5-Fluorouracil Prodrug: Role of Anabolic and Catabolic Pathway Modulation in Therapy of Colorectal Cancer

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ABSTRACT

Following p.o. administration to rats bearing advanced colorectal carcinoma, Ftorafur (FT) is converted to 5-fluorouracil (FUra) by microsomal P450 in the liver. To optimize the therapeutic selectivity of the FUra generated from FT, three approaches were utilized: (a) inhibition of FUra degradation to dihydrofluorouracil by uracil as an alternative substrate for uracil reductase in the molar ratio of 4 uracil:1 FT (UFT); (b) modulation of drug inhibition of thymidylate synthase by levamisole (LV); and (c) by increasing the level of FUra incorporation into cellular RNA by N-(phosphonomethyl)-L-aspartate (PALA), an inhibitor of aspartate transcarbamylase. The maximum tolerated dose (MTD) of FT and UFT, administered 3 times a day for 28 days, was 150 mg/kg/day and 60 mg/kg/day, respectively. The MTDs were not significantly modified by LV (150 or 600 mg/kg/day), administered by the p.o. route with the drugs, or by PALA (100 mg/kg) administered weekly by the i.v. route. The dose-limiting toxicity of FT alone and in combination with the modulators was stomatitis. The severe alopecia observed with FT alone was reduced significantly by uracil. At the MTD, the antitumor activity of UFT was superior to those of FT and FUra alone and in combination with LV and/or PALA. The 3-month sustained complete tumor regression for UFT, FT, and FUra was 38%, 0%, and 13% (for the weekly schedule), respectively. Although uracil, LV, and PALA individually increased the antitumor activity of FT at its MTD, the combination of the three modulators produced the highest therapeutic efficacy in rats bearing advanced colorectal carcinoma, in which 100% of the treated animals achieved complete and sustained tumor regression. The therapeutic efficacy observed with FT modulation could not be achieved with FUra administered by different schedules, each at its MTD alone or in combination with either LV or PALA.

In brief, modulation of FT produced greater therapeutic efficacy and selectivity than FUra. Furthermore, the combined use of modulators capable of inhibiting the degradation pathway of FUra and potentiating the effects of the anabolic metabolites action appears to offer the greatest therapeutic potential.

INTRODUCTION

FUra\(^3\) is a widely used chemotherapeutic agent for the treatment of patients with gastrointestinal cancers. FT is a FUra prodrug converted to FUra by microsomal P450 in the liver (1). To maximize the therapeutic selectivity of FUra generated from FT, the molar ratio of uracil:FT of 4:1 (UFT) was determined to be the optimal therapeutic ratio in model systems (2–7). Uracil inhibits the degradation of FUra formed from FT by inhibiting uracil reductase. Phosphorylation of FUra, on the other hand, was not inhibited, and the 4:1 ratio was optimal tumor:serum FUra levels in rats bearing A130-130 tumors (3).

Fujii and colleagues (2–4) found that the coadministration of uracil enhanced the concentration of FUra in the tumor and antitumor activity of FT. In in vitro studies, uracil strongly inhibited the degradation of FUra to 2-fluoro-beta-alanine. In animal experiments, UFT and FT gave a comparable distribution of FUra in blood and other normal tissues, but UFT resulted in 5–10 times greater distribution of FUra in tumor. UFT was more effective than FT in Lewis lung carcinoma, B-16 melanoma, and human mammary, gastric, and pancreatic carcinoma transplanted in nude mice.

Recent studies were initiated with EU (776C85), a potent mechanism-based inhibitor of uracil reductase (8, 9). Using a low and nontoxic dose of EU, potent and prolonged inhibition of uracil reductase was achieved. Furthermore, the therapeutic efficacy and selectivity of FUra was enhanced by EU (10–13). The combination of FUra with EU at the MTD produced 100% complete and sustained tumor regression in both i.v. daily \(\times 4\) and weekly \(\times 3\) schedules in rats bearing colorectal carcinoma. EU also increased the therapeutic index of FUra by 6-fold (12).

The antitumor activity of UFT was demonstrated in patients with a variety of solid tumors (14–19). Phase I clinical trials of p.o. UFT (3 times/day for 28 days) with p.o. low or high doses of LV (5 or 50 mg, 3 times/day for 28 days for a total dose of 15 or 150 mg/day for 28 days, respectively)

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\(^3\) The abbreviations used are: FUra, 5-fluorouracil; TS, thymidylate synthase; FT, Ftorafur; UFT, uracil/FTorafur in a molar ratio of 4:1; EU, 5-ethyluracil; LV, leucovorin; PALA, N-(phosphonomethyl)-L-aspartate;FdUMP, 5-fluorodUMP; CR, complete tumor regression that is sustained for 90 days after therapy; PR, partial tumor regression, \(\geq 50\%\) reduction in tumor mass; MTD, maximum tolerated dose; c.i., continuous infusion.
Fig. 1  Dose-response profile of FT, UFT (A), and FUra (B) in rats bearing colon carcinoma. FT and UFT were given via the p.o. route and FUra was given via the i.v. route. A, FT (●) and UFT (○) p.o. three times a day for 28 days. B, FUra 28 days. (●), FUra 4 days (△), FUra i.v. push daily for 4 days (●), and FUra i.v. push weekly for 3 weeks (▲). Rats were observed for a period of 3 months after therapy. At each dose, 12–20 rats were used. Values are the average of three to five separate experiments.

Table 1  Toxicity profiles of FT and UFT with/without modulators at the MTD

<table>
<thead>
<tr>
<th>MTD (mg/kg/day)</th>
<th>Incidencea (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Stomatitis</td>
</tr>
<tr>
<td>FT (150)</td>
<td>63</td>
</tr>
<tr>
<td>FT (150) + LV (150)</td>
<td>88</td>
</tr>
<tr>
<td>FT (150) + LV (600)</td>
<td>75</td>
</tr>
<tr>
<td>FT (600) + PALA (100)</td>
<td>75</td>
</tr>
<tr>
<td>UFT (600)</td>
<td>19</td>
</tr>
<tr>
<td>UFT (600) + LV (150)</td>
<td>25</td>
</tr>
<tr>
<td>UFT (600) + LV (600)</td>
<td>25</td>
</tr>
<tr>
<td>UFT (600) + PALA (100)</td>
<td>38</td>
</tr>
<tr>
<td>UFT (600) + LV (600) + PALA (100)</td>
<td>25</td>
</tr>
</tbody>
</table>

a No diarrhea was observed at the MTD or lower dose.

MATERIALS AND METHODS

Rats

Six- to 8-week-old female Fisher 344/HSD rats (body weight 150–200 g) were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN) and kept four rats per cage with water and food ad libitum according to an institutionally approved animal protocol.

Tumor

The chemically induced Ward colorectal carcinoma, which has been extremely investigated in this laboratory, was used (26, 27). Nonnecrotic tumor pieces (0.1 g) were transplanted s.c. via trocar under slight ether anesthesia. Treatment was initiated 14–16 days later when tumor sizes were approximately 3 g.

Chemotherapy

FT and UFT were obtained from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). One capsule of UFT contains 224 mg uracil and 100 mg FT (molar ratio of 4:1). LV was obtained from Lederle Co. (Pearl River, NY) and PALA was obtained from U. S. Biosciences (West Conshohocken, PA). FUra was purchased from Hoffmann LaRoche Inc. (Nutley, NJ). All drugs were dissolved in 0.9% NaCl solution. FT and UFT were administered via the p.o. route, 3 times a day for 28 days. LV (150 and 600 mg/kg/day) was administered p.o. along with FT or UFT. PALA (100 mg/kg) was administered i.v. push weekly for 4 weeks with the first dose administered 24 h prior to FT or UFT. FUra was administered by: (a) i.v. push daily for 4 days; (b) 4 days of continuous i.v. infusion (23); (c) 28 days of continuous i.v. infusion; and (d) i.v. push weekly for 3 weeks. Each treatment group had four rats per experiment, and each experiment was repeated three to five times. Data points represent the average of all experiments including CR.

MTD

The MTD was defined as the maximum dose that could be administered to tumor-bearing rats without causing drug-related lethality.
Fig. 2 Growth inhibition of FT, UFT, and FÜra at the MTD. ○, saline control; ●, FT 150 mg/kg/day for 28 days (p.o.); ▽, UFT 60 mg/kg/day for 28 days (p.o.); ■, FÜra 12 mg/kg/day for 28 days (i.c.); and ▼, FÜra 100 mg/kg/week, i.v. push, weekly for 3 weeks. Data represent an average of three to five experiments with at least four rats in each experimental group.

**Tumor Measurements and Body Weights**

Two axes (mm) of tumor \((L, \text{longest axis})\) and \((W, \text{shortest axis})\) were measured with the aid of a Vernier caliper. Tumor weight (mg) was estimated as:

\[
\text{Tumor weight} = \frac{1}{2} (L \times W^2)
\]

Tumor measurements were taken every day during the therapy, three times a week the first 2 weeks after therapy, then once a week thereafter. For each experiment the same observer made all measurements to minimize variations in caliper measurements. As a general policy, rats were sacrificed when the tumor size exceeded 6–10 g. Body weights of the animals were recorded at the time of tumor volume measurement.

**Tumor Response**

CR was defined as complete disappearance of tumor at the sites of tumor transplant for more than 90 days after therapy, when the animals were sacrificed. PR was defined as ≥50% reduction in tumor size during or after therapy with subsequent regrowth of tumors. All studies were performed in accordance with IAUC Guidelines and under the approved Roswell Park Cancer Institute protocol.

**Pharmacokinetics**

**Incannulation of Rat’s Jugular Vein for Repetitive Blood Sampling.** During slight ether anesthesia, the rat’s jugular vein was exposed, and a catheter with saline and heparin (100 units/ml) was inserted into the right atrium. The opposite end of the catheter was brought out of the back of the neck by a trocar needle. Blood (250–300 μl) was taken with a 22-gauge needle attached to a 1-ml syringe prior to initiation of therapy and at various times after the p.o. administration of FT or UFT.

**Extraction of Rat Plasma.** To a 100-μl plasma, 30 μl 10 μg/ml bromouracil (internal standard) and 1.5 ml ethyl acetate were added. Samples were vortexed and centrifuged at 3000 × g for 10 min at 4°C. Following two extractions, the supernatants were removed, lyophilized to dryness, resuspended in 1 ml methanol, evaporated, and resuspended in 200 μl HPLC buffer (see below) for either immediate analysis or stored at −20°C. Standard samples were prepared in an identical manner.

**HPLC Analysis of FT, FÜra, and Uracil.** We used a Perkin Elmer/Cetus Model 410 Bioprogrammable pump, equipped with a LC 95 UV/V detector set at 265 nm, and an interface connected to a Sigma 10 Data System. A Spherisorb S-3-ODS52 (15 cm × 4.6 cm inside diameter) and a Spherisorb S-3-ODS52 (10 cm × 4.6 cm inside diameter) column system in series was used. Following injections of 20 μl sample, materials were eluted by an isocratic buffer of 2.5 mm ammonium acetate containing 1.25% methanol (pH 5), at a flow rate of 1 ml/min. Retention times for uracil, FÜra, BU, and FT were 3.4, 3.6, 9.4, and 47.7 min, respectively.
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Fig. 4 CR (□) and PR (■) of FT, UFT, and FUra alone and in combination with LV and/or PALA. Data represent an average of three to five experiments with at least four rats in each experimental group.

TS Activity Assay

Frozen tumor tissues were weighed, hand homogenized in extraction buffer (20 mm Tris-HCl, 250 mm sucrose, 2 mm DTT, 1.5 mm MgCl₂, and 1 mm EDTA-Na₂, pH 7.5, 1 ml/g), and then centrifuged. Tissues extract (30 µl) and 20 µl reaction cocktail (200 mm Tris-HCl, 4 mm DTT, 1.5 mm EDTA-Na₂, 2.25 mm MgCl₂, 150 mm NaF, 4.5 mg/ml BSA, 60 mm HCHO, and 225 µM 11.25 µCi/ml [³H]dUMP) were prewarmed for 3 min at 37°C. Ten µl 4.5 mm H₃PteGlu (in 100 mm Tris-HCl, pH 7.5, and 10 mm DTT) were added to the tubes to start the 30-min reaction, and 200 µl 10% charcoal (suspended in 2% trichloroacetic acid, ice cold) were added to stop the reaction. The mixture was vortexed and centrifuged at 8800 × g for 15 min at 4°C. One hundred µl of the supernatant were counted for radioactivity.

RESULTS

Determination of MTD

The MTDs of FT, UFT administered p.o. 3 times daily for 28 days, and FUra administered by 4 days i.v. push, 4 days and 28 days continuous i.v. infusion, and weekly i.v. push for 3-week schedules were identified as shown in Fig. 1. The MTDs of FT and UFT were 150 and 60 mg/kg/day for 28 days, respectively (Fig. 1A). The MTD of FUra by daily i.v. push and by continuous i.v. infusion was 35 mg/kg/day for 4 days, 12 mg/kg/day for 28 days c.i., and 100 mg/kg/week for 3 weeks by the weekly schedule (Fig. 1B).

Toxicity of FT, UFT ± LV, and/or PALA at MTD

The effect of LV and PALA on the FT and UFT induced toxicity at the MTD were evaluated, and the results are shown in Table 1. Toxicity was expressed in terms of maximum weight loss, alopecia, and mouth ulceration (stomatitis). The data indicated that neither low- nor high-dose LV or PALA produced significant alteration of the MTD of the agents. Uracil, however, potentiated the toxicity of FT, requiring about 2.5-fold reduction in FT dose. Furthermore, the incidence of stomatitis and alopecia induced by FT were reduced by uracil. The MTD of FUra in the presence of LV or PALA had to be reduced by approximately 25–30%, from 35 mg/kg/day to 25 mg/kg/day for daily schedules and from 100 mg/kg to 75 mg/kg for the weekly schedule (data not shown).

Tumor Growth Inhibition

FT, UFT, and FUra. Comparative tumor growth inhibition by the MTDs of FT, UFT, and FUra were carried out, and the results are summarized in Fig. 2. The data in Fig. 2 indicate that UFT was the most active agent, with 38% of the animals disease-free (CR) for more than 3 months. The observed regrowth of tumors in animals treated with UFT were in those which achieved PR. With the FUra administrated weekly schedule, 13% of animals produced CR. No CR or PR was observed with the MTD (12 mg/kg/day) of FUra administrated by a 28-day c.i. schedule.

FT, UFT, and FUra Modulation. The antitumor activity of FT and UFT administered p.o. 3 times a day for 28 days alone and in combination with LV (150 or 600 mg/kg/day for 28 days p.o.) or with PALA i.v. push (100 mg/kg weekly for 4 weeks and first dose 24 h prior to FT or UFT) was evaluated. At the MTD of each treatment modality, although tumor growth delay by FT was evident (Fig. 3A), the effect was not highly significant with no evidence of CR or PR. Although tumor growth inhibition was potentiated by LV and PALA, the effects were not sustainable (Fig. 3A). In contrast to the results obtained with FT, 38 and 62% of rats treated with UFT achieved CR and PR, respectively (Fig. 3B).

The data in Fig. 4 summarize the results of the antitumor activity of FT, UFT, and FUra alone and under conditions of modulation by LV and PALA all at the MTD. With FT no CR was observed. Partial responses were seen only when FT was modulated by either LV or PALA. In contrast, more than 3 months of sustained CR (cure) was achieved in 100% of the animals treated with UFT modulation by LV and PALA in combination. In this model system, a higher dose of LV in combination with UFT was essential for the conversion of PR to CR. In combination with PALA, however, a lower dose of LV was equally effective.

The data in Fig. 4 are an outline of the antitumor activity of FUra ± modulators administered by i.v. push, i.v. infusion, and the weekly schedule. The results indicate that the therapeutic efficacy of these treatments is in the order of weekly ≥ c.i. > i.v. push. Furthermore, the data in Fig. 4 demonstrate the supe-
Pharmacokinetics of FT and UFT

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg/day)</th>
<th>Peak (µM)</th>
<th>AUC (µM·h)</th>
<th>t₁/₂ (h)</th>
<th>Peak (µM)</th>
<th>AUC (µM·h)</th>
<th>t₁/₂ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>150</td>
<td>320 ± 81</td>
<td>5939 ± 781</td>
<td>5.8 ± 1.4</td>
<td>2.8 ± 0.9</td>
<td>49 ± 10</td>
<td>4.0 ± 1.3</td>
</tr>
<tr>
<td>UFT</td>
<td>60</td>
<td>487 ± 69</td>
<td>6729 ± 961</td>
<td>6.2 ± 1.2</td>
<td>19.4 ± 2.8</td>
<td>368 ± 58</td>
<td>7.5 ± 1.6</td>
</tr>
</tbody>
</table>

Fig. 5  Plasma concentration of FT (●), FUra (○), and uracil (□) in rats treated p.o. with FT 150 mg/kg (A) or UFT 60 mg/kg (B). Values represent an average of three separate experiments (mean ± SD).

Table 3  TS activity in tumors treated with FT or UFT with or without LV

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT(150)</td>
</tr>
<tr>
<td>4</td>
<td>84 ± 12</td>
</tr>
<tr>
<td>24</td>
<td>78 ± 10</td>
</tr>
</tbody>
</table>

Pharmacokinetics of FT and UFT

Using therapeutic doses of FT (150 mg/kg) and UFT (60 mg/kg), the pharmacokinetics properties of these agents in Fisher rats were evaluated (Table 2 and Fig. 5). In rats treated p.o. with FT, the peak plasma concentration and plasma areas under the concentration time curve of FT were 320 ± 81 µM and 5939 ± 781 µM·h, respectively. The same parameters for FUra derived from FT were 2.8 ± 0.9 µM and 49 ± 10 µM·h, respectively (Fig. 5A and Table 2). For rats treated p.o. with UFT, the peak plasma concentration and AUC of FT were 487 ± 69 µM and 6729 ± 961 µM·h. FUra derived from UFT was 19.4 ± 2.8 µM and 368 ± 58 µM·h, respectively (Fig. 5B and Table 2). There were similar elimination rates of FT when either FT or UFT was given at the MTD (t₁/₂, 5.8 versus 6.2 h). However, there was a significantly longer half-life of FUra when derived from treatment with UFT than that from FT (t₁/₂, 4.0 versus 7.5 h). In addition, the plasma FUra concentrations derived from UFT were higher than those derived from FT. An approximately 10 µM FUra concentration was maintained for up to 24 h. This level is comparable to that achieved by a continuous infusion of low-dose FUra.

TS Inhibition

The effects of FT, UFT alone, and UFT in combination with LV on the activity of TS in rats bearing colon tumor were evaluated. The data in Table 3 indicate that after treatment with FT, TS inhibition was greater under the conditions of modulation by uracil and LV. No significant enzyme recovery was observed at 24 h after treatment with UFT + LV.

DISCUSSION

In this investigation, using in vivo model systems we have tried to answer five questions: (a) Does uracil modulate the antitumor activity of FT? (b) Do LV and PALA modulate further the antitumor activity of UFT? (c) Is there additional therapeutic benefit from the combined use of modulators affecting the anabolic and catabolic pathways of FUra generated from FT? (d) Are the therapeutic efficacy and selectivity of UFT different from those of FUra? (e) What are the mechanisms associated with therapeutic efficacy of FT modulation?

FT is a produg of FUra and is well absorbed after p.o. administration with less side effects than FUra (28). Clinical studies have demonstrated that FT is effective against several tumors, including gastrointestinal and breast cancers (29). Uracil inhibits uracil reductase, the first enzyme of the catabolic pathway of FUra. UFT was reported to have superior efficacy to FT in animal model systems (2, 3, 6). The data reported herein demonstrated that UFT has greater therapeutic efficacy and less toxicities than FT or FUra (Table 1 and Figs. 2–4). The improved efficacy may be due in part to the higher and a longer half-life of plasma FUra concentration derived from UFT alone and under condition of modulation.

Pharmacokinetics of FT and UFT

Using therapeutic doses of FT (150 mg/kg) and UFT (60 mg/kg), the pharmacokinetics properties of these agents in Fisher rats were evaluated (Table 2 and Fig. 5). In rats treated
from UFT than those derived from FT (Table 2 and Fig. 5). Other explanations may include an increased amount of FdUMP and TS inhibition as well as an increased level of 5-fluorouridine triphosphate incorporation into cellular RNA. The observed decrease in host toxicity may be due to a decreased level of FUra catabolic products, namely, 5-fluorodihydrouracil and β-fluorolalanine.

LV is a source of intracellular reduced folates that increases the inhibition of TS by FdUMP through the stabilization of a ternary complex of the enzyme, reduced folate, and FdUMP. Phase III randomized clinical trials have demonstrated that FUra in combination with LV significantly increases response rates over FUra alone (30, 31). The question of the optimum dose of LV for modulation of FUra antitumor activity, however, remained unresolved. Following p.o. administration of (6R,S)LV, the biologically active isomer (6S)LV is selectively absorbed. The peak plasma concentrations of (6S)LV, however, are in the nm range (32). In the study reported herein we demonstrated that high-dose LV potentiated significantly the antitumor activity of UFT. High-dose LV (600 mg/kg/day) achieves a plasma concentration $>1 \mu M$ (data not shown), a concentration determined to be optimal for modulation of FUra (33). Under these conditions, the duration of TS inhibition was prolonged.

PALA is a potent inhibitor of de novo pyrimidine biosynthesis and potentiates the efficacy of FUra by increasing the level of drug incorporation into cellular RNA (34, 35) and also decreases the pools of UTP and CTP (36). We demonstrated that low-dose PALA significantly improves the antitumor activity of FT and UFT. It is likely that the observed increase in the therapeutic efficacy of FUra by PALA is due in part to the decrease in dUMP, a competing metabolite with FdUMP for TS inhibition and the likely increase in the levels of FUra incorporated into cellular RNA. The data reported herein demonstrate that greater therapeutic efficacy (cure) of FT can be achieved when LV, PALA, and uracil are used in combination (Fig. 4). Thus, through inhibition of the degradation pathway of FUra generated from FT by uracil and modulation of the anabolic pathway of FUra-nucleotide by LV and PALA, improved therapeutic selectivity was achieved. The use of modulators alone and in combination produced greater therapeutic efficacy of FT than could be achieved with the MTD of FT, UFT, or FUra. The toxicity of FT, at its MTD, was not altered by LV or PALA modulation. In contrast, modulation of FUra by LV and PALA increased the drug-induced toxicity of FUra, requiring approximately 25–30% reduction in the FUra dose. These findings point out the need for clinical confirmation of the concept developed in this model system.

UFT alone or with modulators produced a greater antitumor activity than FT or FUra alone or with modulators. Although high-dose LV and low-dose PALA when used alone potentiated the antitumor activity of UFT, modulation of UFT by PALA and LV in combination was most effective where 100% CR was achieved. Pharmacokinetics results demonstrated that higher peak plasma concentrations and a longer half-life of FUra derived from UFT than those derived from FT. Plasma FUra levels were similar to those achieved with protracted continuous infusion of low-dose FUra (300 mg/m$^2$) to patients. UFT was superior to FT in its ability to maintain the potent inhibition of TS activity. These data suggest that UFT in combination with LV and PALA should be evaluated in the clinic.

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