (Peters *et al.*, 1982b) were performed in cells grown in suspension. These cells do not show a large variation in PRPP concentration. With monolayers, we observed in the present study a large variation in PRPP concentration using standard harvesting methods (Fig. 1). These findings correspond with those observed with fibroblasts which also grow in monolayers and in which PRPP concentrations depend on the composition and exhaustion of the culture medium (Torrelio *et al.*, 1982; Gay and Amos, 1983). Depletion of substrates for PRPP utilizing enzymes, such as hypoxanthine, and of substrates for PRPP synthesis, may occur. Resuspension of monolayer cells in fresh medium might lead to a new equilibrium between synthesis and consumption of PRPP. This equilibrium appeared to be reached after 1 hr since longer incubation did not cause significant additional changes in PRPP concentration. Therefore these cell suspensions appear suitable to measure modulation of PRPP, but other metabolic parameters can also be monitored.

The increase in PRPP concentration caused by methotrexate is comparable with that reported for leukemic cell lines (Buesa-Perez *et al.*, 1980; Cadman *et al.*, 1981; Kufe *et al.*, 1981). Sequential methotrexate and 5FU show synergistic inhibition of cell growth (Benz *et al.*, 1980; Kufe *et al.*, 1981). PALA, an effective inhibitor of aspartate transcarbamylase, causes depletion of orotic acid (Casper *et al.*, 1983), a substrate for OPRT. In our experiments this depletion resulted only in M5 cells in a slight increase of PRPP concentration. 5FU alone had little effect on PRPP but reduced PRPP levels significantly when it was added after PALA. This is possibly due to the depletion of orotic acid leading to a lack of competition between orotic acid and 5FU for the active site on OPRT. Since 5FU is added in a higher concentration than the original orotic acid concentrations, the conversion of 5FU to FUMP by OPRT might result in a decrease of PRPP levels. In leukemic cells this was accompanied by enhanced incorporation of 5FU into RNA (Kufe *et al.*, 1981). In B16, M5 and WiDr cells PALA has also been shown to increase 5FU incorporation into RNA (Peters *et al.*, 1984). A decrease in

PRPP levels was only found in those cell lines, which contain a relatively high activity of OPRT.

WiDr cells are 3–20 times more sensitive to 5-fluoro-5′-deoxyuridine than the other cell lines. Pyrimidine phosphorylase will cleave this prodrug to 5FU. However OPRT appears to be important for subsequent metabolism of 5FU, since in WiDr cells 5-fluoro-5′-deoxyuridine added after PALA lowers the PRPP concentrations.

In conclusion these results show that suspension of monolayer cells enables short term studies on modulation of PRPP levels by various drugs. The method appears useful not only in further studies on 5FU and 5-fluoro-5′-deoxyuridine metabolism, but also in investigations of other drugs involved in PRPP metabolism.

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