For more than 30 years, 5-fluorouracil (5-FU) has been the drug of choice for both single-agent and multiagent chemotherapy of colorectal cancer (CRC; ref. 1). Significant improvements in response rates and overall survival have been achieved with the introduction of new cytotoxic agents such as irinotecan and oxaliplatin or, most recently, with targeted therapies such as bevacizumab or cetuximab (2, 3). However, 5-FU still represents the backbone in the majority of regimens for advanced CRC. Since the early eighties attempts have been made to increase the efficacy of 5-FU by modulating its dose, schedule, or route of administration (4–6). Likewise, modulation of 5-FU by folinic acid (FA) has been shown to enhance the antineoplastic activity in vitro and in vivo. In patients with metastatic CRC, the combination of 5-FU and FA resulted in higher response rates compared with 5-FU alone with some studies also demonstrating a benefit in survival (2, 5, 6). A recent meta-analysis showed, on a large data set, a 2-fold increase in tumor response rates (21% for 5-FU/FA combination versus 11% for 5-FU alone) and a small but significant overall survival benefit for 5-FU/FA over 5-FU alone (median survival, 11.7 versus 10.4 months; ref. 2). Nevertheless, the optimal dose of FA as 5-FU–modulating agent is still a matter of debate.

One metabolic pathway of 5-FU in the cell is the conversion toFdUMP, a cytotoxic compound that forms a covalent ternary complex with thymidylate synthase and 5,10-methylenetetrahydrofolate (5,10-mTHF), the reduced folate cofactor required for the normal catalytic reaction of thymidylate synthase. Despite covalent binding, the reaction is reversible and the dissociation half-life of the complex is inversely proportional to the concentration of unbound 5,10-mTHF. It does, however, not correlate with the amount of free FdUMP (7). Enhancement of 5-FU cytotoxicity by FA is based on the optimal inhibition of thymidylate synthase resulting from an increase in the intracellular pool of 5,10-mTHF, which, in turn, stabilizes the inactive ternary complex (8, 9). In extracts from 5-FU–insensitive human adenocarcinoma xenografts, the amount of ternary complex isolated in the presence of endogenous cofactor concentrations has been shown to be just ~50% of

Abstract

Purpose: In patients with colorectal cancer (CRC), modulation of 5-fluorouracil (5-FU) by folinic acid (FA) improves response rate and overall survival compared with 5-FU alone. However, the optimal dose of FA is still debated. We investigated reduced folate pools in various tissues from patients with CRC without and after prior administration of FA.

Experimental Design: A total of 186 specimens (normal colorectal mucosa, primary colorectal tumor, normal liver, and liver metastases) from 86 consecutive patients with CRC were obtained and investigated for levels of reduced folates. Before surgery, patients did (n = 52) or did not (n = 34) receive FA as 15-minute i.v. infusion. FA-dose levels chosen were 20, 200, or 500 mg/m². Tissue lysates were analyzed for reduced folate levels by means of the tritium release assay.

Results: In normal mucosa, combined pools of tetrahydrofolate and 5,10-methylenetetrahydrofolate were significantly elevated at all FA dose levels compared with untreated controls. In primary tumor, only 200 and 500 mg/m² FA resulted in a significant increase of reduced folates with highest values measured after 500 mg/m² FA. In specimens from normal liver, folate levels did not increase after administration of FA. By contrast, in specimens from liver metastases, reduced folate levels were low without FA pretreatment compared with levels from normal liver samples. Infusion of 500 mg/m² FA caused a significant increase of reduced folate levels in liver metastases.

Conclusions: From a pharmacologic point of view, high-dose FA should be recommended for optimal modulation of 5-FU in patients with mCRC.

Cancer Therapy: Clinical
Translational Relevance

In patients with colorectal cancer (CRC), modulation of 5-Fluorouracil (5-FU) by folic acid (FA) improves response rate and overall survival compared with 5-FU alone. Because the optimal dose of FA is still debated, we investigated reduced folate pools in various tissues from patients with CRC without and after prior administration of FA at various dose levels ranging from 20 to 500 mg/m². In tissue from primary tumor, only 200 and 500 mg/m² FA resulted in a significant increase of reduced folates with highest values measured after 500 mg/m² FA. In specimens from liver metastases, only infusion of 500 mg/m² FA caused a significant increase of reduced folate levels. Thus, from a pharmacologic point of view, high-dose FA should be recommended for optimal modulation of 5-FU in patients with mCRC rather than FA at a dose of 20 mg/m². In current 5-FU-based regimens such as FOLFOX or FOLFIRI, the dose of FA varies from 200 to 500 mg/m². Given our results with 500 mg/m² FA resulting in higher CRC tissue levels than lower doses of FA, higher doses such as 400 or 500 mg/m² FA should be preferred.

the maximal level determined if 5,10-mTHF was present in excess (10).

Given the complex nature of FA and 5-FU metabolism, serum pharmacokinetics can only be used as a rough estimate for the concentration of the activated metabolite at its molecular target (11, 12).

Because the concentration of reduced folates is important for optimal inhibition of thymidylate synthase (9), several studies have looked at intracellular concentrations of 5,10-mTHF and tetrahydrofolate (THF) in human tumor biopsies obtained at time of surgery (13–19). In two of these studies, reduced FA levels were also measured after pretreatment with FA at doses of 500 mg/m² given either as a 2-hour infusion (n = 4) or as continuous infusion over 5 days (cumulative dose, 2,500 mg/m²; n = 2; ref. 13) and at doses of 45 mg/m² given as a single bolus injection (n = 2) or divided in 3 bolus injections (n = 3; ref. 14). Pretreatment with FA resulted in an increase of reduced folate pools in primary CRC by 2- to 8-fold and 0.5- to 5-fold, respectively (13, 14). However, due to the very limited number of patients, no firm conclusions can be made with respect to the optimal dose of FA to be used in the clinical setting. Furthermore, there are no data available that systematically relate different FA-doses to expansion of reduced folate pools in human tissue.

In the present study, tissue levels of combined pools of 5,10-mTHF and THF were measured in normal colorectal mucosa, primary tumor, liver, and liver metastases from CRC patients who had or had not received FA at different dose levels before surgery.

Materials and Methods

Patients and drug delivery. Tissue was obtained from patients undergoing surgery for either newly diagnosed CRC and/or liver metastases from CRC. All patients gave their written informed consent to take part in the study. The study was approved by the ethics committee of Ludwig-Maximilians-University, Munich.

Patients did or did not receive FA (Ruscovolin; Medac GmbH Hamburg) before surgery. FA dosages chosen were either 20, 200, or 500 mg/m² diluted in 250 mL 0.9% saline given as short-term infusion over 15 min (11) at initiation of general anesthesia. Tissue was obtained from at least one of the following organs: (a) normal colorectal mucosa, (b) primary tumor (CRC), (c) normal liver, (d) liver metastases.

The specimens were removed a median of 90 min (range, 60-130 min) after the infusion of FA. The excised tissue measured 0.5 to 1 cm³ corresponding to a wet weight of 0.5-1 grams. The tissue was immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Tissue processing. Tissue was processed and reduced folate pools were determined according to a modification of the method described by Houghton et al. (20). Specimens (1 gram per 3 mL buffer) were minced in extraction buffer (Tris-MA) containing trishydroxymethylaminomethane (Sigma Chemicals), 25 mmol/L (pH 7.4) with 0.5% 1,4-dithioerythritol (DTE; Fluka BioChemika AG), and 37 mmol/L formaldehyde on ice using an Ultra-Thurax-mincer (Janke & Kunkel GmbH) at 25,000 rpm. The suspension was boiled for 3 min to inactivate endogenous metabolizing enzymes, centrifuged at 2°C and 15,500 x g for 13 min in a Beckman J16 ultracentrifuge (Beckmann Instruments GmbH). After recovery of the supernatant, the pellet was reextracted with 1.5 mL extraction buffer per gram of pellet and centrifuged again. The supernatant was then combined with that from the first extraction procedure. The recovery rate, determined with radiolabeled FA, was >80%. Excess formaldehyde was added to stabilize 5,10-mTHF, which is otherwise converted to the more labile THF if heated at 100°C in the absence of formaldehyde (8). As THF is chemically converted to 5,10-mTHF in the presence of excess formaldehyde, only combined pools of reduced folates could be determined with the catalytic enzyme assay used.

Tritium release assay. For the tritium release assay, 50 μL reaction mixture containing 0.25 units thymidylate synthase from L. casei (27.4 μmol/h/mL; 24.0 mg/mL; Biopure), 5-[3H]-dUMP (Amersham Buchler; specific activity, 19 Ci/mmol) diluted with unlabeled 2’-deoxurytidine-5’-monophosphate (dUMP; Serva Fine Biochemicals) to a specific activity of 0.54 Ci/mmol, and a radioactive concentration of 11.26 μCi/mL in 25 mmol/L Tris-buffer (pH 7.4). 0.5% 1,4-dithioerythritol, and MgCl₂ 40 mmol/L (E. Merck) were mixed with 50 μL of the lysate and incubated for 3 min at 25°C. The reaction was terminated by addition of 10 μL ice-cold 0.75 mol/L perchloric acid (E. Merck).

Unreacted 5-[3H]-dUMP was adsorbed on ice with 900 μL of a 7.5% charcoal suspension containing 0.5% bovine serum albumin and 0.05% dextran for 20 min. After centrifuging twice and careful removal of the supernatant, [3H]O in 500 μL of the final supernatant was determined with a liquid scintillation counter (Beckman). Standard curves were prepared with every run using the stereochromically pure (>97% based on high performance liquid chromatography) biological isomer of 5,10-mTHF (MEDIAC GmbH).

Protein determination. Protein in lysates was quantified according to the method of Bradford using a Bio-Rad protein assay (Bio-Rad Laboratories GmbH). Protein was determined in the final lysate used for the tritium release assay to avoid errors caused by the boiling step with an unaccountable loss of volume by evaporation and by several dilutions. Before having established this procedure, the loss of protein due to boiling was constant, i.e., the amount of protein in the boiled lysate was proportional to the amount before boiling. The boiling-induced loss of protein was 3.1-fold higher in lysates from liver tissue and 2.4-fold higher in tissue from liver metastases compared with normal mucosa and primary tumor tissue. Given that the same concentration of 5,10-methylene-THF per milligram protein is present in the lysate before boiling, the boiling step—leaving the amount of 5,10-methylene-THF unchanged—would change the concentration per milligram protein
due to differences in the protein concentration after boiling in the final lysate. Thus, the same amount of 5-10-methylene-THF in liver tissue would result in a 3.1-fold higher concentration per milligram protein after boiling than in colorectal mucosa and primary tumor. To generate comparable values from different tissues, a correction factor of 3.1 for liver specimen and 2.4 for tissue from liver metastases was calculated. The actually determined concentration was divided by the correction factor. Final results were expressed as pmol reduced folates as determined by the tritium release assay per milligram protein.

**Statistics.** Statistical analyses were done using the statistical software SAS for Windows, Version 9.1. THF concentrations were skewed to the right with a mean of 82.7 and a median of 41.5. Therefore, we chose a nonparametric procedure using the Mann-Whitney U test to compare combined pools of THF and 5,10-mTHF between different subgroups. A P value of <0.05 was considered statistically significant.

**Results**

**Tritium release assay**

A linear correlation between the radioactivity determined in 500 μL of the supernatant treated with activated charcoal and the limiting substrate of the enzymatic reaction was obtained for a 6R-5,10-mTHF concentration range of 1.25 to 50 pmol. The measured dpm-values were corrected for the background activity determined in a reaction mixture containing enzyme and [3H]-dUMP but not 5,10-mTHF. The values measured were in the range of 1,000 to 1,500 dpm.

**Tissue samples obtained.** A total of 186 specimens from a cohort of 86 consecutive patients were obtained and investigated for levels of reduced folates as outlined in Table 1.

**Reduced folate levels in tissue without prior administration of FA**

Reduced folates were below the detection limit of 1 pmol/mg protein in 16 of the 23 normal intestinal mucosa samples and 13 of 22 primary tumor specimens. In the remaining 7 normal mucosa and 9 CRC specimens, reduced folate levels were found to be in the range of 3 to 40 pmol/mg protein and 7 to 117 pmol/mg protein, respectively. The median value for all normal rectal mucosa and all primary tumor specimens was 0 pmol/mg protein (Table 1).

Median levels of 5,10-mTHF and THF were 135 pmol/mg protein (25-75% quantile, 66-209) in normal liver tissue and 19 pmol/mg (3-54) protein in liver metastases (Table 1; Fig. 1), which was a significant difference (P = 0.002). Reduced folate levels in liver metastases were significantly higher than in normal colorectal mucosa (P = 0.005), but there was no significant difference when compared with primary tumor (P = 0.09).

**Reduced folate levels in tissue after administration of FA**

**Normal colorectal mucosa.** After pretreatment with 20 mg/m² FA, median levels of 5,10-mTHF and THF in normal colorectal mucosa were significantly higher than in untreated controls (32 versus 0 pmol/mg, P < 0.0001). After administration of 200 mg/m² FA, no further increase in tissue levels was observed (22 pmol/mg) compared with 20 mg/m² FA (P = 0.598). In contrast, pretreatment with 500 mg/m² FA resulted in a substantial increase to a median of 48.5 pmol/mg. This median concentration was of significant difference when compared with the 20 and 200 mg/m² dose levels (P = 0.003 and P = 0.005; Table 1).

**Fig. 1.** Tissue levels of combined pools of 5,10-mTHF and THF in normal colorectal mucosa, primary tumor, normal liver, and liver metastases without prior administration of FA displayed by a box-and-whisker plot with median, 25% and 75% quantile (box), minimum and maximum (whisker), and mean (+).
Primary tumor (CRC). After infusion of 20 mg/m² FA, median-reduced folate levels determined in primary tumor were 13 (0-46) pmol/mg, which was not significantly different from those measured without prior FA ($P = 0.133$; Table 1). Of note, the folate levels were below the level of detection in 3 of 11 samples from primary tumor after infusion of FA at a dose of 20 mg/m². However, after infusion of 200 mg/m² FA, the median concentration of 5,10-mTHF and THF was 39 pmol/mg (21-76), which was significantly higher compared with the untreated control group ($P = 0.001$). Moreover, after administration of 500 mg/m² FA, median-reduced folate levels were found to be 100 pmol/mg (69.5-235.5), which was significantly higher than those measured without prior FA ($P < 0.0001$), after 20 mg/m² FA ($P = 0.0002$), and after 200 mg/m² FA ($P = 0.005$; Table 1).

Normal liver tissue. In the FA-naive group of normal liver specimens, median concentration of reduced folates was 135 pmol/mg protein (66-209) as outlined in Table 1 and Figure 2. Folate levels were below the level of detection in 11 samples without prior infusion of FA. Administration of 20, 200, and 500 mg/m² FA did not result in a significant increase of the median 5,10-mTHF and THF levels ($P > 0.21$; Fig. 2), indicating saturation of the reduced folate pool in normal liver tissue.

Liver metastases. Without prior administration of FA, folate levels were below the level of detection in 3 of 12 samples from liver metastases. Compared with reduced median folate levels measured without prior FA infusion (19 pmol/mg protein), pretreatment with 500 mg/m² FA resulted in a significant increase to a median of 127.5 pmol/mg protein ($P = 0.002$) as outlined in Fig. 2. Pretreatment with 20 and 200 mg/m² FA did not significantly alter the median folate level ($P = 0.079$ and 0.162).

Discussion

In patients with metastatic CRC, the modulation of 5-FU by FA leads to higher response rates and a small but statistically significant benefit in survival (2). The optimal dose of FA is, however, still debated. Only sparse data are available that relate different FA-doses to expansion of reduced folate pools in human tissue. Thus, we investigated concentrations of combined pools of 5,10-mTHF and THF in various tissues from CRC patients without and after prior administration of FA.

Concentrations of combined pools of 5,10-mTHF and THF were surprisingly high in tissue from liver metastases with highest levels determined in specimens from normal liver. This observation may be explained by the entero-hepatic circulation of 5-methylTHF, which contributes to high folate levels in the liver. Values of reduced folates measured in liver metastases were similar to what has been reported in a previous study by Chéradame et al. (15).

Notably, the administration of 500 mg/m² FA significantly increased reduced folate pools in liver metastases but not in normal liver. This indicates saturation of the reduced folate pool in normal liver tissue. Thus, high doses of FA may be required to achieve an optimal inhibition of thymidylate synthase in liver metastases.

A dose-dependent increase in the concentration of reduced folates in primary CRC tissue was observed with the administration of 200 and 500 mg/m² FA. However, the difference between the 20 and 200 mg/m² dose level did not reach statistical significance ($P = 0.107$). In contrast, reduced folate levels in normal colorectal mucosa significantly increased only after infusion of FA at a dose of 500 mg/m². This observation also suggests the use of high doses of FA for optimal modulation of 5-FU in CRC patients treated with 5-FU based chemotherapy.

The high SDs observed in all groups indicate a considerable interpatient variability. For example, some of the FA-untreated or 20 mg/m² FA pretreated patients showed tissue levels of reduced folates similar to those found in the patients who had received 200 and 500 mg/m² FA. Likewise, reduced folate levels in primary tumor varied from 13 to 158 pmol/mg and 44.2 to 263.7 pmol/mg protein after infusion of 200 and 500 mg/m² FA, respectively. Remarkable different levels of reduced folates after administration of FA were also reported in xenografts (21). Moreover, wide variations in the concentration of reduced folates were also noted in recent studies with resected CRC specimens (17, 18).

Several factors may contribute to these high interindividual differences. Apart from variances in the folate nutritional status, differences in tissue concentrations of folylpolyglutamates synthase (FPGS), the enzyme responsible for converting the monoglutamate forms of reduced folates to higher order polyglutamates, which are retained inside the cells, may at least in part explain high interindividual differences in levels of combined folate pools (9, 15). As recently reported by Odin and coworkers (16), patients with high levels of FPGS in colorectal mucosa were shown to have significantly higher total folate concentrations and higher expression levels of reduced folate carrier, compared with patients with low FPGS levels. In the present study, we were not able to determine FPGS levels due to a limited availability of tissue specimens. However, as shown in a previous study on CRC patients, FPGS activity in primaries is not significant different from that in liver metastases (22), indicating that differences in the increase of reduced folate levels after infusion of FA may not merely be explained by differences in FPGS activity. Furthermore, it has
recently been shown that the concentrations of reduced folates in colorectal tumors are directly related to the presence of frequent DNA hypermethylation and inversely related to the presence of a common polymorphism in the *methylene-tetrahydrofolate reductase* (*MTHFR*) gene (17). Other studies observed a significantly lower concentration of THF in patients with *MTHFR* genotype CT or TT compared with patients having the CC genotype [19], whereas no difference was found in the total folate concentration in human HCT116 colon cancer cells expressing the mutant 677T and those expressing wild-type (23).

The present study is limited in that various factors possibly influencing the folate status in CRC patients were not determined. However, given the high number of samples analyzed together with the highly significant increase in liver metastases and primary tumor folate levels measured after infusion of high doses of FA, our results seem valid.

We are aware of three trials comparing different doses of FA while leaving the dose and schedule of administration of 5-FU unchanged (5, 24, 25). The results of these studies, initiated two decades ago with 5-FU given as i.v. bolus, which is no longer standard today, were inconsistent. Thus, one should be careful with firm recommendations on the optimal dose of FA.

However, a number of clinical trials on CRC compared different FA-doses along with different schedules of 5-FU. In these studies, regimens with FA at doses of 200 or 500 mg/m\(^2\) were associated with an improved progression-free survival compared with FA given at doses of 20 mg/m\(^2\) (26, 27). Notably, the 20 mg/m\(^2\) dose of FA is usually part of 5-FU i.v. bolus regimens, whereas FA at higher doses is chosen for modulation of infusional 5-FU. Given our results with 500 mg/m\(^2\) FA resulting in higher CRC tissue levels than lower doses of FA, higher doses such as 400 or 500 mg/m\(^2\) FA should be preferred.

In conclusion, as 500 mg/m\(^2\) FA achieved higher tissue levels of 5,10-mTHF and THF in the majority of specimens from primary tumor and liver metastases, high dose FA may more effectively modulate 5-FU than low-dose FA.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Bettina Engemann and Cornelia Riedelsheimer for their technical assistance and Jens Baumert, PhD, for his expert statistical support.

## References

Tissue Levels of Reduced Folates in Patients with Colorectal Carcinoma After Infusion of Folinic Acid at Various Dose Levels

Marcus Schlemmer, Michael Kuehl, Andreas Schalhorn, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/14/23/7930

Cited articles
This article cites 27 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/14/23/7930.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/14/23/7930.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.