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Prolonged retention of high concentrations of 5-fluorouracil in human and murine tumors as compared with plasma

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Summary. Concentrations of 5-fluorouracil (5-FU) and its active metabolite 5-fluoro-2′-deoxy-5′-monophosphate (FdUMP) were measured in biopsy specimens of tumor tissue, normal mucosa, metastatic liver nodules, and normal liver tissue obtained from 39 patients and in two murine colon tumors (colon 26 and colon 38) after a single injection of 5FU at a therapeutic dose (500 mg/m^2 and 100 mg/kg, respectively). These data were compared with plasma concentrations. Peak plasma concentrations (300–500 μM) of 5FU were comparable in human and murine plasma. The half-life of plasma elimination (during the period from 15 to 120 min) in both mouse and man ranged from 10 to 20 min, whereas at between 2 and 8 h, plasma concentrations varied from 0.1 to 1 μM, the half-life being about 100 min. In both species, 5FU could be measured in plasma at concentrations ranging from 0.01 to 1 μM for several days after 5FU treatment. 5FU concentrations in tissue samples obtained from 14 patients were measured during the time range of 1–6 h, those in samples taken from 7 patients, during the interval of 19–27 h; and those in samples obtained from 18 patients, within the interval of 40–48 h after injection. 5FU tumor concentrations varied between 0.78–21.6, 0.44–6.1, and 0.17–10.8 μmol/kg wet wt., respectively. Some of the 48-h samples were obtained from patients who had received leucovorin plus 5FU; coadministration of leucovorin did not alter 5FU tissue concentrations. At between 4 and 48 h, the tissue concentration/plasma concentration ratio was at least 10. 5FU concentrations in murine tumors were measured for up to 10 days after 5FU administration, with plateau 5FU tumor concentrations being about 50 μmol/kg wet wt. in colon 38 and about 200 μmol/kg wet wt. in colon 26 at 2 h after treatment; after 4 days, values of 0.5 and 4.8 μmol/kg, respectively, were obtained and after 10 days, respective concentrations of 0.1 and 0.07 μmol/kg were detected. The FdUMP concentrations measured in colon 26 and colon 38 tumors were 214 and 46 pmol/g, respectively, at 2 h after 5FU administration, and these values subsequently decreased to about 15 pmol/g in both tumors. In human tumors the initial FdUMP concentration ranged from 10 to 1000 pmol/g; at later time points the level of FdUMP was just above the detection limit of the assay. In liver metastases, high 5FU concentrations seemed to be related to high levels of FdUMP, which was likely of importance for the antitumor effect. The prolonged retention of 5FU should be taken into consideration in the design of biochemical modulation studies.

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Introduction

5-Fluorouracil (5FU) has been in use as a antineoplastic agent for more than three decades, but little is known about its long-term retention in plasma and tissues or the possible implications of such retention for its therapeutic efficacy. The drug has to be converted into a nucleotide, either the triphosphate (FUTP) or 5-fluoro-2′-deoxy-5′-monophosphate (FdUMP), in the tumor before it can exert its antitumor effect [29]. The mechanisms of action of 5-FU are complicated; effects on both DNA and RNA have been demonstrated. Strong evidence has been obtained that incorporation of FUTP into RNA, leading to altered translocation from the nuclear RNA to the cytoplasmic forms, may be responsible for the cytotoxicity of 5FU [9, 29]. In addition, inhibition of the key enzyme thymidylate synthase by FdUMP leads to inhibition of DNA synthesis [4, 29] as well as to incorporation of FdUTP into DNA, resulting in strand breaks [17]. It has been suggested that the metabolism of 5FU and the RNA and DNA effects may depend on the type of tissue involved and on the concentrations of 5FU and its metabolites. Drug transport to and from the tissues after 5FU injection may be an important determinant of the in vivo effects [22]. The retention of high concentrations of 5FU in the tissue may be important in terms of the sustained release of toxic metabolites near or on the cellular target sites. In addition, the blood flow in

the tissues and the cellular pharmacokinetics may determine the metabolism of 5FU [34].

The prolonged presence of high 5FU concentrations is likely to enhance the antitumor effect. However, data on the retention of 5FU in tissues are usually limited to short periods after its administration. The selectivity and detection limit of the procedure for analysis of the drug is usually a limiting factor. The use of radiolabeled 5FU (either tritiated or tagged with ^{14}C) has the advantage of enabling the separation of 5-FU from its metabolites [10, 20]; however, a high specific radioactivity is usually required. We recently described the retention of ^{18}F-labeled 5FU and 5FU metabolites in colon 26 and colon 38 tumors [38]. One of the main advantages of this method is the possibility of accomplishing very rapid sampling and analysis of a number of different tissues without the necessity of extracting radioactivity from the tissues. Furthermore, this technique might be useful for positron emission tomography (PET). However, a major disadvantage is the rather short half-life of ^{18}F, which precludes the measurement of 5FU and its metabolites over longer periods [32, 38]. Using [^{19}F]-nuclear magnetic resonance (NMR), it is possible both to measure 5FU and its metabolites in tissues without the necessity of removing the tissue, while it is possible to repeat measurements over time [14, 18, 20, 30, 31, 35, 40]. Metabolites, predominantly catabolites, have been demonstrated in tissues, fluoro-β-alanine being one of the major catabolites. One major disadvantage of this method is its insensitivity. Using high-performance liquid chromatography (HPLC), concentrations of 5FU have been determined in colon tissues and tumors [11]; however this method has a complicating factor, namely, the interference of other compounds in the tissue with the separation.

In a recent study we determined the clinical pharmacokinetics of 5FU in patients using a sensitive gas chromatography-mass spectrometry (GC-MS) method [15, 37], which enabled the demonstration of a prolonged presence of 5FU at a concentration of between 1 and 10 nM in plasma, even after 24 h [37]. This almost horizontal plateau in 5FU concentrations was thought to represent efflux of the drug from tissues to plasma. The GC-MS method also showed optimal sensitivity and specificity for the determination of 5-FU in tissues. Herein we describe 5FU concentrations in normal and tumor tissues as compared with plasma of patients and mice treated with 5FU as measured using the sensitive and selective GC-MS methodology. These concentrations were related to FdUMP concentrations in the same tissues.

Materials and methods

Chemicals. 5FU for the treatment of patients and animals was obtained from Hoffmann-La Roche (Mijdrecht, the Netherlands) and was formulated as a 50-mg/ml solution. 5FU, FdUMP and 5-chlorouracil for use in analytical procedures were obtained from Sigma (St. Louis, Mo., USA) and pentafluorobenzylbromide, from Pierce Chemicals (Rockford, Ill., USA). [^{15}N,^{15}N]-FU (purity, 99.9%) was supplied by Merck-Sharp and Dome (Montreal, Canada). [6-^{3}H]-5-Fluoro-2′-deoxyuridine-5′-monophosphate (spec. act., 20 Ci/mmol) was acquired from Moravek Biochemicals Inc., (Brea, Calif., USA). Partially purified thymidylate synthase from *Lactobacillus casei* was obtained from Biopure, (Boston, Mass., USA). All other chemicals were of analytical grade. Solutions were made in water purified by a Millipore Reagent Q system (Millipore, Bedford, Mass., USA).

Patients. A total of 39 patients with either primary colorectal cancer and/or colorectal cancer metastasized to the liver were included in the study (21 women and 18 men; median age, 61 years; range, 34–78 years). Nine patients had both a primary tumor and liver metastases. Patients were asked for their consent to participate in the study, which was approved by the ethical committee of the Free University Hospital. Patients received an i. v. bolus injection of 5FU at 500 mg/m^2 prior to surgery; 6 patients received a 2-h infusion with leucovorin (500 mg/m^2), with an i. v. bolus injection of 5FU at the same dose being given mid-infusion. Surgery was performed at 1–48 h after treatment for removal of the primary tumor or of liver metastases; biopsy specimens of the tumor tissue and adjacent healthy tissue (either mucosa or liver) were immediately frozen in liquid nitrogen and stored in liquid nitrogen or at –70° C.

Plasma and tumor sampling. The plasma pharmacokinetics of 5FU was studied in normal BALB/c and C57Bl/6 mice, both tumor-bearing and non-tumor-bearing animals. The mice were obtained at 6–8 weeks of age from Harlan-Olac-CPB (Zeist, the Netherlands). All mice were kept in an area maintained on a standardized light-dark cycle for at least 10–14 days prior to the beginning of an experiment. Mice had access to food and water ad libitum. Accumulation of 5FU was examined in two murine colon adenocarcinomas, colon 26 and colon 38, maintained in female BALB/c and C57Bl/6 mice, respectively. Their sources and growth characteristics have been described elsewhere [25, 26]. Tumors were transplanted s. c. as 1- to 5-mm^3 fragments in the flanks of animals aged between 2 and 3 months.

Mice were treated by i. p. injection of 100 mg/kg 5FU. Blood samples (about 50 µl) were obtained by retro-orbital bleeding of mice under slight ether anesthesia using heparinized hematocrit capillaries at 5, 10, 15, 30, 60, and 120 min after 5FU administration. From several mice, plasma samples were obtained at later time points; however, since more blood (>100 µl) would be needed at the later time points, to prevent excessive blood sampling, blood was also taken from other mice at these times. Capillaries were emptied into heparinized Eppendorf vials and centrifuged at 4° C for 5 min at 4000 *g*; supernatants could be stored for at least 28 months [37] at –20° C until batch analysis.

The 5FU concentration in tumors was measured at several time points after 5FU administration. Tumors were allowed to reach volumes of >200 mm^3 before treatment but did not exceed 5% of the total body weight. Mice were killed by cervical dislocation and tumors were immediately excised and directly frozen in liquid nitrogen. Under these conditions, 5FU concentrations in tumors remained stable for several years. Repeated measurement of 5FU concentrations in the same piece of tissue at an interval of 3 years did not reveal significant differences.

Measurement of 5FU in plasma. High plasma concentrations of 5FU (>1 µM) were measured by HPLC as described elsewhere [37]. Low plasma concentrations of 5FU were measured using GC-MS essentially as previously described for plasma samples obtained from patients [15, 37]. For the GC-MS method, plasma samples were mixed with 0.2 M TRIS-HCl (pH 6.0) and the internal standard 5-chlorouracil (ClU) and further processed as described below.

Briefly, the mixture was extracted twice with 4 ml diethylether/isopropanol (78 : 22; v/v) during vortexing for 5 min. The organic layers were collected and mixed, 1 ml 0.2 M phosphate buffer (pH 10.5) was added, and the mixture was vortexed for 5 min. After centrifugation, the organic layer was removed by suction and tetrabutylammoniumhydroxide (pH 10.5) was added to reach a final concentration of 0.5 M. Dichloromethane (5 ml) and pentafluorobenzylbromide (10 µl) were added and the mixture was vigorously shaken for 60 min, after which the organic layer was washed with 0.1 *N* hydrochloric acid. The organic layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 200 µl 30% acetone/hexane. This sample was injected into the GC-MS system, and the ions were recorded with negative-ion chemical ionization detection using methane as the moderating gas at m/z -309 and m/z -325. 5-FU and the other internal standard (see

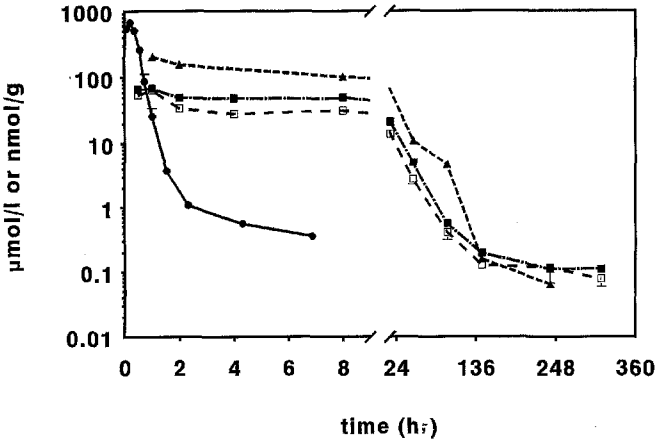

Fig. 1. Average plasma-elimination curve (●——●) generated for 5FU in mice as compared with tissue concentrations of 5FU measured in colon 26 (▲- - -▲) and colon 38 (■, tumor; □, necrosis) tumors. Data on plasma concentration represent mean values ±SD for 6 mice, and each data point for tumor concentration is based on at least 4 tumors. Values are expressed as means ±SD; for several points the SD is within the symbol

below) [^{15}N,^{15}N]-FU were recorded at m/z -309 and -311, respectively, and had the same retention times; the MS sensitivity was similar for both isotopes of 5FU. Details of the GC-MS conditions used have been reported elsewhere [15, 37]. The resolution of the mass spectrometer was increased to 3000 (10% valley), resulting in a better signal-to-noise ratio. By the use of this higher resolution, the absolute sensitivity of the method was decreased to 1 pg for injection into the GC-MS system. The accuracy and sensitivity was improved via suppression of the chemical noise.

Measurement of 5FU and FdUMP in tissues. For measurement of the 5FU concentration in tissue samples, [^{15}N,^{15}N]-5FU was used as the internal standard. A comparison of several plasma and tissue samples analyzed using both internal standards did not reveal any difference. First, 0.10–0.25 g tissue was pulverized with a micro-dismembrator as previously described [23, 24]. The advantage of this procedure is that tissues remain frozen during the procedure, preventing any degradation of nucleotides and/or nucleosides to 5FU. After transfer of the frozen powder to a chilled 12.5-ml polypropylene tube, [^{15}N,^{15}N]-5FU solution (3×10^{-6} M of the same weight as the tissue) and 1 ml ice-cold saline were added to the powder and subsequently extracted by the addition of trichloroacetic acid (final concentration, 5%; 20 min at 4° C). Denaturated high-molecular-weight material was precipitated, and the supernatant was neutralized by the addition of 2 vol. of a mixture of trioctylamine/1,1,2-trifluorotrichloroethane (4:1, v/v) [24]. This mixture was centrifuged, and 0.5 ml of the neutralized upper aqueous layer was subsequently mixed with another 0.5 ml water and 100 µl TRIS buffer (2 M, pH 6.0) and thereafter treated as described for the plasma samples. Recovery of 5FU was determined through the addition of 5FU to tumors from untreated animals just before or after pulverization and was >95% in both cases. The above-mentioned amount of [^{15}N,^{15}N]-5FU relative to 5FU had to be adapted when the ratio of 5FU [^{15}N,^{15}N]-5FU was <0.1 or >10; in this case the extraction procedure had to be repeated using adapted concentrations of the internal standard. The resulting detection limit for tissue samples was 1 pmol/g. FdUMP concentrations in tissues were determined using the isotope-dilution assay essentially as described elsewhere [36]. [6-^{3}H]-FdUMP was used as the substrate.

Pharmacokinetic and statistical calculations. Calculations of pharmacokinetic parameters were performed essentially as described previously [24, 37] using the PC-NONLIN computer program (Statistical Consults, Lexington, Mass., USA). The area under the concentration versus time curve (AUC) for the interval of 0–90 min was calculated by the trapezoidal method. Statistical evaluation was carried out using either Student's *t*-test (for paired samples) or the Mann-Whitney two-tailed *U*-test for samples with abnormal distribution.

Results

Plasma pharmacokinetics of 5FU

The use of a sensitive and specific GC-MS analytical procedure for 5FU enabled us to study the distribution and elimination phases of 5FU in the same mouse. Figure 1 shows the average plasma concentration versus time curve generated for mice that had been treated with 100 mg/kg FU in comparison with that plotted for both colon tumors. This dose is the maximum tolerated dose (MTD) for these mice as given on a weekly (for 4 weeks) administration schedule. The plasma-elimination curves plotted for the two mouse strains were comparable. The pharmacokinetic parameters of 5FU in patients and mice are summarized in Table 1. The total AUC as well as the peak plasma concentrations observed in mice treated with 100 mg/kg 5FU were comparable with those measured in patients treated at 720 mg/m^2. The $t_{1/2}$ values obtained for mice (between 2 and 8 h) were somewhat lower than those calculated for patients. The plasma concentration measured during this phase varied between 0.05 and 1 µM. In several mice, 5FU levels were also measured after 2 and 3 days, when the tumors were removed; the plasma concentration of 5FU was 0.17 µM after 48 h and 0.044 µM after 95 h.

Table 1. Comparison of several plasma pharmacokinetics parameters of 5FU in patients and mice

Parameter	Patients		Mice (100 mg/kg)
	500 mg/m^2	720 mg/m^2	
AUC (µmol h l^{-1}):			
0–90 min	121 ±28 [15]	268 ±44 [7]	287 ±55 [6]
2–8 h	0.73 (0.31–14) [10]	1.1 (0.37–12) [7]	3.8 ±1.8 [14]
$t_{1/2\beta}$ (min)	9.8 ±2.4 [12]	14.4 ±2.5 [7]	13.6 ±4.1 [6]
$t_{1/2\gamma}$ (h)	3.2 (1.8–18.7) [12]	2.6 (1.5–19) [7]	1.8 ±0.6 [14]

Data represent mean values ±SD for the numbers of individuals indicated in brackets. For the gamma phase of elimination in patients, the median values and ranges (in parentheses) are given because of the large interindividual differences. Values were calculated from data on the patients reported previously [37]. The beta phase lasted from 10 to 90 min and the gamma phase, from 2 to 8 h

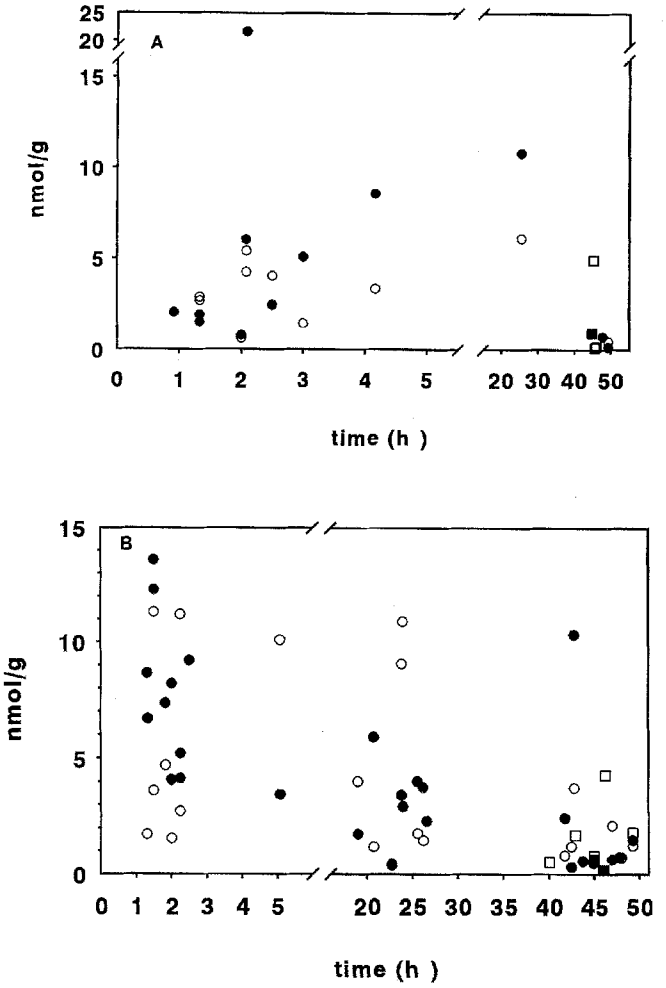

Fig. 2 A, B. 5FU tissue concentrations measured in **A** colon tissues (primary colon cancer and normal mucosa) and **B** liver tissues (metastases and normal liver) after the administration of a 5-FU dose of 500 mg/m^2. *Closed symbols* (●, ■) indicate tumors and *open symbols* (○, □) represent the corresponding normal tissues. *Circles* (○, ●) represent 5FU-treated patients and *squares* (□, ■) indicate patients who also received leucovorin as a 2-h infusion, with 5FU being given as a bolus midway infusion. Symbols plotted at the same time point generally represent samples (tumor and normal tissue) taken simultaneously from the same patient. 5FU concentrations measured in liver metastases within the time range of 1–4 h were significantly higher ($0.02< P <0.05$) than those recorded in primary tumors during the same interval. 5FU concentrations measured in liver metastases within the time range of 1–5 h were significantly higher than those recorded during the intervals of 19–27 h ($P <0.002$) and 40–46 h ($P <0.002$); 5FU concentrations measured in samples obtained at between 19 and 27 h were significantly higher ($0.02< P <0.05$) than those recorded in samples obtained at 40–46 h. In normal liver samples, no such differences were observed

5FU concentrations in murine tumors

Measurement of 5FU concentrations in tumors, especially relatively low concentrations (<10 μmol/kg), using conventional HPLC methodology was difficult because of the presence of a large number of interfering peaks in extracts derived from tissues (data not shown). Similar problems also prevented accurate measurement of 5FU anabolites such as the triphosphate FUTP. The use of GC-MS enabled selective measurement of 5FU. 5FU concentrations were rather high in the two colon tumors as compared with plasma. After 2 h, at which time plasma concentrations were ≤1 μM, tissue concentrations were >20 μmol/kg. Initially, higher concentrations were observed in colon 26 as compared with colon 38 (Fig. 1), but after 1 week the concentrations were lower in colon 26. 5FU concentrations measured in different (viable) parts of one tumor were comparable (<5% variation). In colon 38, concentrations of 5FU in both the viable rim of the tumor and the inner necrotic part were measured; high concentrations were also observed in the necrotic part (Fig. 1). At >4 h, a consistently >2-fold) higher 5FU concentration was observed in the viable rim of the tumor; this difference was found in each separate tumor. After the plateau phase (>6 h until 96 h) the elimination half-life of 5FU in the sensitive colon 38 tumor was about 12 h. In the relatively resistant colon 26 tumor, no plateau phase was observed; the 5FU concentrations decreased with a half-life of about 6 h during the period from 2 to 96 h after injection.

5FU concentrations in human tissues

5FU concentrations determined in colon tumors (from 12 patients) and normal mucosa (12 patients), in liver metastases (29 patients), and in liver tissue (26 patients) are shown in Fig. 2. All concentrations ranged from 0.1 to 25 μmol/kg. Values obtained in liver metastases were significantly higher than those measured in primary tumors within the same interval (Fig. 2). Values obtained in liver metastases at 1–6 h were significantly higher than those measured at 20–27 and 40–48 h after 5FU injection (Fig. 2). At these time points, several samples taken from patients receiving leucovorin and 5FU were also analyzed; no difference between the two groups was observed. Standard deviations of the 5FU concentrations measured in different samples collected from the same patient are given in Table 2. The standard deviation was about 50%. This

Table 2. Variation of 5FU concentrations in tissues obtained from the same patient

Patient number	Time after 5FU (h)	5FU concentrations (nmol/g)			
		Colon tumor	Colon mucosa	Liver metastasis	Liver
5	2.35	2.4±2.0	4.0±1.1		
10	2.25			4.1±2.4	
17	24.00			13.7±2.0	2.9±1.6

5FU concentrations were measured in 3 different parts of the same tissue; data represent mean values ±SD

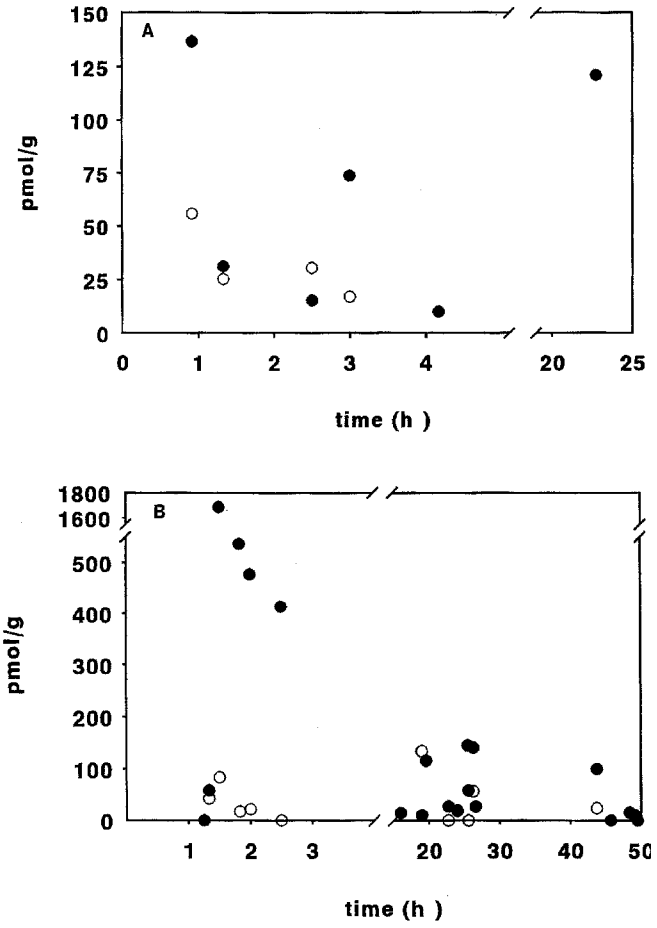

Fig. 3 A, B. Concentrations of FdUMP measured in **A** colon tissues (primary colon cancer and normal mucosa) and **B** liver tissues (metastases and normal liver) after the administration of 5FU at 500 mg/m^2. *Closed symbols* represent tumors and *open symbols* indicate normal tissues. FdUMP concentrations measured in liver metastases within the time range of 1–5 h were significantly higher ($0.02 < P < 00.5$) than those recorded in primary tumors during the same interval. FdUMP concentrations measured in liver metastases within the time range of 1–5 h were significantly higher ($P < 0.02$) than those recorded during subsequent intervals (19–27 h and 40–48 h)

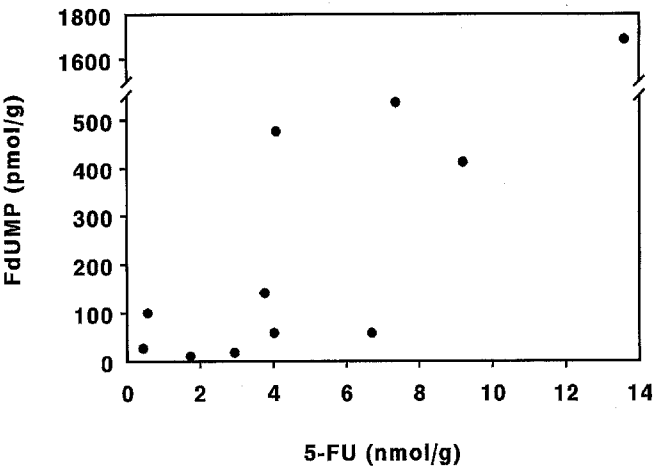

Fig. 4. Relationship between 5FU and FdUMP concentrations in liver metastases. Data points represent concentrations measured in the same tissue samples collected at all time points. Only patients who received 5FU were evaluated. The correlation coefficient was 0.84

spread makes a comparison of average 5-FU concentrations measured in different tissues relatively difficult. When the sample was first homogenized and then split into different portions, the standard deviation was found to be <5%. The average variation between 5-FU mucosa and colon-tumor concentrations within the same patient did not significantly differ from zero ($n = 13$, Student's paired t-test for differences in the percentage of the mean value). Also for paired liver and liver-metastasis tissue samples ($n = 23$), no significant difference was found.

FdUMP concentrations in tissues

FdUMP concentrations in murine tumors have been published elsewhere [36]. After 2 h, high concentrations of FdUMP (214 ± 23 pmol/g) were observed in colon 26 tumors, in contrast to the much lower concentrations (46 ± 16 pmol/g) measured in colon 38 tumors. After 24 h, FdUMP levels in both tumors were comparable (14 and 16 pmol/g, respectively), whereas after 48 h, FdUMP concentrations were measurable only in colon 38 (19 pmol/g) and were below the detection limit (12 pmol/g) in colon 26.

The concentrations of FdUMP measured in tissue samples obtained at the same time points showed large interindividual variation (Fig. 3) both in tumors and in normal tissues. Despite these variations, it was clear that the highest concentrations were observed shortly after 5FU injection. After 48 h, FdUMP was measurable only in a few samples, but in most samples the levels were below the detection limit of the assay. FdUMP concentrations measured in normal tissues (liver and mucosa) were lower than those observed in tumors from the same patients. Liver metastases were evaluated separately, and FdUMP concentrations were compared with 5FU levels in the same extract (Fig. 4). In this tissue as opposed to the other tissues, high 5FU concentrations appeared to be related to high FdUMP levels (data not shown).

Discussion

This study demonstrates that plasma 5FU concentrations do not reflect tumor concentrations in humans or mice. 5FU is retained for a much longer period in tissues than in plasma, with tissue concentrations being at least 10 times higher than plasma concentrations. These differences were observed within several hours of drug administration. We have previously observed a short half-life for 5FU in plasma followed by barely detectable levels over a period of several days in both humans [37] and mice. In the tumors, initial (at 1–5 h) FdUMP concentrations seemed to be related to the 5FU levels.

Although details of plasma 5FU pharmacokinetics in patients and animals have been extensively described by a number of authors (reviewed in [6, 29]), most studies were limited to the first period of 5FU elimination. 5FU is elim-

inated very rapidly from the plasma, with the $t_{1/2}$ value varying between 10 and 20 min for both patients and animals [3, 5, 8, 12, 16]. The plasma peak concentrations and AUC values (when reported at comparable doses) described by these authors were comparable with those reported in the present study. The characterization of a third phase of elimination was possible using the sensitive and specific GC-MS method. This detection method appeared to be very suitable for sequential measurement of 5FU plasma concentrations in the same mouse, since only a limited amount of blood was required. In addition, selective measurement of tissue concentrations was possible. The use of [^{15}N,^{15}N]-5FU as the internal standard further improved the reliability of the assay, as has also been demonstrated by Bates et al. [1].

We recently described the tissue and plasma distribution and elimination of [^{18}F]-5FU injected into mice using various doses and schedules [38]. The total tumor concentration of ^{18}F-labeled compounds measured at between 2 and 6 h after the administration of 100 mg/kg was about twice the 5FU concentration reported in this paper; combined with the present data, this would indicate that about 50% of the 5-fluorolabeled compound is present in another form. These forms consist predominantly of 5FU nucleosides and nucleotides (including FUTP and FdUMP) and 5FU metabolites bound to or incorporated into a macromolecule such as RNA. Degradation products in tumors are likely to originate from normal tissues [20]. A higher tumor/blood ratio of 5FU metabolites after 24 h has previously been observed in tumors as compared with plasma of mice analyzed for total 5FU and metabolites [16]. A relatively long-term retention of 5FU in rat plasma (5FU concentration, about 0.7 μM at 24 h after the injection of 90 mg/kg 5FU) has also been reported by Finn and Sadée [12], who estimated a half-life of about 20 h. The 5FU concentrations measured in tumors were comparable with those determined in samples collected at 48 h after injection as reported by Finan et al. [11].

In mice, the relative concentration of 5FU in tissues corresponded with that of the active metabolite FdUMP, which was about 4 times higher in colon 26 as compared with colon 38 [36]. The 5FU concentration was more than 1000-fold that of FdUMP in both tumors. Moreover, the elimination profile of 5FU in both tumors corresponded with that observed for all ^{18}F-labeled compounds; a higher amount of label was initially observed in colon 26 as compared with colon 38, which was eliminated faster from colon 26 [38]. However, neither the higher 5FU level nor the FdUMP concentration corresponded with a better antitumor effect of 5FU in colon 26, but both compounds were more rapidly eliminated from the insensitive colon 26 tumor. Colon 38 was more sensitive to 5FU than was colon 26 [25, 36].

In addition to the elimination profile, we found strong evidence that this difference in sensitivity was also related to the activity and the inhibition of the target enzyme thymidylate synthase in these tumors [36]. Higher thymidylate synthase activity was present in both untreated and 5FU-treated colon 26 tumors as compared with colon 38 tumors. Also, relatively high concentrations of FdUMP were observed in patients immediately after 5FU administration. In liver metastases, these 5FU concentrations seemed to be related to FdUMP levels when the initial concentrations were evaluated. The extent of inhibition of thymidylate synthase in these patients [28] did not correlate with the concentration of 5FU or FdUMP in these tissues. It seems very likely that 5FU has to exceed a certain threshold level for FdUMP to be formed. Subsequently, the FdUMP level has to exceed at least the inhibition constant of FdUMP for thymidylate synthase to result in sufficient inhibition. Very high concentrations of FdUMP would not add to the inhibition, whereas FdUMP levels lying just above the detection limit would be sufficient. The relatively low concentrations of FdUMP found in normal mucosa might have been related to the relatively low activity of 5FU activating enzymes in this tissue [27]. It is not yet clear as to whether the concentration of 5FU might be related to its incorporation into RNA. Reliable measurement of 5FU incorporation into RNA of human tissues continues to be a limiting factor in the evaluation of this potential 5FU target.

Transport of 5FU into the cell is mediated by the mechanism that is responsible for uracil transport [7, 39]. For transport across the colon mucosa, Na^+ may play a role [33]. It is possible that 5FU may be retained at a higher concentration in tissues than in plasma due to a concentrative uptake of drug from the plasma into the tissues that exceeds the efflux rate [13]; this may depend on extracellular Na^+ and intracellular adenosine 5′-triphosphate (ATP) [41]. Recently it has also been demonstrated in vivo that at a pH of <6.9, the $t_{1/2}$ value for 5FU is enhanced [13] 3-fold as compared with the value obtained within the physiological pH range of 7.0–7.4. The long-term retention of 5FU might also be related to trapping [40] of 5FU in polar metabolites such as nucleotides and to 5FU incorporated into RNA, which cannot pass the cellular membrane. Thus, initially high free 5FU concentrations in tissues would be the result of uptake of the parent drug, which would subsequently be converted into the nucleotides for incorporation into RNA and/or binding to thymidylate synthase. Turnover of RNA and protein degradation would lead to new of formation 5FU, which would either be metabolized again or be partially excreted into plasma, which would explain the long-term retention of low 5FU concentrations in plasma. In addition, compartmentalization of free 5FU in the cell may occur either within cellular organelles or via binding to proteins. During the extraction procedure, the drug in this compartment would appear as free 5FU. Future in vivo studies on 5FU retention should address these questions. Although the concentrative uptake of 5FU might be an important mechanism, it remains unclear as to which of the mechanisms described above might be the major factor contributing to the high intratumor concentration of 5FU.

The clinical relevance of the prolonged presence of high 5FU concentrations in tissues has yet to be determined. They may lead to the maintenance of FdUMP concentrations at levels high enough to inhibit thymidylate synthase. However, the data are important for the design of better schedules for biochemical modulation. For example, from preclinical studies it has become evident that methotrexate (MTX) should preceed 5FU [29], as the reverse schedule resulted in antagonism. The long-term retention of high

5FU concentrations might cause this antagonism, explaining the lack of efficacy when MTX is given to soon after 5FU. Only recently has more evidence for the potentiation of 5FU by MTX been demonstrated [19]. The prolonged retention of 5FU might also favor the weekly bolus schedule, which is one of the preferred schedules for the combination of 5FU with leucovorin [22]. A prolonged presence of 5FU seems to be essential for adequate modulation by leucovorin [21]; this allows stabilization of the ternary complex responsible for inhibition of the target enzyme thymidylate synthase. 5FU is also used as a radiosensitizer [2], and it has been demonstrated that 5FU should be present before radiation starts. Sensitization is observed for a few days after 5FU treatment, which might be explained by the long-term retention of relatively high concentrations of 5FU. It is very likely that the retention of 5FU for up to several days after treatment with the drug not only is of pharmacokinetic interest but may have important clinical implications for the design of better schedules for combination regimens that include biochemical modulation or radiosensitization.

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