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DISTRIBUTION OF PURINE RIBONUCLEOSIDE KINASE AND SELECTIVET OXICITY OF 6-METHYLTHIOPURINE RIBONUCLEOSIDE*

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Abstract—6-Methylthiopurine ribonucleoside (MMPR) and several other adenosine analogues have important antitumor properties. These agents must be converted to the nucleotide by a purine ribonucleoside kinase in order to be biologically active. MMPR is converted to the nucleotide by an enzyme indistinguishable from adenosine kinase. Accordingly, a technique for determining adenosine kinase by using MMPR as substrate was employed to examine cofactor requirements, stability, localization, and kinetics of adenosine kinase and its distribution in various normal and neoplastic tissues of man and mouse.

With enzymatic and thin-layer chromatographic techniques, the only product of the reaction in these tissues was the mononucleotide (MMPR 5′-phosphate). Polyphosphates or other products were not found. With succinate buffer, the optimal pH for both human and mouse adenosine kinase was 5·4. Omission of either adenosine triphosphate or manganese resulted in almost complete loss of adenosine kinase activity. Under our assay condition, the optimum temperature of adenosine kinase was 49° for human and 43° for mouse. The enthalpy of activation was 1·7 kcal/mole for the adenosine kinase of both human erythrocytes and mouse liver. The Michaelis constant for adenosine kinase of human red blood cells was 8×10^{-5} M.

The enzyme was widely distributed in both normal and neoplastic tissues. The activity was present almost exclusively in the supernatant fraction of homogenates centrifuged at 46,000 *g* for 1 hr and was not present in the membranes of erythrocytes. The activity was particularly high in liver and kidney tissue, but was also relatively high in the oral and gastrointestinal mucosa, as well as in the bone marrow. These organs are subject to selective toxicity after MMPR administration. However, there is little correlation between the magnitude of toxicity and adenosine kinase activity. There was no correlation between cell turnover and adenosine kinase activity, nor was there a consistent variation in adenosine kinase activity for normal and neoplastic elements of the same tissue.

There was, on the other hand, excellent correlation of adenosine kinase activity with selective toxicity for experimental tumors sensitive and rendered resistant to MMPR. The resistant lines had markedly reduced activity which was sustained in the absence of MMPR.

Adenosine kinase is an obligatory step in the activation of MMPR and most other adenosine analogues, but selective toxicity, except in the induced resistant cell lines, is largely determined by other factors.

A Number of adenosine analogues with antitumor activity in animal systems have been identified. These include synthetic derivatives such as 6-methylthiopurine ribonucleoside (6-methylthio-9-β-D-ribofuranosylpurine, NSC-40774, MMPR)[1] and

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antibiotics such as tubercidin.[2-4] MMPR is of considerable interest as an antitumor agent because in experimental systems, cell lines resistant to 6-mercaptopurine (NSC-755,MP) are not cross-resistant with MMPR,[1] and also because in mouse tumor systems it is synergistic in combination with MP.[5,6] Several of these agents are in clinical trial. In preliminary studies, MMPR in combination with MP is active in inducing remission in adults with acute myelogenous leukemia,* and tubercidin is effective for pancreatic tumors.†

In experimental systems purine analogues must be converted to the nucleotide in order to be biologically active.[7-11] In fact, drug-induced resistance to the thiopurines usually results from the selection of a cell line lacking inosinic acid pyrophosphorylase (EC 2.4.2.8). Similarly, the adenosine analogue MMPR must be converted to the ribonucleotide by a purine nucleoside kinase which has been demonstrated to be the same as adenosine kinase (EC 2.7.1.10).[12-14] Although MMPR is used as a substrate, the enzyme will hereafter be referred to as adenosine kinase.

Adenosine and adenosine analogues, unlike the purine bases, tend to concentrate within cells. Thus, in human RBC and white cells, both *in vivo* and *in vitro*, intracellular-extracellular concentration gradients approach 50:1. This concentration within the cell almost certainly results from diffusion of the adenosine analogues into the cell and conversion by adenosine kinase to the more polar nucleotide, which does not readily cross the cell membrane. In Ehrlich ascites carcinoma cells, an excellent correlation between adenosine kinase activity, cellular uptake of MMPR‡ and the effectiveness of MMPR against the tumor cells has been found. Cell lines resistant to MMPR derived from a human epidermoid carcinoma and from the Ehrlich ascites carcinoma have depleted adenosine kinase, as evidenced by a marked decrease in capacity to phosphorylate MMPR.[12,15]

Thus, adenosine kinase is essential to the biological activation of MMPR and some other adenosine analogues. Since these are potentially useful antitumor agents, it is important to examine this enzyme in man as well as in experimental systems. In this study, the kinetics and cofactor requirements of adenosine kinase were determined for human tissues and compared to experimental systems. The distribution of adenosine kinase activity in normal and tumor tissues of man and mouse was determined. Its relation to selective toxicity was examined.

MATERIALS AND METHODS

MMPR and MMPR-S^{35} were kindly supplied by the Cancer Chemotherapy National Service Center, Bethesda, Md; 6-methylthiopurine ribonucleotide (MMPR-P) was a gift of Dr. John A. Montgomery, Southern Research Institute; ATP and other chemicals were obtained commercially. Enzyme preparations and assay procedures were essentially those already described.[12,13] Human tissues were supplied by the Department of Surgery, M. D. Anderson Hospital. Mature male DBA/2 mice weighing 23 ± 3 g were supplied by Texas Inbred Mouse Co.

Fresh tissues were rinsed with saline, homogenized in a 0·02 M Tris buffer, pH 7·4, and centrifuged at 46,000 *g* for 60 min. The above procedure was carried out at 4°.

* G. P. Bodey, personal communication.

† A. B. Curreri and R. O. Johnson, personal communication.

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The supernatant was used immediately for assay of enzyme activity. The assay mixture consisted of: 2 μmole $MnCl_2$; 30 μmole potassium succinate buffer, pH 5·4; 0·12 μmole MMPR-S^{35} (sp. act., approximately 5×10^5 cpm/μmole); and an appropriate amount of enzyme in a total volume of 0·2 ml. The reaction was started by adding the substrate, and incubating for 10 min at 49° for human tissues and 37° for mouse tissues. The incubation was stopped by heating the mixture in a boiling water bath for 5 min. The suspensions were centrifuged and 30-μl aliquots of the supernatant were plated on cellulose MN 300 TLC plates (Mann Research Laboratories, New York). The following two solvent systems were used with ascending techniques: equal volumes of 93·8% *n*-butanol and 44% propionic acid; 5% disodium hydrogen phosphate (w/v).

Fluorescent spots were located under ultraviolet light of 253·7 mμ and 366 mμ and radioactive spots were identified with authentic compounds by a Packard radiochromatogram scanner. For routine assays, the compounds were detected by their intense fluorescence and the flourescent areas were scraped into 10 ml of a counting solution (4 g 2,5-diphenyloxazole, PPO, 0·2 g 1,4-*bis*-2-(5-phenyloxazolyl) benzene, POPOP, and toluene to make 1 liter) and counted directly for S^{35} in a Packard liquid scintillation spectrometer.

Blood was drawn from normal human subjects and patients with leukemia at the M. D. Anderson Hospital. Red blood cells were separated from the heparinized blood by centrifuging it at 700 *g* for 10 min, aspirating off plasma and buffy coat, and washing once with saline. The method of Fallon *et al.*[16] was used for the separation of leukocytes. The contamination of RBC and viability of separated leukocytes were determined on each preparation.[16] The preparations of leukocytes and red blood cells were sonicated for 15 sec, and the above procedure was followed for enzyme assay.

Protein concentration was determined by the Lowry method with bovine albumin as a standard.[17]

The stability of the enzyme in frozen tissues was tested. Thirty-five per cent of the enzyme activity was lost after the tissues were kept frozen at — 10° for 14 days. Therefore, the human tissues obtained at operation were always assayed for enzyme activity without freezing.

The stability of the enzyme in fresh tissues was also tested. The enzyme was stable in the fresh tissues, which were kept at room temperature for 1 hr, as well as in those placed in an ice bath for an additional 2 hr. This result eliminated the question of any possible loss of enzyme activity in fresh human tissues by the delay, if any, of shipping the tissues from the operating room to our laboratory for the assay of the enzyme.

RESULTS AND DISCUSSION

Identification of products of the enzyme reaction. The reaction mixture was examined by thin-layer chromatography. Two peaks of radioactivity were obtained. One peak corresponded in its chromatographic behavior to authentic MMPR 5′-phosphate and the other peak corresponded to the substrate MMPR. R_f values are summarized in Table 1. MMPR 5′-phosphate was further identified by its conversion to a compound migrating like MMPR upon incubation with alkaline phosphatase of calf intestinal mucosa (Sigma, Type I) or with 5′ nucleotidase of *Crotalus adamanteus*

venom (Sigma, Grade II) in 0·05 M glycine buffer, pH 9·5, at 37° for 90 min. In all of the experiments performed in this study, no evidence for the formation of MMPR polyphosphates or other products was obtained. In addition, the summation of the substrate left over and the product formed (MMPR 5′-phosphate) was equal to the amount of the substrate added for the reaction. This formation of a single product was true for all tissues whether human or rodent, normal or neoplastic.

TABLE 1. R_f VALUES OF MMPR AND MMPR 5′-PHOSPHATE

Compound	Equal vol. 93·8% *n*-butanol and 44% propionic acid	5% Na_2HPO_4
MMPR	0·80	0·64
MMPR 5′-phosphate	0·46	0·90

TABLE 2. COFACTOR REQUIREMENTS FOR ADENOSINE KINASE REACTION OF HUMAN ERYTHROCYTES

Reaction mixture	Enzyme activity (mμmole product/min)	% Control
Complete	5·88	100
Omit ATP	0·45	7·7
Omit Mn^{2+}	0·36	6·1

Cofactor Requirements. The requirements for ATP and Mn^{2+} for the formation of MMPR 5′-phosphate were examined in human RBC and liver. As shown in Table 2, omission of ATP or Mn^{2+} resulted in almost complete loss of activity. Therefore, for routine assays, ATP and Mn^{2+} were always used. These requirements are similar to those reported by Caldwell *et al.*[13] and Bennett *et al.*[12] for mouse tumor cells and cultured human cells respectively. In addition, Mg^{2+} was shown to be as good as Mn^{2+}.[13,14]

Effect of pH. The effect of pH on the rate of phosphorylation was examined with potassium succinate and phosphate buffers. The optimum pH for both human RBC (Fig. 1) and mouse liver was 5·4. This was similar to that found by Caldwell *et al.*[13] in cell-free extracts of Ehrlich ascites cells. The phosphate buffer generally gave very low activity regardless of pH. Since the enzyme reaction involves the transfer of a phosphate group and requires divalent ions as cofactors, phosphate buffer is perhaps not a good choice. However, Bennett *et al.*[12] and Schnebli *et al.*,[14] using phosphate buffer, reported high kinase activities in H. Ep. No. 2 cells in culture and in yeast cells. The optimum pH was reported to be 6·8 at 25° with a 15-fold purified enzyme.

Effect of temperature. The kinase activities of human RBC and mouse liver were measured at a wide range of temperatures. As shown in Fig. 2 under the assay conditions, 49° gave the highest activity for human RBC and 43° for mouse liver. For a variety of human tissues, the ratio of enzyme activity at 49° and 40° was the same. However, the effects of temperature on the velocity of enzyme reactions is complex.[18] The optimum temperature is determined by the balance between the effect of temperature on the rate of denaturation of the enzyme and its effect on the rate of the enzyme

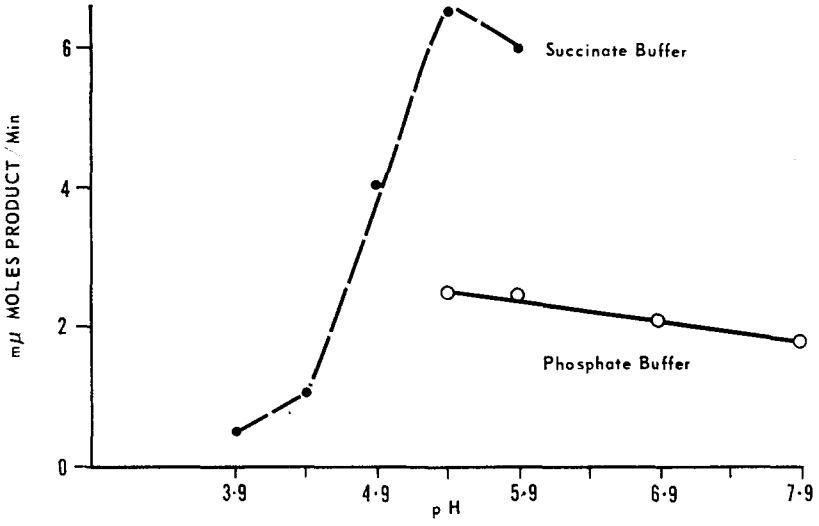

FIG. 1. The effect of pH on adenosine kinase activity of human erythrocytes.

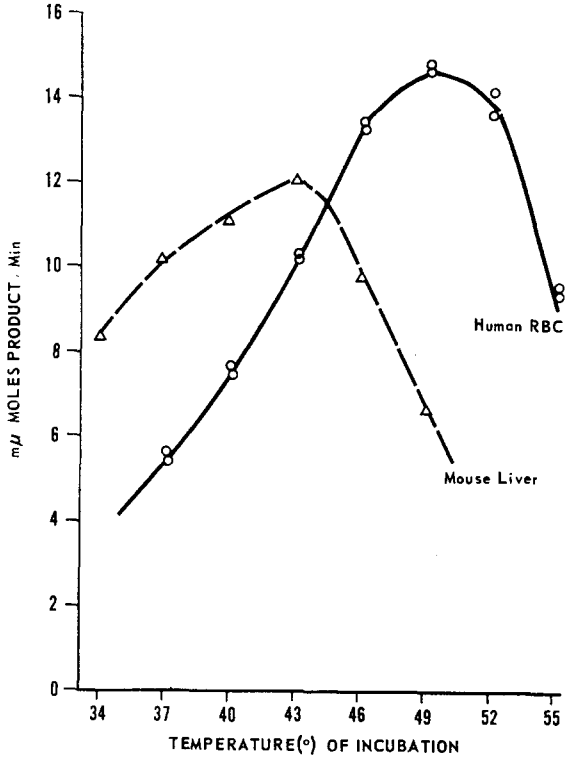

FIG. 2. The effect of temperature on adenosine kinase activity of human erythrocytes and mouse liver.

reaction. The enzyme reaction of human RBC was examined as a function of time at 37° and 49° (Fig. 3). It can be seen that although the true initial velocity increases as the temperature is raised from 37°–49°, the amount of substrate transformed first rises and then falls as the time intervals increase.

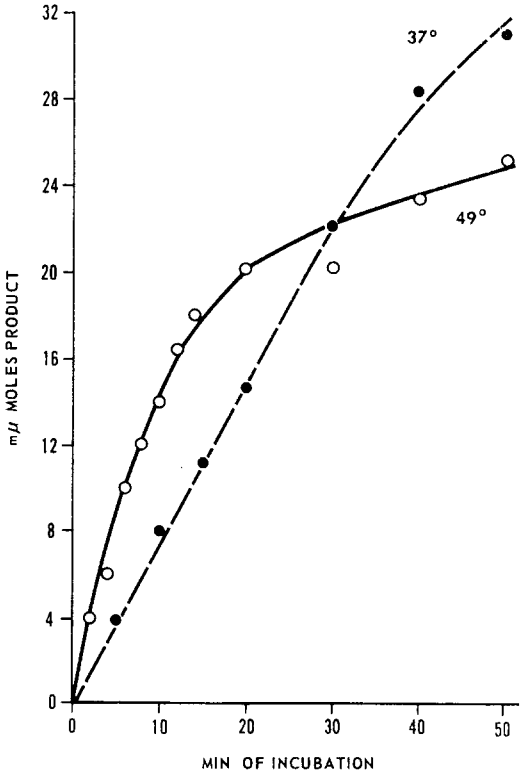

FIG. 3. The effect of time of incubation on adenosine kinase activity at 37° and 49°.

The velocity of the adenosine kinase was determined at 5° intervals from 20°–45°. The logarithms of the velocity were plotted against the reciprocal of the absolute temperature. Two parallel straight lines were obtained for the enzyme of both human RBC (20–45°) and mouse liver (20–40°) (Fig. 4). The enthalpy of activation was calculated from the slope of these lines,[19] and a value of 17,000 cal/mole was obtained from both enzyme preparations.

K_m determination. The K_m value for human RBC calculated from the Lineweaver-Burke plot was $8{\cdot}4 \times 10^{-5}$ M at 49° (Fig. 5), as compared with $4{\cdot}0 \times 10^{-5}$ M reported for Ehrlich ascites cells at 37°[13] and 5×10^{-5} M for H. Ep. No. 2 human culture cells at 25°.[14]

Effect of enzyme concentration. The correlation between the amount of human RBC enzyme and the rate of phosphorylation was studied (Fig. 6). At 49°, the activity was at first linear with enzyme concentration and then leveled off. For every tissue studied, an enzyme curve was always performed. The specific activity was then calculated from the linear portion of the curve and was expressed as mμmoles of product per mg protein per min.

Localization of the enzyme. The homogenates of mouse livers and leukocytes of a patient with chronic lymphocytic leukemia were centrifuged at 46,000 *g* for 1 hr, and the enzyme activity was determined for both supernatant and precipitate. The activity was 10 mμmole product/1 mg liver/min at 37° for the supernatant, and 0·35 for the precipitate; this is also true for the leukocytes.

Red blood cells separated from fresh blood and 27-day-old blood from normal human subjects were lyzed with water to obtain a membrane preparation.[20] The

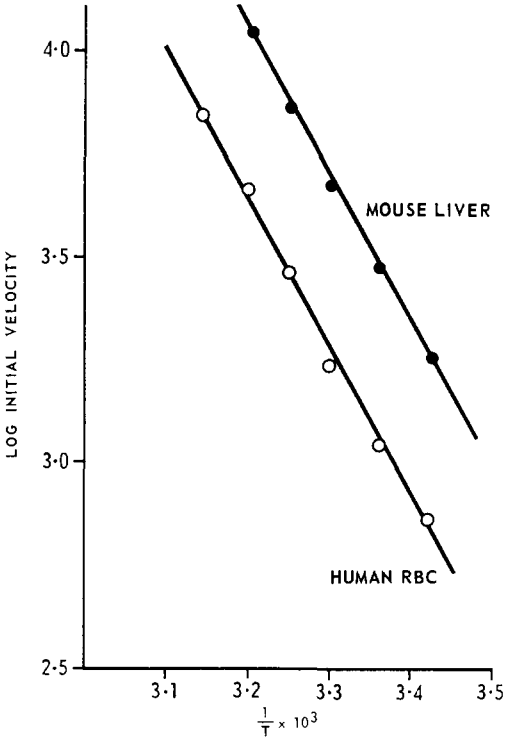

FIG. 4. Plot of logarithm of initial velocity versus reciprocal of absolute temperature (T).

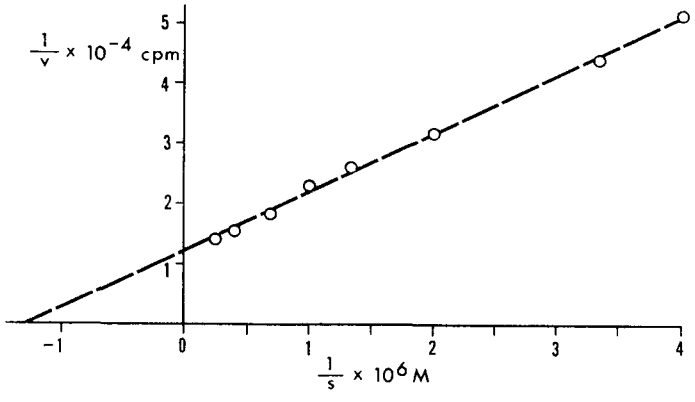

FIG. 5. K_m determination of adenosine kinase of human erythrocytes.

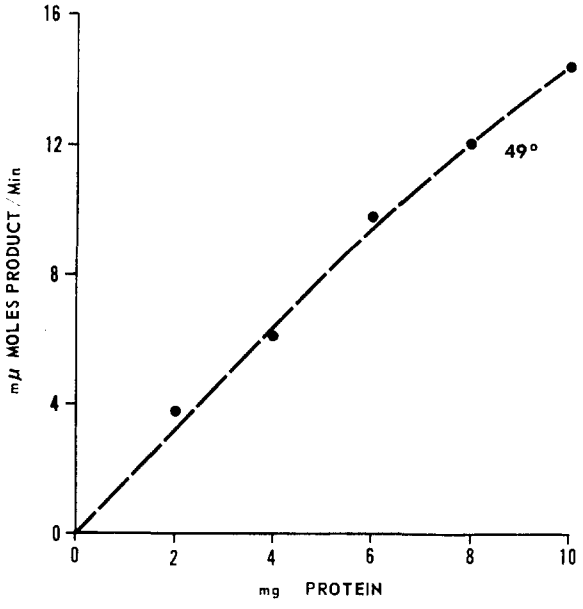

Fig. 6. The effect of enzyme concentration on adenosine kinase activity of human erythrocytes.

cell "ghosts" were examined under a phase contrast microscope and no cell ghosts in pieces were found. The preparation contained 95 per cent round and approximately 5 per cent biconcave intact ghosts which did not contain hemoglobin. As shown in Table 3, the enzyme activity is expressed as mμmoles of product formed from the

Table 3. Adenosine kinase activity of hemolysate and ghosts of human erythrocytes

Enzyme (av. of 2 samples)	mμmole product/ml RBC/min at 37°
Hemolysate from 1 ml RBC	138·20
Ghosts from 1 ml RBC	2·12

hemolysate or ghosts obtained in 1 ml of packed RBC per min at 37°. In terms of relative amount, the adenosine kinase was present mainly in the hemolysate.

Distribution of adenosine kinase. Adenosine kinase activity was determined in various normal and neoplastic human and mouse tissues (Tables 4 and 5). The selective toxicity of MMPR for a given tissue is presented in the right-hand columns on Tables 4 and 5. In man, the major toxicity of MMPR relates to the oral mucosa and gastrointestinal tract where erythema and ulceration occur. Minor and reversible elevations of transaminases and alkaline phosphatase indicating liver damage also occurred. Patients rarely developed leukopenia or thrombopenia.[21]

Adenosine kinase activity is widely distributed in human tissues (Table 4). It is highest in the liver and moderately high (between 8 and 10 mμmole product/mg protein/min) in the oral and gsstrointestinal mucosa, kidney, spleen and bone marrow. In general, those organs subject to toxicity have relatively high levels of enzyme

activity, but there is little correlation between the magnitude of selective toxicity and enzyme activity. Muscle, nerve and red blood cells have low activity. This latter is of interest in view of the avid uptake of MMPR by red blood cells as well as by other cells both *in vitro* and *in vivo*.

TABLE 4. ADENOSINE KINASE ACTIVITY IN HUMAN TISSUES

Tissues	No. of determinations	Specific activity (av.) (mμmole product/mg protein/min at 49°)	Activities (range)	Relative selective toxicity (0–4 +)
Oral mucosa:	5	10·2	4·2 –12·6	4 +
Tongue	2	12·3	12·0 –12·6	
Anterior buccal	1	10·2	—	
Larynx	2	8·0	4·2 –11·8	
Stomach mucosa	3	8·8	8·0 – 9·0	
Ileum mucosa	3	7·6	6·8 – 9·2	3 +
Colon mucosa	2	8·5	8·0 – 9·0	2 +
Liver	3	39·9	37·0 –42·8	0–1 +
Kidney	3	9·6	9·0 –10·8	0
Adrenal gland	2	6·9	5·2 – 8·6	0
Spleen	3	9·3	8·2 –11·2	0
Nerve, peripheral	1	2·6		0
Muscle, striated	1	3·8		0
smooth (digestive tract)	3	3·9	1·6 – 4·0	
Bone marrow	1	8·0		0–1 +
Plasma	1	0		0
Red Blood cells:	5	1·28	1·02– 1·58	0
normal	3	1·2	1·02– 1·30	
banked (22 days)	1	1·1		
Chronic myelogenous leukemia	1	1·58		0
Neoplastic tissues				
Acute myelogenous leukemia	1	12·2		0–1 +
Chronic myelogenous leukemia	1	13·8		
Clear cell carcinoma of kidney	2	5·4	4·8 – 6·0	
Adenocarcinoma of colon	2	10·4	7·6 –13·20	
Melanoma of breast	1	6·4		
Fibrosis of pelvis	1	5·2		
Squamous carcinoma of larynx	1	3·4		
Squamous carcinoma of lung	1	9·2		

The range of activity for neoplastic tissues is similar to that of normal tissues. The activity in adenocarcinoma of the colonic tissue was only slightly greater than in normal colonic mucosa, whereas in carcinoma of the kidney, enzyme activity was somewhat lower than that in normal kidney. The activity in the relatively mature leukemic cells of chronic myelogenous leukemia was essentially similar to that of the highly undifferentiated leukemic cells of a patient with acute myelogenous leukemia. Thus, within the same tissue, the levels of differentiation did not result in a consistent or a significant change in adenosine kinase activity. This was also true for the normal and neoplastic cells of the same tissue.

Analyses of mouse tissues gave somewhat similar results. Again, there was no good correlation between selective toxicity and adenosine kinase activity in the normal tissues. However, Caldwell *et al.*[15] reported that there was a marked reduction in

adenosine kinase activity in MMPR-induced resistant Ehrlich ascites cells. We have confirmed this observation in that the specific activity of the sensitive line was 10 mμmole product/mg protein/min, whereas in the resistant line, whether maintained or not maintained on MMPR, activity was less than 1 mμmole product/mg protein/minute (Table 5). This resistant line appeared to be stable in the absence of continued MMPR treatment. There was no increase in adenosine kinase activity after 27 transplant generations, nor was there any increase in tumor sensitivity to MMPR. This was also found to be the case in cultured human cells with induced resistance to MMPR.[12]

Table 5. Adenosine kinase activity of mouse tissues

Tissues	No. of determinations	Specific activity (av.) (mμmole product/mg protein/min at 37°)	Activities (range)	Relative selective toxicity (mouse or rat)
Intestinal mucosa	4	7·64	6·76 – 8·72	4 +
Liver	4	85·30	74·0 –91·1	1–2 +
Kidney	5	29·20	23·1 –33·2	0–1 +
Spleen	4	7·55	7·2 – 7·84	1 +
Brain	2	7·54	7·44– 7·64	0
Plasma	1	0		0
RBC	4	1·14	0·98– 1·38	0 -
L1210 ascites	2	8·0	7·0 – 9·0	3 +
Ehrlich ascites carcinoma (EAC) sensitive to MMPR 2-27 generations	2	9·92	9·2 –11·08	3 +
EAC-resistant, maintained 2 generations with MMPR	2	0·67	0·66– 0·68	0
EAC-resistant, maintained 10 generations with MMPR	1	0·56		0
EAC-resistant, maintained 27 generations with MMPR	1	0·24		0
EAC-resistant, maintained 2 generations without MMPR	2	0·53	0·52– 0·54	0
EAC-resistant, maintained 10 generations without MMPR	1	0·60		0
EAC-resistant, maintained 27 generations without MMPR	1	0·49		0

In general, the level of activity in mouse tissues was similar to that of human tissues save for the liver and kidney. The activity in these organs of the mouse was considerably higher than in the human. The enzyme activity of human tissues was measured under our assay conditions at the optimum temperature, 49° (Fig. 2), whereas mouse tissues were studied at 37° (optimum temperature, 43°). For various mouse tissues the activity measured at 37° is about 86 per cent of that at 43°. When correction was made for temperature, the level of activity in mouse tissues, except for the liver and kidney, was quite similar to that of human tissues.

Thus, although adenosine kinase activity (i.e. conversion of the adenosine analogue to the nucleotide) is an obligatory first step to biological activity, the degree of selective toxicity would appear to be determined largely by other factors. Tubercidin (7-deazaadenosine) also requires purine nucleoside kinase for activation, but it has a markedly different spectrum of selective toxicity than does MMPR. In intact cells, MMPR produces an inhibition of an early step of purine synthesis *de novo*,[1,22] presumably

by the action of its nucleotide on phosphoribosylpyrophosphate amidotransferase. This site is considered by some to be the major target in terms of biological activity for MP.[23] On the other hand, tubercidin is converted largely to the nucleoside triphosphate (in contrast to MMPR which is converted only to the monophosphate) and is extensively incorporated into RNA and DNA. In addition, and as has already been emphasized, selective toxicity of tubercidin is much different from that of MMPR.

Adenosine kinase has a distribution of activity dissimilar to that of enzymes exclusively concerned with DNA synthesis, such as thymidylate synthetase and thymidine kinase. These enzyme activities correlate closely with the magnitude of cell turnover, that is, the synthesis of DNA. They are high in the bone marrow, gastrointestinal tract and regenerating liver. Adenosine kinase differs in that there is high activity in normal liver and relatively high activity in brain and other organs where there is little or no cell turnover.

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