In Vitro Synergism between 5-Fluorouracil and Natural β Interferon in Human Colon Carcinoma Cells

Alessandra Guglielmi, Carlo Aschele, Ave Mori, Chiara Baldo, Patrizia Russo, Domizia Debernardis, Monica Valenti, Silvia Bruno, Monica Taverna, Riccardo Rosso, and Alberto Sobrero

Medical Oncology, Istituto Nazionale per la Ricerca sul Cancro, V.le Benedetto XV, 10, 16132 Genova, Italy

ABSTRACT

The combination of 5-fluorouracil (FUra) plus IFN-β was studied in vitro using a human colon carcinoma cell line, HCT-8. Continuous exposure to high concentrations of IFN-β is cytotoxic to these cells (ED₅₀ 600 ± 50 IU/ml). A strong synergism (P < 0.002) was observed when a short-term (1-h), high-concentration exposure to fluoropyrimidine (300 or 1000 μM) was followed by IFN-β given continuously. In fact, the mean ratio between the expected (product of the survival of each agent alone) and the observed clonogenic cell survival rates of the combination was 3.4 (range, 2.4–4.9). Longer exposures to the fluoropyrimidine (24 h, 7 days) produced less than additive effects with IFN-β, indicating strong schedule dependency for this synergism. The mechanism of interaction was studied at four levels. First, thymidylate synthase (TS) activity, inhibition, and recovery were measured by an in situ assay in cells treated with FUra, IFN-β, and their combination. When the prolonged infusion of IFN-β followed a 1-h exposure to FUra, the observed TS inhibition and recovery, at each exposure time, were similar to the expected values, indicating that the interactions between these drugs at the level of TS are not the determinant of the synergism. Second, cell cycle analysis was done. During the continuous exposure to IFN-β, a significant accumulation of HCT-8 cells in S-phase was observed at 24, 48, and 72 h compared to untreated controls (P = 0.003); however, under the same experimental conditions producing synergy in the clonogenic assay, no significant cell cycle perturbations were produced by the combination of FUra followed by IFN-β compared to those caused by each agent alone. Third, using the alkaline elution test, we also demonstrated that the synergism is not due to enhanced FUra-induced DNA single-strand break frequency in high molecular weight DNA. Finally, nucleic acid incorporation experiments, using tritiated FUra, showed that the cytokine, given continuously (300 IU/ml), enhanced the amount of FUra incorporated into nucleic acids 24 h after a 1-h exposure to 300 and 1000 μM of FUra. The median percentage of enhancement values were 31.6 ± 11.5% for the lower drug concentration and 18.4 ± 8.1% for the higher drug concentration tested. These results suggest that the mechanism of this synergism may be related to the ability of IFN-β to promote the incorporation of intracellular FUra metabolites into nucleic acids and/or to inhibit the cleavage of FUra nucleotides from RNA/DNA.

INTRODUCTION

Chemotherapy of colorectal cancer has met a phase of moderate success in the last decade. Biochemical modulation of FUra has improved the treatment of this disease substantially in the adjuvant setting (1, 2), but only marginally in the advanced stage (3). In this connection, IFN could be an additional powerful modulating agent of fluoropyrimidines, as suggested by the initial clinical report on this drug combination (4). Unfortunately, the enthusiasm elicited by such report has not been confirmed by subsequent studies (5, 6) that have pointed out the high toxicity of this regimen. In addition, recent randomized comparisons of FUra + IFN-α versus FUra alone (7) or FUra + leucovorin (8) have shown no advantage for the combination containing the cytokine. Therefore, FUra plus IFN-α is now meeting a phase of decline.

These negative results may reflect a lack of potentiation between these two agents. However, they might represent only a consequence of empirically designed regimens lacking a biochemical basis for the selection of drug schedules and doses. The interactions between FUra and modulating agents are very complex. FUra metabolism involves different activation pathways and this drug has multiple cellular targets. In addition, both experimental (9) and clinical data (10) suggest that the fluoropyrimidine may have different mechanisms of action depending on the schedule of administration. In general, this complexity has been overlooked in most clinical trials of biochemical modulation of FUra.

Despite a large number of data, derived from different cell lines, indicating that FUra cytotoxicity could be potentiated by IFNs in a dose- and schedule-dependent manner, the mechanism(s) of interaction of these two drugs is not known.

A review of the literature indicates that IFNs could increase FUra toxicity by: (a) depleting the cellular thymidylate pool (via reduction of thymidine uptake or thymidine kynase activity; Refs. 11 and 12); (b) increasing the formation of FdUMP (13);
The lack of a well-defined mechanism(s) of interaction of these two drugs may explain the difficulty to select a clinically promising regimen of IFN plus fluoropyrimidine and justify the failure of many clinical trials.

Prior to clinical testing, we have investigated the optimal in vitro scheduling of FUra and IFN-β (a molecule derived from human fibroblasts). The combination of short-term exposure to the fluoropyrimidine (1-h) followed by the continuous exposure to the cytokine showed strong synergism in terms of cell kill in a human colon carcinoma cell line, HCT-8. The mechanism of this interaction was investigated at the biochemical level, and results were obtained suggesting that IFN-β may enhance the activity of the antimetabolite by increasing its incorporation into nucleic acids.

MATERIALS AND METHODS

Chemicals. FUra and charcoal (activated, neutralized) were purchased from Sigma Chemical Co. (St. Louis, MO). 2’-5-[3H]deoxyuridine and [6-3H]FUra were purchased from Moravek Biochemicals (Brea, CA). Media and sera for cell culture were obtained from Grand Island Biological Co. (Grand Island, NY), and plasticware was obtained from Corning Glass Works (Corning, NY). IFN-β was supplied by Serono Pharma S.p.A. (Rome, Italy).

Cytotoxicity Assays. The human colon adenocarcinoma cell line HCT-8 was grown as a monolayer in 25-cm² sterile plastic flasks (Costar, Cambridge, MA) in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% horse serum and subcultured weekly. Under these conditions, the doubling time was approximately 18 h, and the cloning efficiency in monolayers was about 30%.

Drug-induced cell lethality was measured with a monolayer clonal growth technique as previously described (17). Each experimental point was determined in triplicate with four replicate controls; experiments were repeated at least twice.

The duration and sequence of exposures to different schedules of fluoropyrimidines and IFN-β are shown in Table 1. In combination experiments, care was taken to select concentrations producing a cell kill between 20 and 80%, so that synergy could be observed. The ratio between the product of the survival fraction of each agent alone (expected survival) and the survival fraction observed with the drug combination allowed detection and classification of the interactions between these two agents (18). Values greater than one were considered to show synergism, those less than one, antagonism, and those of about one, an additive effect.

To quantify the probability that synergism or antagonism assessed using this method is significant, these data were analyzed according to the method of Fleiss (19), which simultaneously evaluates differences between trends.

In Situ TS Activity. Inhibition of TS activity by either or both FUra and IFN-β was measured in intact cells according to the in situ assay of Yalowich and Kalman (20) as previously described (9). The assay is based on the displacement of tritium from 2’-5-[3H]deoxyuridine, since it is methylated by TS after cellular uptake and phosphorylation to deoxyuridylylate. When the effects of IFN-β on the stability of TS inhibition were investigated, cultures were provided with the cytokine (300 IU/ml) during the 48-h incubation following exposure to FUra. After background subtraction (i.e., medium without cells), the counts were fitted to a straight line by linear regression, and the percentage of inhibition was calculated by comparing the slope of treated cultures to that of controls (without the drug). Slopes in all assays fitted the linear regression model with a correlation coefficient of at least 0.9.

Incorporation of FUra into Nucleic Acids. Cells were cultured in 75-cm² flask for 72 h (approximately 10⁷ cells/flask) before carrying out the assay. The experiment was started replacing old medium with complete medium containing a range of concentrations (300–1000 μM) of radiolabeled FUra (specific activity, 1.5–5 μCi/mmol). Triplicate cultures for each experimental condition were set up for cell counting and received tissue culture medium containing cold FUra at the same concentration as in radiolabeled samples. At the end of the desired incubation time (1-h), cells were washed three times with normal saline and either immediately harvested or further incubated in drug-free-medium, with or without IFN-β (300 IU/ml) for 24–48 h. Cells were detached by 5-min trypsinization, washed with normal saline, and centrifuged for 5 min at 1000 × g. Nucleic acid extraction was started, resuspending the resulting pellet in 1 ml of 10% trichloroacetic acid (three washes in 1.5-ml Eppendorf tubes) followed by overnight incubation in 400 μl 1 N NaOH at 37°C. One hundred μl of each sample were counted with 5 ml scintillation cocktail (Picofluor 40; Packard, Meridian, CO) in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Results were converted to pmol of FUra and normalized for cell number as determined by counting triplicate cultures plated and treated at the same time as the cultures receiving the radioactive fluoropyrimidine. Additional cultures were also set up, on the day of initial plating, to test the effect of the interaction between short-term FUra and IFN-β on total cell growth inhibition measured on day 7 after drug exposure. This provided an internal control to ensure that synergy was present in the same experiment in which the pharmacodynamics was being studied.

The separation of label incorporated into DNA and RNA fractions was based on the Schmidt-Thanauser technique as modified by Perez et al. (21). Results were normalized to DNA and RNA content as determined using a variant of the Burton-diphenylamine reaction (22) and a modified orcinol reaction (23), respectively. Both RNA and DNA hydrolyzates were subjected to a Bradford protein assay (24). In no instance were contaminating proteins detected.

Flow Cytometric DNA Analysis. Flow cytometric DNA measurements were performed to determine the percentage of the cells in the various phases of the cell cycle. Cells were treated with FUra (1-h, short pulse), IFN-β (300 IU/ml continuously), and their combination as described for the in situ TS assay. At each time point, cells were harvested with trypsin and processed as previously described (25). Cell cultures were performed in duplicate and, from each culture, two samples for flow cytometry were prepared.

Measurement of DNA SSBs. DNA fragmentation was evaluated using the alkaline elution technique as described by...
Three schedules of FUra administration were selected for combination experiments: a 1-h treatment pulse, a 24-h exposure, and a prolonged exposure (7 days). These experimental conditions mimic the schedules of FUra administration most commonly used in the clinic: the bolus, the 24-h infusion, and the continuous infusion for longer than 2 weeks. The ED$_{50}$ values for the three schedules were 200 ± 14, 3 ± 0.5, and 1.5 ± 0.4 μM, respectively, indicating strong schedule dependency.

**Cytotoxicity of the Combination.** Table 1 shows that the pharmacological interactions between FUra and IFN-β are strongly dependent on the schedule of administration of the fluoropyrimidine. Synergistic cytotoxic interactions were detected only when a short-term (1-h) exposure to FUra was followed by a prolonged incubation with the cytokine. This phenomenon was also dependent on the FUra dose: at drug concentrations producing <50% of cell kill, the two agents were additive. When higher concentrations of FUra were used, very strong synergism was seen (Fig. 2), and the mean ratio between the expected and the observed clonogenic cell survival was 3.4 (range, 2.4–4.9; $P < 0.002$).

Longer exposures (24-h, 7-day) to the fluoropyrimidine resulted in less than additive interactions with IFN-β (given continuously following 24-h exposure to FUra or concurrently with 7-day exposure to FUra); the mean expected/observed survival ratios were 0.7 ± 0.08 and 0.4 ± 0.008 for the 24-h and 7-day exposure, respectively (data not shown).

Other fluoropyrimidines (5-fluorodeoxyuridine, 5-fluorouridine) failed to synergize with IFN-β when clinically relevant schedules of administration of the antimetabolites (prolonged exposure) were used (Table 1).

**In Situ TS Activity.** An in situ assay was used to study the effects of IFN-β on the time course of TS inhibition and recovery at different time points (0, 24, and 48 h) following a short term (1-h) exposure to high concentrations of FUra.

Complete inhibition of TS activity was not achieved at the end of a 1-h exposure to FUra alone, even when very high concentrations were used (50 and 60% inhibition at 300 and 1000 μM, respectively). Almost complete recovery of enzyme activity was observed at both concentrations after 24- and 48-h incubation in drug-free medium (Fig. 3).

Exposures to IFN-β alone for 24 and 48 h at concentrations producing synergism in cloning experiments (100 or 300 IU/ml) did not show any effect on TS activity. Exposure to IFN-β (300 IU/ml continuously) following short-term (1-h) FUra treatment (300 and 1000 μM), failed to affect the recovery of TS activity, as evidenced by the observed values of TS inhibition, approximating the expected values (Fig. 3, dashed lines). These results indicate that interactions at the level of TS inhibition do not play a relevant role in determining the synergism observed between these two agents.

**Incorporation of FUra into Nucleic Acids.** Incorporation of FUra nucleotides into nucleic acids of HCT-8 cells increased with increasing concentrations of the fluoropyrimidine. The amount of drug incorporated after a 1-h exposure to 1000 μM was approximately 2.5-fold greater than that obtained with 300 μM (524.25 ± 3.18 and 247.5 ± 46 pmol/10⁶ cells, respectively; data not shown). Specific fractionation of [³H]FUra incorporated into each species of nucleic acid resulted in undetectable levels of incorporation into DNA (<50 fmol/mg DNA).
Table 1  Effect of different schedules of fluoropyrimidines and IFN-β on clonogenic HCT-8 cell growth

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fluoropyrimidine concentration (μM)</th>
<th>Exposure time (h)</th>
<th>Concentration (IU/ml)</th>
<th>Exposure time</th>
<th>Synergism</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUra</td>
<td>100, 300, 1000</td>
<td>1</td>
<td>100, 300</td>
<td>Continuous</td>
<td>Yes (P &lt; 0.002)</td>
</tr>
<tr>
<td>FUra</td>
<td>0.3, 1, 3, 100</td>
<td>24</td>
<td>100, 300, 1000</td>
<td>Continuous</td>
<td>No</td>
</tr>
<tr>
<td>FUra</td>
<td>0.3, 1, 3</td>
<td>Continuous</td>
<td>100, 300, 1000</td>
<td>Continuous</td>
<td>No</td>
</tr>
<tr>
<td>FdUrd</td>
<td>0.001, 0.003</td>
<td>Continuous</td>
<td>30, 100, 300</td>
<td>Continuous</td>
<td>No</td>
</tr>
<tr>
<td>5-Fluorouridine</td>
<td>0.003, 0.01</td>
<td>Continuous</td>
<td>30, 100, 300</td>
<td>Continuous</td>
<td>No</td>
</tr>
</tbody>
</table>

Fig. 2  Survival of HCT-8 cells exposed to FUra for 1 h, IFN-β continuously, and their combination (FUra pulse followed by IFN-β). Columns, means ± SE of seven experiments. -- -- --, expected survival values of the combination, i.e., the product of the survival produced by each agent alone (see **Materials and Methods**).
obtained using a lower FUra concentration (300 μM; data not shown), indicating that even at lower levels of cell kill, no major kinetic interactions occurred between the two agents.

**Interaction of FUra and IFN-β at the Level of DNA.**
Since the synergism between the fluoropyrimidine and the cytokine appeared to be independent from an interaction at the level of TS and the enhanced incorporation was largely inferior to the extent of the synergism observed in the cytotoxicity experiments, we examined the possible effects of the cytokine on FUra-induced fragmentation of high molecular weight DNA using the alkaline elution.

No DNA SSBs in high molecular weight DNA were observed after treatment with FUra, IFN-β, or their combination under the conditions producing strong synergism in the proliferative assay (Table 3).

To rule out the elution artifact, the same experiments were repeated in the presence of proteinase K. Equivalent results were obtained. Fragmentation of high molecular weight DNA was not observed even when more prolonged elution conditions, allowing alkaline labile sites in DNA to become manifest, were used.

**DISCUSSION**
Enhancement of the growth inhibitory activity of FUra by IFN has been documented in several *in vitro* studies. However, drug concentrations were often selected to maximize synergistic interactions rather than to reproduce the clinical setting. More importantly, no attempts were made to study the possible relationship(s) between the development of synergistic interactions and the schedule of FUra administration.

In the present study, the cytotoxicity experiments were planned to reproduce the clinical situation. The duration of the exposures to the fluoropyrimidine was selected to reproduce the three schedules of FUra most commonly used for treating patients, i.e., a short-term bolus, a 24-h infusion, and prolonged infusion. In addition, care was taken to use FUra doses within the clinical range (25). Under these clinically relevant experimental conditions, strongly synergistic antiproliferative interactions were detected when a short-term, high-dose exposure to the fluoropyrimidine was followed by long-term exposure to IFN-β. Only additive, or less than additive, cytotoxic effects were seen when longer exposures to FUra were used.

The strict dependence of these synergistic interactions on FUra schedule is not surprising. We recently hypothesized that the fluoropyrimidine may have different mechanisms of action depending on the schedule of administration and that bolus FUra may work via incorporation into nucleic acids, while continuous infusion FUra may kill cells via a TS-dependent mechanism (9). As a corollary of this, we have also found that strong synergism occurs with the combination of bolus FUra followed by continuous exposure to the same agent (25). FUra should thus be regarded as two different drugs depending on the schedule of administration, and modulating/synergistic agents may develop specific interactions depending on the time of exposure to the fluoropyrimidine.

Inhibition of TS has been considered the primary site of interaction between FUra and IFN in several *in vitro* studies. Both an increased activation of the fluoropyrimidine to FdUMP, leading to enhanced TS inhibition, and a blockade of the salvage
pathway for thymidylate production (decreased uptake of thymidine and reduced thymidine kinase activity) have been reported to occur following IFN-α, -β, and -γ treatment (11–14). Although neither FdUMP levels nor thymidine kinase activity and thymidine uptake were directly measured in this study, in situ TS activity data rule out that major perturbations occur at this level. Higher FdUMP levels would result in more marked or more prolonged TS inhibition, while a decreased thymidine kinase activity would impair the uptake and metabolization of tritiated deoxyuridine leading to artifactual higher rates of TS inhibition.

Another study by Chu et al. (28) suggested that IFN-γ may abrogate the acute TS induction that has been reported to follow FURA exposure and that may restore thymidylate synthesis. We found no such phenomenon.

These contrasting results may rely on the different types of IFN used: β in the present study, and α and γ in the previous experiments. In addition, this discrepancy may reflect differences in the mechanisms of FURA action in HCT-8 cells as compared to the other cell lines.

Alternatively, these contrasting results may be explained by a difference in the schedules of drug administration. In the present study, exposure to FURA was followed by incubation with IFN-β, whereas both Elias and Crissman (14) and Chu et al. (28) used a concomitant exposure to both agents. More importantly, the previous studies involved long-term exposures
to FUra, while the present study focuses on the interactions between IFN-β and pulse FUra.

Major cytokinetic perturbations were reported to occur following treatment with the combination FUra plus IFN-β in two different human colon cancer cell lines (15). In addition, using the HCT-8 tumor model, we recently demonstrated that cytokinetic effects are the basis for the strongly synergistic cytotoxic interactions between pulse FUra and continuous exposure FUra (25). Based on these data, we analyzed the cell cycle distribution of cells treated with either of both FUra and IFN-β at concentrations producing synergistic interactions in cell survival experiments. No further increase in the S-phase fraction was detected following sequential treatment compared to that produced by each agent alone. These results indicate that the cytokine does not affect the cytokinetics of FUra-treated cells.

A tentative explanation for the synergism is provided by studies of FUra incorporation into RNA and DNA. Prolonged exposures to IFN-β were shown to enhance the amount of FUra incorporated into RNA. This effect was detected at the end of a 24-h exposure to the cytokine that followed a short-term treatment with FUra, suggesting that IFN-β may promote the incorporation of intracellular metabolites of FUra into nucleic acids and/or inhibit the cleavage of FUra nucleotides from RNA.

This issue is of great importance in view of a recent report showing that IFN-α may modulate incorporation/excision of FUra into DNA, resulting in enhanced DNA breaks and increased cytotoxic activity (29). The complete absence of DNA SSBs in high molecular weight DNA following treatment with FUra or FUra → IFN-β argues against the possibility that, in this experimental model, the fluoropyrimidine causes DNA lesions and that IFN-β may influence this phenomenon.

An enhancing effect on the uptake of FUra into RNA would represent a novel mechanism of interaction between the cytokine and the fluoropyrimidine. Support to this model is given by the strict dependence on a short-term, high-dose schedule of the fluoropyrimidine for the synergistic cytotoxic interactions to develop. In fact, we recently demonstrated that, in this cell line, pulse FUra leads to resistance through a decreased incorporation into RNA. It is thus conceivable that FUra incorporation into RNA is also a major determinant of pulse FUra cytotoxicity. As a consequence, potentiation of the antiproliferative effect of pulse FUra by modulating agents is likely to depend on the enhancement of the RNA uptake of this drug.

These results have important clinical implications. If the strict schedule dependency for this synergism holds under clinical circumstances, one would suggest that modulation with IFN-β might be advantageous only in combination with bolus FUra. In addition, the lack of interaction at the level of TS inhibition would suggest that IFN-β may not further improve the cytotoxicity of the combination FUra plus leucovorin. Our recent clinical experience using double modulation of FUra with IFN-α plus leucovorin in patients with advanced colon cancer is compatible with this speculation (30).

Finally, the enhanced FUra incorporation into nucleic acids obtained with IFN-β supports the combined use of the cytokine with other modulating agents that may channel the fluoropyrimidine into RNA, such as methotrexate and N-phosphoacetyl-L-aspartate. Based on these results, following our encouraging experience with sequential bolus FUra, modulated by methotrexate, alternated to continuous infusion FUra modulated by leucovorin in advanced colorectal cancer (31), we have incorporated IFN-β into the “bolus part” of this alternating regimen to further pursue clinically our new concept of schedule-oriented biochemical modulation (25).

**REFERENCES**


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**Table 3** DNA SSB frequency observed in HCT-8 cells treated with FUra, IFN-β (300 IU/ml), and their combination

<table>
<thead>
<tr>
<th>No. of hours from the beginning of treatment</th>
<th>Controls</th>
<th>DMSb</th>
<th>VM26c</th>
<th>FUra (1000 μM)</th>
<th>IFN-β</th>
<th>FUra (1000 μM) + IFN-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.6 ± 1.2</td>
<td>3.9 ± 0.8</td>
<td>5.8 ± 1.1</td>
<td>2.3 ± 0.8</td>
<td>2.7 ± 0.8</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>24</td>
<td>4.1 ± 1.2</td>
<td>20.3 ± 5.3</td>
<td>9.4 ± 2.7</td>
<td>5.2 ± 1.4</td>
<td>6.1 ± 3.2</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>48</td>
<td>16.6 ± 2.5</td>
<td>120.4</td>
<td>20.3 ± 5.3</td>
<td>6.5 ± 1.1</td>
<td>6.1 ± 3.2</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>72</td>
<td>13.7 ± 3.8</td>
<td>51.9</td>
<td>9.8 ± 1.0</td>
<td>4.8 ± 0.9</td>
<td>9.8 ± 1.0</td>
<td>11.2 ± 3.0</td>
</tr>
</tbody>
</table>

* Statistical significance according to nonparametric Mann-Whitney U test, was P < 0.002 for dimethyl sulfate or teniposide and P > 0.05 (not significant) for other drugs.

* Cells were treated with dimethyl sulfate (DMS) for 30 min at 2°C; positive control.

* Cells were treated with teniposide (VM26) for 60 min at 37°C; alkaline elution was performed under deproteinizing conditions; positive control.

* Alkaline elution was performed under deproteinizing conditions.


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