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# Elucidation of Pathways of 5-Fluorouracil Metabolism in Xenografts of Human Colorectal Adenocarcinoma*

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**Abstract**—*Hypoxanthine (Hx) and allopurinol (HPP) have been shown experimentally to reduce the conversion of 5-fluorouracil (FUra) to FUMP by orotate phosphoribosyltransferase (OPRTase). This study was designed to elucidate the major pathway by which FUra was metabolized to ribonucleotides by human colorectal tumors. Consequently, the effect of Hx and HPP on the metabolism of* [6-$^3$H]*-FUra was examined in 5 human colorectal adenocarcinomas maintained as xenografts in immune-deprived mice. In 2 tumors the formation of ribonucleotides from FUra was depressed by Hx and HPP in combination during the first hour after treatment, while in 3 other lines ribonucleotide concentrations were not reduced. The data suggested that these 5 xenograft lines may be divided into 2 groups: (1) group 1 tumors formed relatively high levels of FUrd and low levels of fluorinated ribonucleotides after the injection of FUra, with no decrease in ribonucleotide concentrations after the administration of Hx and HPP. These tumors possessed high ratios of uridine (Urd) phosphorylase/orotate phosphoribosyltransferase (OPRTase: 7–24) and ribose-1-phosphate (R-1-P)/5-phosphoribosyl-1-pyrophosphate (PRPP; 5), and thus appeared to metabolize FUra by the Urd phosphorylase and Urd kinase pathway; (2) group 2 tumors formed low levels of FUrd, higher concentrations of fluorinated ribonucleotides and a reduction in levels of these nucleotides after administration of the purine combination. Group 2 tumors demonstrated a lower enzyme ratio (1–2), higher endogenous levels of PRPP, a lower R-1-P/PRPP ratio (1) and appeared to metabolize FUra predominantly by the activity of OPRTase. Hypoxanthine and HPP, alone or in combination, caused a rapid depletion of PRPP in each tumor line examined. In group 2 tumors this may be responsible for the decreased formation of FUra ribonucleotides observed.*

## INTRODUCTION

THE THERAPEUTIC activity of a cytotoxic agent may be increased, potentially, by selectively reducing conversion to an active metabolite in normal limiting host tissues but not in tumor cells. This may be possible where two or more metabolic pathways to the cytotoxic product exist and where one pathway predominates in normal tissue while the alternative route is the major pathway in tumor. It is considered that the antimetabolite FUra must be converted to the ribonucleotide form as an initial step in its lethal synthesis. This may occur via two pathways: (1) via OPRTase that utilizes PRPP or (2) by the sequential activities of Urd phosphorylase (which requires R-1-P) and Urd kinase. Thus if tumors utilized predominantly the latter route for the metabolism of FUra, selectivity of FUra might be enhanced by inhibiting conversion by OPRTase in normal tissues if this was the major metabolic pathway. In tumor cells in culture, Hx reduced the cytotoxic effects of FUra in lines L5178Y [1] and S49 [2], an effect attributed to the depletion of PRPP. The Hx analog, HPP, antagonized FUra cytotoxicity in S-180 and murine leukemic cell

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lines, but not in Walker 256 or HeLa cells in culture [3]; accumulation of orotic acid which could compete favorably with FUra for metabolism by OPRTase [4] was postulated as the mechanism of protection. Both Hx [5] and HPP [6] have reduced the toxicity of FUra in rodents, while HPP has also reduced FUra toxicity in man [7–9]; thus, clinical evaluation of FUra and HPP in combination has been initiated in patients with colorectal adenocarcinoma [8, 9]. The major route for FUra metabolism in human colorectal tumors remains unknown. We have therefore used Hx and the inhibitor of its degradation, HPP, to elucidate by which pathways ribonucleotides of FUra are formed in a series of human colorectal adenocarcinomas growing in immune-deprived mice. The data suggest that two lines metabolize FUra to ribonucleotides predominantly by OPRTase and three lines by Urd phosphorylase and Urd kinase, and that the relative levels of R-1-P to PRPP and relative activities of Urd phosphorylase and OPRTase may determine the predominant pathway for metabolism.

## MATERIALS AND METHODS

*Chemicals*

[6-$^3$H]-FUra (18–20 Ci/mmol) and [8-$^{14}$C]-Hx (56 mCi/mmol) were purchased from Moravek Biochemicals, City of Industry, CA. [$^{14}$COOH]-orotic acid (51.1 mCi/mmol) was obtained from New England Nuclear, Boston, MA. 5-Fluorouracil was purchased from Hoffmann-LaRoche, Inc., Nutley, NJ, while Hx, HPP and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

*Immune-deprivation of mice*

Male and female CBA/CaJ mice were immune-deprived by thymectomy, lethal whole-body irradiation and reconstitution with syngeneic bone marrow as previously described [10].

*Tumor lines*

The characteristics of the 5 human adenocarcinomas of the colon and rectum maintained subcutaneously in immune-deprived mice and their sensitivity to chemotherapeutic agents have been reported previously [10–17].

*Assessment of tumor response*

Two tumor pieces, approximately 5 mm$^3$, were implanted s.c. into the dorsal flanks of each immune-deprived mouse. Mice were randomized into groups of up to 13 and were treated when tumors had reached 8–10 mm in diameter. Tumor-bearing mice received 50 mg per kg of FUra either alone or in combination with Hx (50 mg/kg) and HPP (10 mg/kg) simultaneously, by i.p. injection, at 7-day intervals. Tumor growth was assessed from the measurement of two perpendicular diameters, at weekly intervals, using vernier calipers, and tumor volume calculated by substitution in the formula $\frac{\pi}{6} \times d^3$, where $d$ is the mean diameter. The delay in growth of treated tumors was assessed at a time when they had reached four times their treatment volume as previously described [16, 17].

*Metabolism of* [6-$^3$H]-*FUra*

Tumor-bearing mice received a single i.p. administration of 100 mg of [6-$^3$H]-FUra per kg (5 $\mu$Ci/g body wt), either with or without the simultaneous i.p. injection of 50 mg of Hx and 10 mg of HPP per kg. Excision of the tumors (which were in the order of 300–500 mg in weight) between 1 and 24 hr after treatment, extraction of acid-soluble metabolites, the determination of concentrations of radiolabeled products by thin-layer chromatography and DNA estimations have been described previously [10, 18]. For the purposes of clarity, total ribonucleotides of [6-$^3$H]-FUra are reported, and the standard deviations omitted. In tumor-bearing mice immune-deprived by the techniques described, FUra (100 mg/kg) represents a dose lethal to approximately 5% of mice. (The doses of Hx and HPP, administered either singly or in combination, were previously shown to be optimal in protecting the normal limiting tissues of mice from the toxicity of FUra; [5].)

*Assay of PRPP*

Tumor-bearing mice received a single i.p. administration of 50 mg of Hx or 10 mg of HPP per kg, or both. At various times from 15 min to 4 hr after injection mice were anesthetized using ether. Each tumor was excised and immersed in liquid nitrogen within 10 sec, thus preventing degradation of PRPP, which has been demonstrated to occur in anoxia [19]. Tissues were ground to a fine powder in a mortar under liquid nitrogen and subsequently placed in a dry-ice–acetone bath prior to assay. Powders were assayed fresh, but could be stored for at least 1 week in liquid nitrogen without loss of PRPP. Extraction was effected by 3 cold washes in 50 mM Tris–HCl, pH 7.4, containing 100 $\mu$l of 40 mM 2,3-diphosphoglyceric acid and 133 $\mu$l of 0.7 M NaF per ml, which yielded a recovery of internal standard from 82 to 91% in 3 tumor lines examined [20]. PRPP was assayed from the release of $^{14}CO_2$ from [$^{14}$COOH]-orotic acid as previously described [20].

*Assay of R-1-P*

Powders prepared as above from both untreated and treated tumors were extracted and assayed for their content of R-1-P by a modified method of Barankiewicz and Henderson [21]. Protein from extracted pellets of both PRPP and R-1-P assays was determined using the method of Lowry *et al.* [22]. The μM concentrations of PRPP and R-1-P were calculated in intracellular water, which was determined from the difference between the dry weight and the [$^3$H]-inulin space of tumors.

*Determination of orotic acid*

Tumors were rapidly excised and placed in liquid nitrogen as described. Powders were prepared as for the assay of PRPP and orotate was extracted using 1.0 M perchloric acid (3 extractions) at 4°C. The tissue extracts were placed in a water bath at 100°C for 14 min as described by Moyer and Handschumacher [23]. After hydrolysis the solutions were adjusted to pH 4 with KOH, and $KClO_4$ was removed. Alternatively, after hydrolysis the solutions were carefully neutralized, and after removal of salt were lyophilized and finally redissolved in water (1 g tissue/2 ml; [24]). Chromatography was performed using an Altex Model 110 A pump and a Partisil PXS 10/25 SAX column (Whatman Inc., Clifton, NJ) equipped with a guard column. Elution was achieved using 0.04 M sodium formate, pH 4.1, at a flow rate of 1.0 ml/min. Absorbance was measured at 254 nm and 280 nm. Under these conditions orotate eluted at 12.2 min.

*Assay of orotate phosphoribosyltransferase*

The activity of OPRTase was determined using a modified method of Reyes and Guganig [4]. $HxGC_3$, $HxELC_2$, $HxHC_1$ and $HxVRC_5$ tumors were exised and placed on ice, and subsequently homogenized in 4 vol. of 45 mM Tris–HCl, pH 8.0, containing 0.93 mM dithiothreitol at 2°C. Homogenization was effected by 6 × 30-sec periods each of tissue disruption and cooling using a Potter Elvjheim glass homogenizer fitted with a motor-driven teflon pestle. Homogenates were centrifuged initially at 30,000 ***g*** for 30 min at 4°C and the supernatants were subsequently centrifuged at 105,000 ***g*** for a further 1 hr. Reaction mixtures, in a total vol. of 200 μl, contained the following: 2 mM PRPP, 4 mM $MgCl_2$, 45 mM Tris–HCl, pH 8.0, 45 mM CMP, 0.9 mM [6-$^3$H]-FUra (10.4 mCi/mmol) and 140 μl cytosol. The presence of cytidylate was necessary to prevent the degradation of radiolabelled phosphates to [6-$^3$H]-FUrd due to phosphatase activity, which was present in tumor lines $HxVRC_5$, $HxGC_3$ and $HxHC_1$. For the blank, 1.8 mM orotic acid was present in the reaction mixture to prevent the metabolism of [6-$^3$H]-FUra by OPRTase. After incubation at 37°C for 30 min the assay was terminated by pipetting 150 μl of reaction mixture into 7 μl 4.2 M PCA. After centrifugation of the precipitate, 100 μl of the supernatant was neutralized with 19 μl 1 M KOH and samples were re-centrifuged. In the final supernatant total phosphates, FUrd and FUra were separated by TLC using cellulose chromatogram sheets and a solvent mixture of *N*-butanol, formic acid and water (77:10:13). Nucleotides remained at the origin, while the $R_F$ values for FUra and FUrd were 0.51 and 0.38 respectively. The reaction was linear during the 30-min assay. The protein content of cytosols was determined by the method of Lowry *et al.* [22], and results were expressed as pmol/min/mg protein.

*Assay of uridine phosphorylase*

Tumors were rapidly excised, placed on ice and homogenized at 2°C in 4 vol. of ice-cold Tris–HCl (100 mM, pH 7.5, containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.25 M sucrose) as described. Homogenates were centrifuged for 30 min (30,000 ***g***, 4°C) and supernatants re-centri fuged for 60 min (105,000 ***g***, 4°C). Optimal conditions for the formation of FUrd with respect to pH, substrate concentration and volume of cytosol were determined using cytosol preparations from $HxGC_3$ xenografts. In a final volume of 560 μl, the reaction mixture contained 100 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 mM R-1-P, 2 mM [6-$^3$H]-FUra and 160 μl cytosol. Under these conditions enzyme was limiting in the formation of FUrd, and the reaction was linear for 30 min. The assay was terminated by pipetting 50 μl reaction mixture into 5 μl of ice-cold perchloric acid (6.2 M). The precipitate was removed by centrifuging, and 25 μl of clear supernatant were mixed with 55 μl KOH (0.1 M containing 10 mM FUra, FUrd and UMP). After further centrifugation to remove perchlorate, 10 μl supernatant were chromatographed as described for the assay of OPRTase.

*Statistical analyses*

Differences in the concentrations of fluorinated ribonucleotides (Table 2) and levels of FUrd (Table 3) observed in tumors after the administration of Hx and HPP simultaneously with FUra were subjected to statistical evaluation by means of the *t* test for small samples where more than 2 determinations per point were obtained. Differences between means giving a probability of less than 5% were considered to be statistically significant.

## RESULTS

Lines $HxELC_2$ and $HxHC_1$ have demonstrated sensitivity to treatment with 5-fluoropyrimidines *in vivo*, while HxBR, $HxGC_3$ and $HxVRC_5$ tumors were unresponsive [10, 13, 17]. The sensitivity of $HxELC_2$ and $HxHC_1$ tumors following the weekly administration of FUra to mice both in the presence and in the absence of Hx (50 mg/kg) and HPP (10 mg/kg) have been reported in detail elsewhere [17], and are summarized in Table 1.5-Fluorouracil at a dose level of 50 mg/kg/week was the maximum tolerated dose in tumor-bearing mice. Inhibition of tumor growth for approximately 17 days occurred both in the absence and in the presence of Hx (50 mg/kg) and HPP (10 mg/kg) in $HxELC_2$ tumors. In line $HxHC_1$ the delay in tumor growth was 28 days after the administration of FUra alone, and was reduced to 9 days in the presence of Hx and HPP.

The concentrations of FUra ribonucleotides determined at various times after administration of either the 3-agent combination or FUra alone are shown in Table 2. At 1 hr after administration of FUra alone concentrations of FUra ribonucleotides were higher in lines $HxHC_1$ (3.5 nmol/mgDNA) and $HxVRC_5$ (4.8 nmol/mg DNA) than in $HxELC_2$, $HxGC_3$ and HxBR tumors (Range 180–867 pmol/mg DNA). In addition, in the latter 3 tumors ribonucleotide levels were either increased or remained unchanged in mice treated with Hx–HPP–FUra, whereas the combination caused a 50% decrease in ribonucleotide concentrations in lines $HxHC_1$

*Table 1. Sensitivity of tumors to FUra*

| Tumor line | Volume doubling time (days) | Treatment* | Time to 4 × Rx vol. (days) | Growth delay (days) | Deaths/total |
|---|---|---|---|---|---|
| | | none | 30.6 | — | 0/12 |
| $HxELC_2$ | 15.3 | FUra | 47.6 | 17.0 | 1/12 |
| | | FUra + Hx + HPP | 48.6 | 17.6 | 3/12 |
| | | none | 28.5 | — | 0/6 |
| $HxHC_1$ | 14.3 | FUra | 56.6 | 28.1 | 1/6 |
| | | FUra + Hx + HPP | 37.7 | 9.2 | 1/6 |

*FUra (50 mg/kg) was administered alone or simultaneously with Hx (50 mg/kg) and HPP (10 mg/kg) at weekly intervals by i.p. injection.

*Table 2. Concentrations of fluorinated ribonucleotides* (pmol/mg *DNA*)

| Tumor line | Treatment | 1 hr | 4 hr | 24 hr |
|---|---|---|---|---|
| $HxELC_2$* | combination (A) | 771 | 539 | 361 |
| | FUra alone (B) | 867 | 235 | 322 |
| | A/B | 0.9 | 2.3 | 1.1 |
| $HxGC_3$† | combination (A) | 445 | 483 | 182 |
| | FUra alone (B) | 620 | 449 | 193 |
| | A/B | 0.7 | 1.1 | 0.9 |
| HxBR* | combination (A) | 340 | 222 | 58 |
| | FUra alone (B) | 180 | 111 | 37 |
| | A/B | 1.9 | 2.0 | 1.6 |
| $HxHC_1$† | combination (A) | 1809‡ | 1801§ | 1074‡ |
| | FUra alone (B) | 3479 | 5568 | 2069 |
| | A/B | 0.5 | 0.3 | 0.5 |
| $HxVRC_5$† | combination (A) | 2457‖ | 1514‖ | 1194‖ |
| | FUra alone (B) | 4775 | 5016 | 4168 |
| | A/B | 0.5 | 0.3 | 0.3 |

Results represent the mean of *2 determinations or †4–6 determinations per time point. Differences significant at the level ‡$P<0.05$, §$P<0.01$, ‖$P<0.001$, evaluated using the $t$ test for small samples.

*Table 3. Concentrations of FUrd* (pmol/mg *DNA*)

| Tumor line | Treatment | 1 hr | 4 hr | 24 hr |
|---|---|---|---|---|
| $HxELC_2$* | combination (A) | 53,800 | 48,800 | 22,500 |
| | FUra alone (B) | 74,100 | 9400 | 1440 |
| | A/B | 0.7 | 5.2 | 15.6 |
| $HxGC_3$† | combination (A) | 49,800§ | 50,400‡ | 11,700‡ |
| | FUra alone (B) | 20,900 | 7640 | 1930 |
| | A/B | 2.4 | 6.6 | 6.1 |
| HxBR* | combination (A) | 48,000 | 28,400 | 2400 |
| | FUra alone (B) | 37,300 | 5940 | 1140 |
| | A/B | 1.3 | 4.8 | 2.1 |
| $HxHC_1$† | combination (A) | 19,000§ | 23,800§ | 2450† |
| | FUra alone (B) | 8800 | 4470 | 797 |
| | A/B | 2.2 | 5.3 | 3.1 |
| $HxVRC_5$† | combination (A) | 19,600 | 22,600§ | 5830 |
| | FUra alone (B) | 13,100 | 3660 | 2230 |
| | A/B | 1.5 | 6.2 | 2.6 |

Results represent the mean of *2 determinations, or †4–6 determinations per time point. Differences significant at the level $‡P<0.05$, $§P<0.01$, evaluated using the *t* test for small samples.

and $HxVRC_5$ 1 hr after treatment. Levels of FUra ribonucleotides remained between 30 and 50% of controls in these 2 tumor lines for up to 24 hr.

Concentrations of FUrd determined in each tumor line after administration of [6-$^3$H]-FUra either alone or in combination with Hx and HPP are presented in Table 3. When [6-$^3$H]-FUra was administered alone the concentration of FUrd at 1 hr was lower in $HxHC_1$ and $HxVRC_5$ xenografts (8.8 and 13.1 nmol/mgDNA respectively) than observed in the other 3 tumor lines (range 21–74 nmol/mgDNA). At 1 hr after administration of the combination levels of FUrd were elevated in $HxGC_3$ and $HxHC_1$ tumors. The major effect produced, however, was the apparent retention of this nucleoside in all tumor lines after administration of the 3-agent combination in comparison to the administration of FUra alone.

High concentrations of FUrd, together with the relative ineffectiveness of Hx and HPP in reducing the levels of FUra ribonucleotides, suggested that in tumor lines $HxELC_2$, $HxGC_3$ and HxBR metabolism of FUra to nucleotide forms may be predominantly by the Urd phosphorylase and Urd kinase pathway that utilizes R-1-P as the ribose donor. In lines $HxVRC_5$ and $HxHC_1$, however, low concentrations of FUrd observed after the administration of FUra alone, high concentrations of fluorinated ribonucleotides and depression of formation of the latter after the administration of Hx and HPP with FUra indicated that in these lines FUra metabolism may proceed by the activity of OPRTase, utilizing PRPP as the donor of ribose-5′-phosphate. We examined, therefore, whether the relative concentrations of R-1-P and PRPP may have influenced the route by which FUra was metabolized. Endogenous levels of R-1-P and PRPP for each tumor line are shown in Table 4. Levels of R-1-P were similar in each line, ranging from 1080 to 1660 pmol/mg protein. Concentrations of PRPP, however, varied considerably. In lines $HxELC_2$, $HxGC_3$ and HxBR PRPP levels were similar, ranging from 240 to 330 pmol/mg protein, while in $HxVRC_5$ and $HxHC_1$ tumors PRPP levels were considerably higher (1180 and 1280 pmol/mg protein, respectively). Thus in lines $HxELC_2$, $HxGC_3$ and HxBR R-1-P levels were approximately 5 times that of PRPP, while in $HxVRC_5$ and $HxHC_1$ tumors the two ribose phosphates were present in similar concentrations.

The activities of OPRTase and Urd phosphorylase in $HxGC_3$, $HxELC_2$, $HxHC_1$ and $HxVRC_5$ tumors were also examined and are presented in Table 5. The anabolic activity of Urd phosphorylase was similar in each tumor line, ranging from 1500 to 3860 pmol/min/mg protein; the activity of OPRTase was similar in $HxHC_1$ tumors (2206 pmol/min/mg protein), but was considerably lower in the 3 other lines (108–621 pmol/min/mg protein). In lines $HxGC_3$ and $HxELC_2$, which demonstrated high R-1-P/PRPP ratios (~5), the ratio of Urd phosphorylase/OPRTase was 7–24, while in $HxHC_1$ and $HxVRC_5$ tumors, which demonstrated low R-I-P/PRPP ratios (~1), the ratio of enzyme activities was ~1–2.

The effect of Hx and HPP on levels of PRPP in

*Table 4. Basal levels of PRPP and R-1-P in tumors*

| Tumor line | PRPP (A) (pmol/mg protein) | R-1-P (B) (pmol/mg protein) | B/A |
|---|---|---|---|
| $HxELC_2$ | 240 ± 20 ( 7 μM) | 1210 ± 100 (33 μM) | 5.0 |
| $HxGC_3$ | 330 ± 90 (18 μM) | 1660 ± 649 (88 μM) | 5.0 |
| HxBR | 303 ± 20 (18 μM) | 1570 ± 100 (91 μM) | 5.2 |
| $HxHC_1$ | 1280 ± 190 (76 μM) | 1080 ± 310 (64 μM) | 0.8 |
| $HxVRC_5$ | 1180 + 104 (69 μM) | 1510 ± 582 (88 μM) | 1.3 |

For each assay, results represent the mean ± 1 S.D. of 6–12 determinations.

*Table 5. Activities of OPRTase and uridine phosphorylase (pmol/min/mg protein)*

| Tumor line | OPRTase (A) | Uridine phosphorylase (B) | B/A |
|---|---|---|---|
| $HxELC_2$ | 108 | 2570 | 23.8 |
| $HxGC_3$ | 522 | 3860 | 7.4 |
| $HxHC_1$ | 2206 | 2600 | 1.2 |
| $HxVRC_5$ | 621 | 1500 | 2.4 |

Results represent the mean of duplicate determinations.

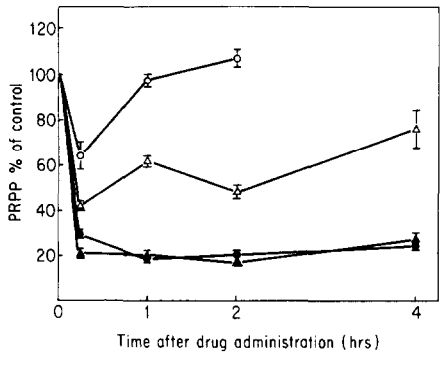


*Fig. 1. The effect of administration of Hx (50 mg/kg) or HPP (10 mg/kg) alone or in combination on the concentations of PRPP in xenografts. $HxELC_2$: Hx (○); HPP (△); Hx + HPP (●). $HxHC_1$: Hx + HPP(▲). Bars: ± 1 S.D. of 5–6 determinations.*

$HxELC_2$ and $HxHC_1$ tumors are shown in Fig. 1. In $HxELC_2$ tumors PRPP levels were rapidly reduced after the administration of either Hx or HPP, or both agents simultaneously. By 15 min after injection both Hx and HPP administered alone had significantly reduced levels of PRPP to 64 and 42% of the control respectively. At this time the purine combination decreased PRPP concentrations by approximately 70%, indicating additivity of the two agents. Recovery of PRPP levels after Hx was rapid, occurring within 1 hr of injection. Allopurinol, however, caused a more prolonged depression in the concentration of PRPP, with levels remaining at 76% of the control 4 hr after treatment. After administration of Hx and HPP in combination, levels of PRPP were lower than had been observed by the injection of HPP alone, and were still only 25% of the pretreatment level 4 hr after treatment. Hypoxanthine administered simultaneously with HPP produced a similar reduction and prolonged depression in the level of PRPP in $HxHC_1$ tumors (Fig. 1).

As accumulation of orotate may interfere with PRPP determinations, causing an apparent decrease in PRPP, we examined levels of orotate in $HxELC_2$ tumors using HPLC techniques. In tumor samples, both untreated and for up to 4 hr after Hx and HPP administration, we were unable to detect orotic acid. Similarly, after concentration of samples by lyophilization orotic acid was undetectable.

The effect of Hx and HPP on levels of R-1-P was examined in $HxELC_2$ and $HxHC_1$ tumors during the first 60 min after treatment, when fluorinated ribonucleotide concentrations were reduced in line $HxHC_1$, but not in $HxELC_2$ tumors (Fig. 2). No decrease in the concentrations of R-1-P in

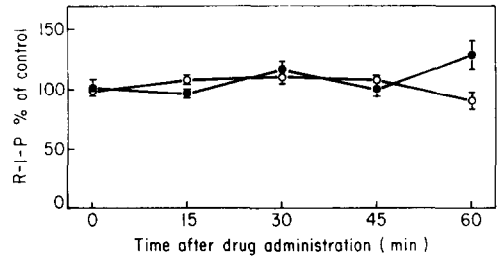


*Fig. 2. The effect of simultaneous administration of Hx (50 mg/kg) and HPP (10 mg/kg) on the concentrations of R-1-P in xenografts. $HxELC_2$ (●); $HxHC_1$ (○). Bars: ± 1 S.D. of 5–6 determinations.*

either tumor was detected. Similarly, Hx and HPP, at concentrations of 520 μM, did not affect the activity of Urd phosphorylase *in vitro* when added simultaneously to the reaction mixture (data not shown).

## DISCUSSION

After the administration of FUra the pattern of metabolism to FUrd and to fluorinated ribonucleotides allowed the 5 human colorectal xenograft lines to be divided into two groups: (1) tumors that formed high concentrations of FUrd and relatively low concentrations of ribonucleotides ($HxELC_2$, $HxGC_3$, HxBR) and (2) tumors that formed relatively low levels of FUrd but greater concentrations of fluorinated ribonucleotides ($HxHC_1$, $HxVRC_5$). Hypoxanthine [1, 2] and HPP [3], which have been shown to reduce the conversion of FUra to FUMP by OPRTase in cultured cells, were subsequently used in combination with FUra to further define the predominant pathway of FUra metabolism to ribonucleotides in human colorectal xenografts. Of interest is the finding that the concentrations of FUra ribonucleotides in group 2 tumors were depressed by combining Hx and HPP with FUra, while those of group 1 tumors were not reduced by administration of the purine combination.

It was apparent that in tumors of both groups ($HxELC_2$, $HxHC_1$) Hx and HPP caused rapid depletion of PRPP within 15 min of injection, with no accumulation of orotic acid for up to 4 hr after treatment. These purines exerted no effect, either on endogenous concentrations of R-1-P during the first 60 min after treatment or on the activity of Urd phosphorylase *in vitro*. Thus depletion of PRPP appears to be the mechanism responsible for depression of the formation of FUra ribonucleotides in group 2 tumors.

One possibility for group 1 tumors was that the OPRTase pathway was not operative, either because of low enzyme activity or low basal levels of PRPP. Alternatively, initial endogenous concentrations of PRPP may have been so high that depleting PRPP by using Hx and HPP would not reduce the PRPP concentration sufficiently to be limiting in the formation of FUra ribonucleotides. The relative activities of the enzymes, OPRTase and Urd phosphorylase, and levels of co-substrates, PRPP and R-1-P, that would be involved in competition for the metabolism of FUra to ribonucleotides were subsequently examined in two lines from each of the two groups. Activities of Urd phosphorylase were similar in all tumors. For OPRTase, activities of this enzyme were considerably lower in $HxELC_2$, $HxGC_3$ and $HxVRC_5$ tumors than in line $HxHC_1$. However, it is apparent after examination of the ratios of activity of Urd phosphorylase/OPRTase that these were high for group 1 tumors (7–24), whereas group 2 tumors possessed similar levels of both enzymes (1–2). The availability of R-1-P has been reported to be limiting in the conversion of FUra to ribonucleotides [25, 26]. Although the metabolism of FUra differed within the 5 tumor lines, concentrations of R-1-P in pmol/mg protein were generally similar. For Urd phosphorylase of rat liver, the $K_m$ for R-1-P in the anabolic reaction was reported to be 71 μm [27]. All tumor lines in this study with the exception of $HxELC_2$ (33 μM) were either similar to, or greater than, this value (64–91 μM). For $HxELC_2$ tumors the protein content per g of tissue was approximately halved in comparison to the other lines, although the interstitial space was similar. This would indicate that $HxELC_2$ tumors may be less cellular and may contain larger cells than the other lines. These findings may relate to the high intracellular accumulation of mucin [11] and may explain the lower R-1-P concentration obtained. However, the data suggest that the availability of R-1-P may not be limiting in the formation of ribonucleotides from FUra.

The only individual parameter that has correlated with the pathway of FUra metabolism has been the basal level of PRPP, which was higher in group 2 tumors. For OPRTase the $K_m$ for PRPP with orotate as substrate was 15 μM in human erythrocytes [28] and 32 μM for OPRTase from P1534 cells [4]; with FUra as substrate the $K_m$ value was 25 μM [4]. For group 1 tumors the intracellular concentration of PRPP was 7–18 μM, lower than the $K_m$ value, and was considerably lower than the available concentration of R-1-P. In contrast, the intracellular level of PRPP was higher than the $K_m$ value in group 2 tumors (69 and 76 μM respectively). By increasing the endogenous level of PRPP in $HxELC_2$ and $HxVRC_5$ tumors through administration of methotrexate, the incorporation of FUra into RNA was increased from 2 to 4 nmol/mgRNA in the former line but was not increased in the latter tumors [20]. Therefore, in group 1 tumors the availability of PRPP may influence the pathway of FUra metabolism until levels of OPRTase become limiting. Thus group 1 tumors possessed low concentrations of PRPP and hence high R-1-P/PRPP ratios (~5), while group 2 tumors possessed PRPP levels that were between 3.6 and 5.3 times higher than those of group 1, and demonstrated R-1-P/PRPP ratios ~1.

These results indicated that in tumors with high R-1-P/PRPP ratios ($HxELC_2$, $HxGC_3$, HxBR) formation of FUra ribonucleotides proceeded predominantly by Urd phosphorylase

and Urd kinase, while the metabolism of FUra in tumors with low R-1-P/PRPP ratios ($HxVRC_5$, $HxHC_1$) proceeded by the activity of OPRTase. One tumor in each group ($HxELC_2$, $HxHC_1$) was sensitive to treatment with FUra. In the presence of the 2 purines a reversal of FUra-induced growth inhibition was observed in line $HxHC_1$ but not in $HxELC_2$ tumors, and thus correlated with the biochemical determinations.

After the administration of Hx and HPP in combination with FUra, FUrd was retained at higher concentrations in each of the 5 xenografts studied. Basal levels of PRPP may influence the pathway of FUra metabolism such that under conditions of PRPP depletion the increase in the R-1-P/PRPP ratio may also increase the efficiency by which FUra is metabolized by Urd phosphorylase. Alternatively, administration of Hx with HPP may cause a rapid increase in the synthesis of purine nucleotides, with subsequent reduction in the conversion of IMP to AMP and to GMP as a result of allosteric regulation by these nucleoside monophosphates on the enzymatic modification of IMP. Accumulation and subsequent degradation of IMP to inosine and finally to Hx may thus occur with the production of R-1-P. Such donors of the ribofuranose moiety, including inosine, adenosine or guanosine, have potentiated the antimetabolic action of FUra in tumor cells [26, 29, 30], where R-1-P has been limiting in the metabolism of FUra. In the present study R-1-P does not appear to be limiting, at least in group 2 tumors. In the absence of FUra R-1-P formed may be rapidly reutilized by endogenous bases, but in the presence of FUra may be utilized in the formation of FUrd. The maintenance of higher concentrations of FUrd may indicate that the conversion of FUrd by Urd kinase is relatively inefficient at the concentrations of the nucleoside that may be achieved in tumors. Activities of Urd kinase in these tumors have in general been found to be lower than the respective anabolic activities of Urd phosphorylase (Dr. N. K. Ahmed, personal communication).

It is apparent from the data presented that human colorectal xenografts are heterogeneous in their pathways of metabolism of FUra to ribonucleotides. It would not be possible to predict the predominant metabolic pathway from the determination of either OPRTase or Urd phosphorylase, or R-1-P alone. However, both the basal levels of PRPP and the ratios of the 2 competing enzymes and co-substrates would appear to be better indicators of the latter.

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## REFERENCES

1. YOSHIDA M, HOSHI A, KURETANI K. Prevention of antitumor effect of 5-fluorouracil by hypoxanthine. *Biochem Pharmacol* 1978, **27**, 2979–2982.
2. ULLMAN B, KIRSCH J. Metabolism of 5-fluorouracil in cultured cells. Protection from 5-fluorouracil cytotoxicity by purines. *Mol Pharmacol* 1979, **15**, 357–366.
3. SCHWARTZ PM, HANDSCHUMACHER RE. Selective antagonism of 5-fluorouracil cytotoxicity by 4-hydroxypyrazolopyrimidine (allopurinol) *in vitro*. *Cancer Res* 1979, **39**, 3095–3101.
4. REYES P, GUGANIG ME. Studies on a pyrimidine phosphoribosyltransferase from murine leukemia P1534 J. *J Biol Chem* 1975, **250**, 5097–5108.
5. HOUGHTON JA, HOUGHTON PJ. 5-Fluorouracil in combination with hypoxanthine and allopurinol: toxicity and metabolism in xenografts of human colonic carcinomas in mice. *Biochem Pharmacol* 1980, **29**, 2077–2080.
6. SCHWARTZ PM, DUNIGAN JM, MARSH JC, HANDSCHUMACHER RE. Allopurinol modification of the toxicity and antitumor activity of 5-fluorouracil. *Cancer Res* 1980, **40**, 1885–1889.
7. CAMPBELL TN, HOWELL SB, PFEIFLE C, HOUSE BA. High-dose allopurinol modulation of 5-FU toxicity: phase I trial of an outpatient dose schedule. *Cancer Treat Rep* 1982, **66**, 1723–1727.
8. FOX RM, WOODS RL, TATTERSALL MHN, PIPER AA, SAMPSON D. Allopurinol modulation of fluorouracil toxicity. *Cancer Chemother Pharmacol* 1981, **5**, 151–155.
9. KROENER JF, SALEH F, HOWELL SB. 5-FU and allopurinol: toxicity modulation and phase II results in colon cancer. *Cancer Treat Rep* 1982, **66**, 1133–1137.
10. HOUGHTON JA, HOUGHTON PJ. On the mechanism of cytotoxicity of fluorinated pyrimidines in four human colon adenocarcinoma xenografts maintained in immune-deprived mice. *Cancer* 1980, **45**, 1159–1167.
11. HOUGHTON JA, TAYLOR DM. Maintenance of biological and biochemical characteristics of human colorectal tumours during serial passage in immune-deprived mice. *Br J Cancer* 1978, **37**, 199–212.

12. HOUGHTON JA, TAYLOR DM. Growth characteristics of human colorectal tumours during serial passage in immune-deprived mice. *Br J Cancer* 1978, **37**, 213–223.
13. HOUGHTON JA, HOUGHTON PJ. Evaluation of single agent therapy in human colorectal tumour xenografts. *Br J Cancer* 1978, **37**, 833–840.
14. HOUGHTON PJ, HOUGHTON JA, TAYLOR DM. Effects of cytotoxic agents on TdR incorporation and growth delay in human colonic tumour xenografts. *Br J Cancer* 1977, **36**, 206–214.
15. REEVES BR, HOUGHTON JA. Serial cytogenetic studies of human colonic tumour xenografts. *Br J Cancer* 1978, **37**, 612–619.
16. HOUGHTON PJ, HOUGHTON JA. Evaluation of cytotoxic agents in human colonic tumor xenografts and mouse gastrointestinal tissues using $^{3}$H-thymidine fractional incorporation into DNA. *Eur J Cancer* 1979, **15**, 763–770.
17. HOUGHTON JA, HOUGHTON PJ. Combinations of 5-fluorouracil, hypoxanthine and allopurinol in the chemotherapy of human colon adenocarcinoma xenografts. *Cancer Treat Rep* 1982, **66**, 1201–1206.
18. HOUGHTON JA, HOUGHTON PJ, WOOTEN RS. Mechanism of induction of gastrointestinal toxicity in the mouse by 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine. *Cancer Res* 1979, **39**, 2406–2413.
19. HISATA T. An accurate method for estimating 5-phosphoribosyl 1-pyrophosphate in animal tissues with the use of acid extraction. *Anal Biochem* 1975, **68**, 448–457.
20. HOUGHTON JA, TICE AJ, HOUGHTON PJ. The selectivity of action of methotrexate in combination with 5-fluorouracil in xenografts of human colorectal adenocarcinomas. *Mol Pharmacol* 1982, **22**, 771–778.
21. BARANKIEWICZ J, HENDERSON JF. Determination of ribose-1-phosphate in ascites tumor cells. *Biochem Med* 1977, **17**, 45–53.
22. LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951, **193**, 265–275.
23. MOYER JD, HANDSCHUMACHER RE. Selective inhibition of pyrimidine synthesis and depletion of nucleotide pools by *N*-(phosphonacetyl)-L-aspartate. *Cancer Res* 1979, **39**, 3089–3094.
24. BROCKMAN RW, SHADDIX SC, ROSE LM. Biochemical aspects of chemotherapy of mouse colon carcinoma. *Cancer* 1977, **40**, 2681–2691.
25. KESSEL D, HALL TC. Influence of ribose donors on the action of 5-fluorouracil. *Cancer Res* 1969, **29**, 1749–1754.
26. LASKIN JD, EVANS RM, SLOCUM HK, BURKE D, HAKALA MT. Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in cell culture. *Cancer Res* 1979, **39**, 383–390.
27. YAMADA EW. Uridine phosphorylase from rat liver. *Methods Enzymol* 1978, **51**, 423–431.
28. SILVA RF, HATFIELD D. Orotate phosphoribosyltransferase: orotidylate decarboxylase (erythrocyte). *Meth Enzymol* 1978, **51**, 143–167.
29. CORY JG, CRUMLEY J, WILKINSON DS. Evidence for role of purine nucleoside phosphorylase in sensitivity of Novikoff hepatoma cells to 5-fluorouracil. *Adv Enzyme Regul* 1977, **15**, 153–166.
30. GOTTO AM, BELKHODE ML, TOUSTER O. Stimulatory effects of inosine and deoxyinosine on the incorporation of uracil-2-$^{14}$C, 5-fluorouracil-2-$^{14}$C, and 5-bromouracil-2-$^{14}$C into nucleic acids by Ehrlich ascites tumor cells *in vitro*. *Cancer Res* 1969, **29**, 807–811.