ABSTRACT

Seven established human cell lines in vitro were, on the average, 8 times less sensitive to growth inhibition by 5-fluorouracil (FURA) than were eight mouse cell lines. Four of the human cell lines (U-cells, HeLa, KB, and Hep-2) and three mouse cell lines (S-180, MMT, and LM) were examined to clarify the basis of the different sensitivities. The Km's and Vmax's for the total cellular uptake of FURA were higher in the more sensitive (Km 300 to 500 μM; Vmax 110 to 140 pmol/min/mg cellular protein) than in the less sensitive cells (Km 30 to 120 μM; Vmax 10 to 70 pmol/min/mg cellular protein). At a fixed concentration of [2-14C]FURA (10 μM), the velocity of incorporation of the analog into acid-soluble and -insoluble material was up to 7 times more rapid in the more sensitive cells. The major acid-soluble metabolite was 5-fluorouridine triphosphate, which after 3 hr incubation with 10 μM FURA comprised 60 to 90% of soluble radioactivity and reached 43 to 130 μM in the cell water of the more sensitive and 11 to 24 μM in the less sensitive cells. No acid-soluble 5-fluorodeoxyuridine-5'-monophosphate was detected. There was no difference in the velocity of accumulation of unaltered FURA. This suggested that the conversion of FURA to nucleotides was slower in the less sensitive cells. Of three enzymes (uridine phosphorylase, thymidine phosphorylase, and FURA phosphoribosyltransferase), at least two had higher activities in the extracts of the more sensitive cells. There was no difference in fluorouridine kinase activity, while the activity of thymidine kinase was consistently higher in the less sensitive cells.

These findings show that sensitivity to FURA of cells in vitro, whether of mouse or human origin, is associated with (a) greater activity of enzymes responsible for converting FURA to 5-fluorodeoxyuridine, 5-fluorouridine, and 5-fluorouridine-5'-monophosphate; (b) lower activity of thymidine kinase; and (c) more rapid intracellular accumulation of acid-soluble FURA nucleotides and their incorporation into RNA.

INTRODUCTION

The antimetabolite, FURA, is one of the more prominent clinical antitumor agents. Its use alone and in combination with other agents has produced objective responses in cancer patients, particularly those with carcinoma of the stomach, colon, pancreas, breast, or ovary (5, 6, 31, 42). However, most clinicians report only a 10 to 30% response rate with the drug, and remissions, once obtained, are generally of short duration (1, 6, 18, 32). Additionally, more than 95% of such tumor types as Hodgkin's, lung, renal cell, and acute myelocytic and acute and chronic lymphocytic leukemias display innate clinical resistance to fluorinated pyrimidines (16). Varying degrees of antitumor activity with FURA have also been observed with mouse tumor systems including Sarcoma 180, Ehrlich ascites carcinoma, and leukemia L1210 (19, 20). While pharmacokinetic and toxicity parameters undoubtedly influence FURA effectiveness in vivo, natural variations in the sensitivity of different mammalian tumors to FURA are also apparent.

A number of mouse and human cells grown in vitro have been compared with respect to their sensitivity to growth inhibition by various antimetabolites (15). No significant differences were noted in sensitivity to methotrexate, vincristine, 6-mercaptopurine, azaserine, or N4-(β-d-isonicotinyl)adenosine. However, human cells as a group were less sensitive than mouse cells to the pyrimidine analogs FURA, FdUrd, and 1-β-d-arabinofuranosylcytosine but, interestingly, not FUrD. The purpose of the present study was to determine the biochemical basis for the differences in natural sensitivity to FURA.

FURA is thought to exert its antitumor effects through its metabolic conversion to FUTP with subsequent incorporation into RNA and/or through the formation of FdUMP, the well-recognized inhibitor of thymidylate synthetase (17, 27). A summary of the metabolism of FURA is shown in Chart 1. Early studies with mouse tumors in vivo indicated that drug-induced and natural resistance to FURA was associated with changes in activities of enzymes involved in the metabolism of the drug (21, 34, 35, 37, 39). These included uridine phosphorylase and kinase and uracil phosphoribosyltransferase. This paper reports on the comparison of mouse and human cells, more and less sensitive to FURA, with respect to the activities of these 3 enzymes and also includes data on thymidine phosphorylase and kinase. The cellular uptake and intracellular metabolism of FURA and its incorporation into RNA were also examined. The role of thymidylate synthetase inhibition in causing growth inhibition of these cells is the subject of our next paper.
MATERIALS AND METHODS

Chemicals. [2-14C]FUra, [2-14C]FUrd, and [2-14C]thymidine (all 56 μCi/μmol, 98% pure) were purchased from Moravek Biochemicals, City of Industry, Calif. FUrd was obtained from Calbiochem, San Diego, Calif., and FUrd was from Terra Marine Bioreresearch, San Diego, Calif. Inulin-[14C]carboxylic acid (13 mCi/mmol) was purchased from Moravek Corp., Cleveland, Ohio. FUra, Escherichia coli alkaline phosphatase, and all other biochemicals were from Sigma Chemical Co., St. Louis, Mo. The powdered Medium 1640 and serum were from Grand Island Biological Co., Grand Island, N. Y.

Cells. The origin and maintenance of the cells has been described previously (40). The mouse cell lines used were: sarcoma, S-180; leukemia, L1210; kidney, MUK; adenocarcinomas, RAG and TA3; fibroblast, LM; mammary tumor, S-180; leukemia, L1210; kidney, MUK; adenocarcinoma, HeLa, KB, and Hep-2. Cells were monitored weekly for the absence of Mycoplasma (14).

Growth Inhibition Studies. These were conducted as described previously (40).

Cellular Uptake and Metabolism of [2-14C]FUra. The methods for studying the uptake of labeled precursors and their incorporation into acid-soluble and -insoluble material have been reported (9). For metabolism studies, T-25 flasks were used. Cell layers were incubated with 3 ml of serum-free Medium 1640 supplemented with 10 μM [2-14C]FUra (56 μCi/μmol). All incubations and preincubations were carried out at 36°C. In experiments on the effect of inosine on the metabolism of FUra, cells were preincubated for 1 hr with 200 to 1000 μM inosine and then incubated with labeled FUrd plus inosine. Ice-cold 5% TCA was used to extract labeled metabolites. For chromatography, TCA was removed with diethyl ether; the samples were frozen in an ice bath and lyophilized. The dried sample was reconstituted in 0.1 ml of distilled water and used for radiochromatographic analysis with Solvent B. The uptake into acid-soluble and -insoluble material was expressed in μg/mg of protein, protein being proportional to the number of cells (33). The data for acid-soluble radioactivity were further converted to molarity in intracellular water.

Cell Extracts for Enzyme Assays. Cells were grown and collected as described (40). The resulting cell pellets, weighing 200 to 300 mg, were stored at −70°C. All extraction procedures were performed at 4°C.

For thymidine and FUrd kinases, the frozen cell pellets were thawed and suspended in 1 ml of buffer which consisted of 10% glycerol, 2 mM dithiothreitol, 0.15 M KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 7.5), and 50 μM thymidine or FUrd, respectively. For thymidine and uridine phosphorylases, frozen cells were thawed in 1 ml of buffer containing 9 mM Tris-HCl and 50 μM KH₂PO₄ (pH 7.5). Each cell suspension was set in ice and sonicated with a Bronson Ultrasonic Sonifier until the cells appeared broken under microscopic examination (usually 1 to 2 sec) and then centrifuged at 100,000 × g for 1 hr in a Beckman L5-50 ultracentrifuge.

Extracts for FUra phosphoryltransferase were prepared by thawing the frozen cells in 1 ml of buffer containing 5% glycerol, 1.5 mM MgCl₂, 3 mM dithiothreitol, 75 mM KCl, and 10 mM Tris-HCl (pH 7.5). The suspension was freeze-thawed 3 times alternating between a dry ice-acetone bath and ice-cold water. The cells appeared broken under microscopic examination. The homogenate was centrifuged as above; the supernatant was dialyzed for 16 hr against 2000 volumes of 2 mM dithiothreitol, 10 mM Tris-HCl (pH 7.5), and 1.5 mM MgCl₂; recentrifuged; and assayed immediately.

Enzyme Assays. The assay mixtures and cell extract were first incubated separately for 5 min at 36°C in a Dubnoff shaker bath. The reaction was started by the addition of enzyme, and the product formation was measured at 5- to 10-min intervals.

FUrd Kinase. This was assayed by a modification of the method of Sköld (39) using [2-14C]FUrd as the substrate. The reaction mixture in a total volume of 0.2 ml contained 2 mM FUrd (1.25 μCi/μmol), 5 mM ATP, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), and cell extract equal to 0.5 to 1.0 mg of protein. To stop the reaction, 30-μl aliquots of the reaction mixture were removed at appropriate intervals for descending chromatography for 14 hr in Solvent C. In this solvent system, the products (FUMP, 5-fluorouridine diphosphate, and FUdP) remained at the origin while the substrate had an Rf of 0.47. The reaction was linear for 30 min.

Thymidine Kinase. The enzyme was assayed as described by Lee and Cheng (28). The reaction was linear for 40 min.

FUra Phosphoribosyltransferase. This was assayed by the method of Reyes (36) using [14C]FUra as the substrate. The reaction mixture in a total volume of 120 μl contained 1 mM [2-14C]FUra (5 μCi/μmol), 5 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5 mM 5-phosphoribosyl 1-pyrophosphate, and enzyme extract equal to 0.2 to 0.3 mg of protein. To stop the reaction, 20-μl aliquots of the reaction mixture were removed at appropriate time intervals for chromatography with Solvent A. In this solvent system, the product remained at the origin while the substrate had an Rf of 0.33. The origins of the chromatograms were cut out and counted for 14C. The reaction was linear for 45 min.

Uridine and Thymidine Phosphorylases. The enzymes
The reaction was stopped at appropriate intervals by transferring 100 μl of the mix to tubes containing 100 μl of ice-cold 60% TCA. The volume was increased to 1 ml by the addition of cold 5% TCA, and the precipitate was removed by centrifugation. One-half of the supernatant was withdrawn and adjusted to pH 12.9 by the addition of 0.5 ml of 0.9 N NaOH. Uracil, the reaction product, shows a characteristic increase of the absorbance at 290 nm while thymine shows an increase at 300 nm. The absorbances were determined using a Zeiss Model PMQ II spectrophotometer. The results were read from the standard curves prepared with known mixtures of either uracil and uridine or thymine and thymidine. In most cases, the reactions were linear for 20 min.

**Protein Determination.** Protein was determined according to the method of Lowry et al. (29) with bovine serum albumin as the standard.

**Measurement of Intracellular Water.** The intracellular water was determined using a modification of the method of Goldman et al. (11). Two T-75 monolayer cultures were drained of medium and rinsed twice with serum-free medium. The cells (about 30 million) were scraped off with a rubber policeman into 12 ml of serum-free medium containing [*4C*]inulin (300,000 to 1,000,000 dpm/ml). Several 1-ml aliquots of the cell suspension were removed and pelleted by centrifuging at 2,000 rpm (1,400 × g) for 10 min in an International PR-6000 centrifuge. The supernatant was aspirated off, and aliquots (0.1 ml) were removed for counting. The wet weight of the pellet contained intracellular and extracellular water and dry cell material. The wet and dry weights of the pellets were determined using a Sartorius microbalance. To determine the extracellular water, the wet pellets of known weight were resuspended in 2 ml of serum and label-free medium. This diluted the extracellular [*4C*]inulin. The cells were centrifuged, and aliquots (0.1 ml) of the supernatant were counted. The extracellular water of the pellet was thus determined, and this together with the wet and dry weights allowed the calculation of intracellular water. The determination of the total cellular protein in the pellet allowed estimation of intracellular water corresponding to each mg of cellular protein.

**Radiochromatographic Techniques.** Descending paper chromatography was used to separate FUra metabolites using: Solvent A, ethyl acetate:formic acid:water (upper phase), 60:5:35 (v/v/v); Solvent B, fert-amy alcohol:formic acid:water, 3:2:1 (v/v/v); and Solvent C, isopropyl alcohol: NH₄OH:water, 7:2:1 (v/v/v). The cell extracts and the aliquots from enzyme assays were spotted onto Whatman No. 3MM paper strips (2.5 × 60 cm) together with unlabeled marker compounds (20 μl of a 10 mm solution) and air dried. After chromatography, the UV-absorbing spots were marked, and the strips were cut into 1-cm sections and counted for radioactivity. Radiolabeled products were identified by comparing their Rₖ with those of the marker compounds. In Solvent B, the Rₖ's of FUra and its metabolites were: FUra, 0.57; FUrd, 0.47; FdUrd, 0.60; FUMP, 0.39; FdUMP, 0.43; and FUTP, 0.15. FUTP was not available as a marker, but its identity was ascertained by: (a) its mobility relative to UTP; and (b) the identification of FUrd after phosphatase treatment. The limit of detection of any metabolite was 2 to 4% of the total acid-soluble radioactivity.

The percentage of distribution of [*14C*]metabolites in the chromatograms was determined. Since the total acid-soluble radioactivity was known, this permitted the estimation of the amount and molarity of each acid-soluble metabolite in cell water.

**Counting.** A Packard Model 2450 Tri-Carb liquid scintillation spectrometer was used to measure [*4C*]. Aqueous samples were counted in 10 ml of aqueous counting scintillant (Amersham/Searle). The counting efficiency, determined with internal standards, was 79% for NaOH solutions and acid extracts of cells and 83% for samples of culture medium. Sections of paper chromatograms were counted in toluene:PPO:POPOP as previously described (10).

**RESULTS**

**Growth Inhibition.** The ID₅₀'s of FUra for 8 mouse and 7 human cell lines are compared in Chart 2. While human cells were on the average 8 times less sensitive than were mouse cells, overlapping of sensitivity between the species was apparent. Thus, human fibroblasts (U-cells), Burkitt's lymphoma cells (BL), and Wilms' tumor were within the range of mouse cells while mouse LM, L1210, and EAC cells were within the range of human cells. Four human cell lines (U-cells, HeLa, KB, and Hep-2) and 3 mouse cell lines (S-180, MMT, and LM), all grown in monolayer, were chosen for further study.

**Enzymes of FUra Metabolism.** FUra must be enzymatically converted to nucleotides before it becomes biologically active (see Chart 1). It was of interest to assay uridine phosphorylase which converts FUra to FUrd, FUrd kinase

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**Chart 2. 5-FUra (FU) as a growth inhibitor of mouse and human cells in vitro.** The cells were grown in the continuous presence of the inhibitor in Medium 1640 supplemented with 5% fetal calf serum as described in "Materials and Methods".
which converts FUrD to FUMP, and FUrA phosphoribosyltransferase which converts FUrA to FUMP. Table 1 lists the specific activities of these enzymes in the extracts of the various cells together with the corresponding ID$_{50}$'s for FUrA. The specific activity of uridine phosphorylase was found to be on the average 4 to 5 times greater in the more sensitive (S-180, MMT, LM, and U-cells) than in the less sensitive cells. There were, on the average, no differences in the activities of uridine kinase in the more and less sensitive cells. The activities of FUrA phosphoribosyltransferase were exceptionally low in KB and Hep-2 cells. It is of interest that MMT cells, which are as sensitive as S-180 cells to inhibition by FUrA, have FUrA phosphoribosyltransferase activity less than one-half that of S-180 cells. It appears that in MMT cells FUrA metabolism may proceed in part through the phosphorylase-kinase pathway, the activity of which was unusually high in these cells.

Two pathways are possible for the synthesis of FdUMP from FUrA. These involve either thymidine phosphorylase and kinase or ribonucleotide reductase (Chart 1). Thymidine phosphorylase catalyzes the conversion of FUrA to FdUrD utilizing deoxyribose 1-phosphate while thymidine kinase catalyzes the conversion of FdUrD to the 5'-monophosphate form utilizing ATP as the phosphate donor. As indicated in Table 1, the activities of thymidine phosphorylase were found to be higher in the more sensitive cells, while thymidine kinase activities were lower.

The data in Table 1 as a whole reveal a negative correlation between the ID$_{50}$'s and the sum of the activities of FUrA phosphoribosyltransferase plus the 2 nucleoside phosphorylases, while a positive correlation is evident between the ID$_{50}$'s and the activities of thymidine kinase.

**Intracellular Water.** The values obtained for intracellular water for MMT was lower than for the other cell lines may be related to their unusual growth pattern in monolayer. These cells are needle shaped and tend to form large ball-shaped clumps while still attached to the substratum. The value of cell water for S-180 obtained here is similar to that previously found by Hakala (13) using a chemical method for analysis.

**Cellular Uptake and Incorporation of [2-14C]FUrA.** The kinetics of cellular uptake was determined from initial velocities which were found to be linear for at least 30 min at 5 to 100 μM FUrA. Table 2 lists the Km's and V$_{max}$'s which describe the total uptake, i.e., all the metabolic products of FUrA in the cells. The Km's varied from 30 to 120 μM and V$_{max}$'s from 11 to 70 pmol/min/mg protein for the less sensitive cells; for the more sensitive cells, the respective values were 300 to 500 μM and 110 to 140 pmol/min/mg protein. Thus, greater sensitivity to FUrA was associated with higher Km's and V$_{max}$'s for FUrA uptake. In S-180 cells, the uptake was strictly linear with concentration within the range used.

The cells were also compared with respect to their content of acid-soluble radioactivity and incorporation into DNA after 30 min exposure to 10 μM FUrA. As can be seen in Table 2, the more sensitive cells, whether mouse or human, had 2 to 4 times larger acid-soluble pools of FUrA and had incorporated 2 to 8 times more FUrA into RNA than did the less sensitive cells.

**Acid-soluble Metabolites of FUrA.** To compare the cell lines with respect to the formation of intracellular FUrA metabolites, they were incubated with 10 μM [2-14C]FUrA for various periods of time up to 3 hr. The total amount of radioactivity and the percentage of composition in the acid extracts were determined. This allowed estimation of the rate of formation of each metabolite in intracellular water.

An example is shown in Chart 3 for S-180 and Hep-2 cells. One can see that the total acid-soluble metabolites of FUrA were 3 to 4 times higher in S-180 cells than in the Hep-2 cells. In all cells, the major intracellular component was FUTP. The second largest component was unaltered FUrA; only traces of FUMP were present. No FdUMP was ever detected in the radiochromatograms. In the next paper, it will be demonstrated that acid-soluble FdUMP is easily detectable if it is present in excess of TMP synthetase.

The molarities of FUTP and FUrA in the cell water were compared after 30 min and 3 hr incubation with 10 μM FUrA.

### Table 1

**Relationship between sensitivity to 5-FUrA and activities of 5 enzymes in mouse and human cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ID$_{50}$ (μM FUrA)</th>
<th>FUrA phosphoribosyltransferase</th>
<th>Uridine phosphoribosylase</th>
<th>Thymidine phosphorylase</th>
<th>FUrA kinase</th>
<th>Thymidine kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-180</td>
<td>0.25 ± 0.06$^a$ (12)$^b$</td>
<td>0.86 ± 0.32</td>
<td>5.7 ± 0.81</td>
<td>5.5 ± 1.1</td>
<td>5.0 ± 2.3</td>
<td>0.40 ± 0.21</td>
</tr>
<tr>
<td>MMT</td>
<td>0.24 ± 0.23 (5)</td>
<td>0.40 ± 0.11</td>
<td>13 ± 0.42</td>
<td>9.2 ± 0.48</td>
<td>7.3 ± 1.6</td>
<td>0.42 ± 0.13</td>
</tr>
<tr>
<td>LM</td>
<td>0.81 ± 0.25 (7)</td>
<td>0.97 ± 0.16</td>
<td>6.2 ± 0.49</td>
<td>2.8 ± 0.84</td>
<td>5.3 ± 1.9</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-cells</td>
<td>0.43 ± 0.07 (4)</td>
<td>0.99 ± 0.48</td>
<td>5.0 ± 0.28</td>
<td>1.5 ± 0.14</td>
<td>3.9 ± 0.78</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>HeLa</td>
<td>2.8 ± 1.2 (11)</td>
<td>0.59 ± 0.10</td>
<td>2.2 ± 0.53</td>
<td>2.4 ± 0.45</td>
<td>4.5 ± 2.3</td>
<td>1.3 ± 0.24</td>
</tr>
<tr>
<td>KB</td>
<td>2.7 ± 0.49 (6)</td>
<td>0.08 ± 0.01</td>
<td>1.1 ± 0.04</td>
<td>4.1 ± 0.3</td>
<td>2.6 ± 1.1</td>
<td>1.1 ± 0.17</td>
</tr>
<tr>
<td>Hep-2</td>
<td>6.8 ± 2.9 (11)</td>
<td>0.17 ± 0.02</td>
<td>1.6 ± 0.04</td>
<td>2.2 ± 0.04</td>
<td>6.1 ± 2.2</td>
<td>2.5 ± 0.20</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D.

$^b$ Numbers in parentheses, number of separate series of experiments, each run in triplicate.
(Table 3). At 30 min, the FUTP pools were higher (15 to 49 μM in cell water) in the more sensitive cells (S-180, MMT, LM, and U-cells) than in the less sensitive cells (3 to 7 μM). Even after 3 hr of incubation, the FUTP level in the less sensitive cells remained low (11 to 24 μM in cell water) as compared with the more sensitive cells (40 to 130 μM). The concentration of FUra in cell water was independent of sensitivity to FUra. At 30 min, it varied in all cells from 2 to 6 μM, and after 3 hr it was approaching the extracellular level (10 μM) in S-180, MMT, HeLa, and KB cells.

FUra in Combination with Inosine. Inosine has been reported to stimulate the incorporation of FUra into the nucleic acids of Ehrlich ascites cells in vitro. (12). This suggests that it might be possible to enhance the cytotoxicity of FUra by the simultaneous application of inosine. Chart 4 shows how inosine potentiated the growth inhibition of S-180 and KB cells by FUra. It was found that 300 to 1000 μM inosine generally stimulated the incorporation of [2-14C]FUra into acid-soluble and -insoluble material and the formation of FUTP up to 2-fold (data not shown). As in incubations with FUra alone, no acid-soluble FdUMP was detected in the presence of inosine.

DISCUSSION

Human cells included in this study were, on the average, 8 times less sensitive than mouse cells to growth inhibition by FUra (Chart 2). This finding prompted us to examine the biochemical basis for this difference to determine if it was species specific. The results have shown that the bases for the variation in sensitivity were the same regardless of whether the cells originated from man or mouse.

Comparison of the more and less sensitive cells with respect to the velocity of uptake of 10 μM [2-14C]FUra (Table 2) revealed that the more sensitive cells, whether of mouse or of human origin, took up FUra faster than did the less sensitive cells. The cell types varied up to 7-fold in accumulation of the drug. Since slower uptake is likely to limit the ability of FUra to inhibit cellular growth, it was important to analyze this process in detail. The “uptake” as measured here included not only unaltered FUra but also its metabolic products, including FUra incorporated into RNA (see Chart 1). Therefore, in order to determine the rate-limiting process(es) involved in the cellular “uptake” of FUra, it was essential to determine the composition of intracellular ra-

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>V_max (pmol/min/mg protein)</th>
<th>30 min at 10 μM FUra (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acid soluble</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-180</td>
<td>310</td>
<td>140</td>
</tr>
<tr>
<td>MMT</td>
<td>300</td>
<td>311</td>
</tr>
<tr>
<td>LM</td>
<td>40</td>
<td>6.7</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-cells</td>
<td>300</td>
<td>140</td>
</tr>
<tr>
<td>HeLa</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>KB</td>
<td>30 min</td>
<td>53</td>
</tr>
<tr>
<td>Hep-2</td>
<td>30 min</td>
<td>75</td>
</tr>
</tbody>
</table>

Results are expressed as molarity of each metabolite in the intracellular water.

Chart 3. Accumulation of [2-14C]FUra and its acid-soluble metabolites in S-180 and Hep-2 cells. Monolayers of cells were incubated with 10 μM FUra (FU) in serum-free Medium 1640, and the analysis was performed as described in “Materials and Methods”. "Total" indicates the total acid-soluble radioactivity. Results are expressed as molarity of each metabolite in the intracellular water.

FEBRUARY 1979

Basis of Sensitivity to 5-FUra

### Table 3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incubation with 10 μM FUra</th>
<th>% of acid soluble</th>
<th>Concentration of in cell water (μM)</th>
<th>FUra concentration in cell water (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-180</td>
<td>30 min</td>
<td>68</td>
<td>15</td>
<td>5.8</td>
</tr>
<tr>
<td>MMT</td>
<td>3 hr</td>
<td>76</td>
<td>80</td>
<td>12</td>
</tr>
<tr>
<td>LM</td>
<td>30 min</td>
<td>78</td>
<td>132</td>
<td>10</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-cells</td>
<td>30 min</td>
<td>79</td>
<td>15</td>
<td>3.2</td>
</tr>
<tr>
<td>HeLa</td>
<td>3 hr</td>
<td>83</td>
<td>43</td>
<td>4.7</td>
</tr>
<tr>
<td>KB</td>
<td>30 min</td>
<td>61</td>
<td>6.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Hep-2</td>
<td>3 hr</td>
<td>52</td>
<td>11</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Results are expressed as molarity of each metabolite in the intracellular water.

on April 15, 2017. © 1979 American Association for Cancer Research.
As with total cellular “uptake,” the radioactivity derived from extracellular labeled FUra accumulated faster in both acid-soluble and -insoluble fractions of the more sensitive cells. In both cell types, acid-soluble radioactivity was mainly in the form of FUTP (Table 3). After 30 min at 10 μM FUra, it comprised 68 to 80% of total acid-soluble radioactivity in the more sensitive cells and 53 to 55% in the less sensitive cells. After 3 hr at 10 μM FUra, the concentration of FUTP in cell water reached 43 to 130 μM in the more sensitive cells and 11 to 24 μM in the less sensitive cells. The prevalence of FUTP as a metabolite of FUra agrees with similar observations of Kessel et al. (24) with mouse leukemias in vitro and those of Wilkinson and Crumley (41) with Novikoff hepatoma cells. In our studies, the efficiency of FUTP incorporation into RNA, when correlated with FUTP pools, was similar in all cells (Tables 2 and 3). Since at a fixed extracellular concentration of FUra the formation of FUTP in the more sensitive cells was more rapid, these cells incorporated FUra into RNA faster than did the less sensitive cells. Kessel et al. (24) also reported that, in mouse leukemias varying in sensitivity to FUra in vivo, larger FUTP pools were accompanied by more incorporation into RNA.

The second largest acid-soluble component was unaltered FUra which after 3 hr at 10 μM FUra comprised 6 to 12% in the more sensitive cells and 25 to 30% in the less sensitive cells. Interestingly, the velocity of intracellular accumulation (μM in cell water) of unaltered FUra seemed to be similar in all cells (Table 3). This suggested that it was not the velocity of the actual penetration of FUra through the plasma membrane, but rather the intracellular metabolism of FUra which was rate limiting for the "uptake" and thus responsible for the varied sensitivity. One must, therefore, conclude that the kinetic data for FUra uptake (Table 2) largely describe the overall chain of events involved in FUra metabolism rather than the plasma membrane passage of FUra.

The active metabolite FdUMP was not detected in acid extracts of any cell line in the conditions used in this study. FdUMP when bound to TMP synthetase is not acid soluble (27). Kessel et al. (24) and Kessel and Hall (23) were also unable to detect FdUMP in acid extracts of 15 mouse leukemia cell lines treated with labeled FUra in vitro. This is in contrast to the observations of Chadwick and Rogers (8) and Chadwick and Chang (7), who, after injecting [14C]FUra into mice bearing L1210 leukemia, found FdUMP in both normal and tumor tissue. Meyers et al. (30) made similar observations in vivo using mouse leukemia P-1534. Bosch et al. (3) also found FdUMP in acid-soluble pools of Ehrlich ascites cells after incubation with [14C]FUra in vitro. These opposing findings could be due to differences in experimental conditions.

The data on the differing activities of enzymes responsible for the metabolism of FUra (Table 1) correlate well with the differences in velocity of uptake and thus with sensitivity to FUra. FUra has been shown to utilize the enzymes of uracil metabolism (36, 38). In the case of the phosphoribosyltransferase, FUra is actually a superior substrate when compared with uracil (36). As seen in Table 1, the activities of at least 2 of the 3 enzymes, FUra phosphoribosyltransferase, uridine phosphorylase, and thymidine phosphorylase, were always higher in the more sensitive cells. These enzymes are responsible for converting FUra to FUMP, FUr, and FdUrd, respectively. The equilibrium of both nucleoside phosphorylases lies far on the side of nucleoside formation, and these reactions appear to proceed in this direction also in intact cells. In our studies, inosine, a donor of ribose 1-phosphate, potentiated the growth inhibition by FUra in both mouse and human cells (Chart 4). This was accompanied by a stimulation of FUra metabolism including increased formation of FUTP and incorporation into RNA. This suggests that the availability of the sugar phosphates is rate limiting for the conversion of FUra to FUr. In an in vitro study of Flexner-Jobling mouse carcinoma, Kung et al. (26) demonstrated that glucose enhanced the incorporation of FUra into RNA and potentiated its antitumor effect. Similarly, Kessel and Hall (23) found that, for a number of mouse cell lines in vitro, both glucose and inosine lead to increased incorporation of FUra into RNA. However, the antitumor effect of FUra on these leukemias in vivo was not improved by concurrent administration of either glucose or inosine. In our studies, despite potentiation of growth inhibition by inosine, the differences in sensitivity between the cells remained. These differences correlate with the higher activity of FUr phosphorylase in the more sensitive as compared with the less sensitive cells.

In mouse tumor systems in vivo, the activities of some of the enzymes listed in Table 1 have been found to correlate with the degree of drug-induced or innate resistance to FUra. For example, Reichard et al. (34), when studying drug-induced resistance in L1210 leukemia and Ehrlich ascites carcinoma, found that FUra resistance was associated with lower activities for uridine and thymidine phosphorylases. In addition, the parent L1210, which was less sensitive to FUra than was the parent Ehrlich ascites tumor, had lower specific activities for both nucleoside phosphorylases. Decreased FUra phosphoribosyltransferase activity was reported by Reyes and Hall (37) to be associated with FUra-induced resistance in L1210 and by Kasbekar and Greenberg (21) in Gardner lymphosarcoma. Also, natural variation in sensitivity to FUra among transplantable mouse leukemias was found to correlate directly with the activity of FUra phosphoribosyltransferase in the target tissue (37). FUr kinase was found not to have a role in natural variation of sensitivity to FUra among these mouse and human cells. This agrees with the observation of Reichard.
et al. (35) who compared the naturally less sensitive L1210 with the more sensitive Ehrlich ascites; uridine kinase was not significantly different. In contrast, when these authors compared the parent L1210 and Ehrlich ascites with their drug-induced FUra-resistant sublines, significantly lower uridine kinase activities were found in the resistant sublines. Thus, resistance to FUra provides another example where the biochemical basis for drug-induced resistance and natural variation in sensitivity to an antimetabolite are not necessarily the same (9, 40). These differences must be considered in chemotherapy since, in the clinic, both innate and drug-induced resistance to FUra are observed.

That thymidine kinase activity was consistently lower in the more sensitive cells suggests that this enzyme is not critical in FUra metabolism, possibly because prior formation ofFdUrd is essential for it to function. Even in the more sensitive cells where thymidine phosphorylase is high, the formation of FdUrd is limited due to a shortage of deoxyribose 1-phosphate. This has been shown in other mammalian cell systems as well (4, 12). Our observations that deoxyinosine, a donor of deoxyribose 1-phosphate, caused ample formation of acid-soluble FdUMP in all cells* attest to the rate-limiting role of this sugar moiety (and not thymidine kinase) in the conversion of FUra to FdUMP by this pathway. Alternative pathways are available that lead to the formation of FdUMP. Kent and Heidelberger (22) found that 5-fluourouridine diphosphate was a substrate for ribonucleotide reductase. The product 5-fluorodeoxyuridine diphosphate would then be presumably dephosphorylated to FdUMP. Wilkinson et al. (42) have presented evidence indicating that Novikoff hepatoma cells in vitro metabolize FUra to FdUMP through this pathway. Lower levels of thymidine kinase have been shown to be associated with greater sensitivity to FdUrd in a series of transplatable mouse leukemias in vivo (25). This observation made sense considering that in vivo the less sensitive leukemias, due to more active thymidine kinase, could be better protected against FdUrd by thymidine present in mouse serum. Such an explanation would not apply in our in vitro system where the traces of thymidine present in medium containing 5% of undialyzed fetal calf serum were far below the levels required (30 μM) to support growth of any of these cell lines in conditions where thymidine was essential for growth. Moreover, even a supplementation of the medium with 30 μM thymidine failed to reduce the growth inhibitory potency of FUra against human cells, a subject of the next paper.6

On the basis of these studies, it appears that the metabolism of FUra proceeds largely through the phosphoribosyltransferase reaction which then becomes critical for cellular sensitivity to FUra. The nucleoside phosphorylase-kinase pathways for FdUr and FdUrd become significant only if ribose 1-phosphate and deoxyribose 1-phosphate, respectively, are available. Similar correlation has recently been found also between greater sensitivity to FUra in vivo and higher activity of FUra phosphoribosyltransferase in samples of several mouse and rat tumors (Y. M. Rustum and M. T. Hakala, unpublished observations).

REFERENCES


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Basis for Natural Variation in Sensitivity to 5-Fluorouracil in Mouse and Human Cells in Culture


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