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EFFECT OF L-GLUTAMINE ANTAGONISTS ON 5-PHOSPHORIBOSYL 1-PYROPHOSPHATE LEVELS IN P388 LEUKEMIA AND IN MURINE COLON ADENOCARCINOMAS *IN VIVO**

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Abstract—The intratumoral content of 5-phosphoribosyl 1-pyrophosphate (PRPP) and the acitivity of the enzymes anabolizing and catabolizing the sugar phosphate were determined following i.p. administration of an LD_{10} dose of an L-glutamine antagonist or saline to tumor-bearing animals. Elevation of PRPP pool size following administration of L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isopazoleacetic acid (NSC-163501) (AT-125) was maximal at 8 hr and returned to pretreatment levels by 24 hr. In P388 leukemia, dose for dose, at 4 hr, 6-diazo-5-oxo-L-norleucine (NSC-7365) (DON) was the most potent of the L-glutamine antagonists in elevating basal PRPP pool size (507% above control) followed by AT-125 and azaserine, 300 and 100% above control respectively. Moreover, such augmentation in PRPP pool size preferentially affected P388 tumor rather than the small intestine. Following i.p. administration of LD_{10} doses of AT-125, DON and azaserine, the specific activities of PRPP anabolizing and catabolizing enzymes were determined. A significant inhibition of PRPP amidotransferase was demonstrated with DON and AT-125 ($P < 0.05$), and no inhibition with azaserine. A similar modulation of PRPP pool size was demonstrated *in vivo* following administration of 250 mg/kg of AT-125 in mice bearing colonic adenocarcinoma lines. It was suggested that a significant increase of PRPP pool size might cause the possible synergism of a selected L-glutamine antagonist and 5-fluorouracil as reported after the appropriately scheduled administration of methotrexate and 5-fluorouracil.

Early observation of low levels of L-glutamine in tumors focused interest on the role of L-glutamine in tumor metabolism [1]. Moreover, L-glutamine occupies an important role in the biosynthesis of several metabolites. Recently, several analogs of L-glutamine with the ability to inhibit one or more steps in the purine biosynthetic pathway have been identified. DON,¶ azaserine, and AT-125 represent the important L-glutamine antagonists. Each one of the agents demonstrates a different spectrum of activity in the several reactions in which L-glutamine is utilized [2–4]. DON is a powerful inhibitor of the reactions by which L-glutamine transfers its amide to L-aspartic acid (L-asparagine synthetase), whereas azaserine has no activity in this reaction. It is the purpose of this manuscript to examine the influence of the three L-glutamine antagonists on the several enzymes involved in the anabolism and the catabolism of a sugar phosphate, phosphoribosyl pyrophosphate.

MATERIALS AND METHODS

[U-^{14}C]Adenine (276 mCi/mmole), [^{14}C-carboxyl]orotic acid (41.3 mCi/mmole), [8-^{14}C]hypoxanthine (55.8 mCi/mmole) and L-[U-^{14}C]-glutamine (50 mCi/mmole) were obtained from the Amersham Corp., Arlington Heights, IL. Aquasol scintillation fluid was purchased from the New England Nuclear Corp., Boston, MA. PRPP, ATP, IMP, AMP and magnesium chloride were obtained from the Sigma Chemical Co., St. Louis, MO. Murine colonic carcinomas 38, 26, 6A and 11A were the gifts of Dr. Thomas Corbett of the Southern Research Institute, Birmingham, AL. P388/S, the native line, was the gift of Dr. Randall K. Johnson, A. D. Little Institute, Cambridge, MA. Protein determinations were made with Bio-Rad dye binding reagent (Bio-Rad Laboratories, Richmond, CA) [5]. All four murine tumors were carried as solid tumors. Colonic tumors 11A, 51 and 26 were maintained in BALB-C mice, the host of origin, while the colonic tumor 38 was carried in C57BL/6 mice. The procedure for inoculation of the tumor was as follows.

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¶ Abbreviations: DON, 6-diazo-5-oxo-L-norleucine (NSC-7365); PRPP, 5-phosphoribosyl 1-pyrophosphate; AT-125, L-[αS,5S]-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501); azaserine (NSC-742); APRTase, adenine phosphoribosyl transferase; HGPRTase, hypoxanthine guanine phosphoribosyl transferase; OPRTase, orotate phosphoribosyl transferase; PCA, perchloric acid; and 5-FU, 5-fluorouracil.

The tumor, 0.5 cm in diameter, was carefully dissected from mice bearing the specific line. The isolated tumors were minced with scissors into small fragments in a sterile, physiological saline. The tumor fragments, measuring 2–3 mm across, were implanted subcutaneously in new hosts with a trocar. P388/S leukemia was carried as a liquid tumor by i.p. administration of 10^6 leukemic cells to BDF_1 mice; for the experimental purposes, however, P388/S cells were injected subcutaneously in a flank of BDF_1 mice.

Preparation of tumor homogenate for determination of PRPP content. Mice bearing subcutaneous P388/S tumors were administered i.p. AT-125 (250 mg/kg), azaserine (100 mg/kg), DON (100 mg/kg) or saline (LD_{10} doses of drugs). Four hours later, the animals were anesthetized with ether and tumors were rapidly removed and flash frozen on dry ice. This procedure was completed in 2 sec. Subsequently, tumor was pulverized with a percussion hammer and homogenized at 4° in 5% PCA (1:3, tumor/PCA, w/v) and immediately neutralized. The neutralization procedure was as follows. To 1 ml of homogenate were added 50 μl of 1 M potassium phosphate, pH 8.5, and 70 μl of 40% KOH to bring it to pH 7.0–7.2. The neutralized homogenate was immediately frozen on dry ice until it could be assayed for PRPP, at which time it was spun at 12,000 *g* for 3 min to thaw and the supernatant fraction was used for PRPP assay. Similarly, mice bearing subcutaneous colonic adenocarcinomas, colonic tumors 11A, 51, 26 and 38, were administered LD_{10} doses of AT-125, i.e. 250 mg/kg, i.p., and 4 hr later were anesthetized and treated as above.

PRPP assay. The following assay method of phosphoribosyl pyrophosphate is the modified method of Tax and Veerkamp [6]. [^{14}C-carboxyl]Orotic acid (5 μl) was added to the bottom of 1.5 ml Eppendorf polypropylene conical centrifuge tubes, and 5 μl of homogenate supernatant fluid was added to this side. The caps of the tubes were gently shut and centrifuged at 12,000 *g* for 10 sec to mix drops. The tubes were opened and 5 μl of 40% KOH was added to the inside of the lids. Twenty microliters of mixed enzyme (orotidine 5′-phosphate pyrophosphorylase and orotidine 5′-phosphate decarboxylase, 3 units/ml; one unit of mix enzymes catalyzes the phosphorylation of 1.0 μmole of orotic acid to O-5′-MP which is then decarboxylated to U-5′-MP) in 10 mM magnesium chloride was added to the bottom of the tubes. The lids were shut immediately and the tubes were incubated overnight at room temperature. The $^{14}CO_2$ released by the reaction was absorbed by the KOH. The lids were cut and carefully put in 10 ml of scintillation fluid, and radioactivity was counted. Standard solutions of PRPP ranging from 4 mM to 1 μM were prepared in 0.1 M Tris–HCl, pH 7.4, or 5% PCA. The latter samples were quickly neutralized with 40% KOH in a manner similar to that of the experimental samples. These solutions were assayed for PRPP alongside the unknowns. The radioisotope count for each standard was plotted against the PRPP concentration. The concentration of the PRPP in the unknown was then read from the standard curve (Fig. 1).

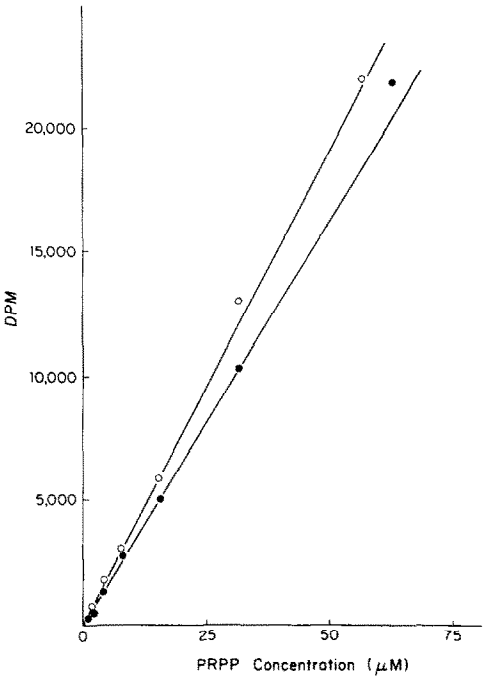

Fig. 1. PRPP assay. Standard solutions of PRPP were prepared in 0.1 M Tris–HCl (pH 7.4) (○——○) or 5% PCA (●——●). The latter samples were quickly neutralized with 40% KOH in a manner similar to that of the experimental samples. The solutions were assayed for PRPP alongside the unknowns as outlined in Materials and Methods. The concentration of PRPP in the unknowns was then read from the standard curve.

Preparation of homogenate for enzyme assays. The tumors were homogenized in 2 × volume 0.1 M Tris–HCl containing 0.5 mM EDTA and 1 mM dithiothreitol, pH 7.6, using a polytron homogenizer. The homogenates were centrifuged at 12,000 *g* for 3 min and the cytosol fraction was kept on ice prior to assays.

Hypoxantine, adenine, and orotate phosphoribosyl transferases. These assays were performed with the modified method of Kelly *et al.* [7]. For HPRTase assay, the following were added separately into 1.5 ml Eppendorf polypropylene centrifuge tubes: 5 μl of PRPP–$MgCl_2$ (10 mM each in 0.1 M Tris–HCl, pH 7.6), 5 μl of tumor homogenate supernatant fluid. In the blank assay, tumor homogenate supernatant fluid was replaced by homogenizing buffer. The reaction was started by a 5-sec centrifugation at 12,000 *g* in an Eppendorf microcentrifuge which brought the reactants together. The tubes were incubated at 37° for 10 min. The reaction was stopped by freezing in dry ice. While the reaction mix was still frozen, 10 μl of 1 N HCl was added to the side of the tube. The tube was centrifuged for 10 sec in the Eppendorf microcentrifuge causing the reaction mix to thaw and the HCl to mix with it and completely stop the reaction. Five μl of supernatant fluid was spotted on Whatman 3-M paper and overspotted with 5 μl of 10 mM IMP. The spots were allowed to dry and the paper was chromatographed overnight using a solvent system of 1 M ammonium acetate, pH 7.0, and ethanol, 30:70 (v/v). The IMP spots

which were visible under u.v. light were encircled, cut, and placed into counting vials containing 200 μl water. Scintillation mixture (10 ml) was added into the counting vial and the radioactivity was measured. Specific activity of the enzyme was calculated based on the radioactive count. In the case of APRTase and OPRTase, [8-^{14}C]hypoxanthine was replaced with [U-^{14}C]adenine and [carboxyl-^{14}C]orotic acid, respectively, and IMP for overspotting was replaced with AMP and OMP, respectively.

PRPP synthetase. The assay method was based on that of Reem [8]. The following were added separately into 1.5-ml Eppendorf polypropylene tubes: 5 μl [carboxyl-^{14}C]orotic acid, 5 μl 40 mM ribose-5-phosphate, 5 μl ATP–$MgCl_2$ (40 mM each in 0.5 M potassium phosphate, 0.1 M Tris–HCl, pH 7.0), and 5 μl tumor homogenate supernatant fluid. The blank assay contains homogenizing buffer in place of tumor homogenate supernatant fluid. The reaction was started by a 5-sec centrifugation in the Eppendorf microcentrifuge which brought the reactants together. The tubes were incubated at 37° for 10 min and then at 95° for 2 min to stop the reaction. After cooling, the tubes were centrifuged to bring down condensation from the lid. Five microliters of 40% KOH was added to the inside of the lide. Twenty microliters of mixed enzyme (orotidine-5-P-pyrophosphorylase and orotidine 5-P-decarboxylase, 3 units/ml) in 10 mM magnesium chloride was added to the bottom of the tubes. The lids were shut immediately, and the tubes were incubated overnight at room temperature, during which $^{14}CO_2$ released was absorbed by the KOH. The lids were cut off and put into 10 ml of scintillation mixture, and radioactivity was measured.

PRPP amidotransferase. The assay was performed with the modified method of Holmes *et al.* [9]. [U-^{14}C]Glutamine (5 μl) was added to the bottom of each Eppendorf tube, 5 μl of PRPP–$MgCl_2$ (30 mM each in 0.1 M Tris–HCl, pH 7.6) to the side, and 5 μl of the extract to the inside of the lid. The caps were closed and the tubes were spun at 12,000 *g* for 5 sec and incubated at 37° for 30 min. Thereafter, the reaction was terminated by heating at 95° for 2 min.

Any L-[U-^{14}C]glutamic acid produced in the incubation step was then quantitated by the addition of 50 μl of 0.66 M acetate buffer (pH 4.2) containing 1 I.U. of glutamate decarboxylase purified from *Escherichia coli* by the method of Shukuya and Schwert [10]. The $^{14}CO_2$ resulting from this reaction was collected in 5-μl droplets of 40% KOH deposited on the underside of the lid of the reaction vessel, as described by Cooney *et al.* [11]. Following an overnight incubation, the caps of the vessels were cut and placed in 10 ml of scintillation fluid and counted.

RESULTS

Tumoral versus small intestinal PRPP concentration. BDF_1 mice bearing subcutaneously implanted P388/S tumors were administered, i.p., LD_{10} doses of DON, AT-125, azaserine and saline control. The mean PRPP concentration of P388/S leukemia line was 507, 300 and 100% above control, respectively, whereas the mean concentration of PRPP in the

Table 1. Intracellular PRPP levels*

Target tissue	Treatment	PRPP (μM)
P388/S	Saline	5.28 + 1.99
	AT-125	21.13 + 5.54
	Azaserine	10.56 + 2.40
	DON	32.10 + 3.00
Small intestine	Saline	2.1 + 1.2
	AT-125	4.3 + 1.5
	Azaserine	2.4 + 0.9
	DON	4.8 + 1.9

* BDF_1 mice bearing subcutaneous tumors P388/S were injected i.p. with AT-125 (250 mg/kg), azaserine (100 mg/kg) or DON (100 mg/kg) (LD_{10} dose for all drugs). Four hours later, the animals were anesthetized with ether. Tumors and small intestine were prepared as outlined in Materials and Methods. The supernatant fractions were used to determine PRPP content. Values are means ± S.D.

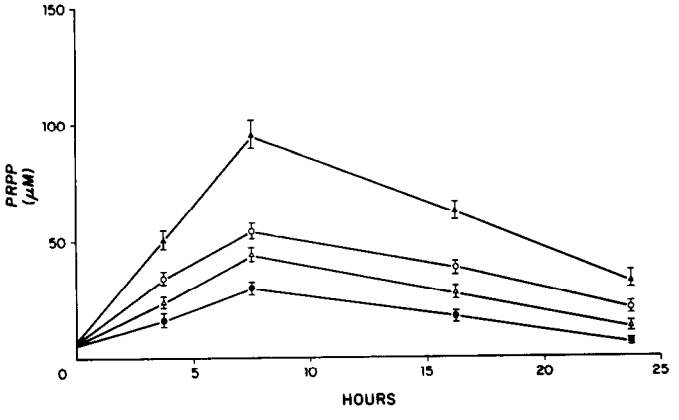

Fig. 2. Time course and dose response of intratumoral concentration of PRPP. BDF_1 mice bearing P388 subcutaneous tumors were injected i.p. with 5 mg (—●—), 250 mg (—△—), 400 mg (—○—), and 800 mg (—▲—) AT-125 per kg body weight. At 4, 8, 16 and 24 hr, five animals in each group were killed, and tumors were rapidly removed and homogenized in 5% PCA as described in Materials and Methods. The supernatant fractions were used to determine PRPP levels.

Table 2. Intracellular PRPP levels in murine colonic adenocarcinoma lines*

Tumor	Treatment	OROO (μM)
6A	Saline	2.8 ± 0.8
6A	AT-125	9.2 ± 3.2
11A	Saline	2.4 ± 0.4
11A	AT-125	3.9 ± 0.9
26	Saline	8.1 ± 2.3
26	AT-125	125 ± 10.2
38	Saline	4.2 ± 1.4
38	AT-125	25.3 ± 5.1

* Mice bearing subcutaneous colonic tumors 6A and 11A (resistant to FU) and 26 and 38 (sensitive to FU) were injected i.p. with AT-125, 250 mg/kg. Four hours later, the animals were killed, and tumors were rapidly removed and homogenized in 5% PCA and subsequently adjusted with 40% KOH to pH 7.0. The supernatant fractions were used to determine PRPP content. Values are means ± S.D.

small intestine was 128, 104 and 14% above control, respectively (Table 1). Moreover, time course and dose–response studies of AT-125 administered at doses of 5, 250, 400 and 800 mg/kg (Fig. 2) demonstrated that there is a rapid increase in intratumoral PRPP levels, as a function of the dose of the drug. Furthermore, following administration of each dose of the drug, maximum PRPP pool size was observed at 8 hr, and by 24 hr the concentration of the sugar phosphate had returned to normal.

Intratumoral PRPP concentration (murine adenocarcinoma lines). Mice bearing adenocarcinoma lines 26 and 38 (5-FU sensitive), and 6A and 11A (5-FU resistant), were treated with 250 mg/kg of AT-125, i.p. Four hours later, the mean PRPP concentration of lines 26 and 38 (5-FU sensitive) had increased 15.1- and 6.2-fold, respectively, whereas the mean PRPP concentration of lines 6A and 11A (5-FU resistant) had increased 3.2- and 1.6-fold, respectively (Table 2).

Enzymes perturbing PRPP pool size in P388 leukemia cells. The specific activities of several enzymes were determined following i.p. administration of L-glutamine antagonists. The drugs were administered at LD_{10} doses, and 4 hr later the specific activities of various enzymes were determined.

L-Glutamine antagonists did not significantly alter the rate of generation of PRPP (Table 3). Moreover, of the PRPP catabolizing enzymes, DON and AT-125 significantly inhibited PRPP amidotransferase activity, whereas azaserine had no effect on this L-glutamine requiring reaaction. The specific activities of the other catabolizing enzymes were not affected by treatment with L-glutamine antagonists.

DISCUSSION

In this manuscript we have examined the effect of L-glutamine antagonists on PRPP pool size and on the enzymes anabolizing and catabolizing this sugar phosphate in a setting of a murine leukemia and murine colonic adenocarcinoma. Moreover, L-glutamine antagonists preferentially enhanced the pool size of PRPP in P388 tumor versus small intestine. At an LD_{10} does, DON was most effective in enhancing PRPP pool size in P388 leukemia, followed by AT-125 and azaserine. Furthermore, AT-125 at LD_{10} substantially enhanced PRPP pool size in colon lines 26 and 38, the most sensitive lines to 5-FU. Similar AT-125 treatment of murine colonic tumors resistant to 5-FU demonstrated a slight enhancement of PRPP pool size ($P < 0.005$). Examination of the specific activities of the several enzymes perturbing PRPP demonstrated that specific activity of PRPP synthetase was not altered by treatment with L-glutamine antagonists, and of the PRPP catabolizing enzymes, only PRPP amidotransferase was inhibited significantly by DON and AT-125. Interestingly, the increase in PRPP pool size in P388 leukemias following treatment with DON and AT-125 was proportional to the inhibition of PRPP amidotransferase by these two drugs.

Table 3. *In vivo* effect of LD_{10} doses of AT-125, azaserine and DON on PRPP anabolizing and catabolizing enzymes in P388 leukemia*

	Anabolism	Catabolism			
	PRPP synthetase [nmoles · hr^{-1} · (mg protein)$^{-1}$]	PRPP amidotransferase	HGPRT	APRT	OPRT
		[nmoles · hr^{-1} · (mg protein)$^{-1}$]			
Saline	103 ± 24	6.06 ± 1.3	255 ± 11	275 ± 20	13.8 ± 0.6
Azaserine	101 ± 31	5.90 ± 2.1	366 ± 15	387 ± 10	17.8 ± 0.5
DON	78 ± 15	2.09 ± 0.5	271 ± 13	290 ± 10	12.8 ± 0.8
AT-125	98 ± 17	2.94 ± 0.9	317 ± 9	353 ± 9	15.7 ± 0.8

* Mice bearing P388 subcutaneous tumors were injected i.p. with LD_{10} doses of azaserine, DON, or AT-125. Four hours later, the animals were killed and tumors were homogenized (1:3, w/v) in 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM EDTA and 1 mM dithiothreitol. The homogenates were centrifuged at 12,000 *g* for 3 min at 4° and the supernatant fractions, after appropriate dilution, were used for enzyme studies. The supernatant fractions were diluted 1:5 for PRPP amidotransferase assay and 1:10 for the rest of the enzyme assays. Values are means ± S.D.

Since regulation of intracellular metabolism does not depend solely upon the specific activities and kinetic properties of enzymes, the concentrations of substrates, products, and metabolic effectors also influence the rates of intracellular reactions. A number of investigators have suggested that the concentration of the cosubstrate PRPP may, in some cases, by rate-limiting for certain reactions utilizing this sugar phosphate [12–15]. Indeed, PRPP K_m for HPRT and OPRT is approximately 20 μM [17], whereas the basal concentration of PRPP in the same neoplastic tissues examined was less than 10 μM.

Recently, Cadman *et al.* [18, 19] have shown that *in vitro* pretreatment of L1210 cells with 0.1 to 100 μM methotrexate for 3 hr increases the intracellular PRPP from 1.5 to 12-fold respectively. Subsequent treatment of the cells with 5-FU produces a synergistic effect on the number of cells killed in culture. In concert with this phenomenon, the intracellular concentration of fluorinated nucleotides was increased, correlating with the enhancement of PRPP pool size. Thus, intracellular PRPP concentration may play an important role in the anabolism of 5-FU in certain tumors.

Moreover, modulation of intracellular PRPP pool size has a profound effect on the regulation of the *de novo* biosynthesis of purine and pyrimidine biosynthesis in mammalian cells [20–29]. For example, Chen and Jones [30] have recently shown that PRPP influences two key enzymes in the pyrimidine biosynthetic: carbamyl synthetase II (EC 2.7.2.9) and orotate phosphoribosyl transferase (EC 2.4.2.10). In this manuscript, we have determined the specific activities of some of the above enzymes.

One of the major highlights of this manuscript is the effect of LD_{10} doses of L-glutamine antagonists on the intracellular levels of PRPP. Moreover, greater than a 12-fold increase in PRPP pool size was achieved in certain murine tumors. Thus, it is concluded that DON and AT-125 are compounds as suitable to combine with 5-FU as methotrexate in its role of increasing PRPP pool size and therefore in "directing 5-FU" to its nucleotides. We are presently conducting studies in several lines of tumor-bearing lower mammals to test our hypothesis.

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