Potentiation of the Antitumor Activity of 5-Fluorouracil in Colon Carcinoma Cells by the Combination of Interferon and Deoxyribonucleosides Results from Complementary Effects on Thymidine Phosphorylase

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ABSTRACT

α-Interferon (IFNα) potentiates the cytotoxicity of 5-fluorouracil (5-FUra) in vitro, and the combination has clinical efficacy in advanced colorectal cancer. We have reported previously an IFNα-mediated elevation in cellular FdUMP levels accompanied by the stimulation of thymidine phosphorylase (TP) activity in extracts from HT-29 human colon carcinoma cells treated with IFNα. We have now found that this effect of IFNα can be measured in vivo as an increase in thymine incorporation in intact cells. The increase was only 3-fold, however, compared to the 10-fold increase seen in TP activity in cell extracts. This suggested that the cosubstrate for TP, deoxyribose-1-phosphate, was rate limiting in the cells. Since the synthetic pathway of TP can also proceed via a transerase reaction, natural and modified deoxyribonucleosides were tested as deoxyribose donors. TP activity was measurable in cell extracts using deoxyinosine as cosubstrate with either thymine or 5-FUra, although activity was only 10% of that measured with deoxyribose-1-phosphate. The pyrimidine analogue 5-propynyloxy-2'-deoxyuridine (PO-dUrd) had 15% of the maximal TP activity in cell extracts and also increased thymine incorporation in intact cells 10-fold. Both 2'-deoxyinosine and PO-dUrd potentiated the cytotoxicity of 5-FUra by 8-11-fold. IFNα potentiated the cytotoxicity of 5-FUra by 1-8-fold, and the combination of IFNα and PO-dUrd produced a 25-fold increase in the cytotoxicity of 5-FUra. Neither the corresponding analogue riboside, 5-propynyloxyuridine, nor the analogue base, 5-propynyloxuracil, had any effect on 5-FUra cytotoxicity. There was a significant correlation between the ability of a nucleoside and/or IFNα combination to increase thymidine incorporation and to reduce the 50% inhibitory concentration for 5-FUra. IFNα and PO-dUrd also potentiated the inhibition by 5-FUra of thymidylate synthase activity. These findings suggest that the use of a deoxyribonucleoside to provide the rate limiting cosubstrate would complement the stimulation of TP by IFNα, and together they should further enhance the antitumor activity of 5-FUra.

INTRODUCTION

IFNα potentiates the antitumor activity of 5-FUra in colon carcinoma in vitro, in animal models, and clinically (1-9). The enhancement of cytotoxicity by IFNα seen in vitro was accompanied by an increased conversion of 5-FUra to its active metabolite, FdUMP (10-11). The elevated metabolic activation of 5-FUra appears to be due to an IFNα-induced increase in TP activity, the first enzyme in the pathway for the direct conversion of 5-FUra to FdUMP and a greater inhibition of TS activity.

The increase in cellular FdUMP levels accompanied by the stimulation of thymidine phosphorylase (TP) activity in extracts from HT-29 human colon carcinoma cells treated with IFNα was only 10% of that measured with deoxyribose-1-phosphate. The pyrimidine analogue 5-propynyloxy-2'-deoxyuridine (PO-dUrd) had 15% of the maximal TP activity in cell extracts and also increased thymine incorporation in intact cells 10-fold. Both 2'-deoxyinosine and PO-dUrd potentiated the cytotoxicity of 5-FUra by 8-11-fold. IFNα potentiated the cytotoxicity of 5-FUra by 1-8-fold, and the combination of IFNα and PO-dUrd produced a 25-fold increase in the cytotoxicity of 5-FUra. Neither the corresponding analogue riboside, 5-propynyloxyuridine, nor the analogue base, 5-propynyloxuracil, had any effect on 5-FUra cytotoxicity. There was a significant correlation between the ability of a nucleoside and/or IFNα combination to increase thymidine incorporation and to reduce the 50% inhibitory concentration for 5-FUra. IFNα and PO-dUrd also potentiated the inhibition by 5-FUra of thymidylate synthase activity. These findings suggest that the use of a deoxyribonucleoside to provide the rate limiting cosubstrate would complement the stimulation of TP by IFNα, and together they should further enhance the antitumor activity of 5-FUra.

MATERIALS AND METHODS

Cell Culture. HT-29 human colon carcinoma cells (25) were maintained in RPMI 1640 with 10% FBS (GIBCO) in 5% CO2 and were free of Mycoplasma contamination.

Synthesis of the 5-(2-Propynyloxy)pyrimidines. PO-dUrd and PO-Urd were prepared as described previously (Refs. 26 and 27, respectively). Essentially, the same approach was used to synthesize the hitherto unreported PO-Ura, i.e., treating an aqueous solution of the monosodium salt of 5-hydroxyuracil with an excess of 3-bromopropyne. The resulting PO-Ura was isolated by preparative high performance liquid chromatography on a C-18 reversed phase silica gel column with 20% methanol as the eluting solvent; m.p. > 250°C (darkens and shrinks above 200°C), 1H NMR (methyl sulfoxide-d6), δ 11.27 (1H, bs, N3-H), 10.57 (1H, bs, N1-H), 7.21 (1H, d, H-6, J6,NH = 3.3 Hz), 4.60 (dr-1-P as a cosubstrate (12); alternatively, the enzyme can transfer a deoxyribose moiety from one deoxyribonucleoside to a pyrimidine base to form a second deoxyribonucleoside (13-15). The deoxyribose transferase activity has been proposed to proceed by both direct and indirect mechanisms; the latter involves an "enzyme-bound" dr-1-P intermediate (14, 16, 17). Kinetic studies with purified TP have shown that the initial velocity of the synthetic reaction is higher than that of the phosphorolytic reaction (17).

The gene for human TP has recently been shown to be identical to that for platelet-derived endothelial cell growth factor, an angiogenic factor that stimulates endothelial cell growth and chemotaxis (18, 19). Expression of TP was found to vary up to 15-fold in different human tissues and was elevated up to 10-fold in biopsies from carcinomas of the stomach, colon, and ovary when compared to normal parenchymal tissue of these organs (20, 21). Despite the presence of this enzyme in most cells, however, the utilization of thymine by cells is low (compared to thymidine incorporation) due to the lack of dr-1-P or deoxyribose donors (22). The addition of deoxyribonucleosides (both purine and pyrimidines) to intact cells greatly increases thymine incorporation. Since FdUMP can readily serve as a substrate for TP, it was anticipated that its conversion toFdUMP would also be suboptimal in IFNα-treated cells, despite the activation of the enzyme activity by IFNα.

5-FUra is thought to be cytotoxic to tumor cells by three potential mechanisms: inhibition of thymidylate synthase by FdUMP; incorporation of FdUTP into DNA; and incorporation of FUTP into RNA (reviewed in Ref. 23). The mechanism by which IFNα potentiates the actions of 5-FUra has been examined, and studies have implicated both DNA-mediated events and indirect or secondary effects on thymidylate synthase as potential targets (3, 4). Other IFNα-mediated activities for which there is experimental evidence include effects on DNA repair and the transcriptional upregulation of the TS gene (4, 24). These biochemical events would all potentially be further enhanced by increased cellular formation of FdUMP. We tested the hypothesis, therefore, that deoxyribonucleoside supplementation would lead to increased 5-FUra cytotoxicity when used in combination with IFNα due to complementary effects on TP, which presumably cause a further increase in the formation of fluoropyrimidine-nucleotides, including FdUMP, and a greater inhibition of TS activity.
Thymidine Phosphorylase Activity. HT-29 cells were treated with recombinant human α2-interferon (Hoffmann-La Roche) for 24 h at 500 units/ml, washed with PBS, and resuspended in 50 mM Tris-HCl (pH 7.5)-1 mM EDTA. The cells were sonicated on ice and centrifuged; the supernatant was stored at -70°C until assayed. TP activity was assayed by incubating cell extracts (100 μg) in 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 2.5 mM dR-1-P, and 0.25 mM [3H]thymine (20 μCi/ml) of either [6-3H]FUra (Moravek Biochemicals) or [methyl-3H]thymine (New England Nuclear). When indicated, deoxyinosine or PO-dUrd (both 2.5 mM) were substituted for the dR-1-P. Reactions were stopped at 40 min by boiling; tubes were briefly centrifuged, and supernatants were spotted on silica gel thin layer chromatography plates. Separation of 3H-bases from dR-deoxyribonucleosides was as previously described (28), and activity was calculated based on the percentage conversion of base to nucleoside. Activity was proportional to both the amount of protein assayed and time.

Incorporation of [6-3H]FUra into RNA and Acid-Insoluble Material. Cells were treated with [6-3H]FUra (2 μCi/ml; 1 μM) for 24 h in the presence of IFNa (500 units/ml) or PO-dUrd (150 μM). Cells were washed with PBS and treated with 1.5% ice-cold PCA; the pellet was washed two times with PCA. The pellet was then hydrolyzed with 1 M KOH (37°C for 16 h). The sample was recrystallized with PCA, and radioactivity in both the RNA (soluble) and acid-insoluble fractions was measured by liquid scintillation counting.

TS Activity. Measurement of TS activity was based on previously described procedures (29-31). Cells were washed twice with cold PBS and removed from the flasks by scraping in PBS. After a brief centrifugation, the cells were suspended in homogenization buffer (50 mM Tris-HCl (pH 7.4), 50 mM 2-mercaptoethanol, 10% glycerol, 0.1% Triton X-100, 15 mM CMP, 10 mM NaF, and 0.5 μg/ml each of leupeptin, aprotinin, pepstatin, and antitrypsin). The extract was sonicated two times for 10 s at 50% power and centrifuged (20 min at 12,000 rpm); the supernatant was used immediately in the assay for TS.

Thymine Incorporation. Cells were plated in 12-well culture plates with RPMI 1640 and 10% dialyzed FBS, and were treated with IFN for periods up to 72 h. The cells were then further incubated with either dino or PO-dUrd for 2 hours; during the last hour, either 3H-thymine or 3H-thymidine (1 μM; 1 μCi/ml) was added. Cells were washed twice with PBS, treated with ice-cold 5% TCA, and washed twice with TCA. The radioactivity in the pellet was determined by liquid scintillation counting.

To evaluate the nature of the interaction of FUra and PO-dUrd, the two compounds were tested alone and in combination at various concentrations but at fixed ratios (i.e., 1:50, 1:100, and 1:200). To facilitate these studies, growth inhibition was measured with continuous drug exposure for 7 days in 96-well plates (4 × 10³ HT-29 cells/well). Cell numbers were quantitated by staining with sulforhodamine B as described previously (32). The data were evaluated by median effects analysis (33-34) to calculate the combination index at each concentration of agents, expressed as the fraction of cells killed (fraction affected). Combination index values equal to 1.0 indicate drug additivity for the combination when compared to the two agents tested alone; values < 1.0 indicate drug synergy, and values > 1.0 indicate drug antagonism.

**RESULTS**

Effect of IFNa on Thymine Incorporation and TP Activity. The cytotoxic actions of FUra are function in part of its conversion to a nucleoside (catalyzed by TP) and ultimately to a nucleotide (by thymidine kinase or directly by a transerase). In a previous study, we had measured TP activity in cell extracts. In the present study, [3H]thymine incorporation was measured in intact cells as a way to assess TP activity in vivo. [3H]Thymidine incorporation (in cells) into acid-insoluble material was also determined relative to [3H]thymidine incorporation to control for potential effects on thymidine catabolism, thymidine kinase activity, and DNA synthesis, as well as unrelated effects on cell proliferation. The thymine:thymidine incorporation ratio would overestimate the change in phosphorylase activity, however, if there were a concurrent decrease in thymidine transport. Hence, the true increase in thymine utilization in vivo likely is somewhere between the values expressed as absolute thymine incorporation and those expressed as the ratio of incorporation of thymine to thymidine.

The utilization of thymine was low in proliferating HT-29 cells, representing just 0.6% of the incorporation of [3H]thymidine (Fig. 1). IFNα treatment for 24 h increased both the [3H]thymine incorporation and the [3H]thymidine:[3H]thymidine incorporation ratio in a concentration-dependent manner. At 500 units/ml IFNα, there was an approximately 3-fold increase in the absolute incorporation of [3H]thymine, and a 4-fold increase in the thymine:thymidine incorporation ratio (Fig. 1). The amount of [3H]thymine incorporation was maximal at 24 h of 500 units/ml IFNα treatment and remained elevated for up to 72 h (Fig. 24). The thymine:thymidine incorporation ratio continued to increase with IFNα treatment from 24 to 72 h, although the thymine:thymidine incorporation ratio also increased in the control cells from 24 to 72 h as the cells reached confluency (Fig. 28). Both the absolute thymine incorporation and the thymine:thymidine ratio...
were significantly greater in IFNa-treated cells compared to control (P < 0.05) at all time points examined.

Treatment of HT-29 cells with IFNa caused an elevation in TP activity in cell extracts that was detectable after 8 h of treatment and remained elevated for up to 72 h (Fig. 3). Enzyme activity was significantly higher in IFNa-treated cells at 24, 48 and 72 h (P < 0.05). The maximal increase was a 12-fold elevation in IFNa-treated cells when compared to control cells at 48 h, and similar increases were observed when activity was measured using either thymine or FUra as substrate in the presence of excess DR-1-P. As illustrated in Fig. 3, the increase in TP activity greatly exceeded that for thymine utilization in the IFNa-treated cells.

Effect of Deoxyribonucleosides on TP Activity. The poor incorporation of thymine in the presence of elevated TP enzymatic capability has been attributed to the insufficient conversion of thymine to thymidine due to an inadequate source of deoxyribose (22). Deoxyribonucleosides have been shown to be able to donate a deoxyribose moiety to a pyrimidine base via a TP-mediated transferase reaction. We first tested the capacity of three deoxyribonucleosides to serve as substrates for TP in cell-free extracts from IFNa-treated cells. The highest activity was observed using DR-1-P as substrate, and the rates of conversion of thymine and FUra to corresponding nucleosides were identical (Table 1). Thymidine had substantial substrate activity, approximately 50% of that of DR-1-P, while the capacity of dlno to donate deoxyribose to either thymine or FUra was found to be lower (approximately 10% of maximal). A deoxyribose analogue, PO-dUrd (Fig. 4), was also found to serve as a substrate for TP, having approximately 50% greater activity than dlno after correction for background activity in the absence of added cosubstrate. Both dlno and PO-dUrd were equally active with either thymine or FUra.

The deoxyribonucleosides enhanced the incorporation of [3H]thymine (Fig. 5A) and the thymine/thymidine incorporation ratio (Fig. 5B) when tested in intact cells (using a 2-h treatment). The increases were concentration dependent, and dlno and PO-dUrd produced statistically significant maximal increases of 17- and 10-fold in [3H]thymine incorporation, respectively. The two nucleosides had differing effects on [3H]thymidine incorporation (38% higher for dlno and 28% lower for PO-dUrd); consequently, the effects of the two nucleosides on the [3H]thymine/[3H]thymidine incorporation ratio were comparable (Fig. 5B). The fact that both agents had only minor effects on [3H]thymine incorporation, however, suggests that the high concentrations of dlno and PO-dUrd used did not compete for thymidine transport into the cells. Although IFNa treatment for 24 h increased thymine incorporation 2-fold, it did not further increase the extent of thymine incorporation produced by dlno and PO-dUrd (Fig. 5A). IFNa did have an additive effect on the thymine/thymidine incorporation ratio when combined with the deoxyribonucleosides (Fig. 5B), reflecting the reduced incorporation of thymidine in the presence of IFNa. Thymidine incorporation exceeded 15% relative to that of thymidine (a 20-fold increase compared to untreated control cells) at the highest concentration of PO-dUrd tested in combination with IFNa.

Potentiation of FUra Cytotoxicity. The effect of IFNa and various nucleosides on the growth inhibitory effects of FUra were evaluated in a clonogenic assay. Under the conditions used (72-h FUra treatment followed by growth in the absence of drug for 10 days), there was a 50% reduction in colonies formed with a concentration of 2 μM FUra (Fig. 6; Table 2). IFNa, used concurrently with FUra, lowered the IC50 for FUra to 1.1 μM. PO-dUrd and dlno (both at 150 μM) increased the cytotoxicity of FUra by 7.7- and 10.5-fold, respectively, and when used in combination with IFNa, by 25- and 17-fold, respectively.

Table 1. Effect of different cosubstrates on TP activity in HT-29 cell extracts

<table>
<thead>
<tr>
<th>Cosubstrate</th>
<th>FUra → FdUrd (%)</th>
<th>Thy → dThd (%)</th>
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<tbody>
<tr>
<td>DR-1-P</td>
<td>79 ± 2.1</td>
<td>74 ± 3.2</td>
</tr>
<tr>
<td>None</td>
<td>2.3 ± 1.2</td>
<td>2.4 ± 1.4</td>
</tr>
<tr>
<td>dThd</td>
<td>38 ± 7.9</td>
<td>41 ± 2.0</td>
</tr>
<tr>
<td>dlno</td>
<td>6.9 ± 0.8</td>
<td>8.4 ± 2.1</td>
</tr>
<tr>
<td>PO-dUrd</td>
<td>11.0 ± 1.4</td>
<td>11.1 ± 2.7</td>
</tr>
</tbody>
</table>

Thymidine Phosphorylase Activity

(pmole/μg/h)

Table 1 Effect of different cosubstrates on TP activity in HT-29 cell extracts

Cell extracts were prepared from HT-29 cells treated with IFNa (500 units/ml) for 72 h. Assays contained 2.5 μM of either [3H]FUra or [3H]thymine and the indicated cosubstrates at 2.5 μM. Enzyme activity was expressed as pmole/μg/h and as a percentage of maximal activity observed. Values are mean ± SE from at least three determinations.

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The cytotoxicity of FUra was blocked by thymidine (Fig. 6; Table 2), suggesting that FdUMP-mediated inhibition of TS activity was the predominant biochemical mechanism of action under the treatment conditions used. Neither IFNα nor PO-dUrd potentiated the cytotoxicity of FUrd (Table 2), suggesting that RNA-directed events were not involved for the actions of these two agents. Furthermore, the lack of effect of IFNα and PO-dUrd on FUrd cytotoxicity was consistent with TP as the site of action of both agents. This observation was strengthened by the observation that neither the riboside nor the base derivatives of PO-dUrd (PO-Urd and PO-Ura, respectively; see Fig. 4) had any effect on the growth inhibition of FUra.

PO-dUrd when used alone did not inhibit HT-29 clonogenicity at the concentrations used in the experiments described above, further suggesting that the net effect of the combination could be attributed to a modulating action of PO-dUrd on the activity of FUra. To examine the interaction in a more rigorous manner, FUra and PO-dUrd were combined at several different concentrations but at fixed molar ratios (i.e., 1:50, 1:100, and 1:200). Drug exposures in these experiments were continuous for 7 days and cell density was determined colorimetrically. Data were evaluated by the median effects analysis method (33–34) to calculate the combination index at each concentration of agents, expressed as the fraction of cells killed (fraction affected).

PO-dUrd alone had a concentration dependent but modest effect on cell proliferation with a maximal 23% inhibition observed at 300 μM PO-dUrd (data not shown). Combination index values obtained for PO-dUrd used with FUra/IFN were less than 1.0 at virtually all drug concentrations tested, providing strong evidence for drug synergy for the combination (Fig. 7). The degree of synergy increased as the molar concentration of PO-dUrd was increased relative to the concentration of FUra, with some suggestion of a maximal effect at a PO-dUrd:FUra ratio of 200:1.

The data obtained for the potentiation of FUra by either IFNα or a deoxycytobinucleoside were all consistent with increased conversion of FUra to FdUrd as the site of action. This hypothesis was evaluated quantitatively by correlating the effect of each of the modulating agents on thymine incorporation with their effect on the IC50 for FUra. There was a statistically significant (P < 0.05) inverse relationship found between the two cellular actions; an increase in the extent of thymine incorporation in intact cells with IFNα and/or deoxycytobinucleoside treatment was correlated (r = 0.86) with the decrease in the IC50 for FUra in the presence of the modulating agents (used at the same concentrations).

**Potentiation of TS Inhibition and [³H]FUra Incorporation.** The correlation observed between the activation of the TP-dependent metabolic pathway and the cytotoxicity of FUra suggest that FdUMP-mediated events are responsible for the growth-inhibitory activities observed. To test this hypothesis, TS activity was measured in extracts from cells treated for 24 h with IFNα, PO-dUrd, or the combination. The concentration of FUra used, 0.5 μM, was chosen to provide a modest inhibition of TS activity (Table 3). The addition of either IFNα or PO-dUrd further enhanced the inhibition of TS by FUra, and when used together, they produced a significantly greater inhibition than did FUra alone. As expected, neither IFNα nor PO-dUrd inhibited TS activity in the absence of FUra, and in fact, there was evidence that the combination increased TS activity by almost 2-fold. This suggests that this experiment may have underestimated the capacity of the combination to modulate the biochemical actions of FUra.

Fig. 4. Structures of the 5-(2-propynyloxy)pyrimidines.

Fig. 5. Effect of deoxyribonucleosides, alone and in combination with IFNα, on [³H]thymine utilization in intact cells. HT-29 cells were treated with (• and △) or without (○ and □) 500 units/ml IFNα for 24 h. The indicated concentrations of dlno (○ and □) or PO-dUrd (△ and △) were added, and after 1 h, either [³H]thymine or [³H]thymidine were added for an additional hour. [³H]Thymine incorporation (A) and the [³H]thymine:thymidine incorporation ratio (B) were determined as described in the legend to Fig. 1. Points, means of at least three experiments.

Fig. 6. Potentiation of the cytotoxicity of FUra by deoxycytobinucleosides alone and in combination with IFNα. HT-29 cells were plated in 24-well plates (150 cells/well) in RPMI 1640 with dialyzed serum. After allowing cell attachment to proceed overnight, the indicated concentrations of FUra in the presence or absence of IFNα and either dlno or PO-dUrd were added. Drug-containing medium was removed and replaced with RPMI 1640 with 10% FBS. Cell colonies were allowed to grow for an additional 10 days, at which point they were stained and counted. Cloning efficiency in the absence of drug averaged 60%. Treatments were: ○, FUra; △, FUra + IFNα (500 units/ml); □, FUra + PO-dUrd (150 μM); △, FUra + dlno (150 μM); △, FUra + IFNα + PO-dUrd; △, FUra + dlno (150 μM); ○, FUra + dUrd (150 μM). Points, mean ± SE from at least four experiments.
Data from three experiments, each assayed in duplicate.

Note: Significantly different from FUra alone, $P < 0.01$.

Table 3 Effect of deoxynucleosides and IFNα on TS activity
TS activity was measured in extracts prepared from cells treated for 24 h as indicated. Data from three experiments, each assayed in duplicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TS activity (pmol/10^6 cells) (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>5.49 ± 0.78 (100)</td>
</tr>
<tr>
<td>IFNα (500 units/ml)</td>
<td>7.46 ± 1.28 (136)</td>
</tr>
<tr>
<td>PO-dUrd (150 µM)</td>
<td>7.83 ± 1.75 (143)</td>
</tr>
<tr>
<td>IFNα + PO-dUrd</td>
<td>10.7 ± 2.93 (195)</td>
</tr>
<tr>
<td>FUra (0.5 µM)</td>
<td>3.51 ± 0.73 (64)</td>
</tr>
<tr>
<td>IFNα + FUra</td>
<td>2.73 ± 0.42 (50)</td>
</tr>
<tr>
<td>PO-dUrd + FUra</td>
<td>2.14 ± 0.58 (39)</td>
</tr>
<tr>
<td>IFNα + PO-dUrd + FUra</td>
<td>1.39 ± 0.32 (25)</td>
</tr>
</tbody>
</table>

* Significantly different from FUra alone, $P < 0.05$.

The incorporation of [3H]FUra into RNA and DNA was also evaluated (Table 4). PO-dUrd caused a modest increase in incorporation into RNA and had a somewhat greater effect on incorporation into acid-insoluble material (which includes DNA and TS-bound fluoropyrimidine). IFNα alone had no effect on incorporation, and when combined with PO-dUrd, enhanced [3H]FUra incorporation into acid-insoluble material but not the incorporation into RNA. Although this was consistent with our previous observations, the changes observed were not statistically significant.

DISCUSSION

In a previous study, we reported that IFNα increased a pyrimidine phosphorylase-like activity in HT-29 cells (10), and other experiments confirmed that TP was the activity stimulated by IFNα (data not shown). Although in these studies we also observed an increase in cellular FdUMP levels in cells treated with IFNα, the increase was only 2-fold compared to the 8-fold increase in enzyme activity found in cell-free extracts. We initiated the present investigation, therefore, to explore the reason for this discrepancy by measuring thymine utilization in both intact cells and cell-free extracts after IFNα treatment. These studies confirmed that IFNα increased thymine incorporation in a dose- and time-dependent fashion that, like FUra, was suboptimal when compared to the TP activity measured in cell extracts.

The use of nucleosides as deoxyribose donors in reactions catalyzed by TP has been previously described, with the rate of thymidine synthesis using pyrimidine deoxynucleosides higher than when purine deoxynucleosides were used (16, 22). In the experiments described in this study, we have shown that both natural and modified deoxyribo-nucleosides increase TP catalytic activity in both control and IFNα-treated cells and cell extracts. Furthermore, identical increases in the rate of conversion of FUra to its respective nucleoside were seen when compared to the endogenous substrate, thymine, under the various conditions examined. In previous studies, deoxyribose donors (e.g., deoxyinosine) have been reported to increase FdUMP formation from FUra and potentiate the inhibitory effects of FUra on TS activity and cell proliferation (35-36). Ribose donors (e.g., inosine) have been shown to increase FUra incorporation into ribonucleotides and RNA (37-39) and also potentiate the cytotoxicity of FUra in vitro (39) and the antitumor activity of FUra in vivo (40), although a second study showed no enhancement in vivo (36). Particu-
larly interesting was the finding that the degree of potentiation of FUra cytotoxicity by dino was similar to the potentiation by folic acid, and the use of dino and folic acid together produced an additive potentiation of the cytotoxicity of FUra (35).

There are many studies in a variety of cell types that implicate three potential sites of FUra action: inhibition of TS by FdUMP, incorporation of FdUTP into DNA, and incorporation of FUTP into RNA (23). Two determinants of the primary mechanism of action of FUra in a particular cell line appear to be the relative activity of enzymatic pathways that activate FUra to either ribo- or deoxyribonucleotides and the concentration and duration of FUra treatment. For example, the high sensitivity of lymphocytic cells to FUra was reported to be due to the high rate of FdUMP formation via thymidine phosphorylase (23). Two determinants of the primary mechanism of action of FUra were shown to be cytotoxic via inhibition of TS in human colon carcinoma cells (41). Similarly, low concentrations of FUra in the cells at relative low concentrations of FUra, while in less sensitive cells, FUra (at higher concentrations) acted through both TS- and RNA-mediated events (41). Potentially high concentrations of FUra were shown to be cytotoxic via inhibition of TS in human colon carcinoma cells, while cell kill with treatment with higher concentrations of FUra (for shorter times) was a consequence of RNA-mediated FUra effects (29). Our studies, in which the modulating agents reduced the IC50 for FUra to as low as 80 nm, suggested that both IFNα and PO-dUrd act by increasing FdUMP-mediated cytotoxic events. Although FdUMP levels were not directly measured, this hypothesis was supported by experiments in which there was potentiation by PO-dUrd but not PO-Urd, by the demonstration that the inhibitory effects of FUra but not FdUrd were increased and by direct evidence of the enhancement of TS inhibition.

The potentiation of the antitumor activity of FUra by IFNα was modest with the cells used in this study; others have shown similar or greater potentiation in other colon carcinoma cell lines (1, 3, 4). Substantially greater potentiation of FUra was seen with the deoxyribonucleoside analogue PO-dUrd, and the combination of IFN and PO-dUrd produced a dramatic increase in the cytotoxicity of FUra. It has been previously suggested that PO-dUrd was not a substrate for thymidine kinase (42); hence, it would not be expected to interfere with biochemical events requiring a nucleotide. The clinical potential of a three-way drug combination (FUra/IFN/PO-dUrd) is supported by the following observations: (a) the elevated levels of TP activity in human tumors suggest that this may be a basis for selectivity of the combination for tumor versus normal tissue. It would be of great interest to determine if the tumors were also selectively sensitive to the activation of TP by IFN; (b) PO-dUrd has no or very modest cytotoxic activity when used alone against a variety of cell lines (42–43), suggesting that there is no a priori reason why it could not be combined with cytotoxic agents clinically; and (c) the combination reduces the IC50 for FUra to a concentration that is readily achievable clinically under most FUra regimens and for an extended period of time.

FUra remains the only active cytotoxic agent for the treatment of advanced colon cancer. Clinical experience with the use of biochemical modulating agents such as leucovorin or interferon in combination with FUra suggest that manipulations designed to partially overcome inherent tumor resistance to FUra provide some therapeutic benefit, although the superiority of a particular regimen has not been unequivocally demonstrated (44, 45). Clearly, there is a need to further develop new agents and approaches and to determine how best to combine them to maximally increase the antitumor activity of FUra.

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