The Mechanism of 5-Fluorouridine Toxicity in Novikoff Hepatoma Cells

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SUMMARY

The mechanism of 5-fluorouridine (FUrd) cytotoxicity in Novikoff hepatoma cells appears to vary under different experimental conditions. Continuous exposure to $1 \times 10^{-7}$ M FUrd results in a simple thymineless death that can be prevented by the addition of $1 \times 10^{-4}$ M thymidine to the culture medium. In contrast, $1 \times 10^{-4}$ M thymidine does not prevent the growth inhibition caused by a 1-hr exposure to $1 \times 10^{-8}$ M FUrd, despite the fact that it does prevent the inhibition of DNA synthesis. Although it has no effect on the inhibition of DNA synthesis, $1 \times 10^{-4}$ M uridine prevents the growth inhibition caused by a 1-hr exposure to $1 \times 10^{-5}$ M FUrd. Since $1 \times 10^{-4}$ M uridine, but not $1 \times 10^{-4}$ M thymidine, prevents the inhibition of ribosomal RNA maturation caused by a 1-hr exposure to $1 \times 10^{-8}$ M FUrd, it seems likely that the effects of the analog on RNA metabolism contribute significantly to the cytotoxic activity of the analog in this specific experimental situation.

INTRODUCTION

FUrd demonstrates a potent in vitro and in vivo antitumor activity against several experimental (12, 13) and human (5) neoplasms. Heidelberger showed that, like the related compounds FUra and FdUrd, FUrd is a potent inhibitor of the incorporation of $[1^4C]$formate into DNA thymine both in vitro (2) and in vivo (6). Presumably, the conversion of FUrd to FdUMP, which is an inhibitor of thymidylate synthetase (10, 24), results in a thymineless death (23). However, Rich et al. (22) found that in H.Ep. 1 cells, thymidine reverses the growth inhibition caused by FUrd, but not that caused by FUrd. Furthermore, FUrd is much more toxic to mice than FUra or FdUrd (12), and a number of transplantable neoplasms that are resistant to either FUra or FdUrd, or both, remain sensitive to FUrd (12, 13). These data suggest additional sites of action for FUrd.

Recent reports (30, 31) have shown that FUrd is a potent inhibitor of rRNA metabolism in Novikoff hepatoma cells. The presence of $1 \times 10^{-8}$ M FUrd inhibits the incorporation of radioactive precursors into mature 18 S and 28 S rRNA.

The initial effect of FUrd is an inhibition of the normal posttranscriptional modification of newly synthesized 4S rRNA precursor. This inhibition occurs within 5 min following the addition of FUrd to the culture medium and appears to depend upon the incorporation of the analog into the 45 S rRNA precursor molecule. Initially, FUrd has no effect on the rate of 45 S rRNA transcription. However, after a 1-hr exposure to the drug, the rate of precursor transcription also decreases.

Thus, FUrd produces at least 2 significant biochemical effects, either one of which alone is sufficient to inhibit cell division. The experiments described herein were designed to determine the relative importance of the DNA and RNA effects in the cytotoxic activity of FUrd in Novikoff hepatoma cells.

MATERIALS AND METHODS

Cells and Media. Suspension cultures of Novikoff hepatoma cells (strain N1-S1) were grown at 37° in Medium S-69 as described previously (17, 31). For enumeration of cells, aliquots of the cell suspension were centrifuged and resuspended in a solution containing crystal violet (0.05 g/100 ml) dissolved in 0.3 M trisodium citrate. Cell counts were obtained on a Bio/Physics Cytograf Model 6300A (Bio/Physics Systems Inc., Mahopac, N.Y.). Cell counts using this procedure were more reproducible and corresponded more closely to counts performed with a standard hemacytometer technique than counts obtained directly from the cell suspension. Furthermore, samples prepared by this procedure were stable for up to 2 weeks when stored in the refrigerator.

Incorporation of Radioactive Nucleosides. Cells were harvested by centrifugation at room temperature and resuspended in one-fifth of the original volume of warm (37°) medium. Incorporation of radioactive nucleosides was carried out at 37° in 10-ml flasks containing 5 ml of the concentrated cell suspension.

Preparation of the Acid-soluble, RNA and DNA Fractions. The procedure described by Munro and Fleck (18) was modified as follows for the preparation of the acid-soluble, RNA, and DNA fractions. After the experimental incubation period, cells were immediately chilled and harvested by centrifugation. The cell pellets were washed twice in cold phosphate-buffered saline (28) or Medium S-69. The washed cell pellets were then suspended in 2 ml of 0.2 N PCA, mixed well, and kept at 0° for 10 min. The precipitate was removed by centrifugation and washed twice by resus-
pension in 1 ml of cold 0.2 n PCA followed by centrifugation. The combined supernatants contained the acid-soluble fraction. The precipitate was dissolved in 2 ml of 0.3 n KOH and incubated overnight at 37° to ensure complete hydrolysis of RNA. The KOH digest was cooled to 0° and acidified with 0.1 ml of 0.6 n PCA. The precipitate was removed by centrifugation and washed twice by resuspension in 1 ml of cold 0.2 n PCA followed by centrifugation. The combined supernatants contained the RNA fraction. The pellet contained the DNA fraction. The RNA concentration was estimated by reading the absorbance at 260 nm of the acidified KOH digest after adjusting the PCA concentration to 0.1 n. We assumed that an A_{260} of 1 corresponded to a RNA concentration of 32 µg/ml. The DNA-containing fraction was dissolved in 6 ml of 0.1 n KOH and the concentration of DNA was estimated by the method of Burton (3). Aliquots of each fraction were assayed for radioactivity by liquid scintillation counting.

**Extraction and Electrophoretic Analysis of RNA.** Phenol extraction (30) and polyacrylamide-agarose gel electrophoresis (29) of RNA were performed as described previously, except that 2.1% acrylamide gels were used in place of 2.4% acrylamide gels for the electrophoresis.

**Materials.** Swim’s Medium 77 and calf serum were purchased from Grand Island Biological Company, Grand Island, N. Y., and International Scientific Industries Inc., Cary, Ill., respectively. Pluronic F-68 was a generous gift from Wyandotte Chemical Corp., Wyandotte, Mich. Scintiverse was obtained from Fisher Scientific, Pittsburgh, Pa. FUrd was a generous gift from Hoffmann-LaRoche Inc., Nutley, N. J.

[8-3H]Guanosine (15.8 Ci/mmmole), [8-3H]deoxyguanosine (3.1 Ci/mmmole), and [6-3H]deoxyuridine (15 Ci/mmmole) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. [2-14C]Uridine (45 mCi/mmmole) was purchased from Calatonic, La., Calif. All radioactive chemicals were shown to be greater than 95% pure by paper chromatography. Specifically, [8-3H]deoxyguanosine was free of labeled guanosine or guanine, [8-3H]guanosine was free of labeled deoxyguanosine or guanine, [2-14C]uridine was free of labeled uracil, and [6-3H]deoxyuridine was free of labeled uridine or uracil.

**Statistical Analysis.** The statistical significance of differences between means was determined by Student’s t test.

**RESULTS**

**Effect of FUrd on the Incorporation of Deoxynucleosides into DNA.** Preincubation of Novikoff hepatoma cells for 1 hr in the presence of FUrd inhibited the incorporation of radioactivity into DNA during a subsequent 30-min exposure to [6-3H]deoxyuridine (Chart 1). Essentially, concentrations of the drug above 1 × 10^{-8} M completely inhibited this process. Since [6-3H]deoxyuridine is incorporated specifically into DNA via thymidylate (1), these data indicate a potent inhibition of thymidylate synthetase activity in Novikoff hepatoma cells by FUrd.

FUrd treatment results in a rapid expansion of the dUMP pool in P1534 ascites cells in mice (19). Since it is reasonable to assume that FUrd would have a similar effect, it is possible that some of the decreased incorporation of [6-3H]deoxyuridine into DNA is due to dilution of the labeled precursor by an expanded dUMP pool. However, if FUrd emulates FUrA effects on precursor pools, one would also expect FUrd treatment to decrease the dTTP pool size (26). This effect would tend to offset to some extent the effect of dUMP pool expansion with respect to [6-3H]deoxyuridine incorporation into DNA. Furthermore, the data of Myers et al. (19) demonstrate that a 20-fold increase in the dUMP pool size is not sufficient by itself to completely suppress [6-3H]deoxyuridine incorporation into DNA. Therefore, despite the possible effects of FUrd on precursor pools, the complete inhibition of [6-3H]deoxyuridine incorporation into DNA at higher analog concentrations probably reflects a complete inhibition of thymidylate synthetase activity.

In contrast, FUrd inhibited the incorporation of [8-3H]deoxyguanosine into DNA much less completely (Chart 2). These data indicate that a significant level of DNA synthesis persists during FUrd treatment, despite the complete inhibition of the target enzyme, thymidylate synthetase. As was the case in utilizing [6-3H]deoxyuridine incorporation into DNA as a measure of thymidylate synthetase activity, one must exercise caution in equating the rate of [8-3H]deoxyguanosine incorporation into DNA with the actual rate of DNA synthesis. However, Tattersall and Harrap (26) have shown that dGTP pools in 5178Y cells change by less than 10% during a 1-hr incubation with 1 µM FUrd. Similarly, the inhibition of thymidylate synthetase by methotrexate causes little or no change in dGTP pool sizes (26). Therefore, the choice of [8-3H]deoxyguanosine as DNA precursor should at least minimize errors due to inhibitor effects on pool size.

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**Mechanism of FUrd Toxicity**

Chart 1: Effect of FUrd on the incorporation of [6-3H]deoxyuridine into DNA. Cells, which had grown to a density of 0.4 × 10^{5}/ml, were harvested and resuspended in one-fifth of the original volume of Medium 5-69 supplemented with various concentrations of either uridine (O, A) or FUrd (●, A). After 1 hr, [6-3H]deoxyuridine (0.1 µCi/ml) was added and the cells were incubated for an additional 30 min. The incorporation of radioactivity was calculated in terms of dpm/10^6 cells for the acid-soluble fraction (O, ●) and dpm/µg for the DNA fraction (A, A). The data points represent the mean of values obtained from duplicate cultures. Bars, range of values at each point. Symbols without bars, range of values that were smaller than the symbol size. The values obtained from cultures that received neither uridine nor FUrd were 605 ± 42 dpm/10^6 cells for the acid-soluble fraction and 1159 ± 20 dpm/µg for the DNA fraction.
Effect of Uridine and Thymidine on the Inhibition of DNA Synthesis by FUrd. Cells were incubated for 1 hr in the presence of various combinations of FUrd (1 × 10^{-6} M), uridine (1 × 10^{-4} M), and thymidine (1 × 10^{-4} M) and were then exposed to [8-^{3}H]deoxyguanosine incorporation into DNA to 9% of that observed in control cultures. The addition of uridine during the preincubation with FUrd had little or no effect on the incorporation of [8-^{3}H]deoxyguanosine into DNA, but the addition of thymidine during the FUrd treatment prevented the inhibition. A similar suppression of the inhibition of DNA synthesis by thymidine has been reported for cells inhibited by FdUrd (20).

When cells that had been inhibited with 1 × 10^{-5} M FUrd for 1 hr were washed, resuspended, and incubated for an additional 1 hr in fresh medium, the inhibition of [8-^{3}H]deoxyguanosine incorporation into DNA was similar to that seen after the initial 1-hr incubation with FUrd (Table 2). The addition of 1 × 10^{-4} M thymidine during the 2nd 1-hr incubation reversed the inhibition of precursor incorporation into DNA while the addition of 1 × 10^{-4} M uridine again had no effect.

Effect of Uridine and Thymidine on the Cytotoxicity of FUrd. The proliferation of Novikoff hepatoma cells was completely inhibited by continuous incubation in 1 × 10^{-7} M FUrd (Chart 3A). The addition of either 1 × 10^{-4} M uridine (Chart 3B) or 1 × 10^{-4} M thymidine (Chart 3C) prevented the growth inhibitory effects of 1 × 10^{-7} M FUrd. Uridine and thymidine only partially prevented the growth inhibition caused by 1 × 10^{-6} M FUrd, and neither nucleoside was effective in the presence of 1 × 10^{-6} M FUrd.

Exposure of cells to 1 × 10^{-5} M FUrd for 1 hr significantly inhibited cell proliferation during a subsequent 3-day incubation in analog-free medium (Table 3). The presence of 1 × 10^{-4} M thymidine during the 1-hr incubation with FUrd had little beneficial effect on cell proliferation during the subsequent 3-day incubation in additive-free medium. In contrast, the presence of 1 × 10^{-4} M uridine during the initial 1-hr incubation period allowed the cells to proliferate to a density equal to 77% of that observed in the control culture.

The presence of uridine during the 3-day growth period reversed the cytotoxic effects of a 1-hr exposure to FUrd less effectively than when it was added during the initial 1-hr...
The inhibition of [8-3H]deoxyguanosine incorporation into DNA by FUrd and its reversal by Urd and dThd

A culture, which contained 1.08 x 10^6 cells/ml, was divided into equal portions. One portion was treated with 1 x 10^-5 M FUrd. Both cultures were incubated for 1 hr. Cells were then harvested by centrifugation, washed twice with fresh medium, and resuspended in one-fifth of the original volume of Medium S-69, supplemented as indicated with 1 x 10^-4 M dThd or 1 x 10^-4 M Urd. The cultures were incubated for 1 hr and then treated with [8-3H]deoxyguanosine (0.1 μCi/ml) for 30 min. The incorporation of radioactivity into the acid-soluble, RNA, and DNA fractions was determined.

Incorporation of radioactivity

<table>
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<tr>
<th></th>
<th>Acid soluble</th>
<th>RNA</th>
<th>DNA</th>
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<tbody>
<tr>
<td></td>
<td>% of control</td>
<td>dpm/μg % of control</td>
<td>dpm/μg</td>
</tr>
<tr>
<td>1st hr</td>
<td>2nd hr</td>
<td>dpm/10^6 cells</td>
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</tr>
<tr>
<td>None</td>
<td>None</td>
<td>4597 ± 148b</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>dThd</td>
<td>3816 ± 143</td>
<td>83</td>
</tr>
<tr>
<td>None</td>
<td>Urd</td>
<td>4615 ± 88</td>
<td>100</td>
</tr>
<tr>
<td>FUrd</td>
<td>None</td>
<td>7328 ± 396</td>
<td>159</td>
</tr>
<tr>
<td>FUrd</td>
<td>dThd</td>
<td>5057 ± 250</td>
<td>110</td>
</tr>
<tr>
<td>FUrd</td>
<td>Urd</td>
<td>5797 ± 463</td>
<td>130</td>
</tr>
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</table>

* Urd, uridine; dThd, thymidine.

** Mean ± S.D. for duplicate cultures.

† Significantly different from control value (p < 0.01).

‡ Not significantly different from each other (p > 0.03).

Table 2

Mechanism of FUrd Toxicity

Table 3. Effect of uridine and thymidine on the incubation of cell proliferation by FUrd. Cultures were inoculated at an initial cell concentration of 0.04 x 10^6/ml. The growth medium contained various concentrations of FUrd. One series of cultures received no other additive (A), a 2nd series received 1 x 10^-5 M uridine (B), and a 3rd series received 1 x 10^-4 M thymidine (C). The points plotted on the curve represent the mean count obtained from triplicate cultures. Bars, magnitude of the S.D. Symbols without bars, the S.D. was smaller than the symbol size. FUrd concentration: 0 (○); 1 x 10^-5 M (△); 1 x 10^-4 M (□); 1 x 10^-5 M (●); 1 x 10^-4 M (△△); 1 x 10^-5 M (□□).

Chart 3. Effect of uridine and thymidine on the incubation of cell proliferation by FUrd. Cultures were inoculated at an initial cell concentration of 0.04 x 10^6/ml. The growth medium contained various concentrations of FUrd. One series of cultures received no other additive (A), a 2nd series received 1 x 10^-5 M uridine (B), and a 3rd series received 1 x 10^-4 M thymidine (C). The points plotted on the curve represent the mean count obtained from triplicate cultures. Bars, magnitude of the S.D. Symbols without bars, the S.D. was smaller than the symbol size. FUrd concentration: 0 (○); 1 x 10^-5 M (△); 1 x 10^-4 M (□); 1 x 10^-5 M (●); 1 x 10^-4 M (△△); 1 x 10^-5 M (□□).

exposure to FUrd (Table 4). In contrast to the data of Chart 3, the reversal of FUrd toxicity of uridine was not significantly different from that achieved with thymidine under these conditions (0.35 ± 0.05 versus 0.19 ± 0.03, p > 0.05). In both experiments, the combination of uridine and thymidine gave essentially the same results as uridine alone.

Although continuous incubation with 1 x 10^-7 M FUrd completely suppressed cell proliferation, a 1-hr incubation with 1 x 10^-7 M FUrd had no effect on the proliferation of cells subsequently cultured in analog-free medium (Table 5).

Effect of Uridine and Thymidine on the Inhibition of rRNA Maturation by FUrd. Since the effects of 1 x 10^-4 M uridine and 1 x 10^-4 M thymidine on the inhibition of DNA
Significantly different from control value (p < 0.001).

absence of 1 x 10^-6 PA FUnd. The cells were then harvested by centrifugation, washed twice, and resuspended in the original volume of fresh, additive-free Medium S-69. Aliquots, containing 1 x 10^6 cells, were then transferred to 25 ml of culture medium, which contained no additives. The cell concentration was determined 3 days later.

Table 3
Effect of Urd* and dThd on the inhibition of cell division by FUrd

<table>
<thead>
<tr>
<th>Treatment</th>
<th>×10^-6 (cells/ml)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.33 ± 0.07^a</td>
<td>100</td>
</tr>
<tr>
<td>FUrd</td>
<td>0.04 ± 0.01^b</td>
<td>3</td>
</tr>
<tr>
<td>FUrd + Urd</td>
<td>1.01 ± 0.04^c</td>
<td>77</td>
</tr>
<tr>
<td>FUrd + dThd</td>
<td>0.09 ± 0.00^c,d</td>
<td>7</td>
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<tr>
<td>FUrd + Urd + dThd</td>
<td>1.18 ± 0.02</td>
<td>89</td>
</tr>
</tbody>
</table>

a Urd, uridine; dThd, thymidine.

b Mean ± S.D. for triplicate cultures.

c Significantly different from control value (p < 0.001).

d Significantly different from each other (p < 0.001).

The inhibition of cell division by FUrd and its reversal by Urd* and dThd

Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>×10^-6 (cells/ml)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urd</td>
<td>0.91 ± 0.15^a</td>
<td>100</td>
</tr>
<tr>
<td>Urd</td>
<td>0.85 ± 0.08</td>
<td>93</td>
</tr>
<tr>
<td>dThd</td>
<td>0.89 ± 0.05</td>
<td>98</td>
</tr>
<tr>
<td>Urd + dThd</td>
<td>1.01 ± 0.18</td>
<td>109</td>
</tr>
<tr>
<td>FUrd</td>
<td>0.12 ± 0.03^c,d,e</td>
<td>13</td>
</tr>
<tr>
<td>FUrd</td>
<td>0.35 ± 0.05^c,d,e,f</td>
<td>38</td>
</tr>
<tr>
<td>dThd</td>
<td>0.19 ± 0.03^c,d,e,f</td>
<td>21</td>
</tr>
<tr>
<td>FUrd + dThd</td>
<td>0.34 ± 0.04^c</td>
<td>38</td>
</tr>
</tbody>
</table>

a Urd, uridine; dThd, thymidine.

b Mean ± S.D. for triplicate cultures.

c Significantly different from control value (p < 0.01).

d,e Significantly different from each other (p < 0.01).

DISCUSSION

The results reported herein indicate that the mechanism of FUrd cytotoxicity may vary under different experimental conditions. Under conditions of continuous exposure, low concentrations of FUrd (e.g., 1 x 10^-5 M) were lethal (Chart 3A). The addition of 1 x 10^-4 M thymidine during both the 1-hr incubation with FUrd and the subsequent 1-hr incubation in analog-free medium had no effect on the inhibition of rRNA maturation (Chart 4B). The presence of 1 x 10^-4 M uridine, however, prevented the inhibition of rRNA maturation caused by FUrd (Chart 4C).

In contrast to the inhibition of rRNA maturation caused by a 1-hr exposure to 1 x 10^-5 M FUrd, even prolonged incubation (e.g., up to 72 hr) in 1 x 10^-7 M FUrd had no effect on the ability of Novikoff cells to synthesize or process rRNA (Chart 5).

The addition of 1 x 10^-5 M thymidine prevented the growth-inhibitory effects of 1 x 10^-4 M FUrd (Chart 3B), suggesting that continuous exposure to low concentrations causes a thymineless death similar to that reported for FUrd (23, 27) and FUra (27). Continuous exposure to 1 x 10^-7 M FUrd did not inhibit rRNA maturation (Chart 5), further indicating that the primary site of FUrd action, under conditions of continuous exposure to low analog concent-

synthesis caused by a 1-hr exposure to 1 x 10^-5 M FUrd did not correlate with their effects on the cytotoxicity of FUrd, it seemed possible that there might be a correlation between cytotoxicity and RNA metabolism. Cells were exposed to 1 x 10^-5 M FUrd for 1 hr, washed, incubated for 1 hr in analog-free medium, and labeled for 90 min with [8-^3H]guanosine. A control culture, which received no additives during the 1st or 2nd 1-hr incubations, was labeled for 90 min with [2-^14C]uridine. ^3H-Labeled RNA from the drug-treated cultures and ^14C-labeled RNA from the control cultures were extracted and coelectrophoresed on polyacrylamide gels. The distribution of radioactivity is shown in Chart 4A. During the 90-min pulse, very little ^3H was incorporated into 28 S and 18 S mature rRNA, whereas in the ^14C-labeled RNA, these were the predominantly labeled species. The addition of 1 x 10^-4 M thymidine during both the 1-hr incubation with FUrd and the subsequent 1-hr incubation in analog-free medium had no effect on the inhibition of rRNA maturation (Chart 4B). The presence of 1 x 10^-4 M uridine, however, prevented the inhibition of rRNA maturation caused by FUrd (Chart 4C).

The inhibition of cell division by FUrd and its reversal by Urd* and dThd

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>×10^-5 (cells/ml)</th>
<th>% of control</th>
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<tbody>
<tr>
<td>None</td>
<td>2.22 ± 0.04^a</td>
<td>95</td>
</tr>
<tr>
<td>FUrd</td>
<td>1.19 ± 0.03^b</td>
<td>97</td>
</tr>
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a Mean ± S.D. for triplicate cultures.

b Not significantly different from control value (p < 0.03).
trations, is the inhibition of thymidylicate synthetase.

Interestingly, $1 \times 10^{-4}$ M uridine also prevented the growth inhibitory effects of continuous exposure to $1 \times 10^{-7}$ M FUrD (Chart 3C). The effect of uridine is difficult to explain, since uridine did not reverse the inhibition of DNA synthesis caused by FUrD (Tables 1 and 2). However, the ratio of uridine to FUrD was $10^3$ in the growth inhibitory studies and only 10 in the nucleoside incorporation studies. Since uridine and FUrD are probably both transported by the same active transport system, the effectiveness of uridine in the growth inhibitory studies may be due to a simple competitive inhibition of FUrD transport into the cell by the large excess of uridine. The fact that $1 \times 10^{-4}$ M uridine does not prevent the cytotoxicity of $1 \times 10^{-7}$ M FUrD (27) supports this interpretation.

Brief (e.g., 1 hr) exposure to $1 \times 10^{-7}$ M FUrD did not affect the subsequent growth of cells cultured in analog-free medium (Table 5). Despite a significant inhibition of thymidylicate synthetase activity after a 1-hr exposure to $1 \times 10^{-7}$ M FUrD (Chart 1), this treatment did not inhibit the incorporation of $[8-3H]$deoxyguanosine into DNA (Chart 2). Even at high concentrations of FUrD (e.g., $1 \times 10^{-5}$ M), where a 1-hr preincubation with the analog completely prevented $[6-3H]$deoxyuridine incorporation into DNA, there was a significant, although depressed, incorporation of $[8-3H]$deoxyguanosine into DNA. Margolis et al. (16) have shown that $[6-3H]$deoxyuridine incorporation into DNA relates better to thymidylicate synthetase activity than to the rate of DNA synthesis. It is possible that there is enough thymidine present in the growth medium to support a decreased level of DNA synthesis, even in the absence of the formation of thymidylicate from deoxyuridylate.

Similar results have been reported by Bosch et al. (2), who found that, in Ehrlich ascites tumor cells, the incorporation of $[2-14C]$uracil into DNA thymine was much more sensitive to the fluorinated pyrimidines than the incorporation of $32P$ (2).

The addition of $1 \times 10^{-4}$ M thymidine reversed the inhibition of $[8-3H]$deoxyguanosine incorporation with DNA caused by a 1-hr exposure to $1 \times 10^{-5}$ M FUrD, regardless of whether it was added during the incubation with the analog (Table 1) or after removal of the analog (Table 2). However, the addition of $1 \times 10^{-4}$ M thymidine did not prevent the growth inhibition caused by a 1-hr incubation with $1 \times 10^{-5}$ M FUrD in either of these circumstances (Tables 3 and 4). These results suggest that, under these conditions, the inhibition of DNA synthesis is not the only important site of FUrD action. The presence of $1 \times 10^{-4}$ M uridine during a 1-hr exposure to $1 \times 10^{-6}$ M FUrD prevented the growth inhibitory effect of the analog (Table 3), despite the fact that this treatment did not prevent the inhibition of $[8-3H]$deoxyguanosine incorporation into DNA (Table 1). Since $1 \times 10^{-4}$ M uridine, but not $1 \times 10^{-4}$ M thymidine, prevented the inhibition of rRNA maturation caused by a 1-hr exposure to $1 \times 10^{-5}$ M FUrD (Chart 4), it seems likely that effects on RNA metabolism may contribute significantly to analog toxicity under these conditions. Transferring cells that had been incubated with $1 \times 10^{-5}$ M FUrD for 1 hr into analog-free medium supplemented with $1 \times 10^{-4}$ M uridine did not effectively reverse the inhibition of cell division (Table 4), suggesting that the effects of the analog on RNA metabolism, once established, are irreversible. Whether the primary target of FUrD is the inhibition of RNA maturation or some other aspect of RNA metabolism cannot be determined from our data. However, the blockade of RNA formation is certainly a sufficient cause for the cessation of cell division (32).

The inhibition of rRNA maturation may also be an important factor in the cytotoxic activity of other fluorinated pyrimidines, especially FUrA. Hahn and Mandel (8) have shown in Bacillus cereus that FUrA inhibits the incorporation of $[14C]$guanine into both DNA and ribosomes. Thymine, which completely eliminates the inhibition of DNA synthesis, has no effect on the decreased doubling time caused by FUrA. Uracil, which has no effect on the inhibition of DNA synthesis caused by FUrA, restores the doubling time to normal. Interestingly, uracil, but not thymine, prevents the inhibition of normal ribosome formation caused by FUrA. Although less effective than FUrD on a molar basis, FUrA also inhibits rRNA maturation in Novikoff hepatoma cells (30). The extent of FUrA conversion to ribonucleotides and RNA correlates well with drug-promoted survival of mice inoculated with a variety of transplantable leukemias (15). It has been assumed that the level of ribonucleotide formation reflects the level of FdUMP formation. However, FdUMP can be formed from FUrA without the intermediate formation of ribonucleotides via deoxouridine phosphorylase and thymidine kinase (25). The increased sensitivity of tumor cells with a high capacity for FUrA conversion to ribonucleotides may be related to increased inhibition of RNA metabolism in the sensitive cells.

The therapeutic effectiveness of FUrD against murine leukemias is related to low levels of uridine phosphorylase (13). This enzymatic deficiency decreases the conversion of FUrD to the less potent FUrA. Resistance to FUrD is characterized by the deletion of uridine kinase, which obviates the conversion of FUrD to ribonucleotides. These FUrD-resistant cells are cross-resistant to FUrA and FdUrD. Four different Ehrlich ascites tumor cell lines, which are cross-resistant to FUrA, FdUrD, and FUrD, are also deficient in uridine kinase activity (21). Both murine leukemia cells (13) and Ehrlich

**Mechanism of FUrd Toxicity**

Chart 5. Effect of $1 \times 10^{-7}$ M FUrd on rRNA maturation. Cells, which had grown to a density of $0.82 \times 10^{9}$/ml, were incubated for 72 hr with $1 \times 10^{-7}$ M FUrd, after which time they were harvested by centrifugation and resuspended in one-fifth of the original volume of Medium S-69. The cells were then incubated with $[8-3H]$guanosine (1 $\mu$Ci/ml) for 90 min. RNA was extracted and analyzed by polyacrylamide-agarose gel electrophoresis.

![Chart 5: Effect of FUrd on rRNA maturation.](chart5.png)
ascites cells (21), which are cross-resistant to FUra and FdUrd but sensitive to FdUrD, contain significant uridine kinase activity.

Despite these data implicating ribonucleotide formation as an important determinant in the antitumor activity of FUra and FdUrd, increasing the conversion of FUra to ribonucleotides by coadministration of ribose donors, such as glucose or inosine, has little effect on the drug-promoted survival of a tumor-bearing host in either mice (14) or man (4, 9). However, these studies may reflect increased host toxicity rather than lack of increased inhibition of tumor cell division. In some of these experiments, drug-treated mice died despite the apparent elimination of all tumor cells (14).

These results have important clinical implications for the potential therapeutic use of FdUrd and possibly the other fluorinated pyrimidines. Heidelberger (11) has suggested that the extreme toxicity of FdUrd, relative to FUra and FdUrd, may be due to its facilitated uptake and efficient incorporation into RNA, whereas most of its antitumor effect is related to the inhibition of DNA synthesis. If this is the case, the administration of uridine may be useful in patients receiving high doses of methotrexate (7). The fact that tumor cells which have developed resistance to FUra or FdUrd often remain sensitive to FdUrd suggests that the latter may be an effective means of treating neoplasms that are refractory to FUra.

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The Mechanism of 5-Fluorouridine Toxicity in Novikoff Hepatoma Cells

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