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Activation pathways of 5-fluorouracil in rat organs and in PC12 cells

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Abstract

Activation of the pyrimidine analogue 5-fluorouracil (5-FU) to the ribonucleotide level may occur through one of the following three pathways: 1) the 5-phosphoribosyl 1-pyrophosphate (PRPP)-mediated direct transfer of ribose 5-phosphate to 5-FU as catalysed by orotate phosphoribosyltransferase; 2) the ribose 1-phosphate (Rib1-P)-mediated addition of ribose by uridine phosphorylase, followed by the action of uridine kinase; and 3) the 2′-deoxyribose 1-phosphate (deoxyRib1-P)-mediated addition of deoxyribose, thought to be catalysed by thymidine phosphorylase, followed by the action of thymidine kinase. Many of the conclusions as to the precise pathways by which normal tissues and different cell lines activate uracil are indirectly derived from drug interactions affecting the availability of the substrates of the three pathways, or from measurement of activities of the enzymes metabolising 5-FU in normal tissues and tumours. In previous papers (Cappiello *et al*. Biochim Biophys Acta 1998;1425:273–81; Mascia *et al*. Biochim Biophys Acta 1999;1472:93–8), we assessed the molecular mechanisms by which the natural base uracil is salvaged *in vitro* to uracil ribonucleotides and deoxyribonucleotides in rat liver and brain. In this paper, we investigated the pathways of 5-FU activation to cytotoxic ribonucleotide and deoxyribonucleotide levels in normal rat tissues and PC12 cell extracts. The results clearly showed that normal rat tissues activated 5-FU mainly via the Rib1-P pathway, and to a lesser extent via the PRPP pathway. The deoxyRib1-P pathway was absent. PC12 cells activated 5-FU mainly via the PRPP pathway and to a lesser extent by the other two pathways.

Keywords: 5-Fluorouracil activation; α-D-ribose 1-phosphate; α-D-deoxyribose 1-phosphate; Pyrimidine salvage pathway; Rat brain; PC12 cells

1. Introduction

The specificity of action of antineoplastic nucleobase analogues depends on the precise pathway by which they are activated [1,2]. One of the most widely used pyrimidine analogue prodrugs, 5-FU, can be activated to cytotoxic phosphorylated nucleotides by three pathways: 1) PRPP-mediated phosphoribosylation to 5-FUMP, generally thought to be catalysed by orotate phosphoribosyltransferase, an enzyme of the *de novo* pyrimidine pathway (the PRPP pathway) [3–5]; 2) Rib1-P-mediated ribosylation to 5-FUrd, catalysed by uridine phosphorylase, followed by phosphorylation to 5-FUMP, catalysed by uridine–cytidine kinase (the Rib1-P pathway) [3,5–8]; 3) deoxyRib1-P-mediated deoxyribosylation to 5-Fdeoxyuridine, catalysed by thymidine phosphorylase, followed by phosphorylation to 5-FdeoxyUMP, the main cytotoxic 5-FU derivative (the deoxyRib1-P pathway) [9]. Multiple phosphorylation steps convert 5-FUMP and 5-FdeoxyUMP to 5-FUTP and 5-FdeoxyUTP, respectively. Finally, 5-FdeoxyUDP can be synthesised from 5-FUDP by ribonucleotide reductase, and either dephosphorylated to 5-FdeoxyUMP or phosphorylated to 5-FdeoxyUTP [see 10 for review].

Because of the complexity of fluoropyrimidine metabolism and the multiple sites of biochemical action of the fluoropyrimidines, 5-FU is also responsible for clinical toxicity [11,12]. The main toxic effects of 5-FU and its metabolites are exerted on rapidly dividing tissues, primary the gastrointestinal mucosa and bone marrow. Neurotoxicity is also frequent when 5-FU is administered in combination with other antimetabolites [13–15].

In this paper, we extend our previous studies on uracil brain salvage synthesis [16–18]. Our purpose was to assess our knowledge on the relative contribution of the three

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Abbreviations: PRPP, 5-phosphoribosyl 1-pyrophosphate; 5-FU, 5-fluorouracil; FUrd, 5-fluorouridine; 5-FUMP, 5-FUDP, and 5-FUTP, 5-fluorouridine 5′-mono-, -di-, and -triphosphate, 5-Fdeoxyuridine, 5-fluoro-2′-deoxyuridine; 5-FdeoxyUMP, 5-FdeoxyUDP, and 5-FdeoxyUTP, 5-fluoro-2′-deoxyuridine 5′-mono-, -di-, and triphosphate; Rib1-P, ribose 1-phosphate; and deoxyRib1-P, 2′-deoxyribose 1-phosphate.

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pathways of 5-FU activation in normal rat tissues and in PC12 cells.

The results show that in rat tissues 5-FU is activated mainly by the Rib1-P-mediated pathway, while PC12 cells activate 5-FU mainly via the PRPP pathway. Contrary to normal tissues, PC12 cells possess the deoxyRib1-P pathway.

2. Materials and methods

2.1. Materials

[2-^{14}C]Uracil (50 mCi/mmol), [2-^{14}C]5-FU (55 mCi/mmol), Rib1-P, dithiothreitol, bases, nucleosides, and nucleotides were from Sigma Chemical Co. HiSafeII scintillation liquid was purchased from Wallac. Polyethyleneimine (PEI)-cellulose precoated thin-layer plastic sheets (0.1 mm thick) were purchased from Merck and prewashed once with 10% NaCl and three times with deionised water before use. All other chemicals were of reagent grade. Three-month-old male Sprague–Dawley rats were killed according to The Guiding Principles in the Care and Use of Animals (DHWEW publication, NIH 86-23). All organs were removed and kept frozen at −80° until needed. Storage times did not exceed 3 months.

2.2. Preparation of rat organ extracts

All organs were cut into small pieces, washed with cold saline, and homogenised with a hand-driven Potter or a Polytron homogeniser in 3 volumes of 100 mM Tris–HCl buffer, pH 7.4, with 20 mM KCl and 1 mM dithiothreitol. The homogenate was centrifuged at 4° at 39,000 × *g* for 1 hr. The supernatant fluid obtained was dialysed overnight at 4° in dialysis bags against 10 mM Tris–HCl buffer, pH 7.4, supplemented with 1 mM dithiothreitol, and is referred to as crude extract.

2.3. Cell cultures and preparation of cell extracts

Rat pheochromocytoma (PC12) cells [19] were cultured in RPMI medium supplemented with 300 mg/L of L-glutamine, 5% foetal bovine serum, 10% heat-inactivated horse serum antibiotics, and 30% conditioned medium to facilitate the propagation. The cells were incubated in plastic dishes at 37° in humidified (96%) air containing 5% CO_2. The medium was changed every 3 days and cells were passed once a week. When cells were subconfluent, the medium was aspirated and the cells were washed twice with PBS, scraped, collected, centrifuged at 800 × *g* for 3 min, and then stored at −80° until use. Cell pellets were resuspended in 2 volumes of 50 mM Tris–HCl buffer, pH 7.4, subjected to ultrasonic treatment, and then centrifuged at 39,000 × *g* for 1 hr at 4°. The supernatant (cell extract) was kept at −80° until use. In preliminary experiments, no difference in salvage enzyme activity levels was observed when extracts were prepared either by sonication or by Potter homogenisation.

2.4. Incubation procedures

Crude extracts or cell extracts, containing 10–50 μg of protein, were incubated in a total reaction volume of 30 μL containing 5 mM Tris–HCl buffer, pH 7.4, 8 mM $MgCl_2$, 0.98 mM [2-^{14}C]uracil (10,000 dpm/nmol) or [2-^{14}C]5-FU (10,000 dpm/nmol), 3.6 mM ATP, and 3 mM Rib1-P to investigate the Rib1-P pathway. The PRPP pathway was investigated by replacing Rib1-P with 3 mM PRPP in the presence or absence of 3.6 mM ATP. Inosine-mediated 5-FU activation was investigated by substituting 6.7 mM inosine and 5 mM inorganic phosphate for Rib1-P. The deoxyRib1-P pathway was investigated by substituting 3 mM deoxyRib1-P for Rib1-P. The reaction was started by the addition of crude extract or cell extract. At different time intervals, the reaction was stopped by rapidly drying 5-μL portions of the incubation mixture on PEI-cellulose precoated thin-layer plastic sheets. A chromatogram was developed in *n*-butanol/glacial acetic acid/water (4:2:1 v/v) to separate uridine and uracil nucleotides or with *n*-butanol/glacial acetic acid/water (8:2:1 v/v) to separate 5-FUrd and 5-FU nucleotides. To separate deoxyuridine and deoxyUMP or 5-FUrd and 5-FdeoxyUMP, the chromatogram was developed in *n*-propanol/NH_3/trichloroacetic acid (100%)/H_2O (75:0.7:5:20). In all separations, appropriate standards were used and detected as ultraviolet absorbing areas that were excised and counted for radioactivity with 8 mL of scintillation liquid.

2.5. Protein concentration

Protein concentration was determined by the Coomassie blue binding assay [20], using bovine serum albumin as standard.

3. Results

We first assessed the relative contribution to 5-FU activation of the Rib1-P and PRPP pathways in rat organs and PC12 cells. Fig. 1 shows the time-courses of labelled FUrd and 5-FU nucleotide formation when crude extracts of rat organs were incubated with [^{14}C]5-FU in the presence of Rib1-P plus ATP (the Rib1-P pathway) or PRPP (the PRPP pathway). For comparison, the results obtained with [^{14}C]uracil are also reported. After a 60-min incubation, the amount of 5-FU nucleotide formed through the Rib1-P pathway ranged between 60 nmol/mg of protein of cerebellum extract and 1 nmol/mg of protein of muscle extract. The contribution of the PRPP pathway was markedly less relevant: the amount of 5-FUMP formed was about 3 nmol/mg of protein only in testis and liver extracts. In addition and

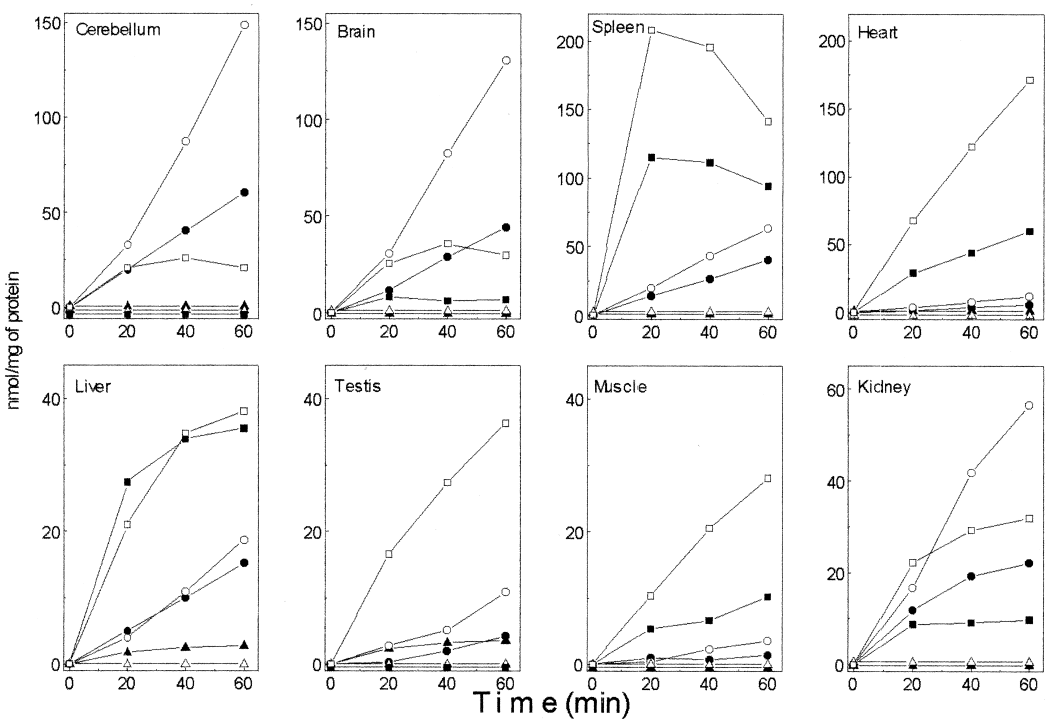

Fig. 1. Time-course of 5-FU activation to 5-FU ribonucleotides (filled symbols) and of uracil ribonucleotide salvage synthesis (open symbols) catalysed by rat organ extracts. The incubation mixtures contained, in a final volume of 30 μL, 30 nmol of either [2-^{14}C]5-FU (10,000 dpm/nmol) or [2-^{14}C]uracil (10,000 dpm/nmol), 90 nmol of Rib1-P, 108 nmol of ATP, 240 nmol of $MgCl_2$, and 10–50 μg of protein in 50 mM Tris–HCl buffer, pH 7.4 (the Rib1-P pathway). The PRPP pathway was investigated by replacing Rib1-P and ATP with 90 nmol of PRPP. (●) 5-FU ribonucleotides, (■) 5-FUrd, (○) uracil nucleotides, and (□) uridine formed by the Rib1-P pathway. (▲) 5-FU nucleotides and (△) uracil nucleotides formed by the PRPP pathway.

extending our observations on rat liver and brain [16], Fig. 1 shows that each of the 8 organs tested, including testis and liver, could not utilise PRPP to salvage uracil to uracil nucleotides, thus confirming the absence of uracil phosphoribosyltransferase in mammals [16,21].

PC12 cells behaved differently. In contrast to rat organs, they activated 5-FU mainly via the one-step PRPP-mediated pathway, even though they were devoid of any uracil phosphoribosyltransferase activity (Fig. 2). 5-FUrd formation observed under these conditions in testis and liver extracts was almost completely prevented by ATP addition (Fig. 3). At the same time, ATP led to an increase in the amount of 5-FU nucleotide formed.

Inosine could be substituted for Rib1-P as a ribose donor. Fig. 4 shows the time-course of labelled FUrd and 5-FU nucleotide formation when rat brain extract was incubated in the presence of [^{14}C]5-FU, ATP, inosine, and inorganic phosphate. Fig. 5 shows that 5-FU inhibited the Rib1-P-mediated salvage of the natural base uracil catalysed by rat brain extract.

The contribution of the deoxyRib1-P pathway to 5-FU activation is shown in Fig. 6 and Table 1. A striking observation here is that only PC12 cell extracts catalysed 5-FU nucleotide formation when incubated with [^{14}C]5-FU, deoxyRib1-P, and ATP. No fluorodeoxyuridine nucleotides were synthesised by rat organ extracts, even though 5-Fdeoxyuridine accumulation was observed.

4. Discussion

The relative contribution of the three pathways for 5-FU activation to normal tissue fluoropyrimidine pools is an important parameter in understanding the molecular mechanism underlying 5-FU toxicity. Our data show that only liver and testis can activate 5-FU via the PRPP pathway, while the Rib1-P pathway is operative in each of the eight organs tested. Cerebellum and liver show the highest Rib1-P activation rate of 5-FU to cytotoxic 5-FU nucleotides (Fig. 1). The lowest rates of Rib1-P-dependent 5-FU activation to 5-FU nucleotides were found in muscle, heart, and testis. The rapid accumulation of uridine and FUrd observed in spleen extracts (Fig. 1) may be explained by a high uridine phosphorylase activity. In normal tissues, this enzymatic activity does not seem to be inhibited by FUrd, as observed earlier [22] in a murine tumour model. Our results might explain why in clinical trials 5-FU administration may lead to acute neurological symptoms [14]. This idea is strengthened by the data reported in Fig. 6 showing that 5-FU has a profound inhibitory effect on the salvage of the natural base uracil in rat brain, most likely by subtracting the Rib1-P needed to synthesise uridine, an obligatory intermediate.

In vivo utilisation of Rib1-P in 5-FU activation via the two-step pathway catalysed by uridine phosphorylase (acting anabolically) and uridine kinase (the Rib1-P pathway) is

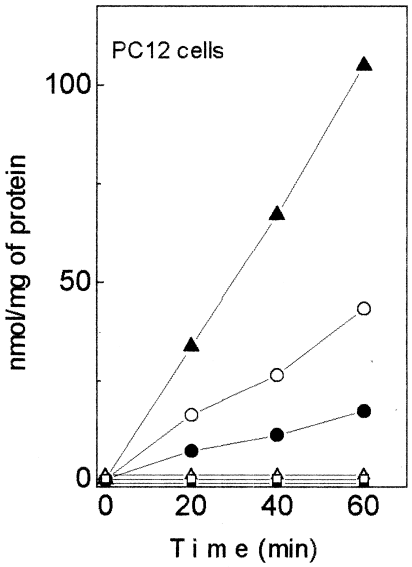

Fig. 2. Time-course of 5-FU activation to 5-FU ribonucleotides (closed symbols) and of uracil ribonucleotide salvage synthesis (open symbols) catalysed by PC12 cell extracts. The assay system was that described under Fig. 1. (●) 5-FU ribonucleotides, (■) 5-FUrd, (○) uracil nucleotides, and (□) uridine formed by the Rib1-P pathway. (▲) 5-FU nucleotides and (△) uracil nucleotides formed by the PRPP pathway.

supported by two considerations. First, the intracellular concentration of Rib1-P in normal tissues and tumour cells, ranging between 30 and 800 μM [4,5,23–25], is sufficiently high with respect to its K_m value for the phosphorylase, ranging between 20 and 60 μM in tumour cells and liver [26,27]. Second, the presence of uridine kinase, catalysing an irreversible reaction, shifts the overall equilibrium towards 5-FU nucleotide formation, even in the presence of excess inorganic phosphate [16].

5-FU shares the same transport system as uracil [28–30]

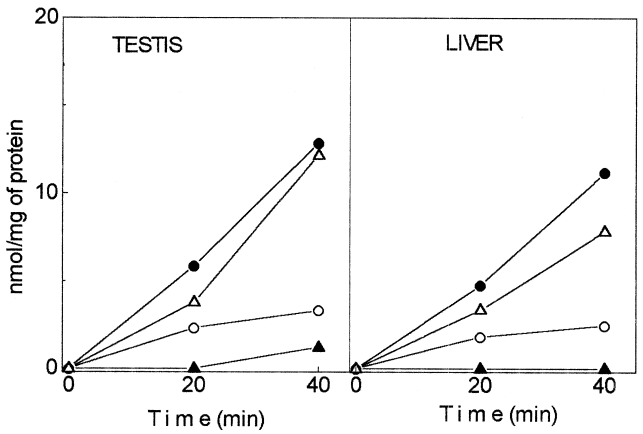

Fig. 3. Effect of ATP on PRPP-mediated 5-FU activation in rat testis and liver extracts. The incubation mixture contained, in a final volume of 30 μL, 30 nmol of [2-^{14}C]5-FU (10,000 dpm/nmol), 90 nmol of PRPP, 240 nmol of $MgCl_2$, and 10–50 μg of protein in 50 mM Tris–HCl buffer, pH 7.4, either in the absence (open symbols) or presence of 108 nmol of ATP (closed symbols). (△) and (▲) 5-FURd, (○) and (●) 5-FU ribonucleotides.

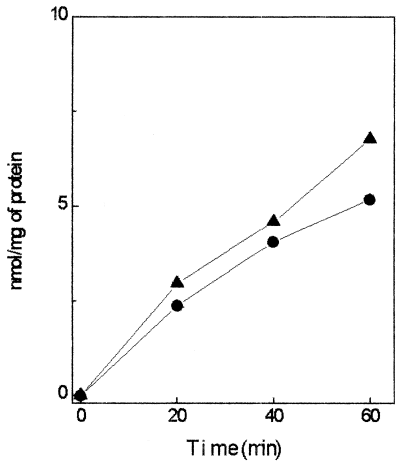

Fig. 4. Inosine-mediated 5-FU activation catalysed by rat brain extract. The incubation mixture contained, in a final volume of 30 μL, 30 nmol of [2-^{14}C]uracil (10,000 dpm/nmol), 200 nmol of inosine, 150 nmol of inorganic phosphate, 108 nmol of ATP, 240 nmol of $MgCl_2$, and 10–50 μg of protein in 50 mM Tris–HCl buffer, pH 7.4. (▲) 5-FURd (●) 5-FU ribonucleotides.

and the same intracellular catabolic and anabolic enzymes, with the exception of orotate phosphoribosyltransferase, which activates 5-FU in the presence of PRPP, but cannot salvage uracil [10]. The enzyme most likely responsible for PRPP-dependent 5-FU activation in liver, testis, and PC12 cell extracts (Figs. 1 and 2) is the orotate phosphoribosyltransferase.

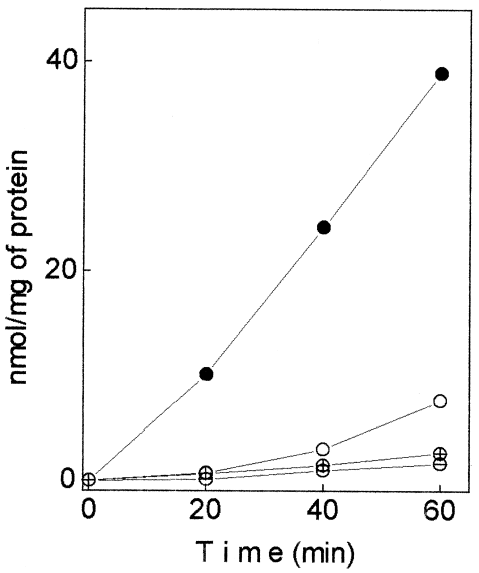

Fig. 5. Effect of 5-FU on Rib1-P-mediated uracil salvage catalysed by rat brain extract. The incubation mixture contained, in a final volume of 30 μL, 30 nmol of [2-^{14}C]uracil (10,000 dpm/nmol), 90 nmol of Rib1-P, 108 nmol of ATP, 240 nmol of $MgCl_2$, and 10–50 μg of protein in 50 mM Tris–HCl buffer, pH 7.4, either in the absence or presence of 5-FU. (●) Uracil ribonucleotides formed in the absence of 5-FU; (○), (⊕), and (⊖), uracil ribonucleotides formed in the presence of 15, 30, and 60 nmol, respectively, of 5-FU.

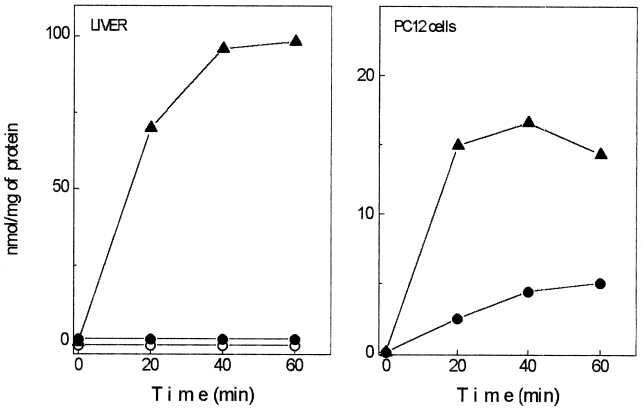

Fig. 6. Time-course of deoxyRib1-P-mediated 5-FU activation to 5-FU 2′-deoxyribonucleotides (filled symbols) and of uracil salvage to 2′-deoxyribonucleotides (open symbols) catalysed by rat liver and PC12 cell extracts. The incubation mixtures contained, in a final volume of 30 μL, 30 nmol of either [2-^{14}C]5-FU (10,000 dpm/nmol) or [2-^{14}C]uracil (10,000 dpm/nmol), 90 nmol of deoxyRib1-P, 108 nmol of ATP, 240 nmol of $MgCl_2$, and 10–50 μg of protein in 50 mM Tris–HCl buffer, pH 7.4. (▲) 5-FdeoxyUrd; (●) 5-FdeoxyUMP; (○) 2′-deoxyuracil ribonucleotides. 2′-Deoxyuridine formed (not shown) was higher than 300 nmol/mg of protein in liver extract after a 60-min incubation.

FUrd is an intermediate in the Rib1-P pathway, but not in PRPP-mediated 5-FU activation, a one-step pathway. It is conceivable that any FUrd formed under the experimental conditions of Fig. 3 arises from 5-FUMP dephosphorylation, catalysed by intracellular nucleoside monophosphatase(s). The effect of ATP in preventing FUrd accumulation and in enhancing 5-fluororibonucleotide formation may be explained by assuming that 5-FUMP synthesised by orotate phosphoribosyltransferase is further phosphorylated to 5-FUDP and 5-FUTP. We suggest that the intracellular ATP/PRPP ratio might be important in the process of 5-FU activation by the PRPP pathway.

Intracellular nucleosides are the most readily available source of Rib1-P for 5-FU activation [25]. Inosine, the best Rib1-P donor, is rapidly taken up by normal and tumour cells by a facilitated transport mechanism [31], followed by intracellular phosphorolysis [32]. Exposure of tumour cells *in vitro* to inosine results in a marked potentiation of growth inhibition by 5-FU, due to its enhanced anabolic conversion to cytotoxic free and polymeric nucleotides [33–37]. We recall that no kinase activities exist in mammals for inosine [38]. Fig. 4 shows that the inosine-mediated 5-FU activation pathway catalysed by rat brain extracts is more efficient than the PRPP pathway catalysed by liver and testis (see Fig. 1).

In addition to Rib1-P, deoxyRib1-P has also been found in mammalian cells [39]. This pentose phosphate is effective in increasing the formation of antineoplastic deoxyribonucleotides in tumour cells [26,27]. A significant increase in cytotoxicity in human colorectal cell lines is observed when 5-FU is associated with 2′-deoxyinosine, a deoxyRib1-P donor [33,40]. The results presented in Fig. 6 and Table 1 show that the deoxyRib1-P pathway is present in PC12 cells, but not in normal rat organs. We could not observe any 5-FU nucleotide synthesis when crude extracts of rat organs were incubated with [^{14}C]5-FU in the presence of deoxyRib1-P and ATP.

Finally, we emphasize that our methods may be applied to tumour extracts to determine those instances in which selective 5-FU chemotherapy should be applied.

Table 1
2′-Deoxyribose 1-phosphate-mediated 5-fluoro-2′-deoxyribonucleotide synthesis catalysed by rat organs and PC12 cell extracts[a]

	5-FluorodeoxyUMP (nmol/mg after 60-min incubation)	5-Fluorodeoxyuridine (nmol/mg after 60-min incubation)
Heart	0	213.14
Cerebellum	0	214.6
Brain	0	138.7
Kidney	0	111.6
Testis	0	52.6
Liver	0	98.3
Muscle	0	67.5
Spleen	0	447.9
PC12 cells	5.03	14.32

[a] The assay system is described under the legend to Fig. 7.

Acknowledgments

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