EFFECT OF URACIL ON METABOLISM OF 5-FLUOROURACIL IN VITRO

Kazuhiro IKENAKA, Tetshuhiko SHIRASAKA, Shizuo KITANO, and Setsuro FUJI
Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University*

The effect of uracil on the metabolism of 5-fluorouracil (5-FU) in vitro was studied. 5-FU was mainly phosphorylated in intact Yoshida sarcoma cells, whereas it was mainly degraded in liver slices. Uracil inhibited degradation of 5-FU much more than its phosphorylation; incubation of 2,500 µM of uracil with 2.5 µM of 5-FU (molar ratio, 1,000:1) inhibited the degradation of 5-FU by 70%, but did not affect its phosphorylation.

With homogenates of Yoshida sarcoma or liver, uracil inhibited degradation of 5-FU greatly, phosphorylation of 5-FU by α-D-ribose 1-phosphate (Rib1P) and ATP to some extent, and phosphorylation by 5-phospho-α-D-riboyl diphosphate (PPRib1P) very little.

The activities of the enzymes involved in the metabolism of 5-FU in various tissues were also determined. Degradation of 5-FU was much faster in liver than in other tissues and was very slow in tumor tissue. Phosphorylation of 5-FU with Rib1P and ATP was rapid in Yoshida sarcoma and bone marrow. Phosphoribosyltransferase activity was high in Yoshida sarcoma and thymus, but low in bone marrow.

1-(2-Tetrahydrofuryl)-5-fluorouracil (FT-207), a masked form of 5-fluorouracil (5-FU), has been widely used as an antitumor agent.1,3,7,8,13 In in vitro studies, we found that FT-207 is converted to 5-FU in the presence of NADPH, mainly in the microsomal fraction of rat liver.14 The active metabolite of 5-FU is generally thought to be 5-fluorodeoxyuridine 5′-monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthetase.5,6 On the other hand, 5-FU is also degraded and excreted in urine as 2-fluoro-3-ureidopropionic acid (F-β-UPA) or 2-fluoro-β-alanine (F-β-Ala).9

Recently, we demonstrated that uracil prevented the growth inhibition of Staphylococcus aureus 209P by 5-FU, but did not affect that of mammalian FM3A/B or HeLa cells, even at 1,000 times the concentration of 5-FU.4 Similar results were reported by Rich et al.10 and by Scheiner et al.11 After coadministration of an appropriate dose of uracil with FT-207 to rats bearing AH130 tumor, the 5-FU level in the tumor became much higher than after administration of FT-207 alone, and this tissue level decreased very slowly, whereas the level in the blood decreased rapidly. Consequently, the antitumor activity of FT-207 on sarcoma-180 and AH130 tumor was enhanced by coadministration of uracil without increasing the toxicity of FT-207. However, when uracil was coadministered with 5-FU, the 5-FU level increased both in blood and in tumor almost to the same extent, and the toxicity always accompanied the increased antitumor activity.4,5

The present work is on the in vitro change in metabolism of 5-FU on addition of uracil.

MATERIALS AND METHODS

Chemicals 5-Fluorouracil (5-FU), 5-fluoro-uracil[3-14C], and 2-fluoro-3-ureidopropionic acid (F-β-UPA) were from Taiho Pharmaceutical Co., Tokushima. Uracil, 3-ureidopropionic acid, β-alanine, NADPH, α-D-ribose 1-phosphate

* 5311 Yamada-Kami, Suita, Osaka-fu 565 (池中一裕, 白坂哲彦, 北野静雄, 藤井節郎).
K. IKENAKA, ET AL.
dicyclohexylammonium salt (RiblP), adenosine 5'-triposphate disodium salt, and other reagents were purchased from Sigma Chemical Co., U.S.A. Donryu and Wistar-King strain rats weighing ca. 120 g were purchased from Kitayama LABES Co., Kyoto.

**Tumor Cells** Yoshida sarcoma cells were maintained by intraperitoneal transfer in Donryu strain rats. Cells were collected 7 days after inoculation, and used for experiments after washing them three times with saline.

**Preparation of Enzyme Solutions** Wistar-King strain rats weighing 150–200 g were decapitated and their liver was rapidly perfused with saline (20 ml) and removed. All subsequent procedures were carried out at 4 °C. Tissues and tumor cells were freeze-thawed twice and homogenized in 3 volumes of 0.25 M saccharose containing 5 mM 2-mercaptoethanol and 0.5 mM EDTA (for experiments on uracil degradation) or with 50 mM Tris-HCl (pH 8.0), containing 5 mM 2-mercaptoethanol (for other experiments). For kinetic studies, the homogenates were centrifuged at 105,000 g for 60 min and the supernatant was submitted to ammonium sulfate fractionation (30–50% for dihydrouracil (DHU) dehydrogenase and 35–55% for other experiments) and then dialyzed against 10 mM potassium phosphate buffer (pH 7.6) (for DHU dehydrogenase) or 10 mM Tris-HCl (pH 8.0) (for other experiments).

**Assay of 5-FU Degradation** For assay of 5-FU degradation, the incubation mixture in a final volume of 1 ml contained NADPH (1.0 μmol), ATP (5.0 μmol), nicotinamide (50 μmol), potassium phosphate buffer (pH 7.6) (45 μmol), 14C-5-FU (0.1 μCi), and the enzyme solution (0.4 ml). The mixture was incubated at 10 or 60 min at 37 °C and the reaction was stopped by immersing the mixture in a boiling water for 2 min. The mixture was centrifuged (3,000 rpm, 10 min) and the supernatant was added to 2 M KOH (0.06 ml, 0.12 mmol), stood at room temperature for at least 30 min to hydrolyze the DHU formed, and then mixed with 60% HClO₄ (0.02 ml, 0.12 mmol) and centrifuged (3,000 rpm, 10 min). A 10-μl aliquot of the supernatant was applied to a thin-layer chromatography (TLC) plate (Merck TLC plates with silica gel 60 F254 pre-coated, 2 × 20 cm, thickness, 0.25 mm), and developed with a mixture of 99% ethanol and 1 M ammonium acetate (5:1, v/v). Samples of 5-FU, F-β-Ala, and F-β-UPA were applied to the plate before the test sample. 5-FU was located by measuring UV absorption at 254 nm, while F-β-Ala and F-β-UPA were located by spraying with ninhydrin and Ehrlich reagent, respectively. The spots of each substance were scraped into vials and extracted with 4 M HCl (0.04 ml). Then 10 ml of scintillator [toluene-Triton X-100 system, containing 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g/liter) and 2,5-diphenyloxazole (4.0 g/liter) in toluene] was added and radioactivity was measured in a Beckmann liquid scintillation spectrometer.

**Assay of 5-FU Phosphorylation** For assay of phosphorylating activity through uracil ribosyltransferase and uridine kinase the incubation mixture in a final volume of 0.5 ml contained RiblP (2.0 μmol), ATP (5.0 μmol), MgCl₂ (2.5 μmol), NaF (5.0 μmol), Tris-HCl buffer, pH 8.0 (25 μmol), 14C-5-FU (0.050 μCi), and the enzyme solution (0.20 ml). For kinetic studies on uracil ribosyltransferase, ATP, MgCl₂, and NaF were omitted from this mixture. For assay of pyrimidine phosphoribosyltransferase, the mixture contained PPRiblP (2.0 μmol), MgCl₂ (2.5 μmol), NaF (5.0 μmol), Tris-HCl (pH 8.0) (25 μmol), 14C-5-FU (0.05 μCi), and the enzyme solution (0.20 ml) in a final volume of 0.50 ml. Mixtures were incubated at 37 °C for 10 min, mixed with 60% HClO₄ (0.025 ml, 0.15 mmol), centrifuged (3,000 rpm, 10 min), and 10-μl aliquots of the supernatant were subjected to silica gel TLC, with a mixture of chloroform, methanol, and acetic acid (17:3:1, v/v). 5-FU and FUrld, applied before the test sample, were located by their UV absorption at 254 nm. Subsequent procedures were the same as in the assay of 5-FU degradation.

**Assay on Intact Cells and Liver Slices** Freshly prepared Yoshida sarcoma cells were used as intact cells. Liver slices were prepared by perfusion of fresh liver with Eagle's MEM, and cutting it up with a razor. The incubation mixture contained 5-fluorouracil-[6-3H] (10 μCi) with or without uracil and 0.2 g of intact cells or liver slices in 0.8 ml of Eagle's minimum essential medium (MEM). Preparations were incubated for 60 min, washed three times with saline, and 5.0% HClO₄ (0.5 ml) was added. The denatured cells or slices were homogenized and the products were analyzed by TLC as described above.

**RESULTS**

It had been found previously that uracil did not prevent the growth inhibition of mammalian cells by 5-FU and enhanced the antitumor activity of FT-207 on sarcoma-180 and AH130 tumors. To determine the reason for these findings, we investigated the metabo-
EFFECT OF URACIL ON METABOLISM OF 5-FLUOROURACIL

Fig. 1. Possible metabolic pathways of 5-FU

Fig. 1. Possible metabolic pathways of 5-FU (Fig. 1) in the presence and absence of uracil. First we examined metabolism of 5-FU in intact Yoshida sarcoma cells and liver slices.

Effect of Uracil on 5-FU Metabolism in Intact Yoshida Sarcoma Cells and Liver Slices  Intact Yoshida sarcoma cells or rat liver slices were incubated in Eagle’s MEM containing 14C-5-FU, with or without uracil. In Yoshida sarcoma cells, 5-FU was mainly phosphorylated and was degraded only slightly, whereas in liver slices, degradation of 5-FU was greater than its phosphorylation, as shown in Table I. Therefore, the effect of uracil on phosphorylation was examined using Yoshida sarcoma cells, while its effect on degradation of 5-FU was examined using liver slices. Various concentrations of uracil were added with three different concentrations of 5-FU, 250, 25, and 2.5 μM.

With the high concentration of 5-FU (250 μM), addition of 2,500 μM uracil (10 times the concentration of 5-FU) decreased phosphorylation to 31% and degradation to 66%; with 25 μM of 5-FU, addition of 250 μM uracil inhibited phosphorylation to 10% and degradation to 39%; with the low concentration of 5-FU (2.5 μM), addition of 25 μM uracil caused no decrease in phosphorylation, but decreased degradation to 6%, as shown in Fig. 2. It seems very interesting that the presence of 2,500 μM uracil with 2.5 μM 5-FU did not inhibit the phosphorylation of 5-FU, but decreased its degradation to 70%.

These findings led us to investigate the metabolism of 5-FU at the enzyme level.

Degradation of 5-FU and Uracil  On the basis of the suggestion by Schumacher et al.,12 that uracil might inhibit the degradation of

Table I. Metabolism of 5-FU in Intact Yoshida Sarcoma and Liver Slices

<table>
<thead>
<tr>
<th>Metabolites formed (%)</th>
<th>Yoshida sarcoma cells</th>
<th>Liver slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorylation</td>
<td>44</td>
<td>3</td>
</tr>
<tr>
<td>Degradation</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

Intact Yoshida sarcoma cells (0.1 g) or liver slices (0.2 g) were incubated in Eagle’s MEM containing 5-FU (25 μM) for 60 min at 37° with shaking. Then metabolites in the cells or slices were analyzed.

Fig. 2. Effect of uracil on metabolism of 5-FU in intact Yoshida sarcoma cells and liver slices

5-FU (250, 25, or 2.5 μM) and the indicated concentrations of uracil were incubated with liver slices or intact Yoshida sarcoma cells in Eagle’s MEM at 37° for 60 min. Phosphorylation of 5-FU was measured in Yoshida sarcoma cells and degradation of 5-FU in liver slices. Activity in the absence of uracil was taken as 100%. ○—○ Yoshida sarcoma, ●—● liver slices.
5-FU, we studied the degradation of 5-FU to 2-fluoro-β-alanine (F-β-Ala) (Pathway 1) in homogenates of tumor and various organs. Fig. 3 shows that the degradation of 5-FU and uracil is much more rapid in liver than in other tissues, and that little degradation occurred in the tumor homogenate. 5-FU was degraded more rapidly than uracil in all the tissues tested.

**Phosphorylation of 5-FU** Next we investigated phosphorylation of 5-FU (Pathways 2 and 3 in Fig. 1) to the active form. We did not investigate Pathway 4, including deoxyribosyltransferase and deoxyuridine (dUrd) kinase, because Wilkinson\textsuperscript{15} reported that this is only a minor pathway in mammalian cells. The phosphorylation of 5-FU in homogenates of tumor and various tissues was determined using either α-D-ribose 1-phosphate

Fig. 3. Degradations of 5-FU and uracil in various tissues

5-FU (80 μM) or uracil (80 μM) was incubated for 10 min (liver) or 60 min (other tissues) with homogenates of various tissues. The rate of degradation of 5-FU was measured. ■ 5-FU, □ uracil

Fig. 4. Phosphorylation of 5-FU in various tissues in the presence of RiblP and ATP

The rate of phosphorylation by Pathway 2 (Fig. 1) was measured by incubating 5-FU (80 μM) with various tissues in the presence of RiblP and ATP for 10 min.

Fig. 5. Phosphorylation of 5-FU in various tissues in the presence of PPRibP

The rate of phosphorylation by Pathway 3 (Fig. 1) was measured by incubating 5-FU (80 μM) with various tissues in the presence of PPRibP.
EFFECT OF URACIL ON METABOLISM OF 5-FLUOROURACIL

(RiblP) and ATP (Pathway 2) or 5-phospho-α-D-ribosyl diphosphate (PPRibP) (Pathway 3) as a cofactor. As shown in Figs. 4 and 5, activities for phosphorylation by Pathway 2 were high in Yoshida sarcoma and bone marrow (Fig. 4), and activities for Pathway 3 were high in Yoshida sarcoma and thymus (Fig. 5). These results suggest that the two pathways are almost equally important in all tissues except the bone marrow.

Effect of Uracil on 5-FU Metabolism

The effect of various concentrations of uracil on 5-FU metabolism was then investigated. Homogenates of rat liver were used in assay of degradation of 5-FU and homogenates of Yoshida sarcoma for the assay of its phosphorylation. Table II shows that degradation of 5-FU (Pathway 1) was markedly inhibited by uracil at concentrations greater than 250 μM, but the phosphorylation of 5-FU with PPRibP (Pathway 3) was not inhibited by uracil. Inhibition of phosphorylation by uracil through FUrd (Pathway 2) was great when the concentration of 5-FU was low, inhibition being less with higher concentrations of 5-FU. Therefore, the FUrd pool in Pathway 2 at high concentration of 5-FU was studied using a homogenate from Yoshida sarcoma cells. As shown in Fig. 6, the FUrd pool increased and phosphorylation of 5-FU decreased when uracil was added to the assay system with RiblP and ATP. This finding suggests that uridine formed from uracil inhibited the phosphorylation of FUrd.

Km and Vmax Values of the Enzymes Involved in Metabolism of 5-FU

The Km and Vmax values of DHU dehydrogenase, uracil ribosyltransferase, and pyrimidine 5'-phosphoribosyltransferase were measured with uracil or 5-FU as a substrate. As shown in Fig. 7, the Km and Vmax values for degradation of 5-FU and uracil (DHU dehydrogenase) were 4.0 × 10⁻⁵M and 0.51, and 1.0 × 10⁻⁵M and 0.22, respectively; the Km and Vmax values for phosphorylation of 5-FU and uracil (uracil ribosyltransferase) were 5.6 × 10⁻⁴M and 1.2, and 4.5 × 10⁻⁴M and 10 (with RiblP and ATP), and 6.3 × 10⁻⁴M and 10, and 9.1 × 10⁻³M and 26 (with PPRibP), respectively. The Km value of DHU dehydrogenase was apparently lower than those of the phosphorylating enzymes, indicating that 5-FU and uracil tend to be used in the degrada-

Table II. Effect of Uracil on 5-FU Metabolism

<table>
<thead>
<tr>
<th>5-FU (μM)</th>
<th>Uracil (μM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degradation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>47</td>
</tr>
<tr>
<td>250</td>
<td>250</td>
<td>69</td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>88</td>
</tr>
<tr>
<td>80</td>
<td>320</td>
<td>86</td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>92</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>43</td>
</tr>
<tr>
<td>250</td>
<td>250</td>
<td>85</td>
</tr>
<tr>
<td>with RiblP and ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>320</td>
<td>320</td>
<td>38</td>
</tr>
<tr>
<td>250</td>
<td>1000</td>
<td>52</td>
</tr>
<tr>
<td>with PPRibP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>250</td>
<td>250</td>
<td>16</td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>320</td>
<td>320</td>
<td>8</td>
</tr>
</tbody>
</table>

Various concentration of uracil was added to the reaction mixture for assay of degradation of phosphorylation of 5-FU. Liver homogenate was used in assay of degradation and Yoshida sarcoma in assay of phosphorylation.

Fig. 6. Effect of uracil on 5-FU ribosyltransferase and phosphorylation

5-FU (80 μM) was incubated with liver homogenate in the presence of RiblP, ATP, and uracil (0, 80, or 320 μM), and then metabolites of 5-FU were analyzed.
K. IKENAKA, ET AL.

The reaction of DHU dehydrogenase, uracil ribosyltransferase, and pyrimidine phosphoribosyltransferase was measured with various concentrations of 5-FU or uracil. $K_m$ and $V_{max}$ values, calculated from double reciprocal plots, are shown in the graphs.

\[ K_m\ (M)\quad V_{max}\ (\text{nmol/mg/min}) \]

- 5-FU: 1.0 x 10^{-5} 0.51
- uracil: 1.0 x 10^{-5} 0.22

On the other hand, phosphorylation of 5-FU was highest in tumor cells, and inhibition of phosphorylation was less than that of degradation. These results suggest that the levels of 5-FU and the active forms of 5-FU in the tumor specifically increased when appropriate dose of uracil was coadministered with FT-207, which releases 5-FU little by little. However when 5-FU was administered, the 5-FU level in blood increased temporarily, and inhibition of 5-FU degrading enzyme by uracil enhanced its toxicity.

Our results showed that the conversion of 5-FU to FUMP can proceed by either phosphoribosylation (Pathway 3) or through FUr (Pathway 2) (Figs. 4 and 5), and suggested that FUr kinase may be inhibited by uridine. We are now investigating the reason for the rapid disappearance of 5-FU from the blood.

(Received January 22, 1979)
REFERENCES