0026-895X/93/020479-07$03.00/0

MOLECULAR PHARMACOLOGY, 44:479-485

Inhibition of 5-Phosphoribosyl-1-pyrophosphate Synthetase by the Monophosphate Metabolite of 4-Amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine: A Novel Mechanism for Antitumor Activity

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Received March 11, 1993; Accepted May 13, 1993

SUMMARY

The aminopyrimidopyrimidine nucleoside 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine (APP), which was previously shown to possess experimental antitumor and antiviral activity, was metabolized within WI-L2 human lymphoblastoid cells to a derivative identified as the β-D-ribonucleotide (APP-MP). In a subline of WI-L2 cells deficient in adenosine kinase, this metabolite was not formed and APP was not cytotoxic, suggesting that APP is converted by adenosine kinase to its 5′-monophosphate. Because no evidence of di- or triphosphates was seen, the monophosphate appeared to be the active species. Treatment of WI-L2 or L1210 cells with APP (10 μM) for 30 min caused extensive depletion of both purine and pyrimidine ribonucleotides. Purine and pyrimidine deoxyribonucleotides were also depleted. Cells were not protected from the cytotoxicity of APP by hypoxanthine plus uridine, but uridine plus adenosine plus 2-deoxycoformycin gave considerable protection. This result was consistent with APP-MP acting as an inhibitor of 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase, a hypothesis that was confirmed by preparing PRPP synthetase from Novikoff hepatoma cells; APP-MP was a noncompetitive inhibitor, with a K_i of 0.43 mM. APP-MP was found to accumulate in APP-treated cells to a concentration of almost 3 mM. The relevance of PRPP synthetase inhibition to the cytotoxic mechanism of APP is indicated by the fact that depletion of the PRPP pool was seen as early as 15 min after treatment, before any change was apparent in cellular levels of ATP or UTP. DNA synthesis was markedly suppressed within 30 min of APP treatment of WI-L2 cells, and a lesser degree of inhibition of RNA synthesis was apparent after 45 min.

The aminopyrimidopyrimidine nucleoside APP (NSC 283867; Fig. 1) was first reported by Berman *et al.* (1) as a novel rearrangement of the purine ring. Srivastava *et al.* (2) reported immunosuppressive and antileukemic activity of APP, and Jackson *et al.* (3) subsequently demonstrated significant activity against a murine mammary carcinoma. Broad-spectrum antiviral activity of APP was shown by Burns *et al.* (4), but different modes of action against different viruses were noted. Other cytotoxic aminoglycosides have been reported, most notably the rearrangement products of triciribine and 8-aza-6-thioinosine; these compounds act through inhibition of *de novo* purine biosynthesis, as evidenced by the protection from cytotoxicity by hypoxanthine (5, 6). The present report describes the inhibition of PRPP synthetase (ribose-5′-phosphate pyrophosphokinase, EC 2.7.6.1) by a metabolite of APP.

PRPP is required by cells for *de novo* biosynthesis of purine and pyrimidine nucleotides, as well as for the salvage of preformed purine and pyrimidine bases. Heinrich *et al.* (7) and Balo-Banga and Weber (8) have reported increased activity of PRPP synthetase in transplanted rat tumors. Natsumeda *et al.* (9) showed that the rat hepatoma 3924A possessed an elevated pool of PRPP, relative to normal rat liver. Based upon these findings, Balo-Banga and Weber (8) suggested that 1) there may be an increased pool and elevated capacity for utilization of PRPP in cancer cells, 2) tumors possess elevated activity of PRPP synthetase, which is linked to transformation and progression, and 3) PRPP synthetase should be an appropriate target for the design of therapeutic agents. The salvage pathways utilizing PRPP as a substrate for various phosphoribosyltransferases are necessary for the activation of several antitumor antimetabolites, including 6-thioguanine and 5-fluorouracil. Consequently, alteration of PRPP levels has been investigated as a means of modulating cellular responses to these agents (10, 11). In addition to its possible utility as an

ABBREVIATIONS: APP, 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine; APP-MP, 4-amino-8-(β-D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine-5′-phosphate; dNTP, 2-deoxyribonucleoside-5′-triphosphate; HGPRT, hypoxanthine/guanine phosphoribosyltransferase; PRPP, 5-phosphoribosyl-1-pyrophosphate; HPLC, high pressure liquid chromatography.

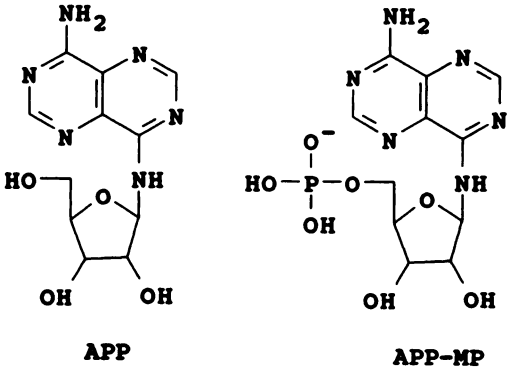

Fig. 1. Chemical structures of APP and APP-MP.

anticancer and antiviral agent, a selective inhibitor of PRPP synthetase might therefore be an effective biochemical modulator and a useful biochemical probe for the investigation of nucleotide metabolism.

Materials and Methods

Chemicals. Purine and pyrimidine nucleotides, nucleosides, and bases, PRPP, and calf thymus DNA were purchased from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* DNA polymerase was purchased from Boehringer-Mannheim (Indianapolis, IN). [*methyl*-^{3}H]dTTP and [8-^{3}H]dATP were purchased from Amersham Corp. (Arlington Heights, IL). [8-^{14}C]Hypoxanthine was purchased from Moravek Biochemicals, Inc. (La Brea, CA). APP was synthesized by the method of Srivastava *et al.* (2).

Synthesis of *N*-(5-*O*-phosphono-β-D-ribofuranosyl)pyrimido[5,4-*d*]pyrimidine-4,8-diamine, disodium salt. A mixture of dry powdered APP (5.91 g, 20 mmol) and dry trimethyl phosphate (120 ml) was cooled to 5° and stirred rapidly as phosphorus oxychloride (9.2 g, 60 mmol) was added dropwise within 30 min. The temperature was kept between 5° and 8° during the addition. The reaction was stirred at 5° for an additional 2.5 hr. The resulting yellow solution was cooled to −20° and added dropwise with rapid stirring to 2.5 liters of ether cooled to −40°. After the addition, the mixture was stirred for an additional 15 min and the ether was then decanted. Cold ether (2 liters, −20°) was added to the syrupy residue and stirring was continued for several minutes. This process was repeated twice. While still cold, the syrupy residue was treated with a mixture of crushed ice (125 g) and saturated $NaHCO_3$ solution (100 ml). The solution was kept at 5° overnight and then extracted with ether (2 × 250 ml). The light yellow solution was evaporated *in vacuo* (20 torr, 50°) to a 75-ml volume and was treated with 150 ml of ethanol. The resulting syrupy precipitate was triturated with ethanol (3 × 150 ml) to effect crystallization. The powdered material was dissolved in water (150 ml), treated with silica gel (50 g), and evaporated under reduced pressure. The residue was placed on a column of silica gel (250 g, packed in acetonitrile/H_2O, 4:1). Elution of the column with 4:1 acetonitrile/H_2O provided a fraction containing only one UV-absorbing and H_2SO_4-charring spot, which was chlorine-free. Evaporation of this fraction at 20 torr and 50° provided 6.5 g (78%) of the aminoglycoside monophosphate disodium salt as an off-white powder, with the following properties: ^{1}H NMR (200 MHz, dimethylsulfoxide-d_6): δ 5.73, 5.76, 5.77, 5.80 (q, 1, H_1, 5.73 and 5.80 disappear on deuteration), 7.76, 8.00 (br d, 2 H, NH_2, exchangeable), 8.20, 8.24 (br s, NH, exchangeable), 8.36, and 8.47 (s, 1 H, C_1H, C_8H); HPLC (C-18 Altex; methanol/0.05 M phosphate buffer, pH 4.5, 5:95; detection at 254 nm): >98.8% pure; analysis: calculated for $C_{11}H_{12}N_6O_7PNa_2 \cdot 2H_2O$ (454.23), C, 29.09; H, 3.77; N, 18.50; P, 6.82; Na, 10.12; H_20, 7.93; found, C, 29.32; H, 3.73; N, 18.46; P, 6.45; Na, 10.06.

Cell culture. WI-L2 human lymphoblasts and L1210 murine leukemia cells were grown in RPMI 1640 medium supplemented with 50 μg/ml gentamycin sulfate and 10% dialyzed fetal calf serum. Growth inhibition studies were conducted using 24-well Linbro plates. Two milliliters of medium containing 10^5 cells were added to each well, and 20-μl aliquots of drug solutions were distributed as appropriate. Plates were incubated at 37° in 95% air/5% carbon dioxide for 3 days, and then cells were counted with a Coulter counter. Under the conditions that were used, WI-L2 cells typically had a doubling time of 18 hr. Therefore, during the 3-day experiment the control cells doubled four times, which represents a 16-fold increase in number.

Incorporation of radioactive precursors into macromolecules. Incorporation of radiolabeled precursors into DNA, RNA, and protein was monitored by exposing logarithmically growing cells to either [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine, or L-[4,5-^{3}H]leucine, respectively, at a concentration of 1 μM and a specific activity of 1 μCi/mmol. At regular intervals the cells from a 1-ml aliquot were injected into 2 volumes of ice-cold 15% trichloroacetic acid, and the precipitate was collected on glass fiber filters. The filters were washed five times with 2-ml aliquots of ice-cold 15% trichloroacetic acid, dried, and placed in scintillation vials with 10 ml of ReadySolv (Beckman, Irvine, CA). Radioactivity was determined in a Beckman LS 6800 scintillation counter.

Nucleotide analysis. Ribonucleotides were assayed in perchloric acid extracts using the anion exchange HPLC procedure described previously (12). A second UV detector monitoring at 320 nm was used to quantify drug metabolites. Deoxyribonucleotides were extracted from approximately 10^8 cells in 60% methanol as described by Tyrsted (13). All four dNTPs were measured by the DNA polymerase method using calf thymus DNA as template/primer and *E. coli* DNA polymerase (14). Standard curves were obtained for each dNTP in each assay. Recoveries were estimated by quantifying extra control samples with standard additions.

Enzyme purification. HGPRT was purified from fresh human erythrocytes by the method of Krenitsky *et al.* (15). PRPP synthetase was purified from Novikoff hepatoma cells as described by Roth *et al.* (16) for the rat liver enzyme, except that the acetone powder step was omitted. This procedure typically produced enzyme preparations with a specific activity of approximately 1000 units/mg of protein, where 1 unit catalyzed the production of 1 nmol of product/min. The purified enzyme was stored in liquid nitrogen in 50 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol.

Quantitation of intracellular PRPP. Approximately 10^7 cells were incubated at 37° in preweighed 50-ml conical centrifuge tubes. At the end of drug treatment the tubes were centrifuged at 250 × *g* for 5 min. The medium was removed, the tube walls were wiped dry, and 250 μl of 50 mM potassium phosphate buffer, pH 7.4, were added. The samples were vortex-mixed and immediately frozen in dry ice/methanol. Additional control samples were prepared containing 0.15 pmol of freshly prepared PRPP as a standard addition. Sample tubes were wiped dry, weighed, placed in vigorously boiling water for exactly 2 min, and then immediately placed on ice. The tubes were centrifuged at 9000 × *g* for 5 min at 0° and the supernatants were assayed immediately for PRPP. This assay was based upon measuring the conversion of [8-^{14}C]hypoxanthine to inosine monophosphate in the presence of human erythrocytic HGPRT. Reaction mixtures containing 120 mM triethanolamine·HCl, pH 7.4, 3 mM $MgCl_2$, 1 nmol of [8-^{14}C]hypoxanthine (57 mCi/mmol), and 200 μl of sample extract, in a final volume of 300 μl, were incubated at 37° for 60 min in 12-ml plastic culture tubes. Reactions were terminated by the addition of 3 ml of ice-cold 1 mM unlabeled hypoxanthine. The entire sample was loaded onto Bond-Elut SAX columns (Analytichem International, Harbor City, CA). Columns were washed three times with 2 ml of water and then [8-^{14}C]IMP was eluted with three 2-ml washes of 0.5 M $NH_4H_2PO_4$, pH 4.8. The column eluant was collected in scintillation vials and counted in 20 ml of ReadySolv scintillation fluid (Beckman Instruments, Fullerton, CA). Standard curves for 0–400 pmol of PRPP were obtained with each assay. The actual PRPP standard concentrations in each

assay were calculated based on the specific activity of the [8-^{14}C] hypoxanthine.

Assay of PRPP synthetase. PRPP synthetase was assayed by a modification of the HGPRT-coupled assay procedure described by Roth *et al.* (16). Reaction mixtures contained 20 mM potassium phosphate buffer, pH 7.6, 5 mM magnesium sulfate, 1 mM disodium EDTA, 50 mM ribose-5-phosphate, 1 mM ATP, and 3.5 mM hypoxanthine (20 μCi/μmol [8-^{14}C]hypoxanthine). Freshly thawed PRPP synthetase (purified as described above) was added to a final concentration of 0.3 enzyme units/ml, and purified HGPRT from human erythrocytes was added to 30 enzyme units/ml, where 1 enzyme unit catalyzes the production of 1 nmol of product/min. For kinetic studies, 35 μl of the aforementioned reaction mixture containing inhibitor but without ATP were preincubated at 37° for 5 min. Reactions were started by addition of ATP and were terminated after 30 min by spotting 25 μl of reaction mixture onto a 1.5-cm square of a polyethylenimine-cellulose thin layer chromatography plate. These squares were dried and then washed three times for 15 min in water and once in methanol. The squares were again dried, placed in 15 ml of scintillation fluid, and counted. Additional unwashed squares were counted to give a measure of the total initial amount of unreacted hypoxanthine.

***In vivo* antitumor evaluation.** The determination of *in vivo* activity was carried out as described previously (17, 18). Briefly, tumors were passaged in the inbred mouse strain of origin (C3H for mammary adenocarcinoma 16C and BALB/c for colon carcinoma C26). Testing for anticancer activity was carried out in an F_1 hybrid mouse derived from the inbred strain of tumor origin (B6C3F_1 for M16C and CD2F_1 for C26). Counted tumor inocula were injected intraperitoneally. All treatments were administered intraperitoneally. Any animals dying within 20 days of the last treatment without obvious signs of tumor burden (ascites, tumor mass, splenomegaly, or hepatomegaly) were judged to have died from drug toxicity.

Results

Effect of APP on intracellular nucleotide levels and nucleic acid synthesis. HPLC analysis of nucleotide extracts from cultured WI-L2 cells treated with APP indicated an almost simultaneous decline of all four ribonucleoside triphosphates, in a time-dependent manner (Table 1). In this experiment the greatest relative decrease was in the GTP pool. The extensive depletion of all ribonucleotide pools raised the possibility that APP may have caused cell lysis. However, microscopic examination of APP-treated cells in the presence of trypan blue showed no visible indication of lysis after 2 or 4 hr in the presence of 10 μM APP. Table 2 shows the effect of a 4-hr exposure to 10 μM APP on dNTP pools in WI-L2 cells. The drug caused significant decreases in dCTP, dTTP, and dATP, while having little effect on dGTP pools.

It was noted that the decreases in purine and pyrimidine nucleotides were accompanied by the accumulation of two possible metabolites of the aminoglycoside that eluted early in the chromatogram, with the nucleoside monophosphates. The two peaks were collected from multiple injections of APP-treated cell extracts and shown to have an UV spectrum similar to that of the aminoglycoside (data not shown). Chemically synthesized standards of the α- and β-monophosphates (see Materials and Methods) coeluted with these two unknowns. The values shown for the metabolite concentrations in Table 1 are based on the 320-nm absorbance of the column eluent using the β-monophosphate standard and are the sums of the α- and β-forms. This summation was necessary because the acid extraction of cellular nucleotides caused rapid equilibration between the α- and β-anomers. No sign of any other metabolites was seen in the chromatograms, at either 254 or 320 nm. Because APP depleted both ribonucleotides and deoxyribonucleotides, an inhibition of both RNA and DNA synthesis might be expected to follow. Fig. 2 shows the incorporation of radiolabeled uridine or thymidine into trichloroacetic acid-insoluble material. Uridine incorporation slowly declined after treatment of cells with APP (10 μM) and by 120 min after the start of treatment was 60% of control. Thymidine incorporation was suppressed earlier and was markedly depleted by 30 min. By 60 min, thymidine incorporation had fallen to 15% of the control value.

Growth inhibition in the presence of protecting agents and in resistant cell lines. Tissue culture studies showed that simultaneous addition of purine bases (adenine and hypoxanthine) or purine or pyrimidine ribonucleosides (adenosine, uridine, and cytidine) did not protect WI-L2 cells from APP growth inhibition (Table 3). A mixture of the four deoxyribonucleosides (deoxycytidine, thymidine, deoxyadenosine, and deoxyguanosine) likewise offered no protection from APP, and the combination of hypoxanthine and uridine had only a marginal effect. However, these experiments were conducted in medium enriched with 10% fetal calf serum, which contains adenosine deaminase. When the adenosine deaminase inhibitor pentostatin (2-deoxycoformycin) was included, adenosine gave partial protection, raising the 72-hr cell count from 6% of control to 38%. The best protection was afforded by the combination of adenosine, pentostatin, and uridine (Table 3).

Because APP is an adenosine analogue, we conducted growth inhibition studies with WI-L2 sublines selected for resistance to other antimetabolites that interact in various areas of adenosine metabolism. Selenazofurin, after conversion to its 5′-phosphate, reacts with ATP to give an analogue of NAD. A selenazofurin-resistant cell line in which the adenylate-coupling reaction no longer occurs was not cross-resistant to APP. Another WI-L2 subline, resistant to pyrazofurin by virtue of a deletion of adenosine kinase, was at least 50-fold resistant to APP (Table 4). This observation suggested that APP is converted to APP-MP by adenosine kinase.

Effect of APP on intracellular PRPP levels. PRPP is a common precursor for *de novo* purine and pyrimidine nucleotide

TABLE 1
Effects of APP treatment on ribonucleotide contents in WI-L2 human lymphoblasts

Treatment	Contents						
	ADP	GDP	UTP	CTP	ATP	GTP	APP-MP
	nmol/10^9 cells						
Control	838	305	628	237	2384	690	0
APP, 15 min	696	247	571	182	2431	559	1426
APP, 30 min	570	210	465	184	2146	421	2013
APP, 60 min	511	180	358	163	1764	311	3047
APP, 120 min	419	146	301	134	1621	269	4566

TABLE 2

dNTP pools in WI-L2 cells treated with APP

Cell cultures were treated for 4 hr and then extracted with 60% methanol as described in Materials and Methods. Numbers in parentheses show values as percentage of untreated control. Values represent the mean of two experiments.

Cell line	Treatment	Pool size			
		dCTP	dTTP	dATP	dGTP
		nmol/10⁹ cells			
WI-L2	Control	9.1 (100)	35.1 (100)	13.0 (100)	10.8 (100)
WI-L2	APP, 10 μM	3.5 (38)	22.8 (65)	7.3 (56)	13.1 (121)

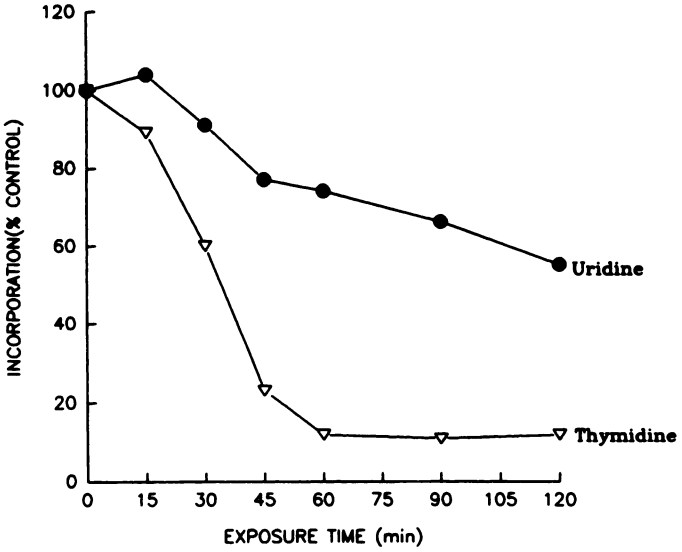

Fig. 2. Effect of APP on thymidine or uridine incorporation into trichloroacetic acid-insoluble material in WI-L2 cells. Cells were exposed to 10 μM APP and samples were removed periodically for analysis of acid-precipitable radioactivity.

TABLE 3

Protection of WI-L2 cells from cytotoxicity of APP by purine and pyrimidine bases and nucleosides

Cells were grown for 72 hr in the presence or absence of APP and the various additives, as indicated, and then counted in a Coulter counter. Values represent the mean of two experiments.

Additive	Concentration	Cell count	
		No APP	+1.5 μM APP
	μM	*% of control*	
None		100	6
Adenine	100	85	20
Hypoxanthine	100	113	17
Adenosine	20	104	15
Adenosine/2′-dCF[a]	20/2	41	38
Cytidine	100	104	13
Uridine	100	96	9
Uridine/hypoxanthine	100/100	113	22
dThd/dCyd/dAdo/dGuo	100/100/100/100	108	13
Adenosine/uridine/2′-dCF	20/100/2	89	56

[a] 2′-dCF, deoxycoformycin; dThd, thymidine; dCyd, deoxycytidine; dAdo, deoxyadenosine; dGuo, deoxyguanosine.

biosynthesis. The effect of APP on PRPP pools was examined to determine whether inhibition of PRPP synthetase was responsible for the drop in nucleotide pools in treated cells (Table 5). In the presence of 10 μM APP a drop in PRPP levels was detected as early as 15 min after drug addition, with further decreases at later times. The drop in PRPP was accompanied by a reduction of ribonucleotides.

TABLE 4

Growth inhibition of parental and drug-resistant WI-L2 cells by antimetabolites

Cells were grown for 72 hr in the presence of inhibitor concentrations covering a 3-log unit range and then counted in a Coulter counter. IC_{50} values were determined by logarithmic interpolation. Values represent the mean of two experiments.

Compound	IC_{50}		
	WI-L2	WI-L2/SZ[r]	WI-L2/PF[r]
		μM	
Pyrazofurin	0.043	0.071	87
Selenazofurin	4.1	90.1	1.7
APP	2.1	2.3	>100

TABLE 5

Time course of changes in pool sizes of ribonucleotides and PRPP in WI-L2 cells treated with APP

Cells were treated for 2 hr with 10 μM APP and harvested by centrifugation. PRPP extracts were made by suspension of cells in cold phosphate buffer, freezing, thawing, and then boiling for 2 min. PRPP was measured as described in Materials and Methods. Nucleotides were measured in separate samples by HPLC analysis of 0.7 M perchloric acid extracts. Numbers in parentheses show values as percentage of control. Values represent the mean of two experiments.

Treatment	Pool size			
	ATP	GTP	UTP	PRPP
	nmol/10⁹ cells			
Control	2056 (100)	388 (100)	377 (100)	71 (100)
APP, 15 min	2015 (98)	330 (85)	388 (103)	55 (78)
APP, 30 min	1871 (91)	275 (71)	317 (84)	32 (45)
APP, 60 min	1727 (84)	194 (50)	268 (71)	28 (39)
APP, 120 min	946 (46)	116 (30)	117 (31)	29 (40)

Table 6 shows the effect of APP on pools of ribonucleotides and PRPP in comparison with two other agents that are known to modulate PRPP pools, adenosine and methotrexate. The three agents gave distinct and characteristic patterns of pool changes. APP depleted PRPP along with purine and pyrimidine ribonucleotide pools. After methotrexate treatment purine nucleotide levels in cells declined, whereas pyrimidine nucleotides and PRPP accumulated, as described previously (11). Adenosine treatment causes cellular accumulation of ATP and ADP via salvage (19). As purine nucleotides accumulate, feedback inhibition of PRPP synthetase causes levels of pyrimidine nucleotides and PRPP to decrease. For all three agents, changes in the concentration of PRPP are reflected in the levels of pyrimidine nucleotides.

Inhibition of PRPP synthetase by APP-MP. Using a partially purified PRPP synthetase from Novikoff hepatoma cells, the β-anomer of APP-MP was shown to be an effective inhibitor at submillimolar concentrations. The nature of this inhibition was studied by varying the concentrations of APP and ATP. The results are plotted in double-reciprocal form in Fig. 3. The data were fitted through nonlinear regression analysis to the kinetic equations described by Cleland (20). The K_i (intercept) and K_i (slope) values were approximately equal, indicating noncompetitive inhibition, and gave an estimated K_i of 0.43 ± 0.03 mM for APP-MP, with a K_m of 27 ± 4 μM for ATP.

Antitumor activity of APP-MP. To confirm that APP-MP retained antitumor activity, the metabolite was tested in two *in vivo* mouse models and its activity was compared with that of the parent glycoside. Initial attempts to synthesize APP-MP resulted in a mixture of the α- and β-anomers, which was tested against intraperitoneal implants of mammary adenocar-

TABLE 6

Comparative study of the effect of agents that modulate PRPP pools in WI-L2 cells

Cells were treated for 2 hr and harvested by centrifugation. Extraction and assay procedures were as described in Materials and Methods. Numbers in parentheses are percentage of control. Values represent the mean of two experiments.

Compound	Concentration	Pool size				
		UTP	CTP	ATP	GTP	PRPP
	μM	nmol/10⁹ cells				
Control		690 (100)	232 (100)	2269 (100)	400 (100)	60 (100)
Methotrexate	1	995 (144)	276 (119)	1770 (78)	199 (50)	275 (459)
Adenosine	10	283 (41)	109 (47)	3472 (153)	368 (92)	17 (29)
APP	10	387 (56)	132 (57)	1906 (84)	144 (36)	18 (30)

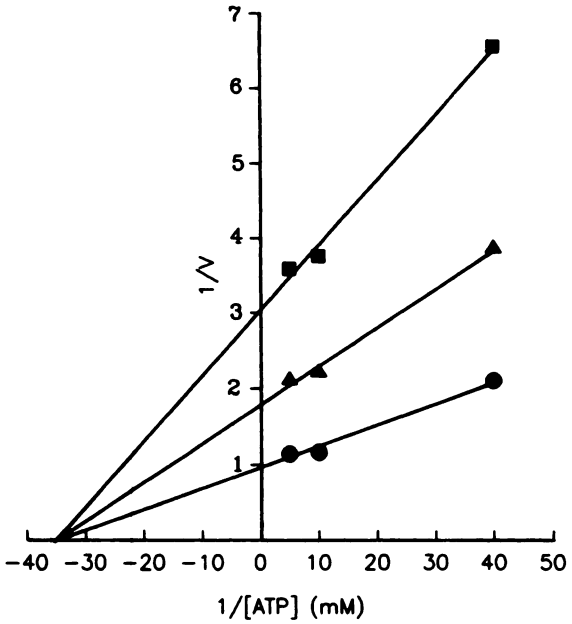

Fig. 3. Inhibition of purified PRPP synthetase by APP-MP. ●, No APP-MP; ▲, 0.384 mM APP-MP; ■, 0.96 mM APP-MP.

TABLE 7

Comparison of *in vivo* antitumor activity between APP and APP-MP

Tumor route and injection site were both intraperitoneal. Experimental details are given in Materials and Methods.

Drug	Tumor	Optimal total dose	Schedule	Increase in life span
		mg/kg		%
β-APP	M16C	23	Days 1–5	80
α,β-APP-MP	M16C	50	Days 1–5	62
β-APP-MP	C26	25	Days 3–7	41

cinoma 16C. Both the glycoside and the monophosphate exhibited considerable activity, increasing the life span by 80 and 62%, repectively (Table 7). Based on a tumor volume doubling time in these experiments of approximately 1 day, these results correspond to an estimated net reduction in tumor burden of 2.5 $\log_{10}$ units over the course of treatment. The pure β-isomer was tested against intraperitoneal implants of colon adenocarcinoma C26 and again moderate activity was obtained, with an increased life span of 41%.

To establish the stereospecificity of this compound, pure preparations as well as a mixture of both isomers were tested in an *in vitro* growth delay assay using L1210 mouse leukemia cells (Table 8). The most potent compound was the β-anomer of APP-MP, followed by the β-anomer of the glycoside. A mixture of α- and β-anomers produced an IC_{50} approximately

TABLE 8

Comparison of *in vitro* growth inhibition between different anomers of APP-MP in L1210 leukemia cells

Cells were grown for 72 hr in the presence or absence of the indicated drugs at varying concentrations. Cells were counted in a Coulter counter. Values represent the concentration of drug that inhibits cell growth by 50% and are the mean of two separate experiments.

Drug	IC_{50}
	μM
β-APP	0.38
α,β-APP-MP	0.72
β-APP-MP	0.28
α-APP-MP	11.2

2.5-fold higher than that of the pure β-anomer, whereas the pure α-anomer was 40-fold less potent than the β-anomer. These results clearly indicate that the β-anomer is the active form of the drug.

Discussion

The structural resemblance of APP to adenosine suggested that it might act as an antipurine agent, and the measurements of ribonucleotide pools in APP-treated cells were conducted in an attempt to determine whether this was the case. In fact, as shown in Tables 1, 5, and 6, extensive decreases were seen in pools of both purine and pyrimidine ribonucleotides. There are two plausible explanations for this observation; APP could block formation of a common precursor required for biosynthesis of both purine and pyrimidine nucleotides (such as PRPP) or, alternatively, it might cause a primary antipurine effect and the drop in pyrimidine nucleotide pools could be a secondary effect of ATP depletion. If the latter explanation is correct, it might be expected that depletion of ATP would precede depletion of UTP; in fact, Tables 1 and 5 indicate that UTP pools decline faster than ATP pools.

The extensive depletion of both ribonucleotides and deoxyribonucleotides raised the question of whether DNA synthesis or RNA synthesis would be more susceptible to the effects of APP. The precursor incorporation studies illustrated in Fig. 2 suggest that DNA synthesis declines more rapidly and more extensively in APP-treated cells than does RNA synthesis. These experiments, however, are notoriously difficult to interpret when the test agent is a compound that perturbs nucleotide pool concentrations, because the intracellular isotope dilution factors are altered so that rates of nucleic acid synthesis are not directly proportional to rates of uridine or thymidine incorporation. Uridine incorporation after 120 min of APP treatment was 60% of the control value; allowing for a UTP pool size of 45% of the control value at this time gives a corrected rate of RNA synthesis of 27% of control. Thymidine incorpo-

ration after 120 min with APP was 16% of the control value; allowing for a dTTP pool size of 39% of the control value gives a corrected rate of DNA synthesis of 6% of control. Although these corrected values are subject to several assumptions and uncertainties, it does seem that DNA synthesis is more extensively and rapidly inhibited by APP than is RNA synthesis.

The cross-resistance studies in antimetabolite-resistant cells (Table 4) provide strong evidence that APP is converted to the nucleoside 5′-monophosphate, APP-MP, through the activity of adenosine kinase, which might be consistent with the structural resemblance of APP to adenosine. A number of other experimental antitumor agents are known to be activated by adenosine kinase, including triciribine and 8-aza-6-thioinosine (21, 22). Because APP causes depletion of the cellular ATP pool, it might be expected that activation of APP would be self-limiting. However, the amounts of APP-MP that accumulate in the cells are quite considerable; based upon a cell volume of 1.55 ml/10^9 cells, the concentration of APP-MP after a 120-min incubation with 10 μM APP (Table 1) would be 2.95 mM. Two features of the regulation of adenosine kinase probably explain why the phosphorylation of APP is so extensive. First, levels of ATP above 1 mM cause excess substrate inhibition of adenosine kinase (23), such that in the initial stages ATP depletion would actually activate adenosine kinase. Second, AMP and ADP are feedback inhibitors of adenosine kinase (23) and these are also depleted by APP. Thus, APP facilitates its own phosphorylation by depleting natural inhibitors of its activating enzyme. It should be noted that, unlike many other adenosine analogues, APP is not a substrate for adenosine deaminase (3). APP-MP appears not to be a substrate for adenylate kinase, because no signs of the corresponding di- or triphosphates were found in treated cells.

The inhibition of PRPP synthetase by APP-MP is not highly potent, but the lack of potency is counteracted by the high levels of APP-MP reached in the cells (nearly 3 mM), which would result in about 87% inhibition of the enzyme. PRPP synthetase was reported to be feedback-inhibited by both AMP and ADP (24). The ADP inhibition was competitive with ATP, but inhibition by AMP was noncompetitive with respect to both substrates and under standard assay conditions 1 mM AMP gave 45% inhibition. Thus, the inhibition of PRPP synthetase by APP-MP appears to resemble that by AMP in mechanism, is more potent, and is an example of "pseudo-feedback" inhibition by an antimetabolite.

At present, the inhibition of PRPP synthetase by APP-MP is its only known site of action. The metabolite reversal studies (Table 3) support this interpretation. Although a minor degree of protection was given by hypoxanthine (especially when uridine was also present), hypoxanthine requires PRPP for its salvage and thus cannot give efficient protection. Adenosine is salvaged by the PRPP-independent adenosine kinase reaction but is rapidly degraded by adenosine deaminase. However, in the presence of an adenosine deaminase inhibitor (pentostatin) adenosine gave partial protection, which was further increased when uridine was also present (Table 3). Although it is possible that adenosine acted by preventing phosphorylation of APP, the fact that uridine increased the degree of protection indicates that APP-MP was being formed.

Manipulation of PRPP pools has been used as an approach to biochemical modulation (10, 11, 25). In principle, normal cells (which tend to have lower PRPP synthetase activity than tumor cells) might be selectively protected by APP from the cytotoxic effects of drugs such as 6-thioguanine and 5-fluorouracil, whose anabolism is dependent on PRPP levels. Alternatively, if an antimetabolite is activated by a PRPP-independent route but is then subject to reversal by hypoxanthine salvage (as is the case for methylmercaptopurine riboside), then APP might enhance the efficacy and potency of such an agent.

The basis for antitumor selectivity of APP is not clear. Tumor cells tend to have reduced adenosine kinase activity and elevated PRPP synthetase (8, 19). Both of these alterations should limit the efficacy of APP in tumor cells, but the higher rate of utilization of nucleic acid precursors in tumor cells may negate these apparent disadvantages. The availability of an effective inhibitor of PRPP synthetase will provide a valuable probe for study of the control of nucleotide metabolism.

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