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5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers

P Noordhuis, U Holwerda, CL van der Wilt, CJ van Groeningen, K Smid, S Meijer, HM Pinedo, GJ Peters

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Abstract

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

Methodology: Patients received 5FU (500 mg/m²) with or without high-dose leucovorin, low-dose leucovorin or l-leucovorin and biopsy specimens were taken after about 2, 24 or 48-hr. Tissues were pulverized and extracted for nucleic acids. 5FU 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/µg-RNA) and DNA (127 fmol/µg-DNA) of 59 and 46 biopsy specimens, respectively, was found at 24 hours after 5FU administration. Incorporation into RNA but not DNA was significantly correlated with intratumoral 5FU 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) or DNA (24 patients) incorporation a significant correlation with response to 5FU therapy was found. However, in the same group of patients response was significantly correlated to TS inhibition (mean TS in responding and non-responding group 45 and 231 pmol/hr/mg protein, respectively, p=0.001).

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

Introduction

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

Insight in the mechanism of action of 5FU might improve the therapies in which 5FU 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 5FU(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 the pyrimidine de novo synthesis, has been studied extensively during the last decades in vitro as well as in vivo. TS inhibition results in a depletion of dTTP and an increase of dUTP followed by a 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 a long term maintenance of TS inhibition.

Incorporation of 5FU into RNA has also been related to the cytotoxic action of 5FU(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 5FU with thymidine, which bypasses the inhibition of TS, increased the anti-tumor activity of 5FU, 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 5FU(19). Both UDPG, a precursor for uridine, and uridine decreased the incorporation of 5FU into RNA of tumors but did not affect the inhibition of TS or the antitumor effect(20). Uridine enabled to increase the dose resulting in increased TS inhibition and antitumor activity. Cytotoxicity by 5FU 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 5FU administration and might be related with incorporation of 5FU into U3-RNA(23, 24). Incorporation of 5FU into different RNA molecules may therefore lead to disturbance of mRNA processing and protein synthesis(25, 26).

5FU 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 5FU(27-31). Misincorporation of 5FU into DNA is associated with the formation of DNA strand breaks(32-34). After incorporation into DNA 5FU can be excised by uracil-DNA-glycosylase followed by apurinic-apyrimidinic endonucleolytic cleavage resulting in DNA strand breaks(35-37). Also when polymerase-α is inhibited by a specific inhibitor like aphidicolin strand break formation after 5FU exposure remains, indicating a
second mechanism of 5FU 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 of dTTP and accumulation of dUTP with a subsequent decrease in DNA synthesis and repair. Misincorporation of dUTP and FdUTP into DNA can be prevented by action of dUTPase(36).

So far, incorporation of 5FU into RNA and DNA was usually determined in preclinical in vitro and in vivo model systems by the use of radiolabeled 5FU. However, in order to determine the incorporation of 5FU into RNA and DNA of patient samples this is not a suitable method. We developed a non-radioactive method using gas-chromatography coupled to mass-spectrometry (GC-MS) to determine 5FU 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 5FU 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 and 31 females) with a median age of 57 years (range 34 to 78) 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 5FU(11). All patients gave informed consent. The used 5FU schedules are routinely being given in our hospital. The study consisted of two parts: a) administration of an experimental dose of 5FU at 500 mg/m² alone or in combination with leucovorin prior to a planned surgery (mostly implantation of a Port-a-Cath); and b) treatment of a selection of these patients with a hepatic artery infusion or an iv schedule. Twenty-two patients were treated with hepatic arterial infusions of 5FU (1000 mg/m²/day x 5, every 3 weeks). From patients in whom only resection of the primary tumor took place, 8 patients were subsequently treated with bolus injections of 5FU (weekly 500 mg/m²), and 10 patients were treated with a 2 hours infusion of high-dose leucovorin and a midway bolus injection of 5FU (weekly 500 mg/m² leucovorin and 500 mg/m² 5FU). From the other patients, one was treated with a 2 hour infusion of leucovorin and midway bolus injection of 5FU followed by oral uridine (500 mg/m² leucovorin, 600 mg/m² 5FU, 5 g/m² uridine every 6 hr for 72 hr, weekly). A total of 21 patients received no further treatment while the subsequent treatment of 6 patients was not fluoropyrimidine related. From the 8 patients who received 5FU bolus injections 3 patients were subsequently treated with hepatic arterial infusions of 5FU; also one of
the 10 patients receiving 5FU with leucovorin was subsequently treated with hepatic arterial infusions of 5FU.

**Chemicals**

5FU, alkaline phosphatase type VII-S (APase, 1000 U/150μl, EC 3.1.3.1) and thymidine phosphorylase (TPase, 600 U/ml, EC 2.4.2.4) were obtained from Sigma (St. Louis, MO, USA). 5FU-15N2 was from Merck-Sharp and Dome (Montreal, Canada) and pentafluorobenzylbromide was from Pierce Chemicals (Rockford, Ill., USA). 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, USA).

**5FU 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 5FU into RNA and DNA. These patients received an experimental dose of 5FU as an iv bolus injection at a dose of 500 mg/m$^2$ with or without a 2 hour infusion of either high dose leucovorin (500 mg/m$^2$), low dose leucovorin (25 mg/m$^2$) or l-leucovorin (250 mg/m$^2$) at approximately 2, 24 or 48 hours before surgical resection of the tissues; biopsy specimens of the tissues were immediately frozen in liquid nitrogen and stored at -80°C or in liquid nitrogen.

The frozen tissue was pulverized as described before(38). To the frozen pulverized tumor tissue three volumes of ice-cold saline was added and mixed thoroughly. After centrifugation for 10 min at 4000 rpm 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 before(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 rpm and 4°C. The RNA containing upper layer was removed and precipitated with an equal volume of 2-propanol at -80°C for 2 hr. After centrifugation at 4000 rpm 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). The RNA concentration and purity was 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. This mixture was incubated at 37°C for 1 hour to completely degrade the RNA to bases. After incubation the samples were stored at -20°C until extraction for GC-MS analysis(39).

**DNA isolation and degradation**

The DNA isolation was essentially performed as described previously(40). Precipitated nucleic acids from tissues were suspended in 7 ml lysis buffer consisting of 100 mM Tris, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 µg/ml Proteinase K (pH=8.5). The suspension was incubated at 55°C overnight and another hour 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 further purified by extractions with an equal volume of phenol, phenol/chloroform/iso-amylalcohol (50/49/1, v/v/v) and chloroform/iso-amylalcohol (49/1, v/v), respectively. 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 and 40 mM KH₂PO₄, pH=7.4) at 55°C. For measurement of the concentration and purity optical density (OD) was measured at 260 and 280 nm. For enzymatic degradation of the DNA, to 340 µl DNA suspension 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. In this composition the reaction mixture was optimal to degrade all DNA to bases after incubation overnight at 37°C. The digest was stored at -20°C until extraction for GC-MS analysis.

**GC-MS extraction and measurement**

The extraction for GC-MS was performed as described before(39). Briefly, to 300 – 350 µl enzyme digestion product 50 µl 5FU-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. The solution was extracted twice with 4 ml di-ethyl-ether/2-propanol (80/20, v/v). The organic fraction was blown to dryness under N₂ at 60°C. The residue was reconstituted in 80 µl acetonitrile and 10 µl triethylamine and 10 µl pentafluorobenzylbromide were added. The mixture was left at room temperature for at least 15 min. After addition of 400 µl 0.1 M HCl the solution was extracted once with 1 ml hexane. The organic layer
Figure 1. Incorporation of 5FU into RNA of human tumor tissue at different time points after administration of 5FU alone (closed circle) or 5FU combined with high dose leucovorin (hd-LV, open circle), low dose leucovorin (ld-LV, closed triangle), l-leucovorin (l-LV, open triangle) and mucosa (closed squares). Each point represents a single sample.

was blown to dryness under N\textsubscript{2} 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 I.D., film thickness 0.2 µm) (Chrompack, Middelburg, The Netherlands). The ions for 5FU and 5FU-\textsuperscript{15}N\textsubscript{2} (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 5FU measurement with GC-MS have been described elsewhere(39).

Thymidylate synthase assays

In the same samples in which we measured incorporation of 5FU 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.
Figure 2. Incorporation of 5FU into RNA 48 hours after administration of 5FU versus response to treatment with either iv LV/5-FU (closed symbols) or ia 5FU (open symbols). Each point represents a single sample. PD/SD = progressive disease/stable disease, PR/CR = partial response/complete remission.

Results

Incorporation into RNA of human tissue

The assay to optimize the incorporation of 5FU into RNA was described previously. Fig. 1 shows 5FU incorporation into RNA of 59 human tumor tissues (17 primary tumors and 42 liver metastasis) and 9 colon mucosa samples obtained at about 2, 24 and 48 hours after 5FU administration and the mean values of the different groups are summarized in Table 1. The incorporation of 5FU into RNA of human tumor tissue showed a variation of 0.01 to 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 hours after administration of 5FU 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 hours the incorporation increased to 0.96 and 1.02 pmol/µg RNA for primary colon tumors and liver metastasis, respectively. No colon mucosa samples were available at this time point. At 48 hours after administration of 5FU the incorporation decreased to 0.20 pmol/µg RNA in primary colon tumor samples, 0.33 pmol/µg RNA in liver metastasis.
Table 1. Time dependence and the effect of leucovorin on incorporation of 5FU into RNA of human tumor tissue and mucosa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hr)</th>
<th>Tumor (mean ± SEM (n)) (pmol/µg)</th>
<th>Mucosa (mean ± SEM (n)) (pmol/µg)</th>
<th>Metastasis (mean ± SEM (n)) (pmol/µg)</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>n.a</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 administration of 5-FU alone or in combination with high dose leucovorin (hd-LV), low dose leucovorin (ld-LV) or l-leucovorin (l-LV). In case of 1 or 2 samples the individual values are given. n.a. = not available.

Table 2. Statistical evaluation of the incorporation of 5FU 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</th>
<th>p-value</th>
<th>DNA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU 48 hr</td>
<td>5FU/hd-LV 48 hr</td>
<td>Mann-Whitney U test</td>
<td>0.344</td>
<td>0.250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5FU 48 hr</td>
<td>5FU/ld-LV 48 hr</td>
<td>Mann-Whitney U test</td>
<td>0.244</td>
<td>0.782</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5FU 48 hr</td>
<td>5FU/l-LV 48 hr</td>
<td>Mann-Whitney U test</td>
<td>0.048</td>
<td>0.039</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5FU/hd-LV 48 hr</td>
<td>5FU/ld-LV 48 hr</td>
<td>Mann-Whitney U test</td>
<td>0.865</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5FU/hd-LV 48 hr</td>
<td>5FU/l-LV 48 hr</td>
<td>Mann-Whitney U test</td>
<td>0.648</td>
<td>0.064</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5FU/l-LV 48 hr</td>
<td>5FU/l-LV 48 hr</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></td>
<td>0.245</td>
<td></td>
</tr>
<tr>
<td>Colon tumor</td>
<td>Colon mucosa</td>
<td>paired T-test</td>
<td>0.396</td>
<td></td>
<td>0.658</td>
<td></td>
</tr>
<tr>
<td>5FU tissue</td>
<td>5FU 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>5FU RNA/DNA</td>
<td>Response (iv 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>5FU RNA/DNA</td>
<td>Response (ia 5FU)</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>

The evaluation was performed on data obtained after administration of either 5FU alone or in combination with high dose leucovorin (hd-LV), low dose leucovorin (ld-LV) or l-leucovorin (l-LV). Correlation with response was evaluated at 48 hours.

and 0.27 pmol/µg RNA in colon mucosa. Addition of high dose leucovorin (hd-LV), low dose leucovorin (ld-LV) or l-leucovorin (l-LV) to 5FU therapy was studied at 48 hr after administration. Incorporation of 5FU after addition of hd-LV and ld-LV showed similar results as treatment with 5FU alone and was not significantly different. Only l-LV seemed to increase the 5FU incorporation as compared with 5FU alone but not with regimens including hd-LV and ld-LV (Table 2). Comparison of the incorporation
of 5FU into DNA of human tissue at different time points after administration of 5FU alone (closed circle) or 5FU combined with high dose leucovorin (hd-LV, open circle), low dose leucovorin (ld-LV, closed triangle), l-leucovorin (l-LV, open triangle) and mucosa (closed squares). Each point represents a single sample.

Incorporation of 5FU into DNA was measured with a novel sensitive assay, allowing to use small tissue samples. Similar to the assay for 5FU incorporation into RNA, this assay was first validated in a cell line and murine tissues. Similar results as with classical methods (radioactive 5FU) were observed (data not shown).

Fig. 3 shows the results of the incorporation of 5FU into DNA of 46 human tumor tissues (13 primary tumors and 33 liver metastasis) and 8 colon mucosa samples. The data are summarized as mean values in Table 3. These samples were obtained at about 2, 24 and 48 hours after 5FU administration. For the first time point, about
Table 3. Time dependence and effect of leucovorin on incorporation of 5FU into DNA of human tumor tissue and mucosa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (hr)</th>
<th>Tumor (Mean ± SEM (n))</th>
<th>Mucosa (Mean ± SEM (n))</th>
<th>Metastasis (Mean ± SEM (n))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(fmol/µg)</td>
<td>(fmol/µg)</td>
<td>(fmol/µg)</td>
</tr>
<tr>
<td>5FU</td>
<td>2</td>
<td>12.0 (1)</td>
<td>4.5 (1)</td>
<td>47.1 (1)</td>
</tr>
<tr>
<td>5FU</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>5FU</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>5FU/hd-LV</td>
<td>48</td>
<td>8.6/8.1 (2)</td>
<td>1.0/6.1 (2)</td>
<td>26.6 ± 24.0 (3)</td>
</tr>
<tr>
<td>5FU/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>5FU/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>

Tumors were excised at different time points after administration of 5FU alone or in combination with high dose leucovorin (hd-LV), low dose leucovorin (ld-LV) or l-leucovorin (l-LV). In case of 1 or 2 samples the individual values are given. n.a. = not available

2 hours after 5FU administration, only 3 samples were available which showed an incorporation into DNA of 11.95 fmol/µg DNA for the primary colon tumor sample, 47.1 for the liver metastasis and 4.5 pmol/µg DNA for the colon mucosa sample (Table 3). The DNA incorporation of 5FU at 24 hours showed the largest variation and ranged from 0.6 to 245 fmol 5FU/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 hours after administration of 5FU 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 the DNA incorporation LV seemed to increase the incorporation compared to 5FU 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 5FU into DNA compared to liver metastases 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 the 5FU concentration in the tissue and the DNA incorporation of 5FU. However, the DNA incorporation was significantly correlated to the RNA incorporation.

The 5FU incorporation into DNA after 48 hr could be evaluated in 24 patients receiving a 5FU containing regimen and in whom response was evaluable (Fig. 4), but similar to RNA, there was no significant relation (Table 3), neither for the whole group or subgroups.

**Thymidylate synthase levels**

Previously we reported a relation between TS levels and response to 5FU(11). In order to determine the relative role of TS levels in the group of patients in which we
Figure 4. Incorporation of 5FU into DNA 48 hours after administration of 5FU versus response to treatment with either iv LV/5-FU (closed symbols) or ia 5FU (open symbols). Each point represents a single sample. PD/SD = progressive disease/stable disease and PR/CR = partial response/ complete remission.relation (Table 2), neither for the whole group or subgroups.

also measured incorporation into RNA and DNA, we evaluated TS levels in this particular subgroup (Fig. 5). The mean residual TS activity in patients responding to 5FU based treatment was 45 pmol/hr.mg protein, while that in the group not responding to 5FU treatment the levels were significantly higher (245 pmol/hr.mg protein; p =0.001). Also using other assays to evaluate TS expression we observed similar differences.

Discussion

This is the first paper which describes the incorporation of 5FU into both RNA and DNA in tumors from patients without the use of radio-labeled drugs. In the same patients we also evaluated TS inhibition(11, 13), which was related to response to 5FU treatment. It is unlikely that TS inhibition is related to 5FU incorporation into RNA but a prolonged TS inhibition might favor 5FU incorporation into DNA. TS inhibition results in dTTP depletion, which favors the incorporation of FdUTP 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 a 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.

The incorporation of 5FU into RNA of human tumor tissue showed different kinetics than those found in murine tumors(12, 20), while 5FU tissue levels(38) were correlated with 5FU incorporated into RNA. The incorporation in human tumors was higher after 24 hours compared to 2 and 24 hr, whereas in murine tumors the incorporation is maximal at 2 hours. The large variation found for the DNA incorporation, especially at 48 hr after administration, shows that incorporation is not only dependent on the intra-tumoral concentration of 5FU but that other mechanisms also play a role.

Other mechanisms involved are dUTPase activity, inhibition of TS and excision of incorporated FdUTP by Uracil-DNA-Glycosylase(35, 36, 41, 42).

The 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, 43) which was also shown in several studies by measurement of mRNA expression.

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**Figure 5.** Relation between TS levels and response to 5FU-based treatment in the same group of patients mentioned in Figs. 2 and 4. TS levels are shown as the residual TS activity measured at 10 μM dUMP(11), in responding (PR/CR, 16 patients) and non-responding (PD/SD, 26 patients). Lines indicate the means, 231 and 45 pmol/hr.mg protein, respectively. TS levels were significantly different between both groups (p=0.001; Mann-Whithey U test). Other assays to evaluate TS levels showed a similar result.
and protein expression(7-10). A relation of 5FU RNA incorporation with response in tumor biopsy specimens was demonstrated in one limited study which evaluated 11 cases for incorporation of 5FU into RNA(44). It was postulated that incorporation at a level higher than 200 ng/mg RNA 5FU was effective. The samples analyzed in our study showed a lower incorporation at a similar time point which might explain that we could not demonstrate a significant relation with response to treatment. Also the DNA incorporation although 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(43, 45).

Measurement of the incorporation of 5FU into RNA and DNA of human tissue enabled the evaluation of the role of 5FU incorporation on efficacy of the treatment of patients. We were able to measure in the tumor samples from the same patients not only 5FU levels(39), 5FU incorporation into both RNA and DNA, but also TS levels and inhibition(11, 43), TS induction(45) and the downstream effects of TS inhibition(11, 43). These are all mechanisms by which fluoropyrimidines are postulated to act. This unique set of data obtained in the same group of patients clearly demonstrates that response to 5FU treatment is related to TS levels and expression and not to 5FU incorporation into RNA. These findings are supported by the observations that TS inhibition and response to treatment are increased by addition of leucovorin(11). Also in animal models(12, 14, 20), in which TS inhibition and incorporation into RNA were evaluated in the same tumor, response was related to TS inhibition. Evidence is also accumulating that incorporation of 5FU into RNA is related to 5FU toxicity(12, 19, 20), which does not exclude a relation between TS inhibition and toxicity of 5FU. It was postulated by Sobrero et al(16), that at a bolus injection 5FU would act predominantly by its incorporation into RNA and that a continuous infusion TS inhibition would be the main target. However, in a recent study the same group demonstrated that both at a bolus injection and at continuous infusion response was correlated with TS levels(46). Only in the methotrexate modulated arm this relation was not found. These data are in line with the current finding and that in animal models which also support TS as the main target. Toxicity caused by antifolate TS inhibitors such as Raltitrexed consists of myelotoxicity and gastro-intestinal toxicity(47). 5FU can inhibit TS in normal gut mucosa and bone marrow(48). Besides novel antifolate TS inhibitors such as Raltitrexed, also other new agents such as oxaliplatin and irinotecan can decrease TS expression in model systems(49, 50). In patients novel combinations or schedules should not aim to increase 5FU incorporation into RNA but these approaches should aim to increase 5FU induced TS inhibition in tumors.
REFERENCES.


5-FU incorporation into RNA and DNA in tumor tissue