J Cancer Res Clin Oncol (1997) 123: 595–601

ORIGINAL PAPER

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5-Fluorouracil/leucovorin-induced inhibition of thymidylate synthase in normal tissues of mouse and man

Received: 9 April 1997 / Accepted: 27 July 1997

Abstract We evaluated the effects of 5-fluorouracil (5FU) and leucovorin (LV) on thymidylate synthase (TS) in normal rapidly dividing tissues, which may contribute to toxic side-effects of treatment with 5FU and LV. TS levels were determined in biopsies of human liver and colon mucosa and murine bone marrow, liver and intestinal mucosa at several time points after administration of therapeutic doses of 5FU or LV/5FU. In murine liver, after treatment with 100 mg/kg 5FU, TS inhibition was significantly higher than after LV/5FU administration ($P < 0.001$). A similar trend was observed in human liver tissue. Murine intestinal mucosa had TS levels below the limit of detection after 5FU or LV/5FU treatment. In human colon mucosa samples, administration of 500 mg/m^2 5FU resulted in a large extent of TS inhibition but the small number of samples did not allow a time- or 5FU-LV/5FU-related evaluation. TS activity in murine bone marrow cells was strongly inhibited to 10% of the control value during 48 h. LV/5FU administration resulted in a slightly higher inhibition. No human bone marrow was available to measure TS levels. Both in mice and humans the most pronounced TS inhibition occurred in the tissue that was involved in dose-limiting toxicity. Therefore it is very likely that TS inhibition in normal tissues contributes to the toxic side-effects of 5FU treatment.

Key words 5-Fluorouracil · Leucovorin · Thymidylate synthase · Colon cancer

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Introduction

Combination chemotherapy of 5-fluorouracil (5FU) and leucovorin (LV) is mainly used for the treatment of colon cancer, but has also been applied for breast and head and neck cancer (Peters and van Groeningen 1991). In this combination 5FU is the active cytotoxic agent. The 5FU nucleotides 5-fluorouridine 5′-triphosphate (FUTP) and 5-fluoro-2′-deoxyuridine 5′-triphosphate (FdUTP) can be misincorporated into RNA and DNA respectively. A third metabolite, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) is a potent inhibitor of the enzyme thymidylate synthase (TS, EC 2.1.1.45; 5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase), an essential enzyme in the synthesis of DNA. The inhibition of TS by FdUMP can be enhanced and prolonged by the LV metabolite 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4-folate). A stable ternary complex is formed consisting of TS, FdUMP and CH_2-H_4-folate (Danenberg 1977). This biochemical effect forms the background for the coadministration of LV with 5FU. Naturally occurring low 5,10-CH_2-H_4-folate levels in tissues, which are limiting for inhibition of TS, can be elevated by LV administration. Administration of LV/5FU to patients has been shown to result in a better inhibition of TS than that produced by 5FU alone (Swain et al. 1989; Peters et al. 1994). The increased TS inhibition observed after LV/5FU resulted in a better antitumour effect, compared to the effects of single-agent 5FU (Swain et al. 1989). These observations reflect the importance of TS inhibition in tumours with regard to antiproliferative effects.

The most frequently seen dose-limiting toxic side-effects of 5FU are myleosuppression after bolus injections and gastrointestinal toxicity after continuous infusion of 5FU (Peters and van Groeningen 1991; Leichman 1994). Coadministration of LV and 5FU did not only improve the antitumour activity of 5FU, as has been shown in both murine and human colon tumours, but also increased the gastrointestinal toxicity compared with bo-

lus 5FU treatment in both species (Nadal et al. 1988; Piedbois et al. 1992). It has not been completely elucidated which of the biochemical mechanisms of action of 5FU is involved in toxic side-effects in normal tissues. For gastrointestinal mucosa, incorporation into the RNA was postulated to be an important mechanism in mice (Houghton et al. 1979; Martin et al. 1982), but not much has been written about the mechanism for bone marrow cells and liver tissue. It has been shown that delayed uridine administration reduces myeloid toxicity (Nadal et al. 1989; van Groeningen et al. 1989, 1993). This observation indicates the type of RNA effects that are related to toxicity, since uridine reduced 5FU incorporation into RNA, but did not affect TS inhibition (Peters et al. 1988; Nord et al. 1992).

Little is known about TS activity and 5FU-induced TS inhibition in normal human tissues. One may speculate that TS levels are relatively high in rapidly dividing and DNA-synthesising tissues, such as the gastrointestinal mucosa and bone marrow. Is TS inhibited in these tissues after 5FU administration? Furthermore is there a relation between TS inhibition and the toxic side-effects of 5FU administration that occur in these tissues?

We studied the effect of i.p. 5FU administration on the inhibition of TS in normal murine bone marrow, intestinal mucosa and liver tissues. The murine liver tissue was included in this study because of the comparison with human liver that could be obtained from patients with liver metastases of colon cancer. A study of Harrison et al. (1978) has shown that toxicity due to 5FU in mice is highly similar to that in humans. The effects of coadministration of LV with 5FU on TS were primarily studied in mice. We compared the preclinical results with TS inhibition measured in liver and normal colon mucosa of patients, who had received a bolus injection of 5FU or LV infusion with a midway bolus injection of 5FU before surgery. The present study, performed in normal tissues of mice and humans, provides an insight into the contribution of TS inhibition to the toxic side-effects of 5FU.

Materials and methods

Materials

5FU was obtained from ABIC (Netanya, Israel) and LV was provided by Wyett-Lederle (Amsterdam, The Netherlands) and the pharmacy department of the Free University Hospital (Amsterdam, the Netherlands). [6-^{3}H]FdUMP (specific activity 20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, Calif., USA) and [5-^{3}H]dUMP (specific activity 10.9 Ci/mmol) from Amersham International (Buckinghamshire, England). All other chemicals were of analytical grade, and were commercially available.

Mice

Female Balb/c mice (age 8–10 weeks, about 20 g) (Harlan/Olac; C.P.B., Zeist, The Netherlands) received a single i.p. bolus injection of 100 mg/kg 5FU. This is the maximal tolerated dose for weekly i.p. injections (Peters et al. 1987). LV was given as an i.p. bolus injection 1 h before and together with 5FU in two doses of 50 mg/kg (total dose LV 100 mg/kg). This schedule was derived from the experiments of Nadal et al. (1988). At this dose the weight loss of mice amounted to 5%–15% as a result of gastrointestinal toxicity, while blood cells decreased to 40%. No liver toxicity was apparent. Delivery by i.p. bolus in small animals such as rats and mice results in pharmacokinetic behaviour similar to that following systemic i.v. bolus treatment in man (De Bruijn et al. 1986; Peters et al. 1993), such as a comparable half-life of 10–15 min.

Liver tissue, intestinal mucosa and bone marrow cells of the mice were removed 3, 24 and 48 h after treatment and immediately frozen in liquid nitrogen. Samples from untreated mice served as controls.

Patients

Patients in this study (Table 1) all underwent surgery for therapeutic purposes, i.e. implantation of a Port-a-Cath to be used for subsequent hepatic arterial infusions with 5FU. Informed consent was obtained from all patients before surgery and drug administration. Control liver samples were obtained from 8 patients with a known malignant disease, namely colon cancer without liver metastases. 5FU or LV/5FU samples (colon and/or liver tissue) were from patients who all had histologically proven colorectal cancer. These patients (36) received a single dose of 5FU at 500 mg/m^2 or (29 patients) were given LV administered as a 2-h infusion, with a bolus injection of 5FU at 500 mg/m^2 in the middle of the infusion. Three doses of LV were used: 25 mg/m^2 or 500 mg/m^2 racemic DL-LV or 250 mg/m^2 purified L-LV, which is believed to be the more active of the stereoisomers. These chemotherapeutic agents were given as a single test dose before surgery in order to examine inhibition of TS in surgical samples. Biopsy specimens were taken between 1 h and 48 h after treatment and immediately frozen in liquid nitrogen.

Thymidylate synthase assays on tissues

TS inhibition was evaluated with two assays, which provided different information. The ligand-binding assay gives an estimate of the amount of (free) TS protein. The assay with [6-^{3}H]FdUMP determined the free binding sites for FdUMP. Tissue homogenate was incubated with [6-^{3}H]FdUMP and the cofactor 5,10-CH_2-H_4-folate at 37°C. After 1 h, free [6-^{3}H]FdUMP was removed by a charcoal wash and [6-^{3}H]FdUMP bound to TS was counted. The other assay, the ^{3}H-release assay, gives information about the catalytic activity of the enzyme. One binding site, as measured in the ligand-binding assay, can serve for one or more catalytic conversions. We used [5-^{3}H]dUMP to measured the catalytic activity of the enzyme in converting dUMP into dTMP. Two substrate

Table 1 Data on patient characteristics and sample numbers. The number of patients in each drug administration group and the number of tissue samples obtained from the whole patient group are summarized. *5FU* 5-fluorouracil, *LV* leucovorin. Subscripts (DL-LV_{25} etc.) indicate the doses used (mg/m^2)

Number (male/female)	73 (37/36)
Median age (years)	55 (34–78)
Control	8 patients
5FU	36 patients
LV/5FU (DL-LV_{25}; DL-LV_{500}; L-LV_{250})	29 (11; 10; 8) patients
Colon mucosa only	5 patients
Liver only	57 patients
Colon mucosa and liver	11 patients
Total colon mucosa	16 samples
Total liver	68 samples

concentrations of dUMP were used in the catalytic activity assay: 1 μM, which is around the K_m, and 10 μM, a saturating substrate concentration. Tissue homogenate was incubated with [5-^{3}H]dUMP and the cofactor 5,10-CH_2-H_4-folate at 37°C. After 30 min [5-^{3}H]dUMP bound to TS and free [5-^{3}H]dUMP were precipitated by an acid charcoal wash. The amount of [^{3}H]H_2O released during the reaction was measured in the supernatant. Details of the assays have been published elsewhere (van der Wilt et al. 1992; Peters et al. 1991, 1994).

The control values of murine tissues were determined by measuring TS in samples of untreated mice. The Balb/c mice that we used are an inbred strain and genetically nearly identical, so variations in TS are small.

We used two methods to determine control values in the human tissues. First, controls for the patient's material were obtained by a dissociation procedure performed on a part of each 5FU or LV/5FU sample, since it was not possible to receive control tissue from the same patient before 5FU injection. The dissociation procedure removed all FdUMP bound to TS in the presence of a high concentration dUMP. The free FdUMP was absorbed with neutral charcoal. Details of the procedure have been described previously (van der Wilt et al. 1992). Second, we obtained control liver samples from untreated patients. These samples underwent the same procedure as the 5FU and LV/5FU samples: one part was used for the dissociation procedure followed by TS assays, the other part was used for direct measurement of TS. This allowed us to check the effects of the dissociation procedure in liver samples. FdUMP binding measured after dissociation is referred to as TS-*tot*, while directly measured FdUMP binding is called TS-*free*. Analogous TS activity measured after dissociation is called TS-*total* and directly measured activity is referred to as residual TS activity. Furthermore, we examined whether addition of 0.01 μM FdUMP in the catalytic activity assay would lead to the same extent of TS inhibition in directly measured samples and samples measured after dissociation. The colon mucosa samples obtained from patients treated with 5FU or LV/5FU were handled like the liver samples. Control values for colon mucosa of untreated patients were derived from previously published experiments (Peters et al. 1991).

We also attempted to measure TS levels in human lymphocytes as an indication of bone marrow TS. Up to 9×10^7 cells were collected from healthy volunteers, but even with this high number of cells per assay, TS levels were below the detection limit.

Statistics

The results were evaluated using Student's *t*-test for paired and unpaired data.

Results

Mice

The i.p. 5FU or LV/5FU treatment affected TS in normal tissues of the mice. TS inhibition in murine liver was evaluated both with the ligand-binding assay and the catalytic activity assay and is summarized in Table 2. Both assays showed that the LV/5FU treatment caused no inhibition of TS in the liver, while treatment with 5FU as a single agent caused a significant inhibition of TS activity at 24 h and 48 h. The ratio calculated from separate values for catalytic activity, measured at 1 μM and 10 μM dUMP, was 2.0 ± 0.03 for the control and is indicative of a difference in the enzyme kinetic properties. A significant decrease of this ratio was observed 48 h after 5FU treatment (1.7 ± 0.1, $P < 0.001$), while the ratio increased significantly after LV/5FU administration (2.2 ± 0.1, $P < 0.05$). LV/5FU had a slight inhibitory effect at 1 μM dUMP, while single-agent 5FU exerted its major effect at 10 μM. This could be, for example, a change in K_i for FdUMP, which was 0.5 nM in control liver.

The control values for TS in intestinal mucosa were about 1.5-fold higher than in liver (6.6 compared to 4.3 pmol/g wet weight for FdUMP binding and 130 compared to 80 pmol h^{-1}mg $protein^{-1}$ for catalytic activity respectively). After treatment, no activity of TS could be observed in mucosa; all values at 3, 24 and 48 h were below the detection limit of both TS assays. FdUMP binding lower than 4 fmol/mg protein and catalytic activity lower than 10 pmol h^{-1} mg $protein^{-1}$ could not be measured reliably in murine mucosa.

FdUMP binding in bone marrow could be measured in pooled control samples and was 1471 ± 293 fmol/mg protein; the catalytic activity of TS at 1 μM and 10 μM was 1081 pmol h^{-1} mg $protein^{-1}$ and 4622 pmol h^{-1} mg $protein^{-1}$ respectively. After treatment, TS inhibition was only evaluable with the ^{3}H-release assay at 1 μM dUMP (Fig. 1). This assay allowed the detection of a high degree of TS inhibition (above 90%) at 3 h and 48 h. LV/5FU treatment resulted in a slightly higher

Table 2 FdUMP binding and thymidylate synthase (*TS*) catalytic activity in murine liver. This was measured at three time points after treatment of the mice with 5FU (100 mg/kg) or LV (100 mg/kg) and 5FU and compared to control murine liver tissue. Values are means ± SD, n = 3–6; protein content of liver: 99 ± 14 mg protein/g wet weight

FdUMP binding (pmol/g protein)			TS catalytic activity (pmol/h^{-1} mg $protein^{-1}$)			
			At 1 μM dUMP		At 10 μM dUMP	
Time (h)	5FU	LV/5FU	5FU	LV/5FU	5FU	LV/5FU
Control	43 ± 7	43 ± 7	20 ± 16	80 ± 16	149 ± 44	152 ± 44
3	34 ± 4	37 ± 5	74 ± 3	74 ± 5	149 ± 17	148 ± 12
24	27 ± 3*	40 ± 5	50 ± 3	68 ± 7	90 ± 25	142 ± 21**
48	31 ± 2*	37 ± 2	39 ± 3	65 ± 2	65 ± 7	142 ± 4**

* Significantly different from control ($P < 0.05$, Student's *t*-test unpaired data)
** Significantly different from 5FU treatment 24 h ($P < 0.02$) and 48 h ($P < 0.001$, Student's *t*-test unpaired data)

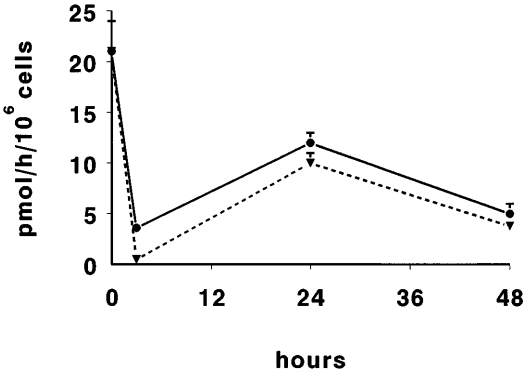

Fig. 1 Comparison of residual thymidylate synthase catalytic activity (at 1 μM dUMP) after i.p. treatment with 100 mg/kg 5-fluorouracil (5FU; ●) or 100 mg/kg leucovorin and 100 mg/kg 5FU (▼) in murine bone marrow cells. Values are means ± SD, n = 3–5, protein content about 18 μg protein/10^6 cells

inhibition of TS activity at the different assay times than did 5FU treatment, but differences were only significant at 3 h ($P < 0.001$). The unexpected higher activity of TS at 24 h, compared to 48 h, was found for both treatments.

Patients

The dissociation procedure was intended to provide an internal control for all liver samples obtained after 5FU or LV/5FU administration. However, the TS catalytic activity measured after dissociation (TS-*total*) was lower than the residual TS activity in 31 of 53 liver samples. This indicated a loss of TS activity during the 3-h dissociation procedure. Control human liver samples were subsequently used to examine the effects of the dissociation procedure. FdUMP binding measured in control samples from untreated patients was not influenced by the dissociation procedure; values were about 70 pmol/mg protein (Table 3). However, these values are much higher than the TS-*tot* measured after 5FU and LV/5FU: 15.3 pmol/mg protein (Table 4). This indicated that 5FU with or without LV influenced TS-*tot* levels in the liver. TS catalytic activity, measured directly or after dissociation, showed that the dissociation procedure clearly diminished the activity of TS and also the K_i of FdUMP seemed to be affected (Table 3).

We could evaluate FdUMP binding in 47 of 60 liver samples obtained after 5FU or LV/5FU administration. TS-*free* values were lower than TS-*tot* values 2 h and 48 h after 5FU or LV/5FU, indicating inhibition of TS, but TS-*free* was comparable to TS-*tot* 24 h after 5FU.

Table 3 FdUMP binding and thymidylate synthase catalytic activity in control liver of patients. The values are means ± SD of 6–8 samples. K_i values were calculated from each separate sample

TS parameters	Direct measurement	Measurement after dissociation
FdUMP binding (fmol/mg protein)	73.8 ± 20.8	70.0 ± 33.9
TS catalytic activity (pmol/h^{-1} mg $protein^{-1}$) at:		
1 μM dUMP	2.92 ± 1.87	1.14 ± 0.89*
1 μM dUMP + 10 nM FdUMP	1.03 ± 0.98	0.62 ± 0.54**
10 μM dUMP	13.4 ± 12.7	7.0 ± 4.3***
10 μM dUMP + 10 nM FdUMP	8.7 ± 7.5	7.6 ± 4.2
K_i FdUMP (nM)	2.7 ± 2.0	10.7 ± 8.3

$^{*-***}$Values significantly lower after dissociation ($^{*}P = 0.029$, $^{**}P = 0.010$ and $^{***}P = 0.048$, t-test for paired data)

Table 4 FdUMP binding to thymidylate synthase (*TS*) in human liver after 5FU or LV/5FU administration. The values of TS-*free* and TS-*tot* are means of evaluable samples ± SD. FdUMP binding to TS-*tot* of 12 patients was below the detection limit and for 2 patients TS-*tot* was extremely high (88 and 1071 fmol/mg protein); these were considered not evaluable and were not included in this table. Protein content liver: 96 ± 21 mg protein/g wet weight. e/n number of evaluable samples compared to the total number of samples

Time (h)				Thymidylate synthase (fmol/mg protein)	
Scheduled	Actual	Drug	e/n	TS-free	TS-tot
Control			8/8		73.8 ± 20.8
2	1.25–5.08	5FU	3/8	7.1 ± 6.8^{*1}	17.7 ± 3.9
24	19.00–26.25	5FU	7/9	18.8 ± 18.6	19.4 ± 14.6
48	41.75–67.50	5FU	12/16	8.7 ± 7.1^{*2}	15.9 ± 11.0
48	39.00–51.00	LV/5FU	25/27	11.3 ± 5.8^{*3}	14.0 ± 9.2
	40.08–48.80	DL-LV_{500}/5FU	6/8	13.6 ± 3.9	16.1 ± 6.7
	39.00–51.00	L-LV_{250}/5FU	8/8	13.4 ± 6.7	17.3 ± 9.6
	41.30–50.00	DL-LV_{25}/5FU	11/11	8.5 ± 5.3	10.6 ± 9.6
Summary	1.25–50.00	5FU and LV/5FU	47/60	11.5 ± 9.4	15.3 ± 10.3^{*4}

$^{*1-*4}$Statistics: TS-*free* is lower than TS-*tot*: $^{*1}P = 0.04$; $^{*2}P = 0.05$; $^{*3}P = 0.02$, paired t-test. TS-*tot* of 5FU and LV/5FU samples is lower than TS-*tot* of control ($^{*4}P = 2 \times 10^{-5}$, unpaired t-test)

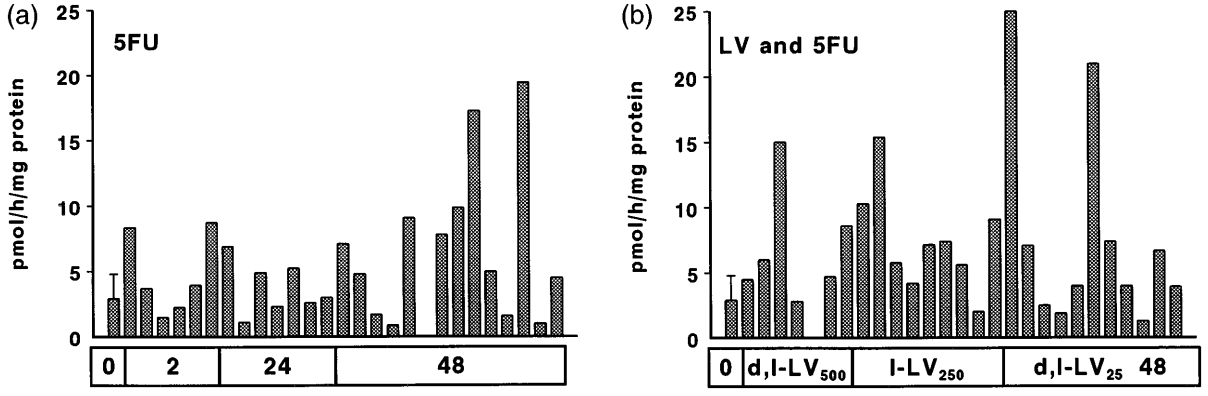

Fig. 2a, b Residual thymidylate synthase (TS) catalytic activity at 1 μM dUMP in biopsy specimens of human liver. Patients received a single dose of 500 mg/m^2 5FU (**a**) or 500 mg/m^2 DL-leucovorin (*d,l-LV*), 250 mg/m^2 L-leucovorin (*l-LV*) or 25 mg/m^2 DL-leucovorin in combination with 500 mg/m^2 5FU (**b**). Biopsy specimens were obtained at the times indicated at the bottom of the figure. For time 0 h, the control value from Table 4 has been used. Statistics: residual TS activity 48 h after drug administration is significantly higher than the control ($P = 0.05$); specified per drug: 5FU 48, $P = 0.12$; 500 mg/m^2 DL-leucovorin +5FU, $P = 0.013$; 250 mg/m^2 L-leucovorin +5FU, $P = 0.054$; 25 mg/m^2 DL-leucovorin +5FU, $P = 0.11$

When measured after 48 h, 5FU samples had TS-*free* values that were comparable to those of LV/5FU samples and also their relative inhibition, calculated by TS-*free*/TS-*tot*, was comparable. So LV did not enhance 5FU-induced TS inhibition in liver tissue.

Figure 2a shows the residual TS catalytic activity in liver samples at about 2, 24 and 48 h after 5FU injection. Figure 2b shows a comparison of the activity at 48 h after injection of 5FU (500 mg/m^2) and infusion of different doses of LV. We considered inhibition of TS activity based on the ratio TS-*residual*/TS-*total* not evaluable in these samples, because it is probable that, as in control samples, dissociation reduced the TS-*total* activity (not shown) by about 50%. Therefore we used the directly measured activity of Table 3 as a reference TS-*total*; TS-*residual* 48 h after 5FU and LV/5FU injection was higher than this control, mainly because of the values of DL-LV_{500} and L-LV_{250}. At the other assay times TS-*residual* was not significantly different from the control.

The enzyme assays in colon mucosa were not perturbed by the dissociation procedure. The total FdUMP binding to TS (TS-*tot*) in human colon mucosa could only be evaluated in 12 of the 17 biopsies (excluding controls) and varied in these samples from 11.3 to 192 fmol/mg protein (Table 5). These TS-*tot* values did not differ significantly from the controls, and both groups showed a large variation. The remaining 4 samples had FdUMP binding below the detection limit of the assay. The number of free sites for FdUMP binding to TS after administration of 5FU or LV/5FU, represented by TS-*free*, were all lower than the corresponding TS-*tot* values. This indicated that FdUMP binding sites were blocked and TS was inhibited in colon mucosa.

TS catalytic activity (Fig. 3) could be evaluated in 15 of the 16 mucosa samples (excluding controls) of treated patients. The TS-*total* value of these samples was significantly higher than TS-*total* of the controls (28.4 ± 20.4 compared to 13.9 ± 10.7). Inhibition of TS catalytic activity after 5FU or LV/5FU was observed in 11 out of 15 samples. An elevation of TS-*total* activity was observed 48 h after administration. The small number of colon mucosa samples and the large variation allowed no further time-dependent or treatment-related evaluation.

Table 5 FdUMP binding to thymidylate synthase (*TS*) in human colon mucosa after 5FU or LV/5FU administration. The values of TS-*free* and TS-*tot* are means of evaluable samples ± SD. FdUMP binding to TS-*tot* of 4 patients (excluding controls) was below the detection limit; these were considered not evaluable. Protein content: 42 ± 10 mg protein/g wet weight. *e/n* number of available samples compared to the total number of samples. Control values from Peters et al. (1991)

Time (h)				Thymidylate synthase (fmol/mg protein)	
Scheduled	Actual	Drug	*e/n*	TS-free	TS-tot
Control		–	7/10		62.7 ± 31.4
2	0.92–4.17	5FU	4/6	7.7 ± 6.2*	21.1 ± 7.3***
24	22.75	5FU	1/1	0	192.0
48	49.25; 67.50	5FU	2/2	17.8; 0	59.0; 20.9
48	43.80–50.30	LV/5FU[a]	5/8	20.2 ± 13.5**	66.9 ± 42.6
Summary	5FU and LV/5FU		12/17		57.6 ± 54.5

[a] Specification LV dose per sample: 500 mg/m^2 DL-LV (1); 250 mg/m^2 L-LV (1); 25 mg/m^2 DL-LV (3). **Statistics: TS-*free* is lower than TS-*tot*: $^{*}P = 0.05$; $^{**}P = 0.02$, paired *t*-test. TS-*tot* 2 h after 5FU is lower than other TS-*tot* values $^{***}P = 0.03$, unpaired *t*-test

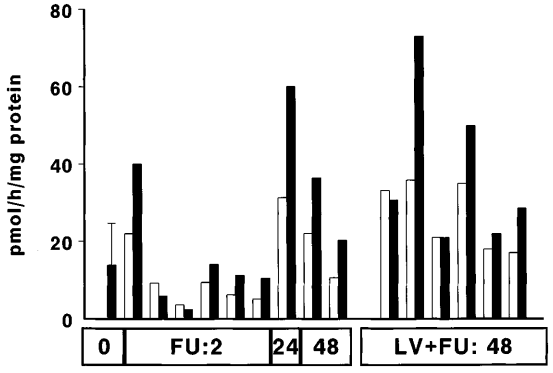

Fig. 3 Inhibition of thymidylate synthase (TS) catalytic activity at 1 μM dUMP in human colon mucosa, obtained from patients who received a single dose of 500 mg/m^2 5-fluorouracil (*5FU*) or 500 mg/m^2 leucovorin (*LV*) and 500 mg/m^2 5FU. The interval between administration of the drug(s) and surgical removal of the tissue is indicated at the bottom of the figure. *White bars* TS-*residual*, *black bars* the corresponding TS-*total* from the same patients. No bar means that the activity has been measured, but was below the detection limit of the assay. At 0 h, data from Peters et al. (1991) were used. This bar represents the mean of TS catalytic activity in colon mucosa of 10 untreated patients. Statistics: TS-*total* activity 48 h after drug administration is significantly higher than TS activity of the control ($P = 0.006$, unpaired *t*-test)

Discussion

TS was inhibited in normal tissues after 5FU administration, but the extent of inhibition varied with the tissue. The most pronounced TS inhibition in mice was observed in the intestinal mucosa, while there was a tendency for higher TS inhibition in human colon tissue than in human liver tissue, when calculated TS-*free*/TS-*tot* values were used as markers for TS inhibition (Tables 4 and 5). A large degree of TS inhibition in bone marrow cells after 5FU administration was observed for mice. This effect appeared to be very similar to that observed for rats (van der Wilt et al. 1995). In murine liver and in about 50% of the human liver samples, TS inhibition was maintained for 48 h after 5FU.

When we added LV to the treatment schedule we expected to enhance the 5FU-mediated TS inhibition. This could not be evaluated in murine intestinal mucosa with the TS assays because single-agent 5FU already caused complete inhibition of the enzyme. Inhibition of TS activity in the bone marrow cells after LV/5FU treatment of the mice was not very different from that observed after 5FU treatment. It was remarkable that, in the livers of these animals, no inhibition of TS was observed after LV/5FU treatment.

We observed a similar trend in human livers. Samples of patients obtained 48 h after LV/5FU usually had higher TS-*free* values (FdUMP binding to TS) than samples obtained 48 h after 5FU. Concentrations of natural folates measured in the liver were higher than those in other tissues (Steinberg et al. 1979). Furthermore it is likely that mice have even higher folate levels in their liver, because of their folate-rich diet. Consequently addition of LV to the 5FU administered is unlikely to increase TS inhibition in normal liver. Differences of TS inhibition in human colon mucosa after 5FU or LV/5FU administration could not be evaluated, because of the small number of samples.

The contribution of TS inhibition in normal tissues to 5FU-mediated toxicity is difficult to assess. Generally, a pronounced TS inhibition was observed in those normal tissues that were involved in the dose-limiting toxicity of 5FU administration. Severe inhibition of TS activity was observed in the intestinal mucosa and bone marrow of treated mice. This corresponded to gastrointestinal toxicity, which has been described for i.p. injection of 5FU (Bagrij et al. 1993), and to the myeloid toxicity described by Nadal et al. (1989). Besides the effects on TS, the biochemical effect of 5FU administration on RNA should be taken into consideration, since it has been shown that this may play a role in gastrointestinal toxicity in mice (Houghton et al. 1979). Bagrij et al. (1993) showed that, in mice, uridine could partially reverse gastrointestinal toxicity. It is believed that uridine metabolites compete with FUTP for incorporation into RNA. Myeloid toxicity in mice and man could also be reduced by delayed uridine administration (Peters et al. 1988; van Groeningen et al. 1989, 1993). TS inhibition and FUTP incorporation into RNA occur in both normal and rapidly dividing tissues affected by the toxic side-effects of 5FU. From this study it can not be concluded which contributes most to the toxic side-effects.

TS inhibition in liver tissue seemed to be of minor importance, since 5FU does not cause toxic side-effects in the liver. However, there are some remarkable features of TS inhibition in liver tissue that might explain why no toxic side-effects occur. First, the extent of TS inhibition, evaluated by FdUMP binding assay, was less than in mucosa, for example. Second, TS inhibition was not enhanced by co-administration of LV, indicating that 5,10-CH_2-H_4-folate was not limiting for TS inhibition in this tissue. Imbalance of the folate homeostasis in the liver, caused by LV administration, induced a reverse effect on TS inhibition. Third, residual TS activity 48 h after 5FU administration was higher than TS activity in the control liver. A similar increase of TS-*total* was observed in rat liver after FU administration (van der Wilt et al. 1995).

The increase of TS activity after 48 h was also observed in human colon mucosa. It appears to be a general mechanism occurring both in vitro and in vivo as a response to 5FU exposure and was first described by Chu et al. (1991). The elevation of TS activity might be a defence mechanism of cells and tissues against 5FU toxicity.

The decrease in TS activity, observed after the dissociation procedure, was specific for liver tissue. Previous tests in colon tumours from mice and men did not reveal such effects. The presence of many proteases in liver tissue might affect the stability of TS during the incubation for 3 h at 30°C. Also the K_i for FdUMP was

altered, so considerable changes of the protein were induced by this procedure.

Parallel to this study in normal tissues we measured TS inhibition in murine and human colon tumours (van der Wilt et al. 1992; Peters et al. 1994). Although TS levels are higher in tumour tissue, the extent and, what is more important, the retention of the inhibition in normal tissues appeared to be less than in tumours obtained from the same animals or the same patients. This implies that, in tumour tissue, a stronger cytotoxic effect of 5FU, measured by TS inhibition, occurs than in normal tissues. So, despite toxic side-effects, 5FU treatment seems to be selective for tumour tissue. This study shows that, in normal tissues, especially bone marrow and intestinal/colon mucosa, pronounced 5FU-mediated TS inhibition could be measured. The same tissues are known to be involved in toxic side-effects of 5FU. Toxicity is probably not only caused by 5FU-related effects on RNA but may also be mediated by TS inhibition.

Acknowledgement This study was supported by the Dutch Cancer Society (Koningin Wilhelmina Fonds by grants IKA-VU 88-20; 92-88 and by Wyett-Lederle, the Netherlands). These experiments were partly performed at the KDL (clinical animal laboratory) of the Free University.

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