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ROLE OF URIDINE PHOSPHORYLASE IN THE ANABOLISM OF 5-FLUOROURACIL

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Abstract—The activities of enzymes responsible for activating 5-fluorouracil (FUra) to 5-fluorouridine-5′-monophosphate (FUMP) were compared in normal and tumor tissues of rodents to assess the potential capacity of uridine phosphorylase to anabolize FUra to the nucleoside in the presence of ribose-1-phosphate (R-1-P). The activity of the alternative pathway to FUMP with a pyrimidine phosphoribosyltransferase [FUra + 1-pyrophosphoribosyl-5-phosphate (PPRP)] was approximately 15 to 17 nmoles/mg protein/hr in bone marrow from mice and rats and ranged from 28 to 47 nmoles/mg protein/hr in tumor tissues. Uridine phosphorylase [measured as the formation of 5-fluorouridine (FUrd) from FUra and R-1-P] was 35–230 nmoles/mg/hr in bone marrow and in two FUra-sensitive solid tumors, colon tumor No. 38 in mice and RPMI colon tumor in rats; the activity of uridine phosphorylase from L5178Y ascites leukemic cells was notably lower, 8 nmoles/mg/hr. Levels of uridine kinase ranged from 55 to 187 nmoles/mg protein/hr. Thus, the activities of the enzymes of the two-step FUra activating pathway were high compared to the PPRP-dependent activity in all tissues except L5178Y; also, the FUra-sensitive tumors yielded extracts with 1.5 to 6.5 times greater enzyme activity than the corresponding activity in bone marrow. Uridine phosphorylase was partially purified from rat liver, RPMI rat tumor and colon tumor No. 38; the apparent K_m of FUra averaged 50 μM, almost 9-fold lower than that of uracil, and the apparent K_m of R-1-P for condensation with FUra was 33 μM. The tissue concentration of R-1-P was >70 μM in kidney and liver of rodents and somewhat less in spleen. Colon tumor No. 38 and RPMI colon tumor had 12 and 20 μM R-1-P, respectively, but these low values may reflect low tumor viability. The high levels of uridine phosphorylase and uridine kinase activities in normal tissues and even higher levels in tissues from FUra-sensitive tumors, as well as the sufficient concentration of R-1-P relative to its kinetic constant, suggest that FUra metabolism by the two-step pathway to FUMP may be a significant factor in the activity and selectivity of FUra.

To be cytotoxic, FUra† must be metabolized to FUMP prior to conversion to other nucleotide forms. Reyes and Guganig [1] isolated a pyrimidine phosphoribosyltransferase that converted FUra (apparent K_m 190 μM) and PPRP (apparent K_m 25 μM) to FUMP in P1534J cells. Since tissue levels of PPRP have been reported to be as low as 4 μM in colon tumor No. 38 [2], the concentration of this sugar donor may be rate-limiting in some FUra-sensitive solid tumors.

An alternative to FUra anabolism via the pyrimidine phosphoribosyltransferase pathway with PPRP is the activation of FUra by the two-step route utilizing uridine phosphorylase and uridine kinase. A role *in vivo* has been ascribed to uridine phosphorylase, which is responsible for catabolism of uridine, in FUra condensation with R-1-P especially in the presence of R-1-P donors such as inosine [3–7]. To compare the potential utilization of the FUra–FUrd–FUMP pathway to the reaction of FUra with PPRP, we measured the activities of the enzymes metabolizing FUra in several tumor and normal tissues of rodents, determined the kinetic constants of a partially purified uridine phosphorylase for FUra and R-1-P, and measured the tissue concentration of R-1-P. These methods may be applied to human tumor and normal tissues to determine those instances in which specific, selective FUra chemotherapy might be achieved.

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† Abbreviations: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FUMP, 5-fluorouridine-5′-monophosphate; Ura, uracil; Urd, uridine; R-1-P, ribose-1-phosphate; and PPRP, 1-pyrophosphoribosyl-5-phosphate.

MATERIALS AND METHODS

Chemicals. FUra, FUrd, FUMP, R-1-P (dicyclohexylammonium salt), PPRP, uracil, uridine and hypoxanthine were purchased from the Sigma Chemical Co. (St. Louis, MO) or Calbiochem (La Jolla, CA). Purine nucleoside phosphorylase was obtained from PL Biochemicals (Milwaukee, WI), Radioactive isotopes were purchased from Moravek Biochemicals (Brea, CA) or the Amersham Corp. (Arlington Heights, IL).

Animals and experimental tumors. Colon tumor No. 38 (T. Corbett, Southern Research Institute, Birmingham, AL) was transplanted every 30–40 days

as a subcutaneous brei in female C57B1/6 mice (Jackson Laboratory, Bar Harbor, ME) 10–18 weeks of age. L5178Y was passed weekly as an intraperitoneal tumor in female CDF_1 mice (Charles River Breeding Laboratories, Portage, MI). RPMI rat colon tumor (Y. M. Rustum, Roswell Park Memorial Institute, Rochester, NY) was maintained in female Fischer rats (Charles River Breeding Laboratories, Kingston, NY) by subcutaneous implantation every 3–4 weeks. Animals without tumors were used as a source of normal tissues.

Preparation of tissue extracts. Solid tumors were obtained either from mice bearing colon tumor No. 38, approximately 1 g of tumor tissue per mouse, or from rats bearing RPMI colon tumor, approximately 5 g of tumor tissue per animal. Solid tumors were carefully excised from surrounding connective tissue and washed free of blood in cold Fischer's medium. The tumor was finely minced in 50 mM Tris buffer (pH 7.4 at 37°) containing NaF (4 mM), dithiothreitol (2 mM) and $MgCl_2$ (2 mM). Ascites L5178Y cells were separated from any red blood cells by hypotonic shock and suspended in the Tris buffer. Bone marrow cells were flushed from the tibia and femur of rodents and gently sieved to obtain a cell suspension. The mononuclear fraction was isolated by centrifugation in a Ficoll–Hypaque gradient (LSM, Litton Bionetics, Kensington, MD), and the cells were washed in Tris buffer.

In an ice-water bath, the tissue preparation was pulse sonified using a Branson Sonifier Cell Disrupter (model 200, Branson Sonic Power Co., Danbury, CT) at 60% power until no whole cells could be seen microscopically, usually about 45 sec. Particulate material was centrifuged at 40,000 rpm (100,000 *g*) at 4° for 1 hr, and the supernatant fraction was used immediately for analysis of enzyme activities and protein content by the Bradford assay [8] using the Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA).

Determination of enzyme activity. Crude tissue extract was incubated in 50 mM Tris buffer (pH 7.4, 37°) containing NaF (4 mM), dithiothreitol (2 mM) and $MgCl_2$ (2 mM) with radioactive substrate ([^{14}C]FUra or [^{14}C]FUrd) and sugar donor (R-1-P or PPRP) or phosphate (ATP or inorganic phosphate) for up to 1 hr at 37°. During the incubation period, 3–5 μl samples were removed with microliter capillary pipets; the sample volume was small relative to the reaction volume which ranged from 50 to 500 μl depending on the experiment. The samples were spotted on TLC plates while being quickly dried by a stream of warm air to stop the reaction. The TLC plates were prepared previously by application of nonradioactive standard compounds. FUra, FUrd and FUMP could be separated on silica gel plates, 0.2 mm thick with fluorescent indicator (silica gel 60 F_{254} EM Science, Darmstadt, Germany) using $CHCl_3$–methanol–acetic acid (17:3:1, by vol.); Ura, Urd and UMP could be separated also by this method. Fractions of the plastic-backed plates were cut into scintillation vials, and extracted with 0.5 ml of 0.1 N HCl in methanol before addition of scintillation fluid. The concentration of protein in the final reaction mixture was varied from 0.5 to 5 mg/ml depending on the activity being measured. The final concentration of [^{14}C]FUra or [^{14}C]FUrd was 200 μM (2.0 μCi/ml). In the standard reaction mixture, the concentration of appropriate sugar or phosphate was: R-1-P, 8 mM; PPRP, 8 mM; ATP, 10 mM; and inorganic phosphate, 25 mM. In the absence of tissue extract or sugar donor, no product formation was observed. In preliminary tests to measure uridine kinase activity using FUrd plus ATP with the tissue extract, various amounts of FUra were detected; this catabolism of FUrd was completely prevented by addition of R-1-P to the routine assay for uridine kinase.

Partial purification of uridine phosphorylase. Tissue from RPMI rat colon tumor, rat liver or mouse colon tumor No. 38 was homogenized in 2–3 vol. of 0.25 M sucrose containing 10 mM 2-mercaptoethanol. The homogenate was centrifuged at low speed (15,000–20,000 rpm for 10 min) and then at 40,000 rpm (100,000 *g*) for 1 hr at 4°. The supernatant fraction was dialyzed against 0.02 M Na_2HPO_4, 1 mM EDTA and 10 mM 2-mercaptoethanol, pH 8, at 4° and then concentrated to 1/2–1/4 the original volume by recycling through an Amicon column eluate concentrator (model CEC-1) with a XM100 ultrafilter (Amicon Corp., Lexington, MA). A sample (10–20 ml) of this preparation containing approximately 10 mg protein was applied to a Fractogel TSK (HW-55F, Manufacturing Chemists, Inc., Gibbstown, NJ) column (1.5 cm × 90 cm) and eluted at 0.8 ml/min with 0.02 M Na_2HPO_4 containing 10 mM 2-mercaptoethanol and 1 mM EDTA. Fractions containing enzyme activity were pooled for isoelectric focusing in a pH 3 to 10 gradient with 1% Pharmalyte (LKB Products, Bromma, Sweden), Active fractions found at pH 5.5 to 6.5 were pooled, and the pH was adjusted to 7.5 prior to storage at −20°. This preparation did not form any detectable products from FUra or FUrd in the absence of R-1-P or phosphate.

During purification, protein concentration was monitored by absorbance at 280 nm or by the Bradford assay [8]. Enzyme activity was routinely assayed by measuring the rate of increase in absorbance at 266 nm as 0.2 mM FUrd was converted to FUra in the presence of phosphate.

Determination of apparent K_m *values.* In the presence of an excess of one substrate, the effect of limiting amounts of a second substrate on the rate of formation of product was determined spectrophotometrically using partially purified uridine phosphorylase. The difference in the millimolar extinction coefficient at 266 nm, pH 8.0, was 3.5 for Ura-Urd and 2.1 for FUra-FUrd. The apparent K_m of Ura or FUra was measured in the presence of 200 μM R-1-P and the apparent K_m of R-1-P was determined in the presence of 200 μM FUra or 1000 μM Ura. The K_m for FUrd in the catabolic direction was determined in 20 mM phosphate buffer, pH 8.0.

Kinetic values for the rat liver enzyme also were determined using partially purified uridine phosphorylase with radioactive substrates by monitoring product formation with TLC as described previously. Using this procedure, the apparent K_m of R-1-P was measured in the presence of [^{14}C]FUra (100 μM, 3.5 μCi/ml) or [^{14}C]Ura (1200 μM, 2.3 μCi/ml). The apparent K_m of FUra was determined using 200 μM

Table 1. Enzyme activities in bone marrow and tumor tissues*

	FUra + PPRP → FUMP	FUra + R-1-P → FUrd	FUrd + P_i → FUra	FUrd + ATP → FUMP
	(nmoles product/mg protein/hr)			
Rat				
RPMI colon tumor	47 ± 5	230 ± 50	228 ± 20	151 ± 2
Bone marrow	15 ± 8	143 ± 31	130 ± 27	55 ± 14
Mouse				
L5178Y	41 ± 6	8 ± 2	50 ± 22	79†
Colon tumor No. 38	28 ± 6	226 ± 30	212 ± 39	187 ± 37
Bone marrow	18 ± 5	35 ± 12	38 ± 5	82 ± 24

* Tissue extracts (100,000 *g* supernatant fraction) were incubated at 37° with radioactive FUra or FUrd (200 μM; ^{14}C, 2.0 μCi/ml) and appropriate co-substrate: R-1-P, 8 mM; PPRP, 8 mM; inorganic phosphate, 25 mM; and ATP, 10 mM. During the interval up to 1 hr, product formation was measured by TLC as described in the text. Values are the mean (± S.E.M.) of three or more determinations except where noted.
† N = 1.

R-1-P; the apparent K_m of Ura was measured in the presence of 400 μM R-1-P.

Determination of tissue concentration of R-1-P. The method of McIvor *et al.* [9] was used to measure tissue concentration of R-1-P. Briefly, tumor and normal tissue were excised rapidly, weighed and boiled in 9 vol. of 10 mM triethanolamine buffer, pH 7.5, containing 1 mM EDTA and 100 mM sucrose for 3 min. After centrifugation at 12,000 *g*, the tissue extract was passed through a column of polyethyleneimine-cellulose (Sigma Chemical Co.) prepared from 50 mg of exchanger. After washing, the column was eluted with 10 mM triethanolamine containing 1 M NaCl. R-1-P was assayed in this extract by conversion of [^{14}C]hypoxanthine (50 μM, 0.02 μCi/assay) to nucleoside; inosine and hypoxanthine were subsequently separated using a boronate column (Affigel 701, Bio-Rad), and radioactivity in the inosine fraction was assayed as a measure of R-1-P.

RESULTS

To permit a comparison between the potentials of normal and tumor tissues to metabolize FUra by the two-step pathway via FUrd, procedures were developed to assess the activities of uridine phosphorylase (both anabolically and catabolically), uridine kinase and orotate phosphoribosyltransferase for the fluoropyrimidine substrates. In a variety of tissues, the activity of the PPRP-dependent conversion of FUra to the nucleotide form was found to be less than half that of uridine phosphorylase except for L5178Y ascites tumor (Table 1). Furthermore, uridine kinase activity present in all tissues would drive the overall reaction to formation of FUMP. The activities of uridine phosphorylase and uridine kinase from the two solid tumors were greater than those activities measured in bone marrow tissue. The low activity of FUra condensation with R-1-P in L5178Y leukemia was notable and, to rule out phosphatase activity that would destroy sugar donors in the crude extract of L5178Y, we measured the effect of a 30-min delayed addition of radioactive FUra on the formation of FUrd. This 30-min preincubation of either R-1-P or PPRP did not reduce the rate of formation of product from FUra compared to reactions done with no preincubation (Table 2); thus, it was unlikely that high phosphatase could account for the low measured activity of uridine phosphorylase in L5178Y cells. Further evidence of the nature of the enzymes metabolizing FUra was the observation that 4 mM orotate completely inhibited the conversion of FUra to FUMP in the presence of PPRP in extracts from colon tumor No. 38 but did not alter the rate of formation of FUrd in the presence of R-1-P (data not shown); Reyes and Guganig [1] had demonstrated previously that FUra competes with orotate for activation by a pyrimidine phosphoribosyltransferase.

Interpretation of the meaning of these determinations of enzyme activities toward FUra also requires information on the kinetic properties of the enzymes and the availability of the substrates. Information about the kinetic properties of the FUra phosphoribosyltransferase and intracellular levels of PPRP has been reported previously by others [1–3, 7]. However, the corresponding data for uridine phosphorylase in the anabolic direction with FUra have not been described. Using a partially purified uridine phosphorylase from rat liver, RPMI colon tumor and colon tumor No. 38 each prepared by gel filtration and isoelectric focusing, the apparent K_m for FUra was approximately 50 μM (Table 3); this was about 9 times lower than the apparent K_m for Ura. The apparent K_m of R-1-P in the presence

Table 2. Assay conditions for L5178Y ascites tumor cells*

Condition	Percent formation of product 30 min after addition of [^{14}C]FUra
FUra	0.1
R-1-P + FUra	8.1, 9.0
R-1-P + FUra†	8.9, 8.7
PPRP + FUra	22.4, 24.2
PPRP + FUra†	26.3, 27.0

* The percent of product formation was determined by TLC as described in the text.
† Addition of radioactive FUra was delayed by 30 min during which time the L5178Y extract was incubated with the sugar donor. Product formation was measured 30 min after [^{14}C]FUra addition.

Table 3. Kinetic constants for uridine phosphorylase*

Reaction	Substrate	Tissue	K_m† (μM)
FUra + R-1-P ⟶ FUrd			
	FUra	Rat liver	36
		RPMI colon tumor	60
		Colon tumor No. 38	46
	R-1-P	Rat liver	20
		RPMI colon tumor	40
		Colon tumor No. 38	38
Ura + R-1-P ⟶ Urd			
	Ura	Rat liver	485‡
		Colon tumor No. 38	367‡
	R-1-P	Rat liver	30§
		RPMI colon tumor	87‡§
FUrd + P_i ⟶ FUra			
	FUrd	Rat liver	58
		RPMI colon tumor	65§
		Colon tumor No. 38	44‖

* Enzyme was partially purified from the specified tissue by molecular weight filtration and isoelectric focusing. Rate of formation of product was determined spectrophotometrically or using a radioactive substrate and TLC as described in the text. The apparent K_m was determined from a least squares fit of the Lineweaver–Burk plot. The apparent K_m of FUra or Ura was measured in the presence of 200 μM R-1-P and the apparent K_m of R-1-P was determined in the presence of 200 μM FUra or 1000 μM Ura. The apparent K_m of FUrd was measured in 20 mM phosphate buffer, pH 8.0.

† Mean of three determinations with four or more concentrations of limiting substrate and r^2 greater than 0.9 unless noted otherwise.

‡ Duplicate determinations.

§ $r^2 = 0.85$.

‖ $r^2 = 0.67$.

of FUra averaged 33 μM for the enzyme isolated from three tissues. In the presence of excess Ura, the apparent K_m of R-1-P was 30 μM for the rat liver enzyme and somewhat higher, 87 μM, for the enzyme from a rat colon tumor. Yamada [10] had described previously a purification of uridine phosphorylase from rat liver and reported kinetic constants for uracil and R-1-P to be 286 and 71 μM respectively.

The marked activities of uridine phosphorylase and uridine kinase in several tissue extracts could result in anabolic conversion of FUra to FUrd only if concentrations of R-1-P were adequate. Analysis of this sugar phosphate donor suggested that it is above the K_m value in several normal tissues (Table 4). The somewhat lower concentrations of R-1-P in the two tumors analyzed may reflect the fact that in histological sections only 30–40% of the tumor was comprised of viable cells. The remaining tissue was either stromal elements or non-viable tumor cells, and tissue concentrations of R-1-P in actively growing tumor areas may be 2–3 times that in the whole tissue specimen. Thus, kinetic properties as well as adequate substrate concentrations suggest the probable activation of FUra by the uridine phosphorylase–uridine kinase pathway in selected tissues.

Table 4. Concentration of ribose-1-phosphate in tissues*

Tissue	R-1-P concn† (μM)
Mouse	
Colon tumor No. 38	12‡
Spleen	19
Kidney	71
Liver	122
Rat	
RPMI colon tumor	20
Spleen	27
Kidney	88
Liver	120

* Concentration of R-1-P was determined by the method of McIvor et al. [9].

† Determined as nmoles/1000 mg tissue and converted to μM by approximating 1 g wet weight of tissue to be 1 ml interstial volume.

‡ Mean of two determinations (one with internal standard) and corrected for standard recovery.

DISCUSSION

The extent to which tissues differ in the balance of alternative pathways of FUra activation to the nucleotide form may permit selective modification of FUra action against specific tumor types *in vivo*. Although many cell lines in culture and several leukemic tumors maintained in mice depended almost exclusively on a pyrimidine phosphoribosyltransferase to activate FUra [2, 11, 12], our data and that of others show that certain tumors have high activities of uridine phosphorylase and uridine kinase relative to that of the PPRP enzyme [7, 13]. Furthermore, the K_m of FUra for the phosphoribosyltransferase, 190 μM [1], is 3-fold higher

than that for the phosphorylase, 50 μM. Although the concentration of FUra is unlikely to be rate-limiting for either pathway, at least initially after drug administration, the circulating concentration of FUra will be a function of the route of administration, dose and time; for example, intravenous, bolus administration of 720 mg FUra/m^2 resulted in a plasma concentration of 400 μM 5 min after injection and decreased to about 50 μM at 30 min [14]. The low K_m of FUra for the phosphorylase explains to some degree its utilization compared to Ura which has a 7-fold greater K_m for that enzyme.

Utilization of the two-step pathway in certain tissues was further supported by the presence of sufficient concentrations of R-1-P with respect to its kinetic constant, approximately 33 μM. Most values reported for this sugar phosphate donor are from cells in culture or ascites fluid where cell viability is high; values range from about 20 μM in Erhlich ascites cells to 42 μM in Novikoff hepatoma cells [3, 15]. That R-1-P may be rate-limiting for FUra activation in such cell lines is suggested by the potentiation of FUra metabolism and toxicity *in vitro* by the ribose donor inosine [3–5]. In contrast to cell suspensions, tissue concentrations of R-1-P are more difficult to measure because of the presence of substances that may interfere with the R-1-P assay and reduced cell viability in tumor tissues. Ipata and Camici [16] reported values as high as 810 nmoles R-1-P/g wet tissue (approximately 810 μM) for rat liver, a value 7 times greater than measured in the present study. Houghton and Houghton [7] reported values of R-1-P for several solid tumor xenographs ranging from 33 to 91 μM. These data as well as our own indicate that the concentration of R-1-P is at least equal to the K_m value and possibly even higher in a variety of normal and tumor tissues. To what extent the R-1-P concentration would persist after FUra administration is unclear since the intracellular concentration of R-1-P could be depleted rapidly, thus reducing the rate of FUra activation by the phosphorylase pathway.

An important difference between FUra-sensitive tumors and normal tissue such as bone marrow where toxicity is dose-limiting was the relative differences in the activities of uridine phosphorylase and pyrimidine phosphoribosyltransferase. The solid tumors displayed a 2- to 7-fold greater activity of these enzymes than bone marrow cells. Means to reduce the activity of these enzymes would have a greater effect on the activation of FUra in normal tissues than on these selected tumors. To find those human tumors where such a selective advantage might be achieved will depend on understanding the activities of FUra metabolizing enzymes in normal tissues that are responsible for dose-limiting toxicity.

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