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Pyrazofurin-Resistant Hepatoma Cells Deficient in Adenosine Kinase*

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Abstract—*A pyrazofurin-resistant subline of the Morris rat hepatoma* 3924*A has been isolated* in vitro. *These cells, designated subline* 3924*A*/1*P*39-3, *were* 100*-fold resistant to pyrazofurin, and showed cross-resistance towards* 6*-methylmercaptopurine riboside and* 6*-chloropurine riboside, but not towards* 6*-azauridine or arabinosyladenine. Measurement of cellular enzyme activities indicated that the level of adenosine kinase in the mutant was decreased to* 2% *of the value in the parental cell line, but activities of uridine kinase, adenosine deaminase, deoxyadenosine kinase and orotidine* 5′*-monophosphate decarboxylase showed no significant change. Despite the extensive deletion of adenosine kinase, the sensitivity of the mutant to adenosine toxicity was not greatly different from that of the parental* 3924*A cell line. Incubation of the cells for* 20 hr *in presence of* 300 μM *adenosine caused an increase of* 43% *in the adenine nucleotide pool of the* 1*P*39-3 *cells, as compared with an increase of* 93% *in the parental* 3924*A cells. In both lines* 300 μM *adenosine caused significant depletion in uridine nucleotides and phosphoribosylpyrophosphate pools. Nevertheless, uridine* (1 mM) *did not reverse the adenosine toxicity in either case. These cells have provided direct evidence that the antipyrimidine agent, pyrazofurin, is activated by adenosine kinase in rat hepatoma cells. The results also show that loss of adenosine kinase does not affect adenosine toxicity in these cells, suggesting that the toxicity may be attributable to free adenosine rather than overproduction of adenine nucleotides.*

INTRODUCTION

THE *C*-NUCLEOSIDE antibiotic, pyrazofurin [1], has been shown to have antitumor activity in a number of experimental systems [2], and a clinical trial has been conducted [3, 4]. The primary mechanism of action of pyrazofurin is believed to be the inhibition of orotidine 5′-monophosphate decarboxylase (EC 4.1.1.23) leading to depletion of pyrimidine nucleotides and concomitant accumulation of orotidylate and orotidine [1, 2]. The active form of the drug is pyrazofurin 5′-phosphate, and it has been suggested that the phosphorylation of pyrazofurin is catalysed by adenosine kinase (EC 2.7.1.20) rather than uridine kinase (EC 2.7.1.48) because adenosine competitively decreased conversion of pyrazofurin to its nucleotide form, whereas uridine did not [5].

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Abbreviations: PRPP, phosphoribosylpyrophosphate; AK^-, adenosine kinase deficient; ID_{50}, inhibitor concentration that gives 50% growth inhibition.

Acquired resistance to pyrazofurin has been studied in hamster cells, and the resistance was attributed to overproduction of the target enzyme, orotidine 5′-monophosphate decarboxylase. Orotate phosphoribosyltransferase (EC 2.4.2.10), which is believed to constitute a multifunctional protein with the decarboxylase, was increased coordinately [6]. In the present study we describe a second mechanism of resistance to pyrazofurin through the loss of cellular adenosine kinase activity. This observation provides strong supporting evidence for the earlier indication that adenosine kinase was responsible for pyrazofurin activation [5].

The availability of an adenosine kinase deficient mutant provided an opportunity to study the mechanism of adenosine toxicity in hepatoma cells. Earlier studies using cultured mouse fibroblasts led to the suggestion that the toxicity of adenosine was the result of inhibition by the adenine nucleotides of the synthesis of UMP [7]. *De novo* biosynthesis of both purines and pyrimidines requires PRPP, and it has been shown that ADP and ATP feedback inhibit PRPP synthetase [8]. Thus in the presence of excess adenosine, the toxic

effect would be the result of pyrimidine starvation. This concept was supported by the observation that adenosine toxicity in these fibroblasts was reversible by uridine [7]. Our present studies indicate that in rat hepatoma cells the adenosine toxicity was neither reversed by uridine nor alleviated by deletion of adenosine kinase, suggesting that a different mechanism of adenosine toxicity may be operative in these hepatoma cells.

MATERIALS AND METHODS

Adenosine, 6-chloropurine riboside, 6-methylmercaptopurine riboside, N^6-ethenoadenosine, uridine, 6-azauridine, 2′-deoxyadenosine, adenine arabinoside, and PRPP were purchased from Sigma Chemical Co., St. Louis, Missouri. Pyrazofurin was generously donated by Eli Lilly & Co., Indianapolis, and 2′-deoxycoformycin was supplied by courtesy of the National Cancer Institute. [^{14}C]-Orotic acid was obtained from New England Nuclear, Boston, Massachusetts. [^{3}H]-adenosine and [^{3}H]-deoxyadenosine were from Amersham Corp., Arlington Heights, Illinois. High pressure liquid chromatography columns were purchased from Whatman, Inc., Fair Lawn, New Jersey. Thin layer chromatography plates coated with polyethyleneimine cellulose were a product of Macherey-Nagel and Co., Duren, Germany. Tissue culture supplies were from Grand Island Biological Co., Grand Island, New York.

Cell culture

Cells of the rapidly growing, poorly differentiated Morris rat hepatoma line 3924A [9] were maintained in McCoy's 5A medium supplemented with 10% dialyzed horse serum, penicillin (100 u/ml) and streptomycin (100 μg/ml). Cultures were maintained in a 95% air and 5% CO_2 atmosphere, and under these conditions grew as monolayers with a mean log-phase doubling time of 14 hr. For selection of resistant cells, 3924A cells were plated at a density of approximately $1.3 \times 10^3/cm^2$ in medium containing 2 μm pyrazofurin. Individual clones that developed were transferred to 21 cm^2 cell culture dishes (Lux Scientific Products, Newbury Park, California) and grown into mass cultures in the presence of pyrazofurin. For growth inhibition experiments, 5 ml cultures were initiated in 21 cm^2 culture dishes at a cell density of 2×10^4/ml. After 2–4 hr the specified concentration of drug was added and the cultures were allowed to grow for 72 hr before the cells were harvested. For studies on adenosine toxicity, cell density was 6×10^3/ml, and the media plus adenosine was changed at 24 and 48 hr. The cell growth was determined by cell count or by measurement of the total nucleic acid content as described previously [6].

Enzyme assays

About 5×10^7 cells were harvested by scraping, collected by centrifugation at 1500 ***g*** for 5 min, and resuspended in 1.5 ml of extraction medium containing 50 mM Tris–HCl buffer (pH 7.2) and 0.5 mM 2-mercaptoethanol. Cells were lysed by two cycles of freezing in liquid nitrogen followed by thawing at 37°C. Lysates were centrifuged at 105,000 ***g*** for 30 min, and assays were performed on the supernatant fraction. Adenosine kinase and adenosine deaminase were assayed as previously described [10]. Deoxyadenosine kinase was assayed using a modification of the adenosine kinase procedure described by Jackson *et al.* [10]. The reaction mixture contained Tris–HCl buffer (pH 8.0) (3.3 μmole), dithioerythritol (0.02 μmole), KCl (1.65 μmole), $MgCl_2$ (1.3 μmole), ATP (0.2 μmole), phosphoenolpyruvate (0.2 μmole), pyruvate kinase (1.0 i.u.), deoxycoformycin (0.015 nmole), ^{3}H-labeled deoxyadenosine (0.06 μmole; 4 mCi/mmole), 40 μl of cell supernatant in a final volume of 0.15 ml. Incubation was for 5 min at 37°C. The reaction was stopped by boiling for 3 min, and precipitated protein was sedimented by a 30 sec microfuge spin. Aliquots of 50 μl were spotted on 2 cm polyethyleneimine squares (polygram Cel 300 PEI), and the unreacted deoxyadenosine was removed by washing as previously described [10]. The squares were dried and the reaction product measured by scintillation counting. Uridine kinase was assayed as described by Weber *et al.* [11], and orotidylate decarboxylase was assayed as described by Suttle and Stark [6].

Ribonucleotide analysis

About 2×10^6 cells were harvested by centrifugation at 1500 ***g*** for 5 min, and cell pellets were extracted in 1 ml of ice-cold 0.7 M perchloric acid. Extracts were centrifuged at 20,000 ***g*** for 20 min to remove precipitated protein. Perchlorate was removed from the supernatant as the potassium salt by addition of 75 mg potassium bicarbonate followed by centrifugation at 1500 ***g*** for 5 min. The extracts were brought to pH 2.6 by addition of

25 μl 4N hydrochloric acid. Aliquots of 200 μl were analysed for ribonucleotides by high pressure liquid chromatography on a Whatman Partisil PSX 10/25 SAX strong anion exchange column (25 × 0.46 cm) using buffer conditions described by Lui *et al.* [12]. Peaks were integrated with a Varian CDS 111L integrator using detector response factors calculated from known amounts of standard nucleotides.

Phosphoribosylpyrophosphate assays

About 5×10^7 cells were harvested by centrifugation at 1500 ***g*** for 5 min, and resuspended in 0.6 ml of extraction medium containing 50 mM potassium phosphate buffer, pH 7.5, 0.25 M sucrose, and 0.1 mM disodium EDTA. Cells were lysed by freezing the suspension in liquid nitrogen, thawing at 37°C, and then boiling for 20 sec. Lysates were cooled on ice, and centrifuged for 20 min at 105,000 ***g***. Assays were then run immediately as follows: to a 400 μl plastic microcentrifuge tube were added 90 μl of triethanolamine chloride buffer, 0.4 M, pH 7.8; 10 μl of $MgCl_2$, 100 mM; 25 μl [^{14}C]-hypoxanthine, 0.8 mM, 20 μCi/ml; 25 μl of hypoxanthine phosphoribosyltransferase (0.8 i.u./ml purified from human red blood cells by the method of Krenitsky *et al.* [13]; and finally 150 μl of the cell extract. Blank tubes were set up using extraction buffer instead of cell extract. Assay standard tubes contained 0.1–2.0 nmole PRPP. Tubes were incubated at 37°C for 30 min during which time the reaction went to completion. 25 μl aliquots of reaction mixture were spotted onto 2 cm squares of polyethyleneimine cellulose thin-layer plate. These squares were washed 3 times in 600 ml of 1 mM sodium formate, and once in 95% ethanol. They were then dried and counted in a scintillation counter. Counting efficiency was determined using unwashed squares. This procedure gave a linear response for up to 15 nmole PRPP, and the lower limit of detection was about 0.05 nmole.

RESULTS

Cells of hepatoma 3924A were cultured in medium containing 2 μM pyrazofurin, which was lethal to over 99.9% of the cells. Some drug-resistant clones emerged under these conditions, and were isolated and characterized. Most of these sublines appeared to be resistant to pyrazofurin by virtue of overproduction of the target enzyme, orotidylate decarboxylase. The properties of these sublines have been described elsewhere [14]. One subline, designated 1P39-3, had normal activity for orotidylate decarboxylase, indicating that drug resistance was attributable to some other mechanism. Dose–response curves to pyrazofurin for the 1P39-3 subline and the parental 3924A cells are shown in Fig. 1. Based upon the pyrazofurin concentration required to give 50% inhibition of growth, the 1P39-3 cells were 105-fold resistant.

It has been suggested that adenosine kinase is responsible for conversion of pyrazofurin to its active nucleotide form, pyrazofurin 5′-monophosphate [5]. Table 1 summarizes the activities of various enzymes of nucleoside metabolism in wild-type 3924A cells and in the 1P39-3 mutants. Adenosine kinase activity in the 1P39-3 cells was less than 2% of that found in the parental 3924A cells. Activity of adenosine deaminase in the mutant cells was normal, and as mentioned above, orotidylate decarboxylase was unchanged. Since pyrazofurin acts at the same site as 6-azauridine, it seemed possible that it might be partially phosphorylated by uridine kinase; however, the activity of uridine kinase was near the normal range in the pyrazofurin-resistant cells.

There has been some uncertainty as to whether deoxyadenosine is phosphorylated by the same enzyme as adenosine in hepatic tissues. Streeter *et al.* [15] found that adenosine kinase activity from rat liver co-purified with deoxyadenosine kinase activity, although inhibitor profiles for the two activities differed. It was thus of particular interest to measure the deoxyadenosine kinase activity of the AK^- hepatoma cells. As shown in Table 1, the deoxyadenosine kinase activity of the 1P39-3 cells was found to be slightly higher than that of the wild-type 3924A cells.

Mammalian fibroblasts deficient in adenosine kinase have been described previously by Ishii and Green [7]; the fibroblasts were highly resistant to adenosine toxicity. It could be concluded that phosphorylation of adenosine by the adenosine kinase reaction was a prerequisite of adenosine toxicity [7]. The adenosine metabolism of hepatomas differs in a number of ways from that of normal cells [10], and hepatomas have greater intrinsic resistance to adenosine toxicity. We used the 1P39-3 cells to determine whether deletion of adenosine kinase results in increased resistance to adenosine in hepatoma cells. A growth inhibition study is shown in Fig. 2. There is no significant difference in the inhibitory effect of adenosine in the 1P39-3 cells compared

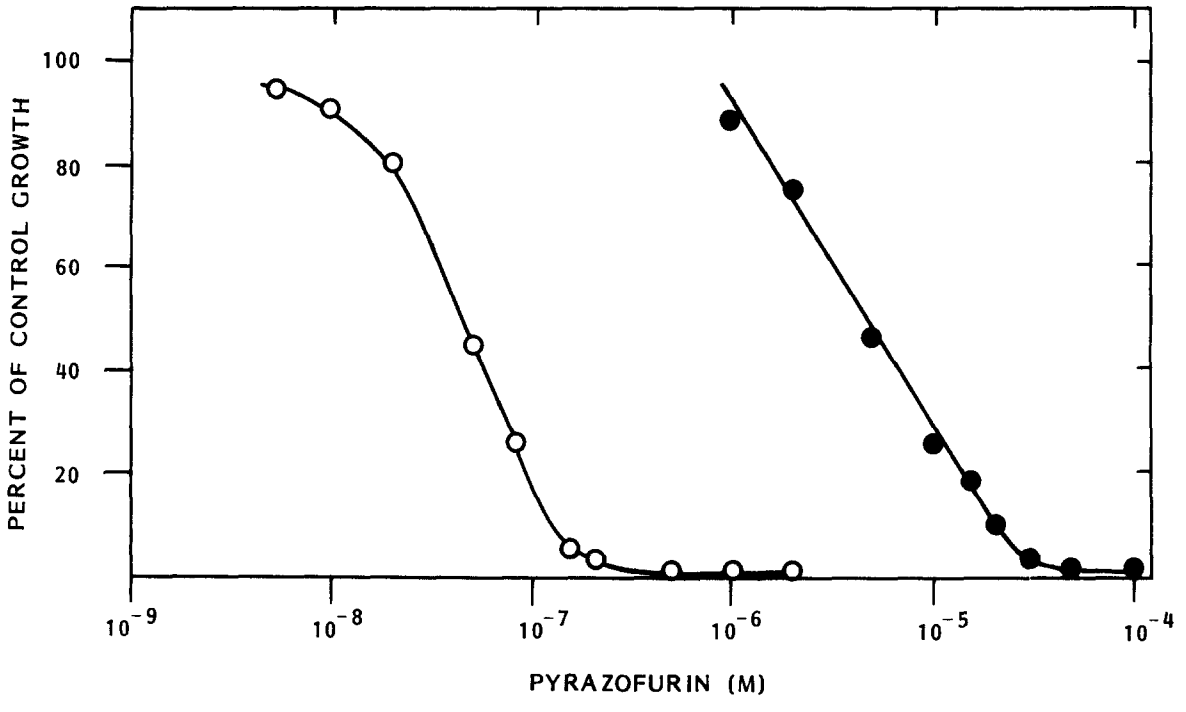

Fig. 1. Inhibition of cell growth by pyrazofurin. The cytotoxicity of pyrazofurin was determined as described in Materials and Methods for 3924A cells (○—○) and 1P39-3 cells (●—●). The total nucleic acid content of cells surviving at the indicated pyrazofurin concentrations after 72 hr exposure is plotted as a percentage of controls grown in the absence of pyrazofurin.

Table 1. Enzymes of nucleoside metabolism in hepatoma cells

Enzyme	Specific activity (nmole/min/mg protein) ± S.E.M.	
	3924A	1P39-3
Adenosine kinase	11.9 ± 0.8	0.21 ± 0.06 (1.8%)*
Deoxyadenosine kinase	8.8 ± 1.50	10.9 ± 1.05 (124%)
Adenosine deaminase	41.8 ± 2.2	39.7 ± 1.7 (95%)
Uridine kinase	10.7 ± 1.1	8.7 ± 0.9 (81%)
Orotidylate decarboxylase	2.56 ± 0.25	2.52 ± 0.1 (98%)

*Significantly different from activity in 3924A ($P < 0.05$ in 2-tail Student's t-test). Extraction and assay procedures are described in the Materials and Methods section. Values in parentheses indicate activity in 1P39-3 as percentage of activity in 3924A.

to the parental cell line. In fact, the ID_{50} for the AK^- cells appears slightly lower than for the wild-type cells (Table 2).

In mammalian lymphoblasts and fibroblasts the toxicity of adenosine has been attributed to pyrimidine starvation. This interpretation is supported by the reversal of adenosine toxicity by uridine [7, 16–18]. Table 2 shows 50% growth-inhibitory concentrations for adenosine in the presence and absence of 0.5 mM uridine. In both the wild-type and mutant cells, uridine increased the adenosine ID_{50} by 2-fold. This minor effect of uridine on the adenosine toxicity is quite different from the approximately 100-fold increase reported by Ishii and Green [7].

It was important to determine whether the pyrazofurin-resistant 1P39-3 cells showed cross-resistance to 6-azauridine, since azauridine 5′-monophosphate acts on the same target enzyme (orotidylate decarboxylase) as pyrazofurin 5′-monophosphate. ID_{50} values for pyrazofurin and 6-azauridine are compared in Table 2; no cross-resistance was

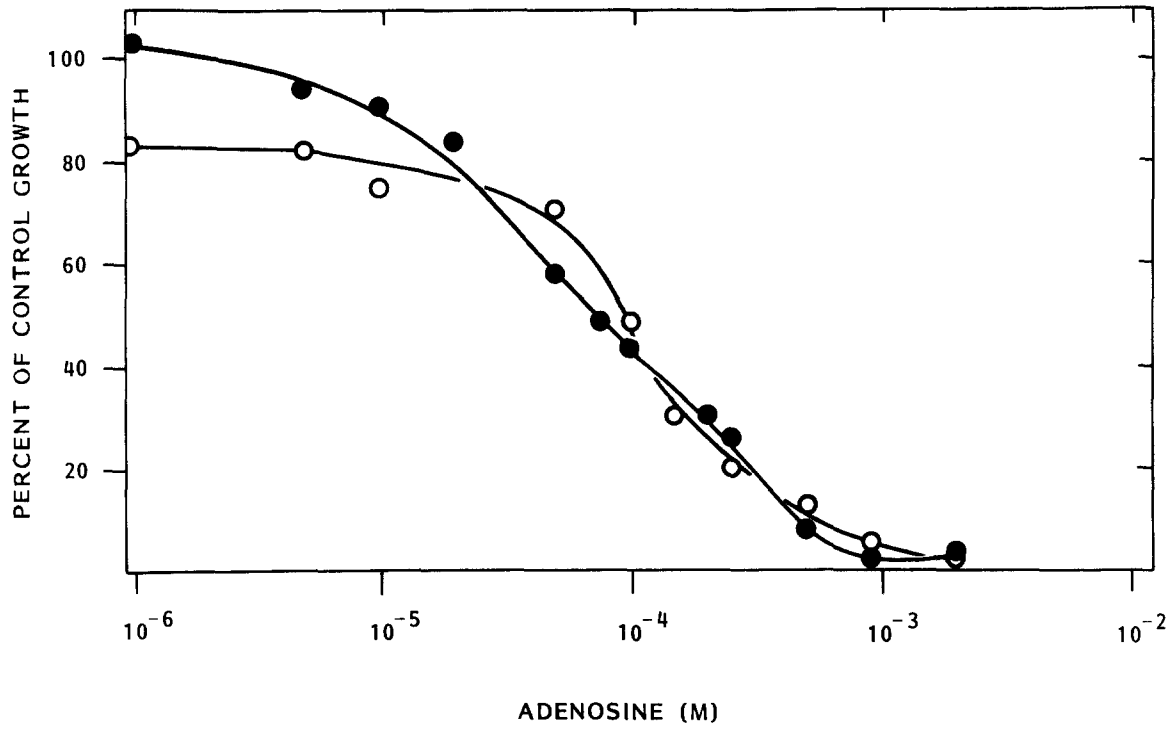

Fig. 2. Inhibition of cell growth by adenosine. The cytotoxicity of adenosine was determined as described in Materials and Methods for 3924A cells (○—○) and 1P39-3 cells (●—●). The total nucleic acid content of cells surviving at the indicated adenosine concentrations after 72 hr exposure is plotted as a percentage of controls grown in the absence of adenosine.

Table 2. Inhibition of growth of hepatoma cells

Inhibitor	ID_{50} concentration (M)		Resistance (-fold)
	3924A	1P39-3	
Adenosine	8.8×10^{-5}	7.0×10^{-5}	0.8
Adenosine + uridine	1.6×10^{-4}	1.4×10^{-4}	0.9
Pyrazofurin	3.9×10^{-8}	4.1×10^{-6}	105
6-Azauridine	4.0×10^{-7}	1.0×10^{-6}	2.5

Cultures were grown for 72 hr in the presence of each inhibitor over a range of concentrations, and the concentration required to inhibit growth by 50% (ID_{50}) was calculated by interpolation on semi-log plots.

evident. Dose–response curves were also run for the antipurine agents N^6-ethenoadenosine, 6-chloropurine riboside, and methylmercaptopurine riboside (Table 3). For each drug, extensive cross-resistance was observed. Arabinosyladenine (ara-A) was also examined. Since ara-A is readily detoxified through deamination catalyzed by the very active adenosine deaminase of hepatoma cells, these experiments were conducted in the presence of the adenosine deaminase inhibitor, 2′-deoxycoformycin, at 0.5 μM. The ID_{50} values, shown in Table 3, indicate no cross-resistance to ara-A in the AK^- cells.

The availability of the adenosine kinase deficient rat hepatoma cells made possible a number of experiments designed to clarify the mechanisms of adenosine toxicity in hepatomas and to determine whether they differed from other mammalian cell types in their response to adenosine. Table 4 shows the effects of adenosine on ribonucleotide pools in parental 3924A cells and in the AK^- subline. In these experiments cells were incubated in the presence of 300 μM adenosine for a total of 20 hr, with fresh adenosine-containing medium added after 16 hr. Adenosine treatment of 3924A cells

Table 3. Inhibition of growth of hepatoma cells by adenosine analogues

Compound	ID_{50} concentration (M)		Resistance (-fold)
	3924A	1P39-3	
N^6-Ethenoadenosine	1.1×10^{-5}	7.0×10^{-4}	64
6-Chloropurine riboside	5.6×10^{-4}	$>5.0 \times 10^{-3}$	>9
6-Methylmercaptopurine riboside	1.6×10^{-5}	1.4×10^{-3}	87
Arabinosyladenine	8.4×10^{-5}	1.3×10^{-4}	1.5

Cultures were grown for 72 hr, in presence of each agent at a range of concentrations, and the concentrations required to inhibit growth by 50% (ID_{50}) were calculated by interpolation on log–log plots.

Table 4. Ribonucleotide pools in adenosine-treated hepatoma cells

	3924A		1P39-3	
	Control	Adenosine treated	Control	Adenosine treated
ATP	9730	19,350 (199)	8820	12,180 (138)
ADP	3225	5540 (172)	3520	5910 (168)
AMP	1175	2370 (201)	1930	2300 (119)
Total A	14,130	27,260 (193)	14,270	20,390 (143)
GTP	1950	2300 (116)	1630	1664 (102)
GDP	873	1124 (129)	958	1158 (121)
GMP	255	310 (122)	329	261 (79)
Total G	3078	3734 (121)	2917	3083 (106)
UTP	2721	1068 (39)	2225	850 (38)
UDP	916	514 (56)	1315	476 (36)
UMP	168	120 (72)	162	121 (75)
UDP-sugars	1915	1451 (76)	2192	1508 (69)
Total U	5720	3153 (55)	5894	2955 (50)
CTP	1744	685 (39)	1230	361 (29)
CDP	798	271 (34)	760	232 (30)
Total C	2542	956 (38)	1990	593 (30)

Nucleotide contents are given in nmole/10^9 cells. Values in parentheses are values for adenosine treated cells as percentage of untreated controls. Values are means for 6 replicate cultures. Adenosine treatment was at 300 μM for 20 hr.

caused an increase in the adenine nucleotide pools of 93% over the untreated control value. In the AK^- cells, adenosine treatment increased the adenine nucleotides by only 43%. The uridine nucleotide pools decreased significantly with the extent of the decrease being similar in both cell lines. The mechanism by which adenosine decreases the cellular pyrimidine nucleotide pools has been attributed to a depletion of the PRPP pool [16–18]. The PRPP content of wild-type and mutant cells in the presence and absence of adenosine is summarized in Table 5. Adenosine caused a significant drop in the PRPP pool of both 3924A wild-type and 1P39-3 AK^- cells. The extent of the decrease was similar in both lines and parallels the effects of adenosine on the pyrimidine nucleotide pools.

DISCUSSION

Pyrazofurin, although acting primarily as an antipyrimidine agent, possesses some structural resemblance to the purines, and it has been shown to have a secondary site of inhibition in the purine *de novo* biosynthetic pathway [19]. The route of phosphorylation of pyrazofurin is another instance of involvement by the enzymes of purine metabolism. Preliminary studies indicated that adenosine competitively inhibited the metabolic activation of pyrazofurin [5], suggesting that the adenosine kinase reaction might be involved. Our present work has shown that deletion of over 98% of cellular adenosine kinase activity in hepatoma cells, with no other discernible abnormalities, caused the cells to become 105-fold resistant to pyrazofurin. This observation has thus provided evidence that pyrazofurin is

Table 5. Phosphoribosylpyrophosphate pools in hepatoma cells

Culture	PRPP (nmole/10^9 cells)	Treated as percentage of control
3924A control	57.6	
3924A, adenosine treated	41.6	72%
1P39-3 control	29.7	
1P39-3, adenosine treated	17.2	58%

Values are means for duplicate cultures. Adenosine treatement was at 300 μM for a total of 20 hr with fresh media + adenosine at 16 hr.

metabolically activated to the nucleotide form by the enzyme adenosine kinase. A recent study reported deletion of adenosine kinase activity in L5178Y mouse lymphoma cells resistant to pyrazofurin [20]; however, the lymphoma cells also produced abnormally large amounts of the target enzyme, orotidylate decarboxylase. Even a small increase in the activity of the decarboxylase can result in a significant increase in cellular resistance not only to pyrazofurin, but also to 6-azauridine [6, 14]. 6-Azauridine is another pyrimidine analog which inhibits orotidylate decarboxylase after phosphorylation by uridine kinase [21–23]. The mutant hepatoma cells described in our present study possessed normal activity for uridine kinase (Table 1), and showed no significant cross-resistance to 6-azauridine (Table 2). These data indicate that pyrazofurin resistance may result from the simple deletion of adenosine kinase without overproduction of the target enzyme.

Examination of the AK^- hepatoma cells (1P39-3) showed that they were cross-resistant to a number of purine nucleoside antimetabolites, N^6-ethenoadenosine, 6-chloropurine riboside, and 6-methylmercaptopurine riboside (Table 3). All these agents are structural analogues of adenosine that are probably metabolically activated through the adenosine kinase reaction. The fact that deletion of adenosine kinase caused resistance to these agents was thus not unexpected. Another interesting result was obtained with the antitumor and antiviral agent, arabinosyladenine (ara-A). Effectiveness of ara-A has been limited by its rapid inactivation through deamination. However, the recent availability of potent adenosine deaminase inhibitors seems likely to greatly increase the usefulness of ara-A. In our experiments we used ara-A in combination with the adenosine deaminase inhibitor 2′-deoxycoformycin. Results showed that deletion of adenosine kinase did not affect the response to ara-A (Table 3). Clearly ara-A must be activated by some other enzyme; the most likely possibility is deoxyadenosine kinase. Table 1 shows that the deoxyadenosine kinase activity of our AK^- hepatoma cells was not significantly altered. This result strongly suggests that adenosine kinase and deoxyadenosine kinase are different enzymes in hepatic cells. This conclusion had been previously suggested on the basis of differential inhibition profiles [15], but detailed studies were not possible because the two activities copurified. The availability of a cell source from which adenosine kinase activity is almost absent will greatly facilitate the study of deoxyadenosine kinase.

Hepatomas appear to have a number of abnormalities in adenosine metabolism, such as unusually high adenosine deaminase activity and low adenosine kinase activity [10]. Perhaps for these reasons, hepatoma cells are rather refractory to adenosine toxicity. Nevertheless, the observation that almost total deletion of adenosine kinase did not alter the sensitivity of the hepatoma cells was unexpected (Fig. 2). Another surprising result was the fact that uridine had very little effect on the sensitivity to adenosine of these hepatoma cells for both the parental and AK^- lines (Table 2). These results indicate that the mechanism of toxicity of adenosine in hepatoma cells may differ from that previously described for fibroblasts and lymphoblasts [7, 16–18]. Studies with the AK^- cells should provide valuable information concerning the control of adenosine metabolism in hepatoma cells. The data presented in Tables 4 and 5 are an initial approach to some of the unanswered questions. From these results it is clear that although uridine does not prevent adenosine toxicity in these cells, adenosine does depress pyrimidine biosynthesis. This may be associated with inhibition of PRPP synthesis. As found in other cell types, adenosine elevated

the cellular adenine nucleotide pools in the 3924A cells. In the AK^- subline some increase was seen in ATP and ADP pools after adenosine treatment, though much less than in the wild-type. However, the depression in the uridine nucleotide pools was just as pronounced in the mutant subline as in the wild-type cells. This is in agreement with the fact that deletion of adenosine kinase did not confer adenosine resistance upon these cells, indicating that the mechanism of adenosine toxicity in hepatoma cells is independent of the activity of adenosine kinase.

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