Metabolism of 5-Fluorouracil in Sensitive and Resistant Novikoff Hepatoma Cells*

(Received for publication, June 23, 1976, and in revised form, September 13, 1976)

DAVID S. WILKINSON AND JEANNE CRUMLEY

From the Department of Biochemistry, University of South Florida, College of Medicine, Tampa, Florida 33620

NI-SI/FdUrd Novikoff hepatoma cells, which lack thymidine kinase activity, are resistant to 5-fluorouracil (FUra) as well as 5-fluorodeoxyuridine (FdUrd), suggesting that the pathway, FUra → FdUrd → FdUMP, is utilized for the conversion of FUra to FdUMP. However, the inhibition of thymidylate synthetase activity, the presumed target of FUra in intact NI-SI Novikoff hepatoma cells, which have significant levels of thymidine kinase activity, is completely eliminated by 5 × 10⁻⁴ M hydroxyurea, which is a potent inhibitor of ribonucleotide reductase. These results imply that the formation of FdUMP from FUra proceeds via the intermediate formation of ribonucleotides and does not involve thymidine kinase. This apparent dichotomy can be explained by the fact that, in addition to the well known lack of thymidine kinase activity, [¹⁴C]FUra conversion to ribonucleotides is greatly depressed in the NI-SI/FdUrd cells. Hence, the formation of FdUMP from FUra in Novikoff hepatoma cells appears to proceed primarily via the intermediate formation of ribonucleotides. The decreased conversion of FUra to ribonucleotides in NI-SI/FdUrd cells decreases not only the ability of the analog to inhibit DNA synthesis, but also its effect on RNA metabolism. FUra, at a concentration of 1 × 10⁻⁵ M, inhibits rRNA maturation in NI-SI cells, but not in NI-SI/FdUrd cells. Since NI-SI/FdUrd cells are completely resistant to 1 × 10⁻⁵ M FUra, whereas NI-SI cells are completely inhibited by 1 × 10⁻⁴ M FUra, even in the presence of 1 × 10⁻⁴ M thymidine, the effects of FUra on RNA metabolism appear to contribute significantly to the cytotoxicity of the analog at higher drug concentrations.

5-Fluorouracil has been used with limited success in the treatment of a variety of experimental (1) and human (2) neoplasms. The active metabolite of FUra is generally thought to be FdUMP (3), which is a potent inhibitor of thymidylate synthetase (4). In mammalian cells, there are several potential pathways by which FUra may be converted to FdUMP. The possible reactions involved are shown in Fig. 1.

The enzymes which catalyze the reactions shown in Fig. 1 are present in numerous cell types. However, the actual pathway for the conversion of FUra to FdUMP in the intact cell is not known with certainty. Furthermore, the importance of the effects of FUra on RNA metabolism is not well understood. One of the factors limiting the therapeutic efficacy of FUra, as well as many other antimetabolite-type agents, is the frequent development of clinical resistance (12). Since the biochemical basis for drug resistance is often related to altered metabolism of the antitumor agent, it is important to establish the pathways involved in drug activation in sensitive cells and possible differences in these pathways in resistant cells if we are to fully utilize the therapeutic potential of the drugs available to us.

The work described in this paper was designed to elucidate the anabolic pathways of FUra metabolism in FUra-sensitive Novikoff hepatoma cells, and to describe the biochemical consequences of alterations in these pathways in FUra-resistant Novikoff hepatoma cells.

EXPERIMENTAL PROCEDURES

Tissue Culture—The Novikoff hepatoma was originally induced as a solid tumor in rats fed 4-dimethylaminoazobenzene (13). The transplantable tumor was subsequently grown in the ascitic form, from which tissue culture lines were derived by Morse and Potter (14). FUra, as well as FdUrd and FdUrd, effectively inhibit the growth of the NI-SI strain of Novikoff hepatoma cells (6, 14). By culturing long term survivors of minimally toxic concentrations of FdUrd, Morse and Potter also developed a FdUrd-resistant strain designated NI-SI/FdUrd. The FdUrd-resistant strain has less than 1% of the thymidine kinase activity present in the NI-SI strain and is cross resistant to FUra (6).

Suspension cultures of the Novikoff cells were grown at 37°C in Medium S 89 as described previously (15). Measurements of cell density were performed with a Bio/Physics Cytograf, model 6300A (Bio-Physics Systems, Inc., Mahopac, N. Y.), after aliquots of the cell suspension had been centrifuged and the cells resuspended in a...
solution containing crystal violet (0.05 g/100 ml) dissolved in 0.3 M trisodium citrate.

Incorporation of Radioactive Nucleotides—Cells were grown to mid- or late log phase density (0.4 to 1.5 × 10^6 cells/ml), harvested at room temperature by centrifugation (500 × g, 20 min), and resuspended in one-fifth the original volume of fresh Medium S-89. Upon completion of the experimental incubations, which were performed at 37°C, the cells were immediately chilled in an ice bath and harvested by centrifugation (500 × g, 5 min) at 4°C. The cells were then washed twice by resuspending the cell pellets from each experimental culture in 10 ml of ice-cold phosphate-buffered saline (16), blending on a vortex mixer, and resuspension the cells. Unless otherwise specified all subsequent procedures were performed at 0-4°C.

Determination of Radioactivity Incorporated into Acid-soluble, RNA, and DNA Fractions—Cell pellets, containing approximately 25 × 10^6 cells, were resuspended in 2 ml of 0.3 M perchloric acid. After 10 min, the precipitate was removed by centrifugation and washed twice with 1 ml of 0.3 M perchloric acid. The combined supernatants contained the acid-soluble fraction. Each pellet of acid-insoluble material was suspended in 2 ml of 0.3 M KOH and incubated overnight at 37°C to hydrolyze the RNA. The KOH digest was neutralized with 0.13 ml of 9.2 M perchloric acid. After 10 min, the precipitate was removed by centrifugation and washed twice with 1 ml of 0.3 M perchloric acid. The combined supernatants contained the RNA fraction. The washed pellet, which contained the DNA fraction, was dried to constant weight in a rotary evaporator (Buchler Instruments, Fort Mich.). Preblend 3a70B complete liquid scintillation cocktail (Research Products International Corp., Elk Grove Village, Ill.) was used for all radioactivity assays. FURA, thymidine, and uracil were purchased from Sigma Chemical Co. (St. Louis, Mo.). [2-3H]Deoxyctydine (29.7 mCi/mmol), [U-14C]cytidine (376 mCi/mmol), and [8-3H1deoxyguanosine (19 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). [6-3H]Deoxyuridine (14 Ci/mmol) and [9-3H]deoxyadenosine (80 Ci/mmol) were obtained from Amer sham/Searle (Arlington Heights, Ill.). 5-Fluoro[2-14C]uracil (55 mCi/mmol) was supplied by Moravek Biochemicals (City of Industry, Calif.).

RESULTS

Effect of Hydroxyurea on Inhibition of [6-3H]Deoxyuridine Conversion to Thymidine Nucleotides by FURA—When N1-S1 Novikoff hepatoma cells were incubated for 1 h with [9-3H]deoxyuridine, essentially all of the intracellular deoxyuridine was converted to thymidine nucleotides (Fig. 2, left panel). Preincubation of the cells with 1 × 10^-4 M FURA for 1 h completely inhibited the conversion of deoxyuridine to thymidine nucleotides (Fig. 2, right panel). These results are consistent with the well known ability of FURA to inhibit thymidylate synthetase as a consequence of its conversion to FdUMP (4). Hydroxyurea, a potent inhibitor of ribonucleotide reductase (20), demonstrated a concentration-dependent ability to prevent the inhibition of deoxyuridine conversion to thymidine nucleotides caused by FURA. At a concentration of 1 × 10^-5 M, hydroxyurea exerted no observable effect, but at a concentration of 5 × 10^-4 M, hydroxyurea completely prevented the inhibition of deoxyuridine conversion to thymidine nucleotides caused by FURA. These results are the hydroxyurea concentrations on the apparent ribonucleotide reductase activity in the intact cell (21) is shown in Table I, which gives the percentage of [U-14C]cytidine converted to deoxyctydine nucleotides during the 1-h incubation. At a concentration of 1 × 10^-5 M, hydroxyurea inhibited the apparent ribonucleotide reductase activity by 22%. At a concentration of 5 × 10^-4 M, hydroxyurea inhibited the apparent ribonucleotide reductase activity by 67%.

Effect of FURA on Incorporation of Deoxynucleotides into DNA of N1-S1 and N1-S1/FdUrd Novikoff Hepatoma Cells—

FIG. 1. Anabolic metabolism of FURA.

FIG. 2. Inhibition of [6-3H]deoxyuridine conversion to thymidine by FURA in N1-S1 cells. Cultures containing 5 ml of N1-S1 cells (3.15 × 10^6/ml) were incubated in the presence or absence of 1 × 10^-4 M FURA for 1 hr, after which time [6-3H]deoxyuridine (1 μCi/ml) was added to each culture. Cells were harvested 1 h after the addition of the radioactive nucleotide. The cell pellet was resuspended in 1 ml of 0.5 M perchloric acid. After 30 min the precipitate was removed by centrifugation (900 × g, 5 min) and the supernatant was neutralized by the addition of 0.075 ml of 5 M KOH and 0.5 ml of 1 M Tris (pH 7.4). The KClO4 precipitate was removed by centrifugation and 150 μl of the supernatant were evaporated to dryness. The residue was dissolved in 30 μl of 0.1 M Tris (pH 9.0), treated with snake venom, and analyzed by paper chromatography, using water-saturated isobutanol:15 N NH4OH (100:1) as solvent. Left, control culture; right, FURA-treated culture.
Metabolism of 5-Fluorouracil

When N1-S1 Novikoff hepatoma cells, which have significant levels of thymidine kinase activity (6), were preincubated for 1 h with various concentrations of FUra, the amount of radioactivity incorporated into DNA during a subsequent 30-min incubation with [8-3H]deoxyguanosine progressively decreased as the concentration of FUra increased (Fig. 4, left panel). Incorporation of [8-3H]deoxyguanosine into DNA was not significantly affected by preincubation with uracil. Neither FUra nor uracil decreased the incorporation of [8-3H]deoxyguanosine into the acid-soluble fraction. In contrast to the results obtained with the N1-S1 cells, FUra did not inhibit the incorporation of [8-3H]deoxyguanosine into DNA in the N1-S1/FdUrd Novikoff hepatoma cells, which are deficient in thymidine kinase activity (6).

The inability of FUra to inhibit DNA synthesis in N1-S1/FdUrd cells was also apparent when [2-14C]deoxycytidine was utilized as the DNA precursor (Table II). When N1-S1 cells

![Graph](https://example.com/graph.png)

**Fig. 3.** Effect of hydroxyurea (HU) on the inhibition of [6-3H]deoxyuridine conversion to thymidine by FUra in N1-S1 cells. Experimental conditions were identical with those described in the legend of Fig. 2 except that various concentrations of hydroxyurea were added together with the FUra.

**TABLE I**

<table>
<thead>
<tr>
<th>Concentration of hydroxyurea (M)</th>
<th>% conversion</th>
<th>% conversion of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.37 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>1 × 10^{-3}</td>
<td>2.47 ± 0.04</td>
<td>78</td>
</tr>
<tr>
<td>5 × 10^{-3}</td>
<td>1.94 ± 0.06</td>
<td>61</td>
</tr>
<tr>
<td>1 × 10^{-4}</td>
<td>1.61 ± 0.02</td>
<td>51</td>
</tr>
<tr>
<td>5 × 10^{-4}</td>
<td>1.06 ± 0.05</td>
<td>33</td>
</tr>
</tbody>
</table>

**Fig. 4.** Differential effect of FUra on the incorporation of [8-3H]deoxyguanosine into the DNA of N1-S1 (NS) and N1-S1/FdUrd (NSF) cells. Cultures containing 5 ml of N1-S1 cells (2.5 × 10^6/ml) and N1-S1/FdUrd cells (2.5 × 10^6/ml) were incubated for 1 h with various concentrations of Ura (open symbols) or FUra (closed symbols), after which time [8-3H]deoxyguanosine (0.1 μCi/ml) was added to each culture. Thirty minutes after the addition of the radioactive nucleoside, the incorporation of radioactivity into the acid-soluble fraction (circles) and DNA fraction (triangles) was determined. The points plotted on the graph represent the mean of values obtained from duplicate cultures. The vertical bars represent the range of values at each point. Symbols without bars indicate that the range of values was smaller than the symbol size.

**TABLE II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[2-14C]Deoxycytidine incorporation</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid-soluble</td>
<td>% (of control)</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td>N1-S1 cells</td>
<td>None</td>
<td>210 ± 60 (100)</td>
</tr>
<tr>
<td></td>
<td>Ura</td>
<td>270 ± 20 (120)</td>
</tr>
<tr>
<td></td>
<td>FUra</td>
<td>490 ± 70 (223)</td>
</tr>
<tr>
<td>N1-S1/FdUrd cells</td>
<td>None</td>
<td>230 ± 20 (100)</td>
</tr>
<tr>
<td></td>
<td>Ura</td>
<td>230 ± 30 (104)</td>
</tr>
<tr>
<td></td>
<td>FUra</td>
<td>240 ± 40 (109)</td>
</tr>
</tbody>
</table>
were preincubated for 1 h with \(1 \times 10^{-4} \text{ M} \) FUra and then incubated for an additional 1 h with \(2^{-14} \text{C} \) deoxycytidine, there was a 95% inhibition of precursor incorporation into DNA when compared to cells preincubated in the presence of either no additive or \(1 \times 10^{-4} \text{ M} \) uracil. FUra had no significant effect on the incorporation of \(2^{-14} \text{C} \) deoxycytidine into the DNA of N1-Sl/FdUrd cells.

**Effect of FUra on Conversion of \(2^{-14} \text{C} \) Deoxycytidine to Thymidine Nucleotides in N1-Sl and N1-Sl/FdUrd Novikoff Hepatoma Cells**—Fig. 5 shows the chromatographic separation of the nucleosides obtained after snake venom treatment of the acid-soluble fractions prepared from cells which had been preincubated for 1 h with no additive, \(1 \times 10^{-4} \text{ M} \) uracil, or \(1 \times 10^{-4} \text{ M} \) FUra and then incubated for an additional 1 h with \(2^{-14} \text{C} \) deoxycytidine. In the control (left panels) and uracil-treated (center panels) cultures, the radioactivity present in the acid-soluble fractions was approximately equally distributed between deoxycytidine nucleotides and thymidine nucleotides in both N1-Sl and N1-Sl/FdUrd cells. Little or no radioactivity was present in the form of deoxyuridine nucleotides. FUra treatment prevented the conversion of deoxycytidine to thymidine nucleotides in N1-Sl cells (upper right panel), but not in N1-Sl/FdUrd cells (lower right panel). Furthermore, a significant amount of radioactivity in the acid-soluble fraction obtained from the FUra-treated N1-Sl cells existed as deoxyuridine nucleotides, whereas no radioactive deoxyuridine nucleotides were apparent in the FUra-treated N1-Sl/FdUrd cells.

**Anabolism of \(2^{-14} \text{C} \) FUra in N1-Sl and N1-Sl/FdUrd Novikoff Hepatoma Cells**—The uptake of \(2^{-14} \text{C} \) FUra into the acid-soluble and RNA fractions of N1-Sl/FdUrd cells was significantly less than that observed in N1-Sl cells (Fig. 6). As the time of incubation with \(2^{-14} \text{C} \) FUra increased, the amount of radioactive FUTP in the acid-soluble fraction of N1-Sl cells also increased (Fig. 7). In contrast to these results, the amount of \(2^{-14} \text{C} \) FUra converted to FUTP was greatly depressed in N1-Sl/FdUrd cells, and the amount of radioactive FUTP did not increase as the time of incubation with \(2^{-14} \text{C} \) FUra increased. Note that the amount of free \(2^{-14} \text{C} \) FUra was about the same at all time points in both N1-Sl and N1-Sl/FdUrd cells.

**Effect of FUra on Maturation of RNA in N1-Sl and N1-Sl/FdUrd Novikoff Hepatoma Cells**—Preincubation of N1-Sl cells with \(1 \times 10^{-4} \text{ M} \) FUra for 1 h significantly decreased the incorporation of \(8^{-3} \text{H} \) guanosine into mature 28 S and 18 S rRNA during a subsequent 90-min incubation with the labeled precursor (Fig. 8, upper panel). FUra had no effect on the incorporation of \(8^{-3} \text{H} \) guanosine into mature 28 S and 18 S rRNA in N1-Sl/FdUrd cells (lower panel).

**Inhibition of Novikoff Hepatoma Cell Growth by FUra**—At a concentration of \(1 \times 10^{-4} \text{ M} \), FUra completely inhibited the proliferation of N1-Sl cells (Fig. 9). Thymidine, at a concentration of \(1 \times 10^{-4} \text{ M} \), overcame the growth inhibition caused by \(1 \times 10^{-4} \text{ M} \) FUra, but not that caused by \(1 \times 10^{-5} \text{ M} \) FUra. The proliferation of N1-Sl/FdUrd cells was not significantly affected by \(1 \times 10^{-4} \text{ M} \) FUra.
Metabolism of 5-Fluorouracil

**DISCUSSION**

The ability of hydroxyurea to completely prevent the inhibition of [6-3H]deoxyuridine conversion to thymidine nucleotides caused by FUrA (Fig. 3) suggests that FUrA is converted to FdUMP via the intermediate formation of ribonucleotides and not via the intermediate formation of FdUrd. However, the inability of FUrA to inhibit either thymidylate synthetase activity (Fig. 5) or the incorporation of radioactive deoxynucleosides into DNA (Fig. 4, Table II) in N1-Sl/FdUrd cells, which lack thymidine kinase activity, suggests that the pathway FUrA → FdUrd → FdUMP may be important. The explanation for this apparent dichotomy appears to reside in the fact that, in addition to the loss of thymidine kinase activity, FUrA conversion to ribonucleotides is greatly depressed in the N1-Sl/FdUrd cells (Figs. 6 and 7). Collectively, these data indicate that the formation of FdUMP from FUrA does proceed primarily via the intermediate formation of ribonucleotides and that the refractoriness of DNA synthesis toward inhibition by FUrA in N1-Sl/FdUrd cells is due more to a suppression of this pathway in the resistant cells than to the well-known lack of thymidine kinase activity. Ribonucleotide reductase, the levels of which are essentially identical in both N1-Sl and N1-Sl/FdUrd cells, requires the nucleoside diphosphate as substrate. Therefore, decreased formation of FdUMP would be a probable consequence of the decreased conversion of FUrA to ribonucleotides in the resistant cells.

The inverse relationship between FUrA conversion to ribonucleotides and FUrA sensitivity, although unrecognized previously in the Novikoff system, is a well-documented phenomenon in other cell types (7, 22-24) and it suggests that the formation of FdUMP via FUMP may be the primary route of FUrA activation in most tumor cells. Since the Novikoff hepato...
Metabolism of 5-Fluorouracil

proliferation is probably due entirely to the inhibition of DNA synthesis in sensitive cells. This hypothesis is supported by the fact that $1 \times 10^{-4}$ M thymidine overcomes the growth inhibition caused by $1 \times 10^{-6}$ M FUra in N1-S1 cells, but not that caused by $1 \times 10^{-5}$ M FUra. Since plasma levels of FUra in the range of $1 \times 10^{-5}$ M to $1 \times 10^{-4}$ M have been reported in human patients following both oral and intravenous administration of FUra (2, 26), RNA effects may be important in the clinical application of this drug.

FIG. 9. Effect of FUra on the proliferation of N1-S1 and N1-S1/FdUrd cells. Flasks containing 25 ml of fresh medium and various concentrations of FUra were inoculated with $1 \times 10^6$ cells. Aliquots were removed daily from each flask for determination of cell density. All cultures were set up in duplicate and the points plotted on the graph represent the mean cell density of FUra-treated cultures (expressed as the percentage of the mean cell density achieved in cultures not receiving FUra) observed on Day 5, since by this time all cultures which grew had achieved maximal density. The mean cell densities achieved by cultures receiving no FUra were: N1-S1 cells in Medium S-69 (NS), 1.87 ± 0.0; N1-S1 cells in Medium S-69 supplemented with $1 \times 10^{-4}$ M thymidine (NS + dThd), 1.68 ± 0.10; N1-S1/FdUrd cells in Medium S-69 (NSF), 1.37 ± 0.11.

toma cells possess thymidine phosphorylase activity (5), the lack of FUra conversion ofFdUMP via FdUrd may be a consequence of the presumed low intracellular concentration of deoxyribose 1-phosphate in mammalian cells (7).

The decreased conversion of FUra to ribonucleotides appears to decrease not only the ability of the analog to inhibit DNA synthesis, but also the effects of the analog on RNA metabolism. Specifically, $1 \times 10^{-4}$ M FUra inhibits rRNA maturation in N1-S1 cells, but not in N1-S1/FdUrd cells (Fig. 8). Since analog incorporation into the 45 S rRNA precursor appears to be a prerequisite for the inhibition of maturation by the fluorinated pyrimidines (25), the decreased incorporation of FUra into RNA in N1-S1/FdUrd cells (Fig. 6) is probably the basis for the inability of the analog to inhibit maturation in these cells.

The differential effect of $1 \times 10^{-3}$ M FUra on rRNA maturation in sensitive and resistant cells raises the possibility that the effects of the analog on RNA metabolism may contribute to the carcinostatic properties of FUra at higher drug concentrations. At lower concentrations of FUra, where effects on RNA metabolism are much less significant, the inhibition of cell proliferation is probably due entirely to the inhibition of DNA synthesis in sensitive cells. This hypothesis is supported by the fact that $1 \times 10^{-4}$ M thymidine overcomes the growth inhibition caused by $1 \times 10^{-6}$ M FUra in N1-S1 cells, but not that caused by $1 \times 10^{-5}$ M FUra. Since plasma levels of FUra in the range of $1 \times 10^{-5}$ M to $1 \times 10^{-4}$ M have been reported in human patients following both oral and intravenous administration of FUra (2, 26), RNA effects may be important in the clinical application of this drug.

REFERENCES
12. Vessely, J., and Cihak, A. (1972) Oncology 26, 204-226
Metabolism of 5-fluorouracil in sensitive and resistant Novikoff hepatoma cells.
D S Wilkinson and J Crumley


Access the most updated version of this article at http://www.jbc.org/content/252/3/1051

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/3/1051.full.html#ref-list-1