Enhancement of the Incorporation of 5-Fluorodeoxyuridylate into DNA of HL-60 Cells by Metabolic Modulations

Masao Tanaka, Kiyoji Kimura, and Shonen Yoshida

Nagoya National Hospital, Department of Medicine, 4-1-1, Naka-ku, Nagoya, 460 [M. T., K. K.], and Department of Biochemistry, Institute for Developmental Research, Aichi Prefecture Colony, Kasugai, Aichi, 480-03 [S. Y.], Japan

ABSTRACT

The exposure of HL-60 human promyelocytic leukemia cells to 0.5 \( \mu M \) 5-fluoro-2'-\([3H]\)deoxyuridine (FdUrd) for 16 hr resulted in the incorporation of 5.14 ± 0.31 (S.D.) \( \times 10^{-7} \) mol FdUrd into DNA per mol of DNA nucleotide, which corresponds to 0.146 ± 0.082 pmol FdUrd per 10\(^7\) cells. Pretreatment with 50 \( \mu M \) deoxythymidine for 24 hr led to a 2.7-fold increase in the incorporation of this analogue into newly synthesized DNA during the ensuing 16-hr exposure to 0.5 \( \mu M \) [\( \text{H} \)]FdUrd. Pretreatment with 0.5 \( \mu M \) methotrexate for 3 hr also increased the [\( \text{H} \)]FdUrd incorporation into newly synthesized DNA approximately 5-fold. The coexistence of deoxythymidine or methotrexate with [\( \text{H} \)]FdUrd, however, led to decreased incorporation of FdUrd into DNA.

INTRODUCTION

FUra\(^3\) and FdUrd are well-known clinical anticancer agents. Their active metabolite is generally thought to be FdUMP, which is a potent inhibitor of thymidylate synthetase (3, 7). On the other hand, FdUrd and FdUrd are converted to 5-fluorouridine monophosphate and subsequently incorporated into RNA of mammalian cells via its triphosphate form (12, 18). This 5-fluorouridine monophosphate-containing RNA is also deleterious to cell growth.

A third possible mechanism, incorporation into DNA, has not been proven until recently, except in the case of a bacteriophage, which normally contains uracil in place of thymine in its DNA. Dannenberg et al. (5), using L1210 murine leukemia cells, demonstrated that detectable amounts of FdUrd can be incorporated into DNA. The presence of FUra residues in DNA has been demonstrated recently in other cell lines (9, 10). Further, the enhancement of FdUrd incorporation into DNA by concurrent incubation with dThd was demonstrated by Major et al. (11) using MCF-7 human breast carcinoma cells. Herrick et al. (9) reported that MTX treatment had little effect on the incorporation of FUra or FdUrd in DNA obtained from MCF-7 cells. Previously, we have shown that FdUTP can be incorporated into DNA as a substrate for mammalian DNA polymerases (17). Here, we extended our work to the incorporation of the fluorinated pyrimidine into DNA in vivo.

We also detected a significant amount of incorporation of FdUMP into DNA of human promyelocytic leukemia cells (HL-60) incubated with [\( \text{H} \)]FdUrd. More importantly, it was found that the incorporation was enhanced by the pretreatment of the cells with either dThd or MTX.

MATERIALS AND METHODS

Cell Culture. Human promyelocytic leukemia cells (HL-60), transferred twice weekly, were maintained as suspension culture in a 5% CO\(_2\) atmosphere at 37°C in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.), 100 units of streptomycin per ml, and 100 \( \mu g \) of penicillin per ml. In all experiments to be described, 75-cm\(^2\) tissue culture flasks (Corning Glass Works, Corning, N. Y.) were inoculated with 30 ml of cell suspension at 10\(^5\) cells/ml.

Chemicals. dThd was obtained from P-L Biochemicals Inc., Milwaukee, Wis. MTX was purchased from Lederle Laboratories, Pearl River, N. Y. FUra and FdUrd were supplied from Mitsui Pharmaceuticals Inc., Tokyo, Japan. 6-\([\text{H}]\)Fu (specific activity, 20 Ci/mmol) was obtained from Moravek Biochemicals, City of Industry, Calif. 6-\([\text{H}]\)Fu (specific activity, 3.83 Ci/mmol) was obtained from Amersham International Ltd. (Amersham, United Kingdom). FdUrd was purchased from Sigma Chemical Co., St Louis, Mo. Thymidylate synthetase, prepared from MTX-resistant Lactobacillus casei by the methods of Crusberg (4), was obtained from New England Enzyme Center, Tufts University School of Medicine, Boston, Mass. The enzyme preparation formed 8.2 \( \mu \)mol TMP per hr per mg protein at pH 7.0 and 30°C. Snake venom phosphodiesterase was from Miles Laboratories, Elkhart, Ind. Bovine pancreatic DNase was from P-L Biochemicals. 5,10-Tetrahydrololate was prepared nonenzymatically by the method of Moran (14).

Incorporation of [\( \text{H} \)]FdUrd and [\( \text{H} \)]Fu into Nucleic Acids. HL-60 cells in logarithmic growth phase were washed twice with 0.01 M Na\(_2\)PO\(_4\) (pH 7.3) containing 0.15 M NaCl (phosphate-buffered saline) and were suspended at 1.5 \( \times 10^7\) cells/ml in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum. The cells were incubated with [\( \text{H} \)]FdUrd (10 \( \mu \)Ci/ml; 0.5 \( \mu M \)) or [\( \text{H} \)]Fu (10 \( \mu \)Ci/ml; 2.6 \( \mu M \)) for a period varying from 4 to 16 hr. To determine the relative rates of synthesis of RNA and DNA, the cells were incubated simultaneously with H\(_2\)PO\(_4\) (10 \( \mu \)Ci/ml; carrier free; New England Nuclear, Boston, Mass.) in the same flasks or in separate flasks incubated in parallel. After incubation, the cells were washed twice with 20 ml of phosphate-buffered saline and then counted in a Model Z Coulter Counter.

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\(^2\) To whom requests for reprints should be addressed, at Nagoya National Hospital, Hematological Disease Center, Nagoya, Japan.

\(^3\) The abbreviations used are: FUra, 5-fluorouracil; FdUrd, FdUrd-5-fluoro-2'-deoxyuridine; FdUMP, 5-fluoro-2'-deoxyuridine monophosphate; dThd, deoxythymidine; MTX, methotrexate; FdUTP, 5-fluoro-2'-deoxyuridine triphosphate.
Relative rate of incorporation of fluorinated pyrimidine into the newly synthesized DNA or RNA was estimated from the ratio of $^{3}H/^{32}P$. The sedimentation patterns were reproducible through 2 or 3 independent experiments, and the representative results are shown in the charts and tables.

Identification of FdUMP in the DNA Region. Hydrolysis of the DNA to nucleotides was carried out with bovine pancreatic DNase and snake venom phosphodiesterase according to the method of Singer (15). FdUMP in hydrolysate was measured by the method of Danenberg et al. (5). In brief, aliquots of the DNA hydrolysates were incubated with 37 nmol of dTMP synthetase, 0.1 M Tris-HCl, pH 7.5, 0.1 mM 5,10-methylene-tetrahydrofolate, 20 mM β-mercaptoethanol in a final volume of 1.0 ml for 2 hr at 32°C. After the incubation, 1 ml of a charcoal suspension (0.1 g of charcoal, 2.5 mg/ml of bovine serum albumin and high-molecular-weight dextran (0.1 mg/ml) in 0.1 N HCl) was added. The mixture was centrifuged for 20 min at 4400 × g, and 1.4 ml of the supernatant was counted for radioactivity in Aquasol (New England Nuclear).

RESULTS

Incorporation of $[3]H$FdUrd into DNA. As shown in Chart 1, a substantial amount of $[3]H$FdUrd was incorporated into DNA by the exposure of logarithmically growing HL-60 cells to 0.5 μM $[3]H$FdUrd. The amount of incorporation increased linearly with the incubation time.

The incorporation was proportional to the amount of DNA synthesized, since the ratio of $[3]H$FdUrd to the newly synthesized DNA ($^{3}H/^{32}P$) in the DNA band was nearly constant through exposure (Chart 1). After 16 hr of continuous exposure, 10⁶ cells incorporated 0.131 pmol of $[3]H$FdUMP into DNA, corresponding to 5.14 × 10⁻⁷ mol $[3]H$FdUMP into 1 mol total DNA nucleotides estimated from UV adsorption at 260 nm. As clearly shown in Chart 1, a larger amount of radioactivity was incorporated into RNA. The incorporation was also time dependent. This incorporation may have resulted from catabolic conversion of FdUrd into FUra, followed by phosphorylation into FUTP, a substrate for RNA synthesis.

Enhancement of $[3]H$FdUrd into DNA by dThd Pretreatment. As shown in Chart 2, the preincubation with 0.1 mM dThd for 3 hr resulted in a 2-fold increase in the incorporation of FdUrd into DNA. The enhancement of incorporation was also measured under various conditions, i.e., longer preincubation time and various concentrations of dThd (Table 1).

As listed in Table 1, the incorporation of $[3]H$FdUrd into DNA per cell increased 3-fold by the preincubation with 0.05 mM dThd for 24 hr. The rate of incorporation of FdUrd into the newly synthesized DNA ($^{3}H/^{32}P$) also increased 2.7-fold by the pretreatment of the cells with 0.05 mM dThd for 24 hr. The incorporation of radioactivity into RNA decreased only slightly by the pretreatment with 0.1 mM dThd (Chart 2B).
Enhancement of Incorporation of \(^{[3]}\text{H}\)FdUrd into DNA

Chart 2. Enhancement of the incorporation of \(^{[3]}\text{H}\)FdUrd into nucleic acids of HL-60 cells by the pretreatment with dThd or MTX. Experimental conditions were the same as in the legend to Chart 1 except that, in B, cells were preincubated with 0.1 mM dThd for 3 hr and, in C, the cells were preincubated with 0.5 mM MTX for 3 hr. After the pretreatment, the cells were washed and then incubated with 0.5 mM \(^{[3]}\text{H}\)FdUrd and \(H_2^{32}\text{PO}_4\) (10 \(\mu\text{Ci/ml})\) for 16 hr. Control experiment without preincubation is shown in A. \(\alpha\) \(^{3}\text{H}\) per 10\(^7\) cells; \(\alpha\) \(^{32}\text{P}\) per 10\(^7\) cells.

Table 1

<table>
<thead>
<tr>
<th>Preincubation with dThd (mM)</th>
<th>(^{[3]}\text{H})FdUrd or (^{[3]}\text{H})Fura Analogue</th>
<th>pmol incorporated into DNA</th>
<th>Viable cell no. ((\times 10^7))</th>
<th>pmol incorporated/10(^7) cells</th>
<th>(^{32}\text{P}/^{32}\text{P}) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (5)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>0.469</td>
<td>2.7</td>
<td>0.174 (1.0)</td>
<td>1.00a</td>
</tr>
<tr>
<td>1.0 (3)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>1.054</td>
<td>2.8</td>
<td>0.375 (2.15)</td>
<td>2.16</td>
</tr>
<tr>
<td>0.05 (24)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>1.335</td>
<td>2.8</td>
<td>0.241 (1.39)</td>
<td>0.96</td>
</tr>
<tr>
<td>0.1 (24)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>0.905</td>
<td>2.0</td>
<td>0.521 (2.99)</td>
<td>2.74</td>
</tr>
<tr>
<td>0.3 (24)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>0.501</td>
<td>2.1</td>
<td>0.245 (4.11)</td>
<td>0.63</td>
</tr>
<tr>
<td>1.0 (24)</td>
<td>(^{[3]}\text{H})FdUrd</td>
<td>0.441</td>
<td>2.1</td>
<td>0.210 (1.21)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

\(^{32}\text{P}/^{32}\text{P}\) ratio

<table>
<thead>
<tr>
<th>(^{[3]}\text{H})Fura</th>
<th>(^{[3]}\text{H})Fura</th>
<th>3.11</th>
<th>2.0</th>
<th>1.505 (1.00)</th>
<th>1.00a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (3)</td>
<td>(^{[3]}\text{H})Fura</td>
<td>3.05</td>
<td>1.2</td>
<td>2.54 (1.68)</td>
<td>1.05</td>
</tr>
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</table>

\(^{32}\text{P}/^{32}\text{P}\) ratio

Enhancement of \(^{[3]}\text{H}\)FdUrd Incorporation into DNA by MTX Pretreatment. Similarly, thepretreatment of HL-60 cells with 0.25 to 0.5 \(\mu\text{M}\) MTX for 3 hr prior to the incubation with 0.5 \(\mu\text{M}\) \(^{[3]}\text{H}\)FdUrd produced an enhanced incorporation of \(^{[3]}\text{H}\)FdUrd into DNA. Up to a 4-fold enhancement per cell and 5-fold enhancement per newly synthesized DNA \(^{[3]}\text{H}^{32}\text{P}\) were produced by 0.5 \(\mu\text{M}\) MTX. The incorporation was rather suppressed by pretreatment with a dose of MTX higher than 1 \(\mu\text{M}\) (Table 2). By pretreatment with 0.5 \(\mu\text{M}\) MTX, the incorporation of radioactivity into RNA was also enhanced (Chart 2C).

Suppression of \(^{[3]}\text{H}\)FdUrd Incorporation into DNA by the Persistent Coexistence of dThd or MTX. When the cells were

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incubated for 16 hr with [3H]FdUrd in the presence of 0.1 to 1.0 μM MTX, the incorporation of [3H]FdUrd into DNA was rather suppressed (Table 3). The coexistence of 0.1 μM dThd with [3H]FdUrd during the incubation did not alter the incorporation of [3H]FdUrd into DNA (Table 3).

Incorporation of [3H]FUra into DNA and RNA. From these experiments, it is clear that [3H]FUra can be incorporated into DNA. Then, a precursor of FdUrd, [3H]FUra, was also tested. As shown in Chart 3, a significant amount of radioactivity was incorporated into DNA fraction, although a much larger amount was incorporated into RNA. After a 3-hr pretreatment with 0.1 μM dThd, the incorporation into DNA per cell increased about 1.7-fold. However, the rate of incorporation into the newly synthesized DNA did not change (Table 1). The incorporation of [3H]FUra into RNA was markedly increased by dThd pretreatment (Chart 3) in agreement with Spiegelman et al. (16).

Identification of FdUMP in DNA. DNA was purified from the cells incubated with [3H]FdUrd for 16 hr. Treatment of the hydrolysat of DNA with excess dTMP synthetase and 5,10-methylenetetrahydrofolate resulted in the binding of at least 55% of the radioactivity found in the hydrolysate to the TMP synthetase. When the increasing amount of exogenous FdUMP was added to the incubation mixture which contained DNA hydrolysate, dTMP synthetase, and 5,10-methylenetetrahydrofolate, the binding of radioactivity to dTMP synthetase was inhibited (Chart 4). The addition of exogenous dTMP, dGMP, or dCMP, however, did not inhibit the binding of radioactivity to dTMP synthetase. The amount of FdUMP required to produce 50% inhibition of the binding of radioactivity to dTMP synthetase was 8 × 10⁻⁶ M.

**DISCUSSION**

We have shown in this paper that a substantial amount of FdUrd was incorporated into DNA of cultured human promyelocytic leukemia cells (HL-60). The rate of incorporation into DNA was calculated at 5.13 × 10⁻⁷ mol of FdUrd per mol of DNA nucleotide. This value is about 70 times higher than that obtained by Danenberg et al. (5), who used murine leukemia L1210 cells. The much higher incorporation into HL-60 DNA compared with that of L1210 cells might be due to the difference in the cells.

### Table 2
Enhancement of the incorporation of [3H]FdUrd into DNA by the pretreatment of HL-60 cells with MTX

<table>
<thead>
<tr>
<th>Preincubation with MTX (μM)</th>
<th>Analogues</th>
<th>pmol [3H]-FdUrd incorporated into DNA</th>
<th>Viable cell no. (x 10⁴)</th>
<th>pmol incorporated/10⁶ cells</th>
<th>³²P/³⁰P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]FdUrd</td>
<td>0.382</td>
<td>2.9</td>
<td>0.131 (1.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>0.25 (3)</td>
<td>[3H]FdUrd</td>
<td>0.323</td>
<td>1.1</td>
<td>0.293 (2.2)</td>
<td>1.79</td>
</tr>
<tr>
<td>0.5 (3)</td>
<td>[3H]FdUrd</td>
<td>0.383</td>
<td>0.8</td>
<td>0.501 (3.6)</td>
<td>5.08</td>
</tr>
<tr>
<td>1.0 (3)</td>
<td>[3H]FdUrd</td>
<td>0.667</td>
<td>0.2</td>
<td>0.353 (2.5)</td>
<td>1.33</td>
</tr>
<tr>
<td>10 (3)</td>
<td>[3H]FdUrd</td>
<td>0.012</td>
<td>0.1</td>
<td>0.120 (0.9)</td>
<td>0.352</td>
</tr>
</tbody>
</table>

Numbers in parentheses, relative increase of [3H]FdUrd incorporation per cell. Numbers in parentheses, hr.

### Table 3
Incorporation of [3H]FdUrd into DNA under the persistent existence of dThd or MTX

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>pmol [3H]-FdUrd incorporated into DNA</th>
<th>Viable cell no. (x 10⁴)</th>
<th>pmol incorporated/10⁶ cells</th>
<th>[³²P/³⁰P] ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³²P]FdUrd</td>
<td>0.443</td>
<td>2.5</td>
<td>0.177 (1.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>0.1 μM MTX + [³²P]FdUrd</td>
<td>0.049</td>
<td>1.5</td>
<td>0.032 (0.16)</td>
<td>0.14</td>
</tr>
<tr>
<td>0.3 μM MTX + [³²P]FdUrd</td>
<td>0.017</td>
<td>0.8</td>
<td>0.021 (0.12)</td>
<td>0.12</td>
</tr>
<tr>
<td>0.5 μM MTX + [³²P]FdUrd</td>
<td>0.018</td>
<td>0.3</td>
<td>0.053 (0.29)</td>
<td>0.16</td>
</tr>
<tr>
<td>0.1 μM dThd + [³²P]FdUrd</td>
<td>0.502</td>
<td>2.8</td>
<td>0.179 (0.01)</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Numbers in parentheses, relative increase of [³²P]FdUrd incorporation per cell. Numbers in parentheses, hr.

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Chart 3. Enhancement of the incorporation of [³²P]FUra into DNA and RNA by the pretreatment with 0.1 μM dThd. Experimental conditions were the same as described in Chart 1 except that, in B, the cells were preincubated with 0.1 μM dThd for 3 hr, washed, and then incubated with 2.6 μM [³²P]FUra and 10 μg/ml of H₃PO₄ for 16 hr, and in A, the cells were incubated with 2.6 μM [³²P]FUra for 16 hr without preincubation. •, [³²P] radioactivity per 10⁶ cells; ○, [³²P] radioactivity per 10⁵ cells.
Materials and Methods."

The binding was measured as described in "Materials and Methods." Also detected FUra or FdUrd residues in L1210 murine leukemia cells and MCF-7 human breast carcinoma cells following exposure to either FUra or FdUrd. They reported approximately 0.8 pmol [3H]FdUrd residues were incorporated into DNA of 10^7 MCF-7 cells, following 12 hr incubation with 0.1 μM [3H]-FdUrd. This amount of FdUrd incorporation is in the same order as that obtained in the present investigation.

By the treatment of the cells with 0.5 μM MTX, the incorporation of [3H]FdUrd into the newly synthesized DNA was enhanced 5-fold. The effect of pretreatment with MTX, a potent inhibitor of thymidylate synthetase by FdUMP, which competes with FdUTP for DNA polymerase. On the other hand, MTX has been reported to increase the level of dTTP up to 1000-fold over that in the control by the pretreatment of the cells with 0.3 mM dThd (data not shown). However, the incorporation of [3H]FdUrd into DNA was enhanced to a higher extent, resulting in a 2.7-fold enhancement of the rate of incorporation of this drug into newly synthesized DNA. At present, the metabolic change which induces this enhancement is not clear. Major et al. (11) observed the enhanced incorporation of FUra into DNA by concurrent incubation with dThd at concentrations much lower than those used here.

In conclusion, FdUrd can be incorporated into DNA, and the incorporation was augmented up to 4-fold by the pretreatment of the cells with MTX and dThd. This magnitude of enhancement might not be dramatic, but the enhanced incorporation of FdUrd and FUra by the metabolic modulation may open up a new viewpoint regarding the combination therapy containing these drugs.

REFERENCES

M. Tanaka et al.


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