Background: The mechanism of action of 5-fluorouracil (5-FU) has been associated with inhibition of thymidylate synthase (TS) and incorporation of 5-FU into RNA and DNA, but limited data are available in human tumor tissue for the latter. We therefore measured incorporation in human tumor biopsy specimens after administration of a test dose of 5-FU alone or with leucovorin.

Patients and methods: Patients received 5-FU (500 mg/m²) with or without high-dose leucovorin, low-dose leucovorin or l-leucovorin, and biopsy specimens were taken after approximately 2, 24 or 48 h. Tissues were pulverized and extracted for nucleic acids. 5-FU incorporation was measured using gas chromatography/mass spectrometry after complete degradation to bases of isolated RNA and DNA.

Results: Maximal incorporation into RNA (1.0 pmol/mg RNA) and DNA (127 fmol/mg DNA) of 59 and 46 biopsy specimens, respectively, was found at 24 h after 5-FU administration. Incorporation into RNA but not DNA was significantly correlated with intratumoral 5-FU levels. However, DNA incorporation was significantly correlated with the RNA incorporation. Primary tumor tissue, liver metastasis and normal mucosa did not show significant differences, while leucovorin had no effect. Neither for RNA (30 patients) nor DNA (24 patients) incorporation was a significant correlation with response to 5-FU therapy found. However, in the same group of patients, response was significantly correlated to TS inhibition (mean TS in responding and non-responding groups 45 and 231 pmol/h/mg protein, respectively; \( P = 0.001 \)).

Conclusions: 5-FU is incorporated at detectable levels into RNA and DNA of human tumor tissue, but no relation between the efficacy of 5-FU treatment and incorporation was found, in contrast to TS.

Key words: 5-fluorouracil, 5-FU incorporation into DNA, 5-FU incorporation into RNA, human colorectal cancer, leucovorin, thymidylate synthase inhibition

Introduction

Since 1957, 5-fluorouracil (5-FU) has played an important role in the treatment of colon cancer and is used for patients with breast cancer and cancer of the head and neck [1]. 5-FU is usually given in combination therapy. So far in colon cancer, response rates of 10–20% after bolus injection of 5-FU as single agent could be improved by leucovorin by up to 30%. A number of schedules are being used, although the choice is often a matter of local preference. For combinations with irinotecan or oxaliplatin, response rates up to 60% have been reported [2–6] for colon cancer.

Insight into the mechanism of action of 5-FU might improve the therapies in which 5-FU has been included. Various mechanisms including inhibition of thymidylate synthase (TS) by 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), incorporation of 5-fluorouridine-5'-triphosphate (FUTP) into RNA and incorporation of 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP) into DNA have been reported. Although TS inhibition and its expression have been related to the antitumor effect of 5-FU [7–13], it has not yet been demonstrated clinically whether incorporation into RNA or DNA contribute to its antitumor effect.

Inhibition of TS, a key enzyme of pyrimidine de novo synthesis, has been studied extensively during recent decades, in vitro as well as in vivo [1]. TS inhibition results in a depletion of dTTP and an increase in dUTP followed by decreased DNA synthesis and DNA repair. The inhibition of TS can be potentiated by leucovorin. Leucovorin acts as precursor for 5,10-methylene-tetrahydrofolate, which is necessary for the formation of the ternary complex with TS and FdUMP, essential for long-term maintenance of TS inhibition.
Incorporation of 5-FU into RNA has also been related to the cytotoxic action of 5-FU [14, 15] and has been postulated to be schedule dependent [16]. In a recent clinical study it was demonstrated that incorporation into RNA was higher after bolus administration of 5-FU, but not significantly different with continuous administration [17]. In an animal tumor model, the combination of 5-FU with thymidine, which bypasses the inhibition of TS, increased the antitumor activity of 5-FU, indicating that incorporation into RNA was the mechanism of action [18]. Evidence that this mechanism contributes to toxicity was obtained from studies with uridine protection. In normal epithelium of the intestine, p53-dependent apoptosis was only inhibited by uridine and not by thymidine after exposure to 5-FU [19]. Both UDPG, a precursor for uridine, and uridine decreased the incorporation of 5-FU into the RNA of tumors, but did not affect the inhibition of TS or the antitumor effect [20]. Uridine enabled an increase in the dose, resulting in increased TS inhibition and antitumor activity. Cytotoxicity by 5-FU incorporation into RNA is possibly mediated by its incorporation into snRNA, especially U2-RNA, which inhibits the splicing of pre-mRNA, resulting in impaired mRNA synthesis [21, 22]. Furthermore, it has been shown that pre-rRNA processing was inhibited after 5-FU administration and might be related to incorporation of 5-FU into U3-RNA [23, 24]. Incorporation of 5-FU into different RNA molecules may therefore lead to disturbance of mRNA processing and protein synthesis [25, 26].

5-FU can also be incorporated into DNA of murine bone marrow cells as well as human tumor cells and this may contribute to the cytotoxicity of 5-FU [27–31]. Misincorporation of 5-FU into DNA is associated with the formation of DNA strand breaks [32–34]. After incorporation into DNA, 5-FU can be excised by uracil-DNA-glycosylase followed by apurinic–apyrimidinic endonucleaseolytic cleavage, resulting in DNA strand breaks [35–37]. Also, when polymerase α is inhibited by a specific inhibitor like aphidicolin, strand break formation after 5-FU exposure remains, indicating a second mechanism of 5-FU-induced DNA strand break formation without incorporation of the drug [34], in which the imbalance of intracellular deoxyribonucleotide pools may play a role. This imbalance might be associated with the inhibition of TS, leading to a decrease in dTTP and an accumulation of dUTP with a subsequent decrease in DNA synthesis and repair. Misincorporation of dUTP and FdUTP into DNA can be prevented by the action of dUTPase [36].

Until now, incorporation of 5-FU into RNA and DNA has usually been determined in preclinical in vitro and in vivo model systems by using radiolabeled 5-FU. However, in order to determine the incorporation of 5-FU into RNA and DNA of patient samples, this is not a suitable method. We developed a non-radioactive method using gas chromatography coupled with mass spectrometry (GC–MS) to determine 5-FU incorporation into RNA [38, 39], which was adapted with minor changes to determine DNA incorporation as well. This is the first report describing the relevance of 5-FU incorporation into both RNA and DNA in a clinical setting and the possible relation with TS and response.

**Patients and methods**

**Patient selection and drug administration**

In this study, tissue samples of 68 patients (37 males) with a median age of 57 years (range 34–78 years) were included. All patients had advanced colorectal cancer. The protocol was approved by the Institutional Review Board and also aimed to evaluate the extent and retention of TS inhibition in relation to response to 5-FU [11]. All patients gave informed consent. The 5-FU schedules used are given routinely in our hospital. The study comprised two parts: (i) administration of an experimental dose of 5-FU at 500 mg/m² alone or in combination with leucovorin before a planned surgery (mostly implantation of a Port-a-Cath); and (ii) treatment of a selection of these patients with a hepatic artery infusion or an intravenous schedule. Twenty-two patients were treated with hepatic arterial infusions of 5-FU (1000 mg/m²/day × 5 every 3 weeks). Of patients in whom only resection of the primary tumor took place, eight were subsequently treated with bolus injections of 5-FU (500 mg/m² weekly) and 10 were treated with a 2h infusion of high-dose leucovorin (hd-LV) and a midway bolus injection of 5-FU (500 mg/m² leucovorin and 500 mg/m² 5-FU weekly).

Of the other patients, one was treated with a 2h infusion of leucovorin and a midway bolus injection of 5-FU followed by oral uridine (500 mg/m² leucovorin, 600 mg/m² 5-FU, 5 g/m² uridine every 6h for 72h, weekly). A total of 21 patients received no further treatment while the subsequent treatment of six patients was not fluoropyrimidine related. Of the eight patients who received 5-FU bolus injections, three were subsequently treated with hepatic arterial infusions of 5-FU. Also, one of the 10 patients receiving 5-FU with leucovorin was subsequently treated with hepatic arterial infusions of 5-FU.

**Chemicals**

5-FU, alkaline phosphatase type VII-S (APase, 1000 U/150 μl, EC 3.1.3.1) and thymidine phosphorylase (TPase, 600 U/ml, EC 2.4.2.4) were obtained from Sigma (St Louis, MO). 5-FU-[¹⁵N₂] was from Merck-Sharp and Dome (Montreal, Canada) and pentfluorobenzylbromide was from Pierce Chemicals (Rockford, IL). DNase I (2000 U/mg; EC 3.1.21.1), Nuclease P1 (300 U/mg; EC 3.1.30.1), RNase A (50 U/mg), RNase T1 (10⁵ U/ml) and proteinase K were acquired from Roche Molecular Biochemicals (Almere, The Netherlands). Uridine phosphorylase [UPase (EC 2.4.2.3), 590 U/ml] was kindly provided by A. Komissarov. All other chemicals were of analytical grade. Solutions were made in water purified by a Millipore Reagent Q system (Millipore, Bedford, MA).

**5-FU incorporation into RNA and DNA of human tumor tissue**

From patients included in a study described elsewhere [11, 13, 38, 39], samples of primary colorectal cancer, liver metastasis and colon mucosa were analyzed for the incorporation of 5-FU into RNA and DNA. These patients received an experimental dose of 5-FU as an i.v. bolus injection at a dose of 500 mg/m² with or without a 2 h infusion of either hd-LV (500 mg/m²), low-dose leucovorin (ld-LV; 25 mg/m²) or l-leucovorin (l-LV; 250 mg/m²) at approximately 2, 24 or 48 h before surgical resection of the tissues; biopsy specimens of the tissues were immediately frozen in liquid nitrogen and stored at −80°C.

The frozen tissue was pulverized as described previously [38]. Three volumes of ice-cold saline were added to the frozen pulverized tumor tissue and mixed thoroughly. After centrifugation for 10 min at 4000 r.p.m.
and 4°C, the resulting pellet, containing the RNA and DNA, was stored at −80°C until RNA and DNA extraction.

**RNA isolation and degradation**

RNA was isolated as described previously [39]. Precipitated nucleic acids from tissues were suspended in 5 ml lysis buffer, which consisted of 4 M guanidine-isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine and 0.1 M β-mercaptoethanol. After suspension, 0.5 ml 2 M sodium acetate (pH 4) was added and mixed. Subsequently, 5 ml water-saturated phenol and 1 ml chloroform/isoamyl alcohol (49/1 v/v) were added. The resulting suspension was shaken thoroughly and centrifuged for 15 min at 4000 r.p.m. and 4°C. The RNA-containing upper layer was removed and precipitated with an equal volume of 2-propanol at −80°C for 2 h. After centrifugation at 4000 r.p.m. and 4°C for 10 min, the RNA pellet was resuspended in 1.8 ml lysis buffer and reprecipitated with 2 ml 2-propanol. The RNA was centrifuged again and reconstituted in 500 μl digestion buffer [40 mM Tris, 1 mM MgCl2, 0.1 mM ZnCl2 and 40 mM KH2PO4 (pH 7.4)]. RNA concentration and purity were determined after measurement of the optical density at 260 and 280 nm. To 360 μl RNA suspension, 20 μl RNase A/RNase T1 (500/500 U/ml), 10 μl APase (1000 U/ml) and 10 μl UPase (590 U/ml) were added. The mixture was incubated at 37°C for 1 h to degrade the RNA to bases completely. After incubation the samples were stored at −20°C until extraction for GC–MS analysis [39].

**DNA isolation and degradation**

DNA isolation was essentially performed as described previously [40]. Precipitated nucleic acids from tissues were suspended in 7 ml lysis buffer [100 mM Tris, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml proteinase K (pH 8.5)]. The suspension was incubated at 55°C overnight and subsequently for 1 h at 37°C with RNase A/T1 10/10 U/ml final concentration (RNase A/T1 was heated at 95°C for 15 min to inactivate DNase). In order to precipitate the DNA, an equal volume of 2-propanol was added and mixed until precipitation was complete. DNA was recovered from the solution and resuspended at 55°C in 2 ml buffer containing 10 mM Tris and 1 mM EDTA (pH 8.0). The suspension was purified further by extractions with an equal volume of phenol/chloroform/isoo-amyly alcohol (50/49/1 v/v/v) and chloroform/iso-amyly alcohol (49/1 v/v). DNA was reprecipitated with an equal volume of 2-propanol and reconstituted in 0.5 ml digestion buffer [40 mM Tris, 1 mM MgCl2, 0.1 mM ZnCl2, 40 mM KH2PO4 (pH 7.4)] at 55°C. For measurement of concentration and purity, optical density was measured at 260 and 280 nm. For enzymatic degradation of the DNA, 20 μl DNase I (1 mg/ml), 20 μl Nuclease P1 (250 U/ml), 10 μl APase (1000 U/ml) and 10 μl TPase (600 U/ml) were added to 340 μl DNA suspension. In this composition the reaction mixture was optimal to degrade all DNA to bases after incubation overnight at 37°C. The digests were stored at −20°C until extraction for GC–MS analysis.

**GC–MS extraction and measurement**

Extraction for GC–MS was performed as described previously [39]. Briefly, 50 μl 5-FU-15N2 (1 μM for RNA and 0.1 μM for DNA samples), 1 ml milli-q water and 100 μl 2 M Tris (pH 6) were added to 300–350 μl enzyme digestion product. The solution was extracted twice with 4 ml di-ethyl-ether/2-propanol (80/20 v/v). The organic phase was blown to dryness under N2 at 60°C. The residue was reconstituted in 80 μl acetonitrile, and 10 μl triethylamine and 10 μl pentfluorobenzylbromide were added. The mixture was left at room temperature for at least 15 min. After the addition of 400 μl 0.1 M HCl the solution was extracted once with 1 ml hexane. The organic layer was blown to dryness under N2 at 45°C and the residue was dissolved in 50 μl hexane/propanon (3/1 v/v). This sample was injected into the GC–MS system (Automass 2; ThermoQuest BV, Breda, The Netherlands). Chromatographic separation was carried out on a CPSil19 CB column (25 m × 0.25 internal diameter, film thickness 0.2 μm) (Chrompack, Middelburg, The Netherlands). The ions for 5-FU and 5-FU-15N2 (m/z 309 and m/z 311, respectively) were recorded with negative chemical ionization detection and methane as the moderating gas. More details of the 5-FU measurement with GC–MS have been described elsewhere [39].

**Thymidylate synthase assays**

Using the same samples in which we measured incorporation of 5-FU into RNA and DNA, we also evaluated the activity of TS. TS was assayed as described previously [11].

**Statistics**

In order to evaluate differences between the various groups of patients, we used SPSS for Windows version 11.5.

**Results**

**Incorporation into RNA of human tissue**

The assay to optimize the incorporation of 5-FU into RNA was described previously [39]. Figure 1 shows 5-FU incorporation into RNA of 59 human tumor tissues (17 primary tumors and 42 liver metastases) and nine colon mucosa samples obtained at approximately 2, 24 and 48 h after 5-FU administration, and the mean values of the different groups are summarized in Table 1. The incorporation of 5-FU into RNA of human tumor tissue showed a variation of 0.01–1.45 pmol/μg RNA. Variation in RNA incorporation within a tissue sample, determined in 11 samples, showed a mean variation of 37% (data not shown). Within 2 h of the administration of 5-FU it was incorporated into RNA, with mean values for primary colon tumors, liver metastasis and colon mucosa of 0.40, 0.74 and 0.21 pmol/μg RNA, respectively. At 24 h the incorporation...
increased to 0.96 and 1.02 pmol/mg RNA for primary colon tumors and liver metastasis, respectively. No colon mucosa samples were available at this time point. At 48 h after 5-FU administration the incorporation decreased to 0.20 pmol/mg RNA in primary colon tumor samples, 0.33 pmol/mg RNA in liver metastasis and 0.27 pmol/mg RNA in colon mucosa.

The addition of hd-LV, ld-LV or l-LV to 5-FU therapy was studied at 48 h after administration. Incorporation of 5-FU after the addition of hd-LV and ld-LV showed similar results as treatment with 5-FU alone and was not significantly different. Only l-LV seemed to increase the 5-FU incorporation as compared with 5-FU alone, but not with regimens including hd-LV and ld-LV (Table 2). The incorporation of 5-FU into RNA is significantly correlated \( (P=0.001) \) to the concentration of 5-FU in the tissue, as was also found previously in murine tumors [12] (Table 2).

The response to therapy with 5-FU was evaluable in 30 patients and was compared to the incorporation of 5-FU into RNA 48 h after administration (Figure 2). No significant relation was found, either when evaluating the whole group or the subgroups receiving different i.v. schedules of 5-FU (Table 2).

### Incorporation into DNA of human tissue

The incorporation of 5-FU into DNA was measured using a novel sensitive assay, allowing the use of small tissue samples. Similar to the assay for 5-FU incorporation into RNA, it was first validated in a cell line and murine tissues. Similar results to those using classical methods (radioactive 5-FU) were observed (data not shown). Figure 3 shows

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**Table 1.** Time dependence and the effect of leucovorin on the incorporation of 5-FU into RNA of human tumor tissue and mucosa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time, h</th>
<th>Tumor [mean ± SEM (n)], pmol/mg</th>
<th>Mucosa [mean ± SEM (n)], pmol/mg</th>
<th>Metastasis [mean ± SEM (n)], pmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>2</td>
<td>0.40±0.19 (4)</td>
<td>0.29/0.12 (2)</td>
<td>0.74±0.24 (3)</td>
</tr>
<tr>
<td>5-FU</td>
<td>24</td>
<td>0.96±0.26 (3)</td>
<td>NA</td>
<td>1.02±0.16 (4)</td>
</tr>
<tr>
<td>5-FU</td>
<td>48</td>
<td>0.20±0.08 (3)</td>
<td>0.27 (1)</td>
<td>0.33±0.10 (10)</td>
</tr>
<tr>
<td>5-FU/hd-LV</td>
<td>48</td>
<td>0.26/0.43 (2)</td>
<td>0.39/0.24 (2)</td>
<td>0.48±0.16 (5)</td>
</tr>
<tr>
<td>5-FU/ld-LV</td>
<td>48</td>
<td>0.30±0.04 (3)</td>
<td>0.47±0.18 (3)</td>
<td>0.38±0.07 (10)</td>
</tr>
<tr>
<td>5-FU/l-LV</td>
<td>48</td>
<td>0.47/0.38 (2)</td>
<td>0.27 (1)</td>
<td>0.53±0.11 (8)</td>
</tr>
</tbody>
</table>

Tumors were excised at different time points after the administration of 5-FU alone or in combination with hd-LV, ld-LV or l-LV. In the case of one or two samples, individual values are given.

5-FU, 5-fluorouracil; hd-LV, high-dose leucovorin; ld-LV, low-dose leucovorin; l-LV, l-leucovorin; NA, not available.

**Table 2.** Statistical evaluation of the incorporation of 5-FU into RNA and DNA of human tumor tissue and mucosa

<table>
<thead>
<tr>
<th>Parameter 1</th>
<th>Parameter 2</th>
<th>Test</th>
<th>RNA Rho</th>
<th>RNA P value</th>
<th>DNA Rho</th>
<th>DNA P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU 48 h</td>
<td>5-FU/hd-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.344</td>
<td>0.250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU 48 h</td>
<td>5-FU/ld-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.244</td>
<td>0.782</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU 48 h</td>
<td>5-FU/l-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.048</td>
<td>0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU/hd-LV 48 h</td>
<td>5-FU/ld-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.865</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU/hd-LV 48 h</td>
<td>5-FU/l-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.648</td>
<td>0.064</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU/ld-LV 48 h</td>
<td>5-FU/l-LV 48 h</td>
<td>Mann–Whitney U-test</td>
<td>0.348</td>
<td>0.053</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon tumor</td>
<td>Liver metastasis</td>
<td>Paired t-test</td>
<td>0.893</td>
<td>0.245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon tumor</td>
<td>Colon mucosa</td>
<td>Paired t-test</td>
<td>0.396</td>
<td>0.658</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU tissue</td>
<td>5-FU RNA/DNA</td>
<td>Spearman correlation</td>
<td>0.439</td>
<td>0.001</td>
<td>0.033</td>
<td>0.838</td>
</tr>
<tr>
<td>5-FU RNA/DNA</td>
<td>Response (i.v. LV/5-FU)</td>
<td>Spearman correlation</td>
<td>−0.070</td>
<td>0.848</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>5-FU RNA/DNA</td>
<td>Response (i.a. 5-FU)</td>
<td>Spearman correlation</td>
<td>0.171</td>
<td>0.511</td>
<td>0.347</td>
<td>0.205</td>
</tr>
<tr>
<td>5-FU RNA</td>
<td>5-FU DNA</td>
<td>Spearman correlation</td>
<td>0.309</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-FU RNA</td>
<td>5-FU DNA</td>
<td>Pearson correlation</td>
<td>0.522</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Evaluation was performed on data obtained after the administration of either 5-FU alone or in combination with hd-LV, ld-LV or l-LV. Correlation with response was evaluated at 48 h.

5-FU, 5-fluorouracil; hd-LV, high-dose leucovorin; ld-LV, low-dose leucovorin; l-LV, l-leucovorin.
the results of the incorporation of 5-FU into the DNA of 46 human tumor tissues (13 primary tumors and 33 liver metastases) and eight colon mucosa samples. The data are summarized as mean values in Table 3. These samples were obtained at approximately 2, 24 and 48 h after 5-FU administration. At the first time point, 2 h after 5-FU administration, only three samples showing incorporation into DNA were available: 11.95 fmol/µg DNA for the primary colon tumor sample, 47.1 fmol/µg DNA for the liver metastasis and 4.5 fmol/µg DNA for the colon mucosa sample (Table 3). The DNA incorporation of 5-FU at 24 h showed the largest variation and ranged from 0.6 to 245 fmol of 5-FU/µg DNA, with mean values of 126.53 and 141.68 fmol/µg DNA for the primary colon tumors and liver metastases, respectively. The incorporation at 48 h after administration of 5-FU decreased to 4.13 fmol/µg DNA for the primary colon tumors, 16.45 fmol/µg DNA for the liver metastases and 0.80 fmol/µg DNA in the only available colon mucosa sample. Also, for DNA incorporation LV seemed to increase incorporation compared with 5-FU alone (Table 2), but was not different compared to that of hd-LV and ld-LV. Primary colon tumors did not show a significantly different incorporation of 5-FU into DNA compared to liver metastasis from the same patients; also there was no difference between the primary tumor and normal mucosa (Table 2). In contrast to RNA incorporation there was no significant correlation between 5-FU concentration in the tissue and DNA incorporation of 5-FU. However, DNA incorporation was significantly correlated to RNA incorporation.

Incorporation of 5-FU into DNA after 48 h could be evaluated in 24 patients receiving a 5-FU-containing regimen, and in whom response was evaluable (Figure 4), but similar to RNA there was no significant relation between 5-FU incorporation into DNA and response (Table 2), either for the whole group or for subgroups.

**Thymidylate synthase levels**

Previously we reported a relation between TS levels and response to 5-FU [11]. In order to determine the relative role of TS levels in the patient group for which we also measured incorporation into RNA and DNA, we evaluated TS levels in this particular subgroup (Figure 5). The mean residual TS activity in patients responding to 5-FU-based treatment was 45 pmol/h.mg protein, while that in the group not responding to 5-FU treatment the levels were significantly higher (245 pmol/h.mg protein; \( P = 0.001 \)). Using other assays to evaluate TS expression, we observed similar differences.

**Discussion**

This is the first paper to describe the incorporation of 5-FU into both RNA and DNA in patient tumors without the use of radiolabeled drugs. In the same patients we also evaluated TS inhibition [11, 13], which was related to response to 5-FU treatment. It is unlikely that TS inhibition is related to 5-FU incorporation into RNA, but a prolonged TS inhibition might favor 5-FU incorporation into DNA. TS inhibition results in dTTP depletion, which favors the incorporation ofFdUTP into DNA due to the lack of competition for DNA polymerase between FdUTP and dTTP. This condition would also favor incorporation of the increased dUTP into DNA. Apparently potential breakdown of FdUTP by dUTPase did not prevent its incorporation into DNA. The relatively long retention of FdUTP in DNA might be due to the favorable incorporation conditions, but also to rather inefficient DNA repair. The latter process is catalyzed by uracil-DNA-glycosylase [36, 37] and apparently functions at different rates in the various tumors, considering the large variation in the incorporation of 5-FU into DNA.

The kinetics of 5-FU incorporation into RNA of human tumor tissue differed to those in murine tumors [12, 20], while
5-FU tissue levels [38] were correlated with 5-FU incorporated into RNA. Incorporation in human tumors was greater after 24 h compared with 2 and 24 h, whereas in murine tumors the incorporation is maximal at 2 h. The large variation found for DNA incorporation, especially at 24 and 48 h post-administration, shows that incorporation is not only dependent on the intra-tumoral concentration of 5-FU, but that other mechanisms also play a role. Other mechanisms involved are dUT-Pase activity, inhibition of TS and excision of incorporated FdUTP by uracil-DNA-glycosylase [35, 36, 43, 44].

Repair may also be dependent on the availability of deoxynucleotides, which is dependent on the extent of TS inhibition. So far in the same patient samples, only TS inhibition, total TS activity and expression could be related to response to therapy [11, 13, 45], which was also shown in several studies by measurement of mRNA expression and protein expression [7–10]. A relationship between 5-FU RNA incorporation and response in tumor biopsy specimens was demonstrated in one limited study that evaluated 11 cases for incorporation of 5-FU into RNA [42]. It was postulated that 5-FU was effective at a 5-FU incorporation level higher than 200 ng 5-FU/mg RNA. The samples analyzed in our study showed a lower incorporation at a similar time point, which might explain why we could not demonstrate a significant relationship with response to treatment. Also, DNA incorporation in a limited number of patients failed to show a significant relationship with response to treatment. Possibly the imbalance of deoxynucleotides by depletion of dTTP after TS inhibition plays a more important role in the response to treatment of patients, leading to the apoptosis observed in these samples [46, 47].

Measurement of the incorporation of 5-FU into RNA and DNA of human tissue enabled evaluation of the role of 5-FU incorporation in the efficacy of patient treatment. We were able to measure in the tumor samples from the same patients not only 5-FU levels [39] and 5-FU incorporation into both RNA and DNA, but also TS levels and inhibition [11, 45], TS induction [46] and the downstream effects of TS inhibition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>Tumor [mean ± SEM (n)], fmol/µg</th>
<th>Mucosa [mean ± SEM (n)], fmol/µg</th>
<th>Metastasis [mean ± SEM (n)], fmol/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>2</td>
<td>12.0(1)</td>
<td>4.5(1)</td>
<td>47.1(1)</td>
</tr>
<tr>
<td>5-FU</td>
<td>24</td>
<td>126.5±59.1(3)</td>
<td>n.a.</td>
<td>141.7±47.9(4)</td>
</tr>
<tr>
<td>5-FU</td>
<td>48</td>
<td>1.4/6.9(2)</td>
<td>0.8(1)</td>
<td>16.5±10.5(9)</td>
</tr>
<tr>
<td>5-FU/hd-LV</td>
<td>48</td>
<td>8.6/6.1(2)</td>
<td>1.0/6.1(2)</td>
<td>26.6±24.0(3)</td>
</tr>
<tr>
<td>5-FU/ld-LV</td>
<td>48</td>
<td>1.6±0.15(3)</td>
<td>18.1±14.8(3)</td>
<td>18.0±10.3(9)</td>
</tr>
<tr>
<td>5-FU/l-LV</td>
<td>48</td>
<td>78.0/56.8(2)</td>
<td>0.60(1)</td>
<td>44.6±24.1(6)</td>
</tr>
</tbody>
</table>

Table 3. Time dependence and effect of leucovorin on incorporation of 5-FU into DNA of human tumor tissue and mucosa

Figure 4. Incorporation of 5-fluorouracil (5-FU) into DNA 48 h after administration of 5-FU versus response to treatment with either i.v. leucovorin (LV)/5-FU (closed symbols) or i.a. 5-FU (open symbols). Each point represents a single sample. PD, progressive disease; SD, stable disease; PR, partial response; CR, complete remission.

Figure 5. Relationship between thymidylate synthase (TS) levels and response to 5-fluorouracil (5-FU)-based treatment in the same group of patients referred to in Figures 2 and 4. TS levels are shown as the residual TS activity measured at 10 µM dUMP [11] in responding [partial response (PR)/complete remission (CR), 16 patients] and non-responding patients [progressive disease (PD)/stable disease (SD), 26 patients]. Lines indicate the means, (231 and 45 pmol/h.mg protein, respectively). TS levels were significantly different between both groups (P = 0.001; Mann–Whitney U-test). Other assays to evaluate TS levels showed a similar result.
should aim to increase 5-FU-induced TS inhibition in tumors. It should not aim to increase 5-FU incorporation into RNA, but oxaliplatin and irinotecan can decrease TS expression in model systems [12, 14, 20]. In the same tumor, response was related to TS inhibition. Evidence is also accumulating that incorporation of 5-FU into RNA is related to 5-FU toxicity [12, 19, 20], which does not exclude a relationship between TS inhibition and toxicity of 5-FU. It was postulated by Sobero et al. [16] that 5-FU given as a bolus injection would act predominantly by its incorporation into RNA and that 5-FU given as a continuous infusion would act by inhibition of TS. However, in a recent study, the same group demonstrated that response to 5-FU given both as a bolus injection and as continuous infusion, was correlated with TS levels [48]. Only in the methotrexate-modulated arm was this relationship not found. These data are in line with the current findings and those in animal models, which also support TS as the main target. Toxicity caused by antifolate TS inhibitors such as Raltitrexed consists of myelotoxicity and gastrointestinal toxicity [49]. 5-FU can inhibit TS in normal gut mucosa and bone marrow [50]. In addition to novel antifolate TS inhibitors such as Raltitrexed, other new agents such as oxaliplatin and irinotecan can decrease TS expression in model systems [51, 52]. In patients, novel combinations or schedules should not aim to increase 5-FU incorporation into RNA, but should aim to increase 5-FU-induced TS inhibition in tumors.

References


27. Schuetz JD, Wallac HJ, Diasio RB. 5-Fluorouracil incorporation into DNA of CF-1 mouse bone marrow cells as a possible mechanism of toxicity. Cancer Res 1984; 44: 1358–1363.


