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INHIBITION OF PHOSPHORIBOSYLPYROPHOSPHATE SYNTHETASE BY 4-METHOXY- (MRPP) AND 4-AMINO-8-(D-RIBOFURANOSYLAMINO)PYRIMIDO[5,4-*d*]PYRIMIDINE (ARPP)*

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Abstract—The basis for the antitumor activities of the exocyclic amino nucleosides 4-amino- (ARPP) and 4-methoxy-8-(D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine (MRPP) was investigated. The primary target of these nucleosides appeared to be 5-phospho-α-D-ribofuranose-1-pyrophosphate (PRPP) synthetase. MRPP-5′-monophosphate was a competitive inhibitor ($K_i = 40\ \mu M$) of the activation of this enzyme by the cofactor inorganic phosphate ($K_a = 2.2$ mM). Consequently, ARPP and MRPP treatment of WI-L2 cultures rapidly inhibited both *de novo* pyrimidine and purine synthesis as well as the nucleotide salvage reactions dependent on PRPP. ARPP or MRPP treatment completely prevented [^{14}C]bicarbonate incorporation into acid-soluble pyrimidine and purine nucleotides. The rate of salvage of [8-^{14}C]hypoxanthine to form IMP was decreased by 85%. Treatment of cells with these agents caused a 50% reduction in the steady-state level of PRPP. When the capacity of the treated cells for sustained synthesis of PRPP was examined by adenine incorporation, the rate of adenine uptake was inhibited by > 50%. *In vivo* treatment of BDF_1 mice with a single dose of ARPP (173 mg/kg) or MRPP (62 mg/kg) extended the mean life span of the mice, which had been inoculated intraperitoneally 1 day earlier with 1×10^6 L1210 murine leukemia cells, by 62 and 82% respectively. These studies indicate that MRPP and ARPP inhibit PRPP synthetase, and that PRPP synthetase may be a viable target in the development of certain antitumor agents.

5-Phospho-α-D-ribosyl-1-pyrophosphate (PRPP)‡ synthetase (EC 2.7.6.1) catalyzes the formation of cellular PRPP, a key intermediate in the formation of nucleoside monophosphates [1]. PRPP is required for the phosphoribosyltransferase-mediated synthesis of phosphoribosylamine and orotidylate in their respective *de novo* purine and pyrimidine pathways and for the salvage synthesis of AMP, IMP and GMP. In addition, the synthesis of the pyridine nucleotide, nicotinamide mononucleotide, requires PRPP [1]. PRPP is also an allosteric activator of carbamoyl phosphate synthetase [2] and phosphoribosylamine synthetase [3].

Regulators of PRPP synthetase activity include inhibitors [4] (2,3-diphosphoglycerate, ADP and other nucleotides) and activators; magnesium ion and inorganic phosphate are necessary cofactors for activity [4]. The greatest degree of sensitivity to phosphate is observed at physiological phosphate concentrations [5].

The regulation of PRPP synthetase is not understood completely [6], but elevated activity of the enzyme can result in uric acid overproduction and gout [7]. Mutations which cause PRPP synthetase superactivity are expressed as catalytic, regulatory, or substrate binding defects or combined catalytic and regulatory defects [8]. Further, the regulation of the enzyme may be tissue specific, for example, the catalytically defective phenotype is expressed in fibroblasts, but not in lymphoblasts [8].

Current understanding of PRPP synthetase regulation has been impeded in part by the lack of specific metabolic inhibitors. Previously reported inhibitors of PRPP synthetase, such as the 5′-phosphate derivatives of 3′-deoxyadenosine (cordycepin) [9], xylosyladenosine [10] and 6-methylmercaptopurine riboside (MeMPR) [11], exert their cytotoxic effects by other mechanisms [12–14]. As yet, the sequence of PRPP synthetase, normal or superactive, from

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‡ Abbreviations: PRPP, 5-phospho-α-D-ribofuranose-1-pyrophosphate; ARPP, 4-amino-8-(D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine; ARPP-5′P, the 5′-monophosphate of ARPP; MRPP, 4-methoxy-8-(D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine; MRPP-5′P, the 5′-monophosphate of MRPP; MeMPR, 6-methylmercaptopurine riboside; HPRTase, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); AKase, adenosine kinase (EC 2.7.1.20); R5P, ribose-5-phosphate; APRTase, adenine phosphoribosyltransferase (EC 2.4.2.7); amidoPRTase, amidophosphoribosyltransferase (EC 2.4.2.14); EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside; FGAR, formylglycinamide ribonucleoside-5′-phosphate; and DTT, dithiothreitol.

humans has not been described, but the sequence of the rat liver enzyme has been obtained from the cDNA sequence and two alleles have been identified [15].

The nucleoside, ARPP, demonstrates antitumor activity against L1210 murine leukemia [16]. In the present study, MRPP was compared with ARPP for L1210 antileukemic activity. ARPP accumulates intracellularly as the 5'-monophosphate [17], but higher phosphate derivatives have not been observed. Studies with salvage enzyme-deficient cell lines show that adenosine kinase is required for phosphorylation [17, 18]. ARPP and the 4-methoxy derivative, MRPP, have been found to be potent inhibitors of *de novo* purine biosynthesis [18]. In this study the mechanisms of action of ARPP and MRPP have been investigated further, and the results are consistent with specific inhibition of PRPP synthetase.

EXPERIMENTAL PROCEDURES

Materials. Sodium [^{14}C]formate, [8-^{14}C]adenine, [8-^{14}C]adenosine, [5,6-^{3}H]uridine, [8-^{14}C]hypoxanthine and sodium [^{14}C]bicarbonate were purchased from ICN Biomedicals. BDF_1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Tri-*n*-octylamine was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and Freon-113 (1,1,2-trichlorotrifluoroethane) was purchased from Matheson. MeMPR was from the Sigma Chemical Co (St Louis, MO).

Cell lines. The B lymphoblast line, WI-L2, and derivatives have been described previously [19]. WI-L2 is the normal lymphoblast phenotype. The enzyme-deficient cell lines selected *in vitro* were: $HPRT^-$, a hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)-deficient line, and AK^-, an adenosine kinase (EC 2.7.1.20)-deficient line. Both deficiencies were $> 0.5\%$ of normal enzyme activity.

HPLC analysis. Sample components were analyzed by HPLC at ambient temperature on a Partisil PXS 10/25 SAX column (Whatman) as previously described [20]. UV absorbance was monitored with either a Kratos model 757 variable wavelength detector or an LKB model 2140 diode array detector in tandem with a Ramona-D radiometric detector.

[^{14}C]*Bicarbonate labeling of cellular nucleotides.* Cultures of WI-L2 cells (20 ml at 4–6 $\times 10^5$ cells/ml) were incubated with test compound for 3 hr at 37°. Cells were collected by centrifugation, and the supernatant fractions removed. The resulting cell pellet was resuspended in one-third volume of bicarbonate-free RPMI-1640 medium containing compound at the same final concentration. [^{14}C]Bicarbonate (20 μCi; 56 Ci/mol) was added, the tube sealed, and incubation continued for 1 hr. The cells were collected by centrifugation and the medium was removed. Nucleotide pools were prepared at 4° by vortexing the cell pellet with 0.4 N $HClO_4$ (100 μl/2×10^6 cells) for 30 sec. Cellular debris was removed by centrifugation, and the aqueous fraction was neutralized with tri-*n*-octylamine/Freon-113 [21] and analyzed by SAX-HPLC.

Inhibition of de novo *purine biosynthesis.* Purine *de novo* synthesis was measured by [^{14}C]formate incorporation as described by Hershfield and Seegmiller [22]. Incorporation of [^{14}C]formate into IMP under feedback-diminished conditions was accomplished by treating cells simultaneously with mycophenolic acid to inhibit IMP dehydrogenase and with alanosine and hadacidin to inhibit adenylosuccinate synthetase [6].

Incorporation of [^{14}C]formate into formylglycinamide ribonucleotide (FGAR) was measured by treatment of WI-L2 cells (1.0 ml, 10×10^5 cells/ml) with azaserine (50 μM) for 45 min before labeling with [^{14}C]formate for a period of 60 min. ARPP was added 5 min prior to the addition of azaserine. Cells were collected by centrifugation, the media fractions were removed by aspiration, and cell-free extracts were obtained from the cell pellet by treatment with 250 μl of 80% EtOH and a 100° water bath for 30 sec. After centrifugation, the supernatant liquid was assayed for FGAR formation by SAX-HPLC using 10 mM potassium phosphate (pH 3.83) as the mobile phase.

Inhibition of salvage synthesis of IMP. WI-L2 cells (10×10^5 cells/ml, 1 ml) were incubated with test compound for 10 min prior to the addition of 2.5 μM [8-^{14}C]hypoxanthine. The rate of incorporation of [8-^{14}C]hypoxanthine into cellular nucleotides was determined by spotting 50 μl of a homogenous suspension of WI-L2 cells onto DE-81 filter paper. The filter papers were washed four times with tap water (4 L), once with 70% EtOH (200 ml), and dried. The radioactivity of each filter was determined using 5.0 ml of a toluene-based scintillation fluid.

Cellular PRPP levels. Intracellular levels of PRPP were determined by a two-step assay [22, 23] as follows. At 4°, to each lyophilized cell pellet or standard sample of PRPP in the same volume of water, 40 μl of buffer A (100 mM Tris–Cl, 10 mM $MgCl_2$, pH 7.5) was added and the tube was immersed in boiling water for 10 sec, then placed in an ice bath. Forty microliters of a mixture of 100 μM [^{14}C]adenine (50 Ci/mol) and APRTase enzyme, at a level sufficient to drive the adenine to AMP reaction to completion in 5 min, was added and the tube was incubated for 30 min at 37°. Two 20-μl aliquots from each sample were spotted onto DE-81 filter papers. The papers were processed as previously described. Samples of known PRPP concentrations were run in parallel with unknown samples to produce the standard curve.

Inosine-driven PRPP synthesis. HPRTase-deficient WI-L2 cells were used for the "inosine-driven" synthesis of PRPP as previously described [24], except that cells were resuspended to 2×10^7 cells/ml.

Enzyme preparations. Erythrocyte PRPP synthetase was purified 400-fold through the ammonium sulfate step as described by Fox and Kelley [25] and stored at −70° in storage buffer (0.3 mM ATP, 6 mM $MgCl_2$, 1 mM DTT, 50 mM sodium phosphate, pH 7.5). Immediately before use, the enzyme preparation was dialyzed for 2 hr against 8 mM potassium phosphate, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$. Adenine phosphoribosyltransferase (APRTase) was purified from erythrocytes as previously described [5] and contained no PRPP synthetase activity. Adenosine

kinase experiments were performed as previously described [17] using protein from WI-L2 lymphoblast lysates.

Determination of PRPP synthetase activity. PRPP synthestase activity was assayed by the PRPP-dependent conversion of [8-^{14}C]adenine to [8-^{14}C]AMP in the presence of adenine phosphoribosyltransferase as previously described [23]. Phosphate concentration was determined as previously described [26].

Reversal of cytotoxicity induced by inhibitors of PRPP synthetase. CHO-K1 cell cultures (0.1×10^5 cells/ml) were established in 24-well plates. After an initial incubation period at 37° under a 5% CO_2 atmosphere to allow cell attachment, combinations of MRPP and the following reversal agents were added to the individual cultures: 25 μM adenosine + 5 μM EHNA, 100 μM uridine, 50 μM AICAR, and 100 μM nicotinamide riboside. Incubation was allowed to continue for 50 hr. Cell growth was quantitated by means of a crystal violet staining procedure [27].

WI-L2 cell cultures were established at 0.5×10^5 cells/ml in 96-well plates. ARPP and combinations of nucleosides were added and incubation continued at 37° under a 5% CO_2 atmosphere for 65 hr; cell growth was quantitated by the MTT dye reduction assay [28]. Absorbance was determined at a test wavelength of 540 nm with a reference wavelength of 620 nm by means of a Titertek MCC scanning well spectrophotometer.

Drug toxicity and antileukemic activity in vivo. Therapeutic evaluations were performed using methods that were described previously in detail [29]. Briefly, BDF_1 female mice weighing about 18 g were treated once on day 1 by intraperitoneal (i.p.) bolus injection of compound 24 hr after intraperitoneal inoculation with 1×10^6 cells of murine leukemia L1210. The drugs were solubilized in water immediately before use and were delivered in uniform volumes of 0.01 ml/g of mouse weight. After treatment, the mice were monitored for post-inoculation life span and also for treatment-induced toxicity or weight change. The incidence of drug- and tumor-induced deaths, the post-inoculation life span of mice that died with tumor, and post-treatment changes in weight were the end points by which responses to treatment were gauged. Concomitantly generated inoculum–response data were used to calculate leukemic cell kill. Temporal patterns of death and observations at necropsy examination were major criteria for assigning deaths to leukemia or to drug toxicity.

RESULTS

Antitumor activity. Administered once at doses that ranged from 37 to 173 mg/kg, both ARPP and MRPP produced biologically significant increases in the mean life span of L1210-inoculated mice (Table 1). A solution of ARPP at maximum solubility, 173 mg/kg, produced a T/C value of 162, which reflects a leukemic cell kill of 99.7%. Comparatively, the maximum non-toxic dose of MRPP, 62 mg/kg, produced a T/C of 182 and a 99.9% leukemic cell kill. The accompanying weight-loss data indicate that the effects of both drugs were somewhat persistent with weight generally being more depressed 72 hr after treatment than at 24 hr. These data indicate that MRPP is a more potent compound than ARPP.

Table 1. Effects of ARPP and MRPP on L1210-inoculated BDF_1 mice

Dose* (mg/kg)	Post-inoculation life span† (% T/C)	Weight change (g/mouse) 24 hr‡	72 hr‡
ARPP			
173	162	−1.67	−2.67
104	145	−1.00	−0.67
62	145	−1.00	−0.67
37	135	−0.67	+1.00
MRPP			
173	TOX 4/6	−2.33	−5.33
104	TOX 1/6	−2.50	−4.17
62	182	−1.67	−1.67
37	167	−0.34	+0.67

* All solutions were delivered i.p. (0.01 ml/g mouse wt) 24 hr after i.p. inoculation of female mice with 1×10^6 L1210 cells.

† Ten control mice lived 6.83 ± 0.58 or 6.60 ± 0.52 days for the ARPP or MRPP experiment, respectively, after injection with a 0.9% solution of NaCl. Treatment responses (six mice/treatment group) are presented as % T/C and were calculated according to the equation, "mean life span of treated mice/mean life span of control mice × 100". A T/C ≥ 125 is considered biologically significant. TOX = number of mice killed by treatment/number of mice treated.

‡ Time after treatment.

Reversal of drug cytotoxicity. Several attempts were made to reverse the growth inhibitory effects of ARPP or MRPP in CHO-K1 adherent cultures or in WI-L2 suspension cultures by providing alternate sources of nucleosides to the growth medium. In each case, growth could be restored only to 30% of control growth by the combination of uridine + adenosine (+ 5 μM EHNA) or uridine + AICAR.

The reversal of MRPP action by drug removal was investigated by treating cells with drug for various times before resuspension in drug-free medium. The cytotoxic effect of MRPP could be reversed up to 8 hr after drug treatment. However, incubation for periods greater than this led to an irreversible inhibition of cell growth.

Intracellular accumulation of MRPP-5′P. The time–course of intracellular MRPP-5′P accumulation in WI-L2 cells is shown in Fig. 1. As MRPP-5′P accumulated intracellularly to levels of approximately 3.7 mM, a concomitant decrease in CTP, UTP, GTP, ATP and the uridine diphosphate sugar peaks was observed. The initial concentration of the ATP pool was approximately 3.5 mM.

Effects on nucleotide synthesis. The mechanisms of action of ARPP and MRPP were investigated initially by measuring the rate of [^{14}C]formate labeling of purine nucleotides formed by *de novo* biosynthesis [18]. On further investigation, ARPP demonstrated potent inhibition of incorporation of formate into either IMP or formylglycinamide ribonucleotide (FGAR) by the use of selective inhibitors to limit the *de novo* purine nucleotide biosynthetic pathway (Table 2). Therefore, the effect of ARPP was identified initially as inhibition of early *de novo*

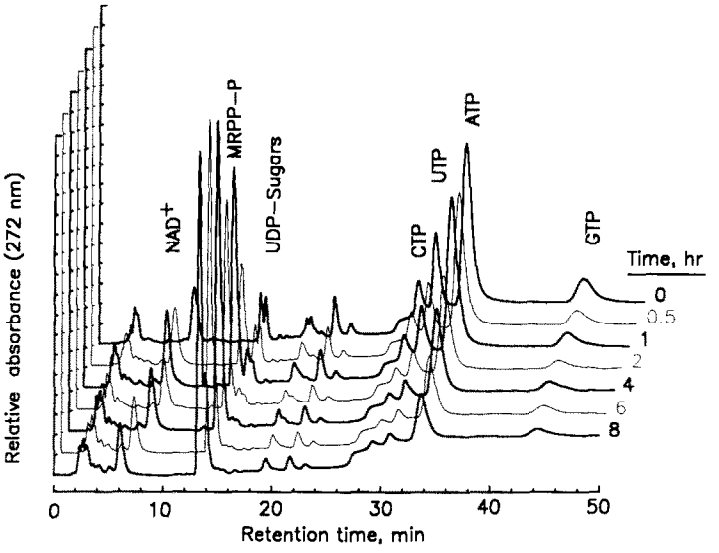

Fig. 1. Accumulation of intracellular MRPP-5'P by WI-L2 cells. WI-L2 cells were incubated in the presence of MRPP-5'P (5 μM) for the times indicated prior to the preparation of acid-soluble extracts and analysis of the extracts by SAX-HPLC. Each trace represents the extract of 2×10^6 cells. The shoulder on the trailing side of the MRPP-5'P peak shown for the chromatogram of the 8-hr sample was identified as the α-anomer. For purposes of comparison, at pH 3.8 the A_{max} (283 nm)/A_{272} ratio for MRPP monophosphate was 1.31; the A_{254}/A_{272} ratio for ATP was 1.61.

Table 2. Effect of ARPP on *de novo* purine biosynthesis

ARPP (μM)	[^{14}C]Formate incorporation (% of control)	
	WI-L2	AKase deficient WI-L2
	(A) Into inosine 5'-monophosphate	
3.2	12.0	99.4
1.0	10.1	96.6
0.3	16.2	97.1
0.1	47.3	94.6
	(B) Into formylglycinamide ribonucleotide (FGAR)	
10.0	4.5	
0.0	100.0	

The incorporation of [^{14}C]formate into purine *de novo* intermediates was observed in separate experiments with WI-L2 cells with (in addition to ARPP): (A) hadacidin (500 μM), alanosine (20 μM) and mycophenolic acid (20 μM) or (B) azaserine (50 μM). The control values, in cpm/min/10^6 cells, were: (A) WI-L2, 4086; AKase deficient, 4874; and (B) 1922.

purine biosynthesis, occurring prior to the formation of FGAR.

The effect of drug treatment on the salvage synthesis of IMP was investigated by measurement of [8-^{14}C]hypoxanthine incorporation into purine nucleotides. When pretreated with 5 μM ARPP or MRPP for 5 min, the incorporation of [8-^{14}C]hypoxanthine was 15 or 22% of the control value (31.2 nmol/min/10^6 cells), respectively, whereas labeling in the presence of 5 μM MeMPR was 98% of the control value. It has been demonstrated previously that, at higher concentrations, MeMPR can inhibit PRPP synthetase [11], but at the lower concentrations, which were used for our studies, MeMPR selectively inhibits amidoPRTase [13, 30], the first enzyme of the *de novo* purine biosynthesis pathway.

These and later results suggested that MRPP and ARPP must be inhibiting an activity prior to amidoPRTase and implicated PRPP synthetase as the site of inhibition. When hypoxanthine was replaced by radiolabeled adenine, adenosine (+ 5 μM EHNA) or uridine, the relative uptake by WI-L2 cells treated with 5 μM MRPP was 18, 80 and 137%, respectively, compared to control, demonstrating a preferential effect on PRPP-utilizing enzymes. The slight inhibition of adenosine uptake was shown later to be a result of direct inhibition of adenosine kinase and was not observed when ARPP was used as the inhibitor.

ARPP and MRPP also completely inhibited the incorporation of [^{14}C]bicarbonate into both pyrimidine and purine nucleotide triphosphates. Cells preincubated for 3 hr in the presence of 5 μM ARPP or MRPP did not incorporate label into the pyrimidine or purine triphosphate peaks as analyzed by SAX-HPLC. The untreated control cells readily incorporated radioactive bicarbonate into the pyrimidine (21,700 cpm/10^6 cells) and purine (59,500 cpm/10^6 cells) nucleoside triphosphate peaks. Drug-treated cells incorporated label into a new peak (1,500 cpm) identified as carbamoylaspartic acid.

Effects on PRPP content and synthesis. The possibility that the formation of the common substrate, PRPP, could be inhibited by ARPP or MRPP treatment was investigated in two ways. A direct effect on the availability of PRPP was observed in dynamic studies with HPRTase-deficient WI-L2 cells supplied with excess ribose-5-phosphate via inosine supplementation. ARPP or MRPP reduced PRPP content to 28 or 50% of the control value (3,400 cpm/10^6 cells), respectively, within 30 min.

In static studies, the PRPP content of WI-L2 cells

Table 3. PRPP levels of WI-L2 cells treated with ARPP or MeMPR

Compound	PRPP pmol/10^6	% Control
Control	170 ± 26	100
ARPP	76 ± 8	45
MeMPR	593 ± 26	349
ARPP + MeMPR*	108 ± 7	64
MeMPR + ARPP†	119 ± 12	70

WI-L2 cells were incubated for 3 hr with drug (10 μM) before determination of PRPP levels.

* Cells were treated with ARPP for 30 min prior to the addition of MeMPR.

† Cells were treated with MeMPR for 30 min prior to the addition of ARPP.

was reduced to 45% of the control value of ARPP treatment (Table 3); whereas MeMPR-treated cells accumulated PRPP to 350% of control. Similar increases of PRPP have been observed in MeMPR-treated L1210 cells [31–33] and result from PRPP accumulation when *de novo* purine synthesis is inhibited at the amidoPRTase reaction. The combination of ARPP and MeMPR resulted in reduction of PRPP and is consistent with PRPP synthetase as the site of inhibition for ARPP treatment.

Effects on PRPP synthetase. The inhibitory activity of ARPP-5′-phosphate was compared with various known inhibitors of PRPP synthetase using partially purified erythrocyte PRPP synthetase. ARPP-5′P (70 μM; anomeric ratio $\alpha/\beta = 1.0$) reduced PRPP formation to 26% of the control value of 22.40 nmol/min/mg protein. This inhibition was comparable to the effect of 2,3-diphosphoglycerate (28% at 4000 μM) and ADP (14% at 100 μM) and considerably more potent than AMP (88% at 200 μM).

The kinetic parameters for inhibition of PRPP synthetase were obtained under pseudo-physiological conditions using anomerically pure β-MRPP-5′P. The α-anomer was inactive in these studies. As indicated in Table 4, with ATP as the variable substrate, non-competitive inhibition by MRPP-5′P was observed. A K_i value of 190 μM was determined with conditions providing an apparent K_m value for ATP of 18 μM. With ribose-5-phosphate as the variable substrate, a complex pattern of inhibition was observed. At 25–75 μM ribose-5-phosphate, this inhibition appeared to be uncompetitive with an apparent K_m value of 130 μM and an apparent K_i value of 27 μM.

Table 4. Kinetic parameters for PRPP synthetase inhibition by MRPP-5′P

Substrates and/or activators		
Constant*	Variable†	Interaction
P_i, R5P	ATP	Non-competitive, K_i = 190 μM
ATP, R5P	P_i	Competitive, K_i = 40 μM
P_i, ATP	R5P	Complex

* Concentrations were: phosphate (P_i), 2 mM; ATP, 500 μM; ribose-5-phosphate (R5P), 350 μM.

† Concentrations varied as follows: phosphate, 0.75 to 4.75 mM; ATP, 10 to 200 μM; R5P, 10 to 100 μM; and MRPP-5′P, 50 to 400 μM. The apparent K_m value for ATP was 18 μM.

Phosphate is an absolute requirement for activation of PRPP synthetase activity [23]. With phosphate as the variable component of the assay, competitive inhibition was observed. A K_i value for MRPP-5′P of 40 μM was determined under conditions providing an apparent K_a value of 2.2 mM phosphate. Similar values for phosphate activation have been reported previously [5, 7, 8].

Activity with adenosine kinase. Previous studies using WI-L2 and adenosine kinase-deficient WI-L2 cells have shown that ARPP is phosphorylated by adenosine kinase [17]. ARPP and MRPP were tested as inhibitors of adenosine kinase. MRPP inhibition was competitive with a K_i value of 40 μM (K_m apparent for adenosine of 6 μM). Under the conditions of the assays, only slight inhibition was detected with ARPP.

These compounds are therefore similar to other exocyclic amino nucleosides which have been shown to be phosphorylated by adenosine kinase but, like ARPP and MRPP, do not accumulate further as the nucleoside di- or triphosphate [34–36].

Activity with adenosine deaminase. ARPP and MRPP are substrates, albeit poor, for calf intestine adenosine deaminase [18]. At high enzyme/substrate ratios, ARPP or MRPP is deaminated or demethoxylated, respectively, to the corresponding inosine analogue, which does not inhibit *de novo* purine biosynthesis [18]. MRPP is less susceptible to enzymatic hydrolysis, the relative substrate activity being: adenosine = 100, ARPP = 1, MRPP = 0.0033. However, cellular adenosine deaminase activity may be of little consequence under physiological conditions due to the rapid formation of the respective 5′-monophosphate derivatives by adenosine kinase. In addition, the corresponding inosine analog has not been observed in either WI-L2 cells or erythrocytes.

DISCUSSION

The above results indicate that ARPP and MRPP, as the monophosphate derivatives, reduce PRPP availability of treated cells by inhibition of PRPP synthetase (Fig. 2). Thus, ARPP-5′P and MRPP-5′P represent novel inhibitors of PRPP synthetase which may have great potential use in the investigation of the regulatory properties of PRPP synthetase and in the chemotherapy of cancer and diseases associated with PRPP overproduction.

Scheduling trials to optimize dosing and to determine the comparative therapeutic indices of the two drugs are in progress. However, the somewhat flat dose response that resulted from a single treatment plus the rebounds in weight that attended treatment with the lowest doses of ARPP and MRPP appear to favor relatively frequent treatment with lower doses of the two drugs. In cell culture studies, the effects of MRPP are observed within a few minutes and over time MRPP-monophosphate continues to accumulate intracellularly in great excess of the concentration required to inhibit PRPP synthetase. The slow release of MRPP from the nucleoside mono-

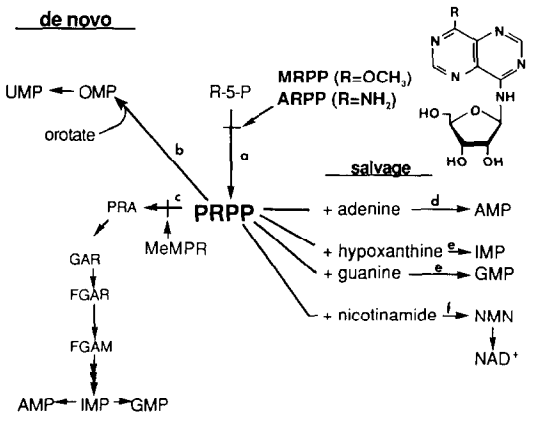

Fig. 2. Schematic diagram of nucleotide biosynthesis by *de novo* and salvage pathways. PRPP serves a central role in *de novo* pyrimidine and purine biosynthesis and in the salvage of preformed nucleobases to form nucleoside 5′-monophosphates. Enzymes involved in PRPP synthesis and use include: (a) PRPP synthetase (EC 2.7.6.1), (b) orotate phosphoribosyltransferase (EC 2.4.2.10), (c) amidophosphoribosyltransferase (EC 2.4.2.14), (d) adenine phosphoribosyltransferase (EC 2.4.2.7), (e) hypoxanthine, guanine phosphoribosyltransferase (EC 2.4.2.8), and (f) nicotinamide phosphoribosyltransferase (EC 2.4.2.12). GAR = glycinamide ribonucleotide; FGAM = formylglycineamidine; and NMN = nicotinamide mononucleotide.

phosphate reservoir may explain the persistence in weight loss of MRPP-treated mice.

It is of interest that a pyridine nucleoside kinase has been identified in CHO cells [37]. Since PRPP is also a required substrate for the synthesis of nicotinamide mononucleotide, nicotinamide riboside was added as a reversal agent to CHO cells in combination with adenosine (+EHNA) and uridine. However, nicotinamide ribonucleoside did not provide any greater reversal of growth inhibition than the combination of adenosine (+EHNA) and uridine. These data (Fig. 1) also indicate that the nicotinamide adenine dinucleotide (NAD) pool is minimally affected by the MRPP-mediated depletion of intracellular PRPP. This is consistent with our reversal data and indicates that, under conditions of PRPP starvation, NAD apparently is not limiting cell growth.

Since MRPP and ARPP as 5′-monophosphate derivatives compete directly with phosphate, they are able to occupy a regulatory site of PRPP synthetase. This site may also recognize adenosine monophosphate and other analogues such as the 5′-phosphate derivatives of 3′-deoxyadenosine (cordycepin) [9], lyxosyladenosine [10] and MeMPR [11], which have been implicated in PRPP starvation. As may be expected for a competitive inhibitor, MRPP 5′-phosphate inhibition of partially purified erythrocyte PRPP synthetase was reversed by addition of high (50 mM) phosphate to the assay reaction (data not shown). The PRPP content of intact erythrocytes can also be regulated by media phosphate concentrations [38]. When such a reversal of WI-L2 cell growth inhibition was attempted by supplementing the growth medium (5 mM phosphate) with additional 5 or 10 mM phosphate, no increase in growth rate was observed.

While mechanistically different, the effect of reduced cellular PRPP content and inhibition of PRPP utilizing enzymes has also been observed in lymphoblasts by Boss [39] as a result of amino acid starvation; the PRPP pool decreases to 40–50% of the control values, but the activity of PRPP utilizing enzymes is inhibited markedly.

The use of specific inhibitors of PRPP synthetase may be useful in a number of applications, such as determining the hierarchy of PRPP synthetase utilization within different cells and tissues. While it is generally assumed that of all the PRPP utilizing reactions [1] purine nucleotide synthesis *de novo* is most sensitive to changes in PRPP availability [40, 41], this is not proven. For example, pyrimidine nucleotide synthesis is limited by PRPP availability in regenerating rat liver [42, 43]. One interpretation of our results suggests that purine nucleotide synthesis by the *de novo* and salvage pathways may be similar in sensitivity to PRPP restriction.

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