Therapeutic Efficacy of Fluoropyrimidines Depends on the Duration of Thymidylate Synthase Inhibition in the Murine Colon 26-B Carcinoma Tumor Model

Jan A. M. van Laar, Clasina L. van der Wilt, Youcef M. Rustum, Paul Noordhuis, Kees Smid, Herbert M. Pinedo, and Godefridus J. Peters

INTRODUCTION

The fluoropyrimidines FdUrd and FuRa are the most effective drugs in the treatment of patients with advanced colorectal cancer (1, 2). These agents exert their action after being metabolized to the nucleotide level. One of these nucleotides, FdUMP, is a potent inhibitor of TS, the key enzyme in pyrimidine de novo synthesis (2–6). The inhibition can be enhanced by the natural cosubstrate of TS, 5,10-methylenetetrahydrofolate (LV). LV is the precursor of this reduced folate, which is essential for the formation of a stable ternary complex with TS and FdUMP (3, 7–10).

FdUrd is metabolized by either phosphorylation into FdUMP catalyzed by thymidine kinase or by cleavage catalyzed by TP into FuRa and can thus act as a prodrug for FuRa (11–14). FuRa is further anabolized via 5-fluoro-UMP into 5-fluoro-UDP and FUTP and subsequently into FdUMP or 5-fluoro-dUTP. FUTP and the latter can be incorporated into RNA and DNA, respectively (2, 15–17). The antiproliferative effects of both drugs are believed to be caused by the inhibition of TS (2, 5, 9, 18, 19), although the RNA incorporation of FUTP has also been associated with the antitumor activity of FuRa therapy (15, 20).

In vitro FdUrd is generally more cytotoxic than FuRa (18, 21); the highest cytotoxicity is seen after prolonged exposure (22). FdUrd was already being given by continuous systemic infusion in clinics in the 1960s; however, it was associated with considerable toxicity, and a 15- to 20-fold dose reduction was required (23). More importantly, these continuous infusions of FdUrd were not more effective than other treatments (24). At present, the use of FdUrd is restricted to selective high-dose regimens by hepatic arterial infusion in patients with colorectal liver metastases (1, 25), or it is given in a chronomodulated modulated schedule (26). When given as a continuous infusion, FuRa does not require dose reduction. It can be administered in different schedules (27).

Recently, our group showed clinically relevant superiority of high-dose i.v. push FdUrd administration in a FuRa-resistant murine colon tumor. Colon carcinoma 26 (here designated Colon 26-B) was considered resistant, because treatment with FuRa did not increase the median life span of mice substantially, and no partial or complete responses had been achieved. Furthermore, in this tumor model, weekly i.v. push administration of FdUrd at its MTD resulted in greater therapeutic activity and selectivity than with continuous infusions during 4 days, three

The abbreviations used are: FdUrd, 5-fluoro-2′-deoxurydine; FuRa, 5-fluorouracil; FdUMP, 5-fluoro-dUMP; TS, thymidylate synthase; LV, leucovorin; FUTP, 5-fluoro-UTP, MTD, maximum tolerated dose; WW, wet weight; TP, thymidine phosphorylase; AUC, area under the concentration curve.
weekly 1-h infusions, or i.v. push daily for 4 days (28). These observations led to the start of a Phase I and pharmacokinetic trial with a high-dose i.v. push of FdUrd at the Roswell Park Cancer Institute (14).

The present study was designed to delineate biochemical mechanisms associated with the observed differences in antitumor activity of FdUrd and FUra in Colon 26-B carcinoma. Using equitoxic doses of FdUrd or FUra, tumor tissue concentrations of FUra and free FdUMP, levels of FUTP incorporation into cellular RNA, and the duration of TS inhibition were measured. The results of this study indicate that the duration of TS inhibition is consistent with the observed differences in antitumor activity between the two fluoropyrimidines.

**MATERIALS AND METHODS**

**Chemicals.** FUra and FdUrd were obtained from Hoffmann-La Roche, Inc. (Basel, Switzerland) [5-3H]dUMP was obtained from Amersham International (Buckinghamshire, United Kingdom), and [6-3H]-FdUMP was from Moravek (Brea, CA). All other chemicals were of the highest quality commercially available.

**Mice.** Female BALB/c mice (Harlan/Cpb, Zeist, the Netherlands), 6–7 weeks old, were kept six per cage with food and water ad libitum. Colon 26-B tumor (28, 29) fragments were inoculated in both flanks of the mice under slight ether anesthesia. Ten days after transplantation, when the tumors had reached a size of about 200 mm³, drugs were administered. The antitumor effects of FUra (80 mg/kg) and FdUrd (400 mg/kg) given as bolus injections were evaluated as described (28).

**Tissue Preparation.** Tumors for biochemical analyses were removed 2, 8, 24, 48, 72, 168, and 240 h after drug administrations. These are the time points when tumor volumes were measured. More points were not considered more informative and would have resulted in needless sacrifice of too many mice. Tumors were removed rapidly during ether anesthesia, immediately frozen in liquid nitrogen, and subsequently kept at −80°C until assayed. Frozen tumors were pulverized with a microdisembrator, and the still-frozen powder was suspended in ice-cold Tris buffer [200 mm Tris, 15 mm CMP, 100 mm NaF, and 20 mm β-mercaptoethanol (pH 7.4)] at a concentration of 0.33 g/ml. The suspension was centrifuged at 4000 × g, and the supernatant was subsequently centrifuged at 7000 × g. This enzyme-containing supernatant (enzyme suspension) was split in several parts for the different assays, thus allowing the use of one tumor sample for all analyses. Fifty μl were used for the estimation of the protein content of the tumor, as measured with Coomassie blue staining (Bio-Rad; according to the method of Bradford; Ref. 30); 400 μl were used for the determination of FUra and FdUrd and free FdUMP levels. The remaining volume was used for the two assays, by which TS inhibition could be evaluated (free FdUMP-binding sites and TS catalytic activity). The 4000 × g precipitate was immediately frozen (−80°C) and later used for RNA incorporation measurements.

**FUra, FdUrd, and FdUMP Measurements.** Because prolonged high concentrations of FUra in human and murine tumors have been observed (31), we evaluated the tumoral FUra concentrations together with FdUrd concentrations.

After extraction of the 7000 × g supernatant with trichloroacetic acid (final concentration, 5%), precipitation, and neutralization with triocetylamine/1,1,2-trifluorotrichloroethane, the measurement of FUra levels in tumors was performed by gas chromatography coupled with mass spectrometry with [15N,15N]FUra as an internal standard (31). FdUrd concentrations were analyzed by further extraction with ethyl acetate and methanol as described for plasma evaluations (14). Tumoral levels of FdUMP were determined in these trichloroacetic acid extracts using the isotope dilution assay with Lactobacillus casei TS. [6-3H]FdUMP was used as the radioactive substrate (8). The detection limit was 10 fmol/mg ww. FdUMP levels were too low for direct measurement by high-performance liquid chromatographic procedures; in addition, in tumor samples, too many interfering peaks preclude selective determination of nonradioactive FdUMP.

**Evaluation of TS Inhibition.** Two assays were used for the evaluation of TS levels in tumors from drug-treated mice. The ligand-binding assay of [6-3H]FdUMP to TS gives an indication of the number of free binding sites for FdUMP of TS after treatment. In the TS catalytic activity assay, the conversion of [5-3H]dUMP into dTMP and 3H₂O is measured. In tumors from early time points (2 h–3 days), TS was measured immediately after preparation of the extracts essentially as described (8, 9). Tumors from untreated mice served as references. Tumor samples from the later two time points (168 and 240 h) were split into two parts. In one part, a dissociation buffer [0.75 mm NH₄CO₃, 100 mm NaF, 20 mm β-mercaptoethanol, and 15 mm CMP (pH 7.8) with 0.05 total volume of 1.6 mm dUMP] was used to remove all the FdUMP from the ternary complex, leaving the total amount of FdUMP-binding sites (TS-tot) or the total activity of the enzyme (TS-total). These samples were incubated for 3 h at 30°C. The other part was frozen (−80°C) during the dissociation procedure, and TS levels were measured thereafter. In these samples, inhibition was measured as the number of free FdUMP-binding sites or residual TS catalytic activity. Values were then calculated as relative to those of untreated tumors. Under these conditions, no loss of enzyme levels were observed (8). Further details on both assays have been described elsewhere (8).

**Determination of RNA Incorporation.** After addition of the Tris buffer to the powder and centrifugation, the precipitated part was stored and used for further analysis of RNA incorporation by FUTP. For this purpose, a recently developed technique, described in detail, was used (32). This assay is based on degradation of purified RNA substituted by FUra, to FUra, by incubation of the RNA with RNase, alkaline phosphatase, and uridine phosphorylase. Hereafter, FUra was extracted and derivatized for measurement by gas chromatography coupled with mass spectrometry as described (31, 32).

**Pharmacokinetic and Statistical Evaluation.** The AUC was determined according to the trapezoidal method. Other pharmacokinetic parameters such as t½ were determined as described previously (28, 31). Statistical evaluation was accomplished with Student’s t test for unpaired data. Statistical significance was assumed when P < 0.05.
RESULTS

Antitumor Activity. Using a weekly schedule, the antitumor activities of FdUrd and FUra at their MTDs, 400 and 80 mg/kg, respectively, were evaluated in mice bearing advanced Colon 26-B carcinoma. The results, shown in Fig. 1, demonstrate that the antitumor activity of FdUrd was superior to FUra not only at the MTD but also at equimolar doses, 200 mg/kg FdUrd versus 80 mg/kg FUra. Administration of 200 mg/kg FdUrd resulted in a tumor-doubling time of 19 days and 13% complete tumor regressions; after 80 mg/kg FUra, these values were 7 days and 0%, respectively.

Tumoral Drug Levels. After 7 days after administration of 400 mg/kg FdUrd, the concentrations of FUra in the tumors tended to be higher than the levels measured in tumors from mice treated with 80 mg/kg FUra at this time point (Fig. 2). At the other time points, these levels were similar. The elimination of FUra derived from FdUrd or FUra appeared to be linear, with similar t1/2 values (Fig. 2 and Table 1). FdUrd could not be detected in any of the treated samples.

Tumoral Free FdUMP Levels. The data in Fig. 3 suggest a biphasic elimination pattern of FdUMP derived from FdUrd. Two h after FUra administration, the free FdUMP levels were 3-fold higher than levels measured in tumors from mice 2 h after FUra treatment (P < 0.01), resulting in a nearly 2-fold higher AUC. The elimination patterns of FdUMP were similar 24 h after drug treatment.

TS Inhibition. TS activity, reflected by the number of free binding sites for [3H]FdUMP, decreased rapidly from 122 ± 33 fmol/mg ww in control samples to 29.6 ± 2.8 and 20.5 ± 2.1 fmol/mg ww at 2 h after administration of FUra and FdUrd, respectively (statistical difference between FUra and FdUrd: Student’s t test, P < 0.01). TS was inhibited at this level in tumors from mice treated with FUra until 72 h but remained inhibited significantly longer in tumors from mice treated with FdUrd, 168 h (7 days). Subsequently, the number of free FdUMP-binding sites increased to 201 fmol/mg ww 7 days after FUra and were higher (P < 0.05) than values of untreated tumors or those of FdUrd-treated tumors (P < 0.001), which were 30.2 fmol/mg ww. An increase of free FdUMP to 205 fmol/mg ww after FUra administration was only seen after 10 days (Fig. 4). The total number of FdUMP-binding sites increased even more. This increase was observed earlier in FUra-treated mice than in FdUrd-treated mice. At 7 days after FUra and 10 days after FdUrd, the TS-tot values were increased 2-fold (Fig. 4, inset).

The data presented in Fig. 5 show similar kinetics of TS inhibition after FUra and FdUrd administration, as determined by the assay measuring the catalytic activity of TS with [5-3H]dUMP. Maximal inhibition of TS activity in the range of 10% of the control values occurred within 2 h after FUra or FdUrd treatment. FUra induced a prolonged TS inhibition compared with FUra (168 versus 72 h, respectively). Subsequently, recovery occurred later after FUra (10 versus 7 days after treatment). Moreover, the TS-tot also increased 2-fold at 7 days after FUra treatment and 10 days after FdUrd treatment (Fig. 5, inset). Interestingly, the ratios between TS values at 10

Table 1 Pharmacological characteristics of the fluoropyrimidines FUra (80 mg/kg) and FdUrd (400 mg/kg) in the murine Colon 26-B carcinoma

<table>
<thead>
<tr>
<th></th>
<th>AUC FUra</th>
<th>FUra: FdUrd ratio</th>
<th>t1/2 (h)</th>
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<tr>
<td>FdUMP_tumor (pmol/min/liter)</td>
<td>114</td>
<td>212</td>
<td>1.9</td>
</tr>
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<td>FUra_plasma (mmol/min/liter)</td>
<td>10.6</td>
<td>2.2</td>
<td>0.2</td>
</tr>
<tr>
<td>FdUrd_plasma (mmol/min/liter)</td>
<td>96</td>
<td>104</td>
<td>1.1</td>
</tr>
<tr>
<td>FdUrd_mac (mmol/μg/min)</td>
<td>32.1</td>
<td>49.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a AUC units are in parentheses.  
b n.e., not evaluable, because not enough points were available in the first part of the curve.  
c Data are from previous experiments (28).  
d Calculated over the β phase of the curves.
Efficacy of Fluoropyrimidines in Colon 26-B Tumors and TS

Fig. 3  Free FdUMP concentrations in Colon 26-B tumors measured at different time points after 80 mg/kg Fura (V) and 400 mg/kg FdUrd (▲) administration. Values are means of at least five tumors. Bars, SD. Only levels at 2 h after FdUrd and Fura treatment are significantly (P < 0.01) different from each other. At later time points, values were below the detection limit.

Fig. 4  FdUMP binding to TS in Colon 26-B tumors at different time points after administration of 80 mg/kg Fura (V) and 400 mg/kg FdUrd (▲). Inset, TS-total values. Values are the means of at least three tumors. Bars, SD. Absolute values of treated samples are significantly lower (P < 0.001) than untreated samples: Fura, 2–72 h; FdUrd, 2 h–7 days. At later time points, values of treated tumors are higher (Fura, 7 days, P < 0.05; Fura and FdUrd, 10 days, P < 0.01). Values at 2 h and 7 days after FdUrd and Fura treatment are significantly different from each other (P < 0.01). TS-total values are significantly different from those of untreated tumors: Fura, 7 and 10 days, P < 0.02 and 0.001, respectively; FdUrd, 10 days, P < 0.001. At 7 days, TS-total values of the Fura-treated tumor samples are higher (P < 0.01) than after FdUrd treatment. Ten days after treatment, no significant difference between treated samples is observed.

Fig. 5  TS catalytic activity (at 10 μM dUMP) in Colon 26-B tumors at different time points after 80 mg/kg Fura (V) and 400 mg/kg FdUrd (▲) administration. Inset, amount of TS-total. Values are means of at least three tumors. Bars, SD. Values of treated samples are significantly (P < 0.001) lower than levels of untreated samples: Fura, 2–72 h; FdUrd, 2 h–7 days. Values at 2 and 168 h (7 days) after FdUrd and Fura treatment are significantly different from each other (P < 0.02 and P < 0.001, respectively). Values of treated samples are significantly (P < 0.001) higher than untreated samples 7 and 10 days after Fura and 10 days after FdUrd treatment. TS-total values of untreated tumors are significantly lower than 7 and 10 days after Fura and 10 days after FdUrd. At 7 days, values of the Fura-treated tumor samples are higher (P < 0.001) than after FdUrd treatment. Ten days after treatment, no significant difference between treated samples is observed.

72 h, but after 72 h, the ratios were comparable (data not shown); this may be related to initially high FdUMP concentrations after FdUrd treatment.

RNA Incorporation. In contrast with the observations on TS inhibition, the kinetics of FUTP incorporation into cellular RNA after treatment with FdUrd or Fura were similar. The RNA incorporation after FdUrd was slightly higher than after Fura but was only significantly different at 48 h (Fig. 6 and Table 1). The elimination of Fura from RNA followed the same pattern for both Fura and FdUrd. The RNA incorporation also correlated very well with the Fura concentration in the tumor (Fig. 6, inset).

DISCUSSION

The present findings are the first to describe a relationship between superior antitumor activity of FdUrd over Fura therapy with maintenance of TS inhibition, without significant differences of RNA incorporation. Several important clinically relevant mechanisms of action of fluoropyrimidines were studied to elucidate the basis for the therapeutic differences between FdUrd and Fura in a murine colon tumor model.

The plasma levels Fura derived from 400 mg/kg FdUrd are about 5-fold lower than those after 80 mg/kg Fura, and the elimination of Fura is similar after both drugs (28). However, tumor tissue concentrations and elimination of Fura derived from both fluoropyrimidines were similar and comparable with those found in previous studies with Fura (31, 33, 34). These observations indicate effective uptake and possibly specific tumor conversion of FdUrd into Fura. Furthermore, TP activity,
that RNA incorporation is merely a result of tumoral FUra incorporation found by Nord and Martin (20) in Colon 26 tumors. This indicates that FUTP formation from FUra is not rate limiting for incorporation into RNA. These observations suggest that RNA incorporation is merely a result of tumoral FUra incorporation by FdUrd to FUra, is high (about 9000 nmol/h/mg protein) in Colon 26 tumors (35), and FdUrd was not detected in the tumors in our studies. Therefore, it is unlikely that the intracellular FUra accumulation after FdUrd administration resulted from extratumoral cleavage followed by uptake of FUra in the tumor. In human colorectal carcinomas, TP levels are lower (20-2000 nmol/h/mg protein) but apparently high enough to cause FdUrd cleavage (36). Previously, such levels were shown to be high enough to catalyze tissue-specific conversion of 5'-deoxy-5-fluorouridine to FUra (35). Consequently, this murine model serves as an excellent tumor model, because the levels of TS, TP, and FUra and FdUrd pharmacokinetics are comparable with those of humans. The differences in tumor and plasma FUra concentrations might be explained by the effect of tissue-specific isoenzymes of TP that are found both in humans and mice (12, 37). This study confirms our previous observations of FUra being trapped in the tumor after FUra therapy (31, 34); apparently, this phenomenon also occurs after FdUrd treatment. FUra in the tumor is not easily exchanged with plasma, resulting in a large concentration gradient existing until several days after treatment, both in mice (31, 34) and humans (31). Similar observations have been made using noninvasive measurements of tumoral FUra by 18F nuclear magnetic resonance (38).

In contrast, the incorporation of FUTP into RNA was only slightly higher after FdUrd administration, and elimination patterns were similar after both drugs. A good correlation was found between tumoral FUra and RNA incorporation, in line with the dose-dependent relationship of FUra with RNA incorporation found by Nord and Martin (20) in Colon 26 tumors. This indicates that FUTP formation from FUra is not rate limiting for incorporation into RNA. These observations suggest that RNA incorporation is merely a result of tumoral FUra derived from FdUrd and does not directly account for the increased therapeutic efficacy of FdUrd in this murine colorectal carcinoma model. In support of this, several studies associate fluoropyrimidine side effects with FUTP incorporation into cellular RNA (35, 39-43). RNA incorporation is possibly cell cycle independent, explaining the relationship of toxicity with FUra concentration.

More significant differences were observed following FUra and FdUrd administration concerning tumoral FdUMP levels and duration of TS inhibition. The higher tumoral FdUMP derived from FdUrd is probably the result of both direct phosphorylation of FdUMP by thymidine kinase and of indirect FUra metabolism to FdUMP. This higher FdUMP accumulation, resulting in immediate saturation of TS, might be associated with prolonged TS inhibition in both assays. Similarly, an early rebound TS was observed after FUra treatment in contrast to FdUrd. This rebound correlated with tumor relapse similar to that in Colon 26-A tumors (8, 34). This elevation of TS levels, observed in vitro and in animals and patients, seems to be a defense mechanism to overcome dTMP depletion (4, 8-10, 17, 44-46) and might be related to a FdUMP-induced disturbance of TS mRNA translation regulation (47). It seems likely that the early increase in TS in FUra-treated tumors is related to a lack of free FdUMP necessary to bind free TS, derived from spontaneous dissociation of the ternary complex, or by new enzyme synthesis. Although FdUMP levels were not measurable anymore after 7 and 10 days, FUra levels, as well as the level of FUra in RNA, seemed higher after FdUrd treatment. A recycling between FUra RNA and free FUra might be sufficient to maintain sufficiently high free FdUMP levels to maintain TS binding and prevent TS synthesis. Only after 10 days did increased TS levels seem too high to be inhibited by such low FdUMP levels after FdUrd therapy. Further investigations at the TS mRNA and protein levels in tumors might reveal the role of the increased TS levels after fluoropyrimidine therapy.

Recent observations have shown that TS induction occurred early in murine Colon 26 tumors after continuous FUra administration compared with bolus administration (34). It is striking that the best antitumor activity was observed with schedules in which the drug was administered again when TS was still almost completely inhibited, such as with FdUrd in this study and with FUra in combination with LV in previous studies in Colon 26-A tumors (8). It should be mentioned that in the Colon 26-B tumors, LV could not enhance the antitumor activity of FUra (48). When FUra alone is administered when TS is high again, the treatment is not effective anymore. This observation may be important for scheduling of therapy with fluoropyrimidines; too frequent (e.g., continuous infusion) administration might result in early TS induction (34), but too infrequent administration might result in TS recovery before the next dose is administered (8).

Rapid uptake of fluorinated pyrimidines is shown in murine colorectal tumors. The superior antiproliferative effect of FdUrd compared with FUra seems to be mediated mainly by TS inhibition and specifically by maintenance of TS inhibition, whereas FUra incorporation into cellular RNA does not appear to be an important factor. This study indicates the importance of scheduling in the clinic; drugs should be administered when TS activity is low to prevent a rebound and, thus, a relapse. Therefore, the aim of therapy of colorectal tumors should be focused on the maintenance of TS inhibition and prevention of TS...
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