Pretreatment of Colon Carcinoma Cells with Tomudex Enhances 5-Fluorouracil Cytotoxicity

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ABSTRACT

The cytotoxic effect of sequence and dose of Tomudex (TX) and 5-fluorouracil (FUra) on an HCT-8 colon carcinoma cell line using a clonogenic assay was evaluated. Synergistic cell kill was obtained with 24 h of exposure to TX followed by 4 h of exposure to FUra. Marginal synergy was obtained with the same sequence but with a 5-day exposure to FUra. The reverse sequence, FUra (either 4 h or 5 days), followed by TX (24 h), resulted in less-than-additive cell kill. The synergistic effect was not due to augmented inhibition of thymidylate synthase, as determined by the measurement of thymidylate synthase activity by tritium release from [5-3H]2'-deoxyuridine. Surprisingly, an increase in intracellular levels of phosphoribosylpyrophosphate was observed after 24 h of exposure to TX, suggesting the possibility of an indirect effect of TX and/or its polyglutamates on purine biosynthesis. Moreover, we observed an increased formation of FUra nucleotides in the cells preexposed to TX, likely due to the increased intracellular levels of phosphoribosylpyrophosphate, that as a consequence led to an enhanced incorporation of FUra into RNA and increased cell killing.

INTRODUCTION

Although the use of agents that modulate FUra, i.e., LV or MTX, have increased the response rate of patients with advanced colorectal cancer, the response rate remains low (20–30%), and survival benefits are minimal (1, 2).

The introduction into the clinic of two new agents with antitumor activity against colorectal cancer, each with activity approximately equal to FUra/LV, TX (D1694), and irinotecan, prompted us to study the combination of one of these agents, TX, an antifolate inhibitor of the enzyme TS, with FUra (3). Studies have shown that like MTX, TX enters cells via the reduced folate carrier and is polyglutamated; this latter process increases its potency as an inhibitor of TS; also, the polyglutamate forms of this inhibitor are retained within the cell, accounting for its potency, even when administered on an every-3-weeks schedule (4). The mechanism of action of FUra is more complex: it can be cytotoxic by virtue of its incorporation into RNA via FUTP formation, into DNA via FdUTP formation, or after conversion to FdUMP, a potent inhibitor of TS. We have suggested that large “pulse” doses of FUra are cytotoxic via incorporation into RNA and inhibition of RNA metabolism, whereas continuous infusion FUra may be cytotoxic, mainly via inhibition of TS (5). In addition, sufficient levels of 5,10-methylene tetrahydrofolate polyglutamates [CH2FH4(glu)n] are necessary to result in optimum and prolonged inhibition of TS, because FdUMP binds tightly to this enzyme only in the presence of this folate coenzyme (6).

Based upon these considerations, combining these drugs might be antagonistic or synergistic: antagonistic if TX decreases the CH2FH4(glu)n pool size by inhibiting polyglutamylation of reduced folates, or perhaps synergistic if inhibition of TS by FdUMP is enhanced in the presence of TX. In addition, TX is a modest inhibitor of dihydrofolate reductase (3), and synergy could result from inhibition of this enzyme and consequent elevation of PRPP levels in the cell via inhibition of purine biosynthesis.

In view of the potential importance of combining two active drugs for the treatment of colorectal cancer, we evaluated the combination of these drugs. We performed these studies using a clonogenic assay using HCT-8 human intestinal adenocarcinoma cells and used the median effect method to analyze the data. Synergy is demonstrated with the sequence TX → FUra. Studies of the mechanism of this interaction are also presented.

MATERIALS AND METHODS

Chemicals. Media, sera, and antibiotics for cell culture were obtained from the Memorial Sloan-Kettering Cancer Center Media Lab. 2'-[5-3H]Deoxyuridine (25 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). TX and [1H]TX (19.1 Ci/mmol) were supplied by Zeneca Pharmaceuticals (Wilmington, DE). 5-[6-3H]FUra (13.3 Ci/mmol) was purchased from Dupont NEN (Boston, MA). [8-14C]Hypoxanthine (58 mCi/mmol) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).
Cell Line and Cell Growth. The human colorectal carcinoma cell line HCT-8 was obtained from American Type Culture Collection and was maintained in monolayer culture in RPMI 1640 supplemented with 10% horse serum, 100 \\mu g/ml penicillin, 100 \\mu g/ml streptomycin, and 1% glutamine at 37\degree C in a 5% CO2 humidified atmosphere and subcultured twice a week. Under these conditions, the doubling time was 18-21 h. The cell line was checked periodically and was found free of Mycoplasma contamination.

Clonogenic Assay. Monolayers of the HCT-8 parental cell line in triplicate 60-mm sterile Petri dishes were exposed for 4 and 24 h to different concentrations of FUra or TX alone, and then, to the sequence schedules of the two drugs. The medium was removed, and the cells were washed twice with PBS. Drug-free medium was added, and growth was followed for 12 days at 37\degree C in a 5% CO2 humidified atmosphere. Colonies were stained with crystal violet and counted, and the results were expressed as a percentage of untreated control colony counts. IC50's were determined by plotting a percentage of colony number (using the average of triplicate determinations for each condition) versus concentrations of drugs.

Determination of Synergism and Antagonism. For each regimen, drug exposure and assays for each drug alone (FUra and TX) and their combination for a particular schedule were carried out under identical experimental conditions. The concentration range for each drug consisted of five or more concentrations of FUra or TX at a fixed ratio, including its IC50 concentration. The ratio of FUra and TX is selected at their IC50 ratio; therefore, the contribution of effect of each drug in the regimen would be about equal at any other ratio.

At the end of 12-day incubation for clonogenic assays, the dose-effect relationships for FUra and TX and their combination were analyzed by the median-effect equation (12):

$$f_{fa}/f_{fu} = (D_{m}/D_{m})^{m}$$

where $f_{fa}$ and $f_{fu}$ are the fraction affected and unaffected, respectively, by a dose, $D_{m}$ is the concentration for the median-effect dose (IC50), and $m$ is the coefficient depicting the shape of dose-effect curve ($m = 1, >1$, and $<1$ indicates hyperbolic, sigmoidal, and negative sigmoidal shape, respectively). The $m$ and $D_{m}$ values for FUra or TX can be readily obtained from the median-effect plot: $x = \log (D_{x}) versus y = \log (f_{fa}/f_{fu})$, based on the logarithmic form of Eq. A:

$$\log (f_{fa}/f_{fu}) = m \log (D_{x}) - m \log (D_{m})$$

in which $m$ is the slope of the plot and $\log (D_{m})$ is the x-intercept. The $m$ and $D_{m}$ values for FUra and TX alone and their combination were obtained by using computer software. These $m$ and $D_{m}$ values are then used to quantitate synergism or antagonism by calculating the CmbI for x-percent inhibition, which is given by:

$$(\text{CmbI})_{x} = \frac{(D_{x})_{1} \times P/(P + Q) + (D_{x})_{2} \times Q/(P + Q)}{(D_{x})_{1} \times (D_{x})_{2} \times (P + Q)} - \frac{1}{(D_{x})_{1} \times (D_{x})_{2} \times (P + Q)} = \frac{1}{(D_{x})_{1} \times (D_{x})_{2} \times (P + Q)} - \frac{1}{(D_{x})_{1} \times (D_{x})_{2} \times (P + Q)}$$

where $(D_{x})_{1}$ and $(D_{x})_{2}$ in the denominator are the dose of the first drug (e.g., TX) and the second drug (e.g., FUra), respectively, each alone for x-percent inhibition, which can be calculated by the rearrangement of Eq. A, i.e., $D_{x} = D_{m}\{f_{fa}/(1 - f_{fa})\}^{1/m}$. $(D_{x})_{1,2}$ is the total dose of TX and FUra in combination also for x-percent inhibition. The combination ratio for TX: FUra = $P/Q$ (e.g., 1:5).

In Situ TS Activity Assay. Inhibition of TS activity in whole cells by TX or FUra was determined by measuring the tritium release from [5-3H]2-deoxyuridine as described previously (9, 10). Briefly, exponentially growing cells were exposed...
Dose effect relationship parameters $C_{mbI}$ at $D_{50}$ (pM) in $R_{1C}$.

<table>
<thead>
<tr>
<th>Schedule</th>
<th>$D_{50}$ (pM)</th>
<th>$m$</th>
<th>$R$</th>
<th>$IC_{50}$</th>
<th>$IC_{75}$</th>
<th>$IC_{90}$</th>
<th>$IC_{95}$</th>
<th>Interpretation</th>
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<td>Single agents</td>
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<td></td>
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<td>TX 24 hr</td>
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<td>0.98</td>
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<td>FUra 4 h</td>
<td>39.2</td>
<td>0.68</td>
<td>0.99</td>
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<td>FUra 24 h</td>
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<td>0.82</td>
<td>0.99</td>
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<td>Combination treatment</td>
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<tr>
<td>(1) TX → FUra (24 h)</td>
<td>0.021/0.104</td>
<td>2.22</td>
<td>0.94</td>
<td>0.84</td>
<td>0.80</td>
<td>0.81</td>
<td>0.82</td>
<td>Moderate synergism</td>
</tr>
<tr>
<td>(2) TX → FUra (4 h)</td>
<td>0.013/6.34</td>
<td>2.11</td>
<td>0.96</td>
<td>0.59</td>
<td>0.52</td>
<td>0.52</td>
<td>0.53</td>
<td>Strong synergism</td>
</tr>
<tr>
<td>(3) FUra (24 h) → TX</td>
<td>8.75/0.018</td>
<td>1.14</td>
<td>0.97</td>
<td>1.07</td>
<td>1.20</td>
<td>1.76</td>
<td>2.42</td>
<td>Antagonism</td>
</tr>
<tr>
<td>(4) FUra (4 h) → TX</td>
<td>1.05/0.21</td>
<td>0.56</td>
<td>0.93</td>
<td>4.98</td>
<td>11.7</td>
<td>27.9</td>
<td>56.9</td>
<td>Strong antagonism</td>
</tr>
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</table>

The median-effect dose $D_{50}$ (or $ED_{50}$) is obtained from the $x$-intercept of the median-effect plot. $m$ is the slope of the plot that signifies the slope, and $R$ is the linear correlation coefficient that signifies the conformity of the data to the median-effect principle (I2). The sequence TX → FUra produced synergy ($C_{mbI} < 1$), especially with schedule (2), whereas the opposite schedule produced antagonism ($C_{mbI} > 1$).

**Fig. 1** Effect of sequence on whole-cell TS activity. Inhibition of TS activity was determined in exponentially growing cells after incubation with FUra alone (4 h); TX alone (24 h); FUra (4 h) → TX (24 h); and TX (24 h) → FUra (4 h). See "Materials and Methods" for details. Values are the average of three experiments; bars, SD.

**Table 1** Schedule dependent synergism or antagonism of the combination of TX and FUra against the HCT-8 adenocarcinoma

Effect of Simultaneous Exposure of HCT-8 cells to FUra and TX. When HCT-8 cells were incubated for 24 h with increasing concentrations of TX and subsequently assayed for cytotoxicity, an $ED_{50}$ of 0.030 ± 0.004 pM was observed ($n = 3$). The $ED_{50}$ for FUra for a 4- or 120-h exposure was 39.2 ± 1.0 pM and 0.79 ± 0.20 pM, respectively ($n = 3$). These times of drug exposure to FUra (4 and 120 h) were chosen to approximate the two commonly used schedules with FUra, i.e., “pulse” and continuous infusion (11). The 24-h schedule with TX was selected because this drug has a 7-h half-life and a single injection is administered every 3 weeks (4). When cells were exposed to both treatments simultaneously for 4 or 24 h, less-than-additive cytotoxicity was observed. The method of Chou and Talalay (12) was used to compare the observed toxicity of the combination and demonstrated that the combination produced less-than-additive effects (data not shown).

**Effect of Sequential Exposure to TX and FUra.** In these studies, cells were exposed to TX for 24 h, either preceded by or followed by FUra, for 4 or 120 h. When HCT-8 cells were exposed to various concentrations of TX, followed by FUra for 120 h (schedule 1), the combination produced additive cytotoxicity (Table 1). When a 4-h exposure of FUra in varying doses followed a 24-h exposure to TX (schedule 2), synergistic cell kill was obtained (CmbI, <1). In a subsequent experiment in which leucovorin was added to FUra, even greater synergism was noted.

In contrast when FUra, either as a 4- or 120-h exposure preceded TX, there was moderate to strong antagonism (schedules 3 and 4; Table 1).
Effect of Sequence of Drug Exposure on Inhibition of TS. To determine whether the sequence TX → FUrA resulted in an increase in inhibition of TS, whole-cell TS activity was measured after cells were exposed to each drug alone and to the drug combinations (TX → FUrA or FUrA → TX). Approximate ED_{50} values of each drug were used as determined in previous experiments, and exposure times were 4 h for FUrA and 24 h for TX. TX alone produced 89 ± 2% inhibition of TS, whereas FUrA alone produced 68 ± 6% inhibition of TS activity. Of interest, however, neither sequence, TX → FUrA, or FUrA → TX resulted in additive inhibition of TS; thus, the synergy observed appeared not to be due to an enhanced blockade of this enzyme activity (Fig. 1).

Effect of TX on Incorporation of FUrA into RNA and FUrA Nucleotide Pool Sizes. The finding that the synergistic cell kill obtained by the sequence TX → FUrA was not due to
increased inhibition of TS turned our attention to the possibility that the pool sizes of FUra nucleotides in the cell were increased as a result of TX pretreatment. To determine whether increased levels of FUTP were generated as well as an increase in FUra incorporation into RNA, FUra nucleotides were measured by high-performance liquid chromatography after incubation with [3H]FUra with or without TX pretreatment. After pretreatment with TX, elevated levels of FUra nucleotides were found, as compared with cells treated with FUra alone (P < 0.001; Fig. 2). In accord with these findings, an increased amount of FUra was also detected in RNA of cells pretreated with TX (P < 0.001; Fig. 3).

**Determination of PRPP Levels in HCT-8 Cells.** Because an increase in FUra nucleotide formation was also observed previously when cells are pretreated with the antifolates MTX and trimetrexate (13–15), we assayed PRPP levels following TX treatment. Surprisingly, PRPP levels increased 5–6-fold after TX treatment, indicating that this antifolate was likely directly or indirectly inhibiting purine biosynthesis as well as thymidylate synthesis (P < 0.001; Fig. 4).

**DISCUSSION**

The present studies show that in a colon carcinoma cell line, HCT-8, the sequence TX → FUra produces synergistic cell kill, whereas simultaneous drug exposure or the opposite sequence produces less-than-additive effects. Exposure to 24 h of an approximately ED50 dose of TX increased the intracellular levels of PRPP 5–6-fold, a surprising finding in that the inhibition of dihydrofolate reductase by this compound or its polyglutamates is 75-fold less as compared with inhibition of TS (3). It is also possible that this effect is an indirect effect of TX polyglutamates on purine biosynthesis, and this possibility is under investigation. Similar results, i.e., an increase in PRPP level and sequence dependent synergy, that was observed previously with MTX and trimetrexate (13–15), was also obtained with Thymitaq (AG337), a specific inhibitor of TS, a compound that is not polyglutamylated (Ref. 16; data not shown). Thus, the elevation of PRPP levels may relate to an indirect effect of TX on purine biosynthesis not mediated by inhibition of dihydrofolate reductase and consequent inhibition of tetrahydrofolate synthesis. Whatever the cause, the increased levels of PRPP result in increased FUra nucleotide formation and an approximately 30% increase in FUTP andFdUMP levels when compared to FUra nucleotide levels in cells not pretreated with TX. As a consequence of this pool size increase, more FdUMP is formed, and more FUra is incorporated into RNA. It is likely that the latter event, in connection with TS inhibition, is the key event leading to enhanced cell death. It is of interest that LV, administered with FUra, did not reverse and possibly enhanced this inhibition.

Although these studies suggest that the sequence TX → FUra may have some therapeutic advantage over FUra or FUra plus LV in the clinic, normal replicating tissues may be as sensitive or more sensitive to the TX → FUra sequence, thus resulting in no therapeutic benefit. A Phase I study has been initiated at our institution to evaluate the toxicity of the TX → FUra sequence, followed by a Phase II study in colorectal cancer patients to determine whether the sequential use of these two active drugs in colon cancer has an additive or synergistic antitumor effect in the clinic.

**REFERENCES**


Pretreatment of colon carcinoma cells with Tomudex enhances 5-fluorouracil cytotoxicity.
