Thymidylate Synthase Inhibition After Administration of Fluorouracil With or Without Leucovorin in Colon Cancer Patients: Implications for Treatment With Fluorouracil

By G.J. Peters, C.L. van der Wilt, C.J. van Groeningen, K. Smid, S. Meijer, and H.M. Rinedo

Purpose: To determine the time-dependence of fluorouracil (5FU)-induced thymidylate synthase (TS) inhibition in colon cancer patients, the effect of leucovorin (LV), and the relation to response.

Patients and Methods: A 5FU injection (500 mg/m²) was given to 47 patients with advanced colorectal cancer; tumor biopsy specimens were obtained 1 to 72 hours after laparotomy. Eleven patients received LV (2-hour infusion of 500 mg/m²) with 5FU midinfusion; biopsies were obtained after 45 hours. TS inhibition was evaluated by comparing the number of total and free 5-fluoro-2′-deoxy-uridine-5′-monophosphate (UMP) (FdUMP) binding sites and the total and residual catalytic activity of TS.

Results: The total catalytic TS activity varied from 0 to 621 pmol/h/mg protein and the total number of FdUMP binding sites varied from 0 to 976 fmol/mg protein. The residual catalytic TS activity after 2, 23, and 45 hours was 41%, 65%, and 74% of the total catalytic activity; the number of free FdUMP binding sites was 12%, 27%, and 49% of the total number, respectively. LV enhanced TS inhibition after 45 hours; the residual catalytic activity decreased from 74% to 49%, and the number of free FdUMP binding sites from 49% to 24%. Eleven of 19 patients treated with hepatic arterial infusion of 5FU had a partial response (PR). In the nonresponding patients, total TS activity was significantly higher (P < .05) than in responding patients. A high TS activity with a poor inhibition correlated with no response.

Conclusion: Residual and total TS activity are predictive for response to 5FU. The findings may be applicable for treatment of patients with advanced disease and TS should be evaluated as a prognostic factor in adjuvant chemotherapy studies.


FLUOROURACIL (5FU) is one of the most widely used chemotherapeutic agents. However, single-agent 5FU has a limited effect in the treatment of colorectal cancer, with a response rate of only 10% to 20%. In combination with leucovorin (LV), this is increased to 30% to 40%.1 The effect of 5FU is mediated by its metabolite 5-fluoro-2′-deoxy-uridine-5′-monophosphate (UMP) (FdUMP), a potent inhibitor of thymidylate synthase (TS), which catalyzes the conversion of dUMP to 2′-deoxythymidine-5′-monophosphate (dTMP), a rate-limiting step in DNA synthesis. Prolonged inhibition of TS has been postulated to be responsible for the increased antitumor effect of the combination of LV/5FU compared with 5FU alone. LV (5-formyl-tetrahydrofolate (SCHOTHF)) is the external source for the cosubstrate of TS, 5,10-methylene-tetrahydrofolate (CH₂-THF). The availability of FdUMP determines the extent of TS inhibition, which is mediated by the formation of a covalent ternary complex consisting of TS, FdUMP, and CH₂-THF.2,4 The stability of this complex is determined by CH₂-THF.5-7 Although dUMP can reverse inhibition, its concentration should then exceed that of FdUMP at least 100-fold.8 An important mechanism of resistance to 5FU is diminished inhibition of TS.5,10,19,10 High pretreatment enzyme levels,9 possibly due to gene amplification, also determine the extent of inhibition and are associated with resistance in both model systems11-13 and patients.14,15 In addition to TS-related resistance mechanisms, resistance to 5FU is also related to a decreased incorporation into RNA, predominantly in preclinical model systems.12,16-20 The mechanism of action of 5FU has also been related to scheduling; at a short pulse, 5FU may exert its cytostatic activity by incorporation into RNA, whereas for continuous exposure or infusion, TS inhibition may be the predominant mechanism. However, for bolus injection of 5FU to both animals and patients, we have demonstrated a prolonged presence of potentially cytotoxic concentrations of 5FU in tumors.21 In mice, this was associated with prolonged inhibition of TS, whereas sensitivity to 5FU was associated with the extent and duration of TS inhibition.13

Currently, many oncologists are reluctant to offer 5FU to their patients with colorectal cancer and resort to experimental chemotherapy. Several clinical studies have been performed in the attempt to correlate 5FU-induced inhibi-

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tion of TS with its antitumor response. Spears et al.\textsuperscript{22} were the first to describe TS inhibition up to 24 hours after 5FU treatment; other studies focused on short-term inhibition, which varied from a few hours to 24 hours after 5FU administration.\textsuperscript{8,15,23} In addition, inhibition of TS was measured using the FdUMP binding assay to evaluate the number of free FdUMP binding sites, but not by studying the catalytic capacity of tumor tissue to convert dUMP to dTMP. Moreover, a disadvantage of the ligand binding assay was its relative lack of sensitivity. Due to the intrinsic low enzyme activity in human tissue\textsuperscript{24} and the additional inhibition caused by 5FU treatment, the number of free FdUMP binding sites was below the detection limit in a number of samples. In the present study, for evaluation of TS inhibition in 58 patients who received one bolus injection of 5FU, we determined the inhibition of TS not only with the FdUMP ligand assay, but also with the catalytic assay, measuring the conversion of dUMP to dTMP. To evaluate the duration of TS inhibition, we obtained biopsy specimens of tumors up to 3 days after drug administration, which enabled us to evaluate the effect of LV on the duration of TS inhibition. The response to subsequent treatment in a limited number of these patients could be related to these pharmacodynamic events. The data of the present study offer a new approach to select patients for treatment with 5FU.

**Increasing Control of TS**

**Patients and Methods**

**Patients**

Patients with histologically proven colorectal cancer were enrolled onto this study (World Health Organization performance status of 0 or 1). Biopsy specimens of primary tumors or metastases were obtained from patients who received 5FU or LV/5FU before surgery. A total of 47 patients received the intravenous (IV) bolus injection of 5FU (500 mg/m\(^2\)) at different time points before surgery. Intervals between 5FU injection and biopsy were planned to be 2, 24 and 48 hours, but due to variations in scheduling at surgery, the actual interval was slightly different and varied for group 1 from 1 to 5 hours (15 patients; median, 2 hours), for group 2 from 16 to 26 hours (14 patients; median, 23 hours), and for group 3 from 42 to 49 hours (16 patients; median, 45 hours). An additional two patients were sampled at 67 and 72 hours. Eleven patients received LV/5FU (LV 500 mg/m\(^2\) administered as a 2-hour IV infusion, with an IV bolus injection of 5FU 500 mg/m\(^2\) midinfusion) between 40 and 49 hours (11 patients; median, 45 hours) before surgery.

Informed consent was obtained from all patients before drug administration and surgery. Patient characteristics are listed in Table 1.

**Tissue Preparation**

Biopsy specimens were obtained from primary colorectal tumors, liver metastases, normal mucosa, and normal liver. In a few patients, biopsies from metastatic sites other than liver were taken. Samples were immediately frozen in liquid nitrogen and subsequently stored at −80°C. Storage of the tissues at −80°C or in liquid nitrogen did not affect the activity of TS. Enough material was obtained from several patients to be divided into two parts; one part was frozen immediately and the other part chilled on ice for 1 hour, before freezing. No difference was observed in activity or extent of inhibition with either the FdUMP binding assay or the catalytic assay.

**Chemicals**

dL-l-Tetrahydrofolic acid was obtained from Sigma Chemical Co, St Louis, MO, and was used for the synthesis of 5,10-methylene tetrahydrofolic acid, essentially as described previously.\textsuperscript{30,24} 5-[\textsuperscript{3}H]-dUMP was obtained from the Radiochemical Centre, Amersham, United Kingdom and [6-\textsuperscript{3}H]-FdUMP from Moravek, Brea, CA. All other chemicals were of analytic grade and commercially available.

**Assays of TS**

Frozen tissues were pulverized using a micro-dismembrator as previously described.\textsuperscript{24,25} Subsequently, the frozen powder was weighed and suspended in ice-cold assay buffer (200 mmol/L tris-HCl, 20 mmol/L β-mercaptoethanol, 100 mmol/L sodium fluoride, and 15 mmol/L cytidine-5′-monophosphate [CMP], pH 7.4) at a concentration of 1 g tissue per 3 to 4 mL buffer. The suspension was centrifuged twice (10 minutes at 4,000 × g at 4°C and the supernatant subsequently for 20 minutes at 7,000 × g at 4°C).

Because no pretreatment samples of the individual patient were available, TS inhibition had to be evaluated by dissociation of the ternary complex. With this procedure, the total amount of TS in these tissues was determined. Inhibition of TS was defined as the TS activity in nondissociated samples relative to dissociated samples. A modification of the method described by Spears et al.\textsuperscript{22,26} and based on limitations and improvements reported by Houghton et al,\textsuperscript{27} Swain et al.,\textsuperscript{19} and Fernandes and Crawford\textsuperscript{28} was used. The total number of FdUMP binding sites and the total TS catalytic activity were determined after dissociation. The number of free FdUMP

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**Table 1. Characteristics of Patients Receiving a Test Dose of 5FU, Including Those Receiving LV/5FU, and Origin of the Tissues**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Patients</th>
<th>LV/5FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no.</td>
<td>58</td>
<td>11</td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>Range</td>
<td>34-78</td>
<td>38-72</td>
</tr>
<tr>
<td>Primary tumors only</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Liver metastases only</td>
<td>38</td>
<td>6</td>
</tr>
<tr>
<td>Primary tumors + liver metastases</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Skin and other metastases</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Total no. of primary tumors</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Total no. of metastases</td>
<td>59</td>
<td>15</td>
</tr>
</tbody>
</table>

NOTE: Nineteen patients (12 women, 7 men; median age, 55 years; range, 34 to 72) received subsequent treatment with hepatic arterial infusions of 5FU. The disease of these patients was limited to the liver and was assessable for response. Eight patients received systemic 5FU.
binding sites and the residual TS catalytic activity were determined in nondissociated samples. The final assay was performed essentially as described for murine tumors\textsuperscript{11} with some slight modifications. Briefly, for the catalytic TS activity, we used a final concentration of 1 \textmu mol/L \textit{[\textsuperscript{6}-\textsuperscript{3}H]-dUMP} (specific activity, 820 mCi/mmol) supplemented with 0.6 \textmu mol/L CH\textsubscript{3}THF and 16 \textmu mol/L CMP and incubated for 1 hour at 37\textdegree C. For measurement of FdUMP binding, 50-\textmu L samples were incubated with 50 \textmu L \textit{[\textsuperscript{6}-\textsuperscript{3}H]-FdUMP/CH\textsubscript{3}THF (50 \textmu mol/L and 2 \textmu mol/L, respectively; specific activity of \textit{[\textsuperscript{6}-\textsuperscript{3}H]-}FdUMP, 20 Ci/mmol) and 10 \textmu L assay buffer (containing 80 \textmu mol/L CMP) for 20 minutes at 30\textdegree C. Substrate dilution might occur due to exchange between nonlabeled FdUMP and labeled FdUMP.\textsuperscript{22} As was previously observed in murine tumors,\textsuperscript{13} correction only resulted in minor changes of the number of free FdUMP binding sites values and was therefore not included. Protein content was determined using the Bio-Rad (Veenendaal, the Netherlands) protein assay as described.\textsuperscript{20}

\textbf{Treatment of Patients}  

Nineteen patients with histologically proven metastases limited to the liver with the primary tumor removed and without extrhepatic disease were treated with hepatic arterial infusion of SFU. During laparotomy, an arterial access device (Port-a-Cath; Pharmacia Deltec, St Paul, MN) was implanted to allow hepatic arterial infusion chemotherapy.\textsuperscript{29} SFU (1,000 mg/m\textsuperscript{2}d for 5 days) was administered as a continuous infusion using the Pharmacia Deltec CADD-1 pump. The treatment was usually initiated 2 to 3 weeks after implantation of the Port-a-Cath and repeated every 3 weeks until disease progression. In case of grade 2 toxicity, the dose was reduced by 25%. Treatment results were evaluated every 3 to 4 months. Partial response (PR) was defined as a reduction of the measurable liver metastasis by = 50% on computed tomographic scan for a period of at least 4 weeks; stable disease (SD) was defined as a tumor reduction less than 50% or an increase of = 25%; progressive disease (PD) was defined as an increase in tumor volume of greater than 25% or the occurrence of new lesions, within or outside the liver.

Eight patients were treated with a weekly IV bolus administration of SFU (500 mg/m\textsuperscript{2}). In six of these patients, SFU was combined with LV. In one of the patients, LV/SFU was combined with uridine.

\textbf{Statistics}  

Statistical evaluation was performed using Student's \textit{t} test and the nonparametric Mann-Whitney \textit{U} test ranking test. The latter test was used for evaluation of the differences between absolute TS activities at several time points and for evaluation of the differences in TS activities between responding and nonresponding patients. Both tests were used for evaluation of differences between inhibited and noninhibited activity.

\textbf{RESULTS}  

\textbf{TS Inhibition in Tumors}  

The inhibition of TS in tissues from patients who received SFU or the combination of SFU/LV was evaluated by comparison of TS levels in dissociated and nondissociated samples (both primary tumors and liver metastasis). In several tissue specimens (most obtained from patients in group 1 within a few hours after drug administration), the total level of TS was below the detection limit (set at 1.5 times the blank) with the FdUMP binding assay in 11 tumor samples. With the catalytic assay, fewer samples (four patients) had nonmeasurable activity. Both assays showed a large interindividual variation (400-fold range) in the absolute level of inhibited TS (Fig 1A and B). The total level of TS in tumor samples as evaluated with both assays was significantly higher at 23 hours than at 2 and 45 hours. The large variation in the total level of TS in these treated tumors was comparable to that found in untreated patients.\textsuperscript{24} No consistent difference in TS levels was found between tumor sites, primary cancers, and metastases, as studied in 14 patients. In seven of these patients, we observed a higher activity in the primary tumor, and in
the other seven patients a higher activity in the liver metastasis. Therefore, data from all tumor sites have been pooled and are represented as separate samples in Figs 1 and 2. The levels of TS using both assays were not significantly different between patients who had not been pretreated with 5FU in the past and 12 patients who had received 5FU-containing regimens before participation in the present study (data not shown).

Inhibition of TS as measured with both assays was more pronounced at 2 hours than at later time points. The values after 2 days in samples after LV/5FU administration were significantly lower than after single-agent 5FU. These differences were more pronounced and better assessable when a comparison of the percentage of inhibited versus total activity was made (Fig 2). The percentages were calculated from separate experiments; in those cases in which the inhibited activities were not detectable, the ratio was considered to be zero, and consequently inhibition was 100%.

**TS Inhibition in Relation to Response**

After surgery, 27 patients were treated with 5FU with or without LV. They received single-agent 5FU either as a hepatic artery infusion (19 patients) or systemically (Tables 2 and 3). In patients who received hepatic arterial infusion, the total level and inhibition of TS correlated with the outcome of therapy (Table 4). In samples of patients who had received a test dose of 5FU only, a high number of total FdUMP binding sites and a high total TS catalytic activity were associated with no response, whereas a low TS was associated with a PR. The difference in TS levels between the two groups was significant (Table 4, group A; and Fig 3). However, when the patients who received LV/5FU as test drugs were included, the difference was not significant (Table 4, groups F and G). Almost no free FdUMP binding sites were detected in responding patients who had received only 5FU as a test drug, and measurable binding was observed in nonresponding patients (Table 4, group A; and Fig 3). The overall picture of the separate values was important to predict whether a patient would respond or not; a combination of either high FdUMP binding (> 100 fmol/mg protein), high total catalytic activity (> 90 pmol/h/mg protein), or a poor inhibition with either assay (< 90%) was related to no response (patients no. 11, 18, 34, and 49). Biochemically, patient no. 5 was not completely assessable, while samples from patient no. 56 were obtained.
Table 3. Inhibition of TS in Tumors After Administration of SFU or LV/SFU as Test Drugs, and Response to Subsequent Systemic Treatment With SFU or LV/SFU

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Tumor Site</th>
<th>Time (hours-minutes)</th>
<th>FdUMP Binding (pmol/mg protein)</th>
<th>Catalytic TS (pmol/h/mg protein)</th>
<th>Treatment†</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>T</td>
<td>0-55</td>
<td>98</td>
<td>ND</td>
<td>90</td>
<td>19</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>1-18</td>
<td>976</td>
<td>378</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>S</td>
<td>26-35</td>
<td>759</td>
<td>296</td>
<td>425</td>
<td>246</td>
</tr>
<tr>
<td>54</td>
<td>S</td>
<td>50-10</td>
<td>58</td>
<td>29</td>
<td>34</td>
<td>25</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>42-50</td>
<td>20</td>
<td>12</td>
<td>14.0</td>
<td>8.2</td>
</tr>
<tr>
<td>43*</td>
<td>S</td>
<td>48-00</td>
<td>59</td>
<td>24</td>
<td>13.6</td>
<td>5.2</td>
</tr>
<tr>
<td>40*</td>
<td>T</td>
<td>48-50</td>
<td>ND</td>
<td>ND</td>
<td>14.1</td>
<td>9.1</td>
</tr>
<tr>
<td>47*</td>
<td>M</td>
<td>45-00</td>
<td>120</td>
<td>55</td>
<td>53</td>
<td>40</td>
</tr>
</tbody>
</table>

Abbreviations: T, primary tumor; M, liver metastasis; S, skin metastasis.
*TS activity measured after LV-SFU.
SFU, weekly SFU; LV/SFU, weekly SFU and LV (both as described in Materials and Methods); LV/SFU/UR, weekly SFU and LV as described, every 3 weeks, followed by uridine (UR), with SFU dose escalated every 4 weeks.

after 3 days. Since administration of LV/SFU can cause changes in the activity and amount of TS different from that of SFU alone in mice, patients who received these drugs in a test dose in combination are difficult to evaluate.

Eight patients whose disease was not limited to the liver received systemic SFU (Table 3). When the patients who received systemic single-agent SFU treatment following a SFU test dose (patients no. 12 and 20) were included in the analysis of data in Table 2, the difference in total TS catalytic activity between responding and nonresponding patients reached a higher level of significance (Table 4, group B): a significant difference between the free FdUMP binding sites was found (Table 4, group C).

Table 4. Statistical Evaluation of the Relation Between Inhibition of TS and Response to Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Test Dose* (drug[s], time [hours])</th>
<th>Treatment†</th>
<th>PR/PD + SD‡</th>
<th>FdUMP Binding§</th>
<th>TS Catalytic Activity§</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SFU 2, 23, 45</td>
<td>IA SFU</td>
<td>9/6</td>
<td>0.0449</td>
<td>0.0248</td>
</tr>
<tr>
<td>B</td>
<td>SFU 2, 23, 45</td>
<td>IA SFU</td>
<td>9/8</td>
<td>0.0123</td>
<td>0.0071</td>
</tr>
<tr>
<td>C</td>
<td>SFU 23, 45</td>
<td>IA SFU</td>
<td>7/5</td>
<td>NS</td>
<td>0.0236</td>
</tr>
<tr>
<td>D</td>
<td>SFU 23, 45</td>
<td>IA SFU</td>
<td>10/0/0</td>
<td>0.0113</td>
<td>0.0103</td>
</tr>
<tr>
<td>E</td>
<td>SFU 23, 45</td>
<td>IA SFU</td>
<td>8/6</td>
<td>NS</td>
<td>0.0131</td>
</tr>
<tr>
<td>F</td>
<td>SFU 23, 45</td>
<td>IA SFU</td>
<td>11/8</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>G</td>
<td>SFU 23, 45</td>
<td>IA SFU</td>
<td>12/14</td>
<td>NS</td>
<td>0.0461</td>
</tr>
</tbody>
</table>

Abbreviations: IA, intraarterial; sys, systemic; NS, not significant (P > .05).
*Test dose of SFU given as single-agent SFU at various time points (median, 2, 23, or 45 hours) or as LV/SFU at 45 hours before surgery.
†Treatment with SFU alone or in combination with LV.
‡Number of responding patients (PR) that was compared with that of nonresponding patients (PD and SD) according to the way SFU was given as a test dose and/or according to treatment protocol with SFU alone (groups A, B, C, and F) or with SFU and LV/SFU as treatment (IA SFU, sys SFU, and LV/SFU; groups D, E, and G). When LV/SFU given as a test dose was included in the evaluation (group F) and LV/SFU also as treatment (group G), the differences were less significant.
§Statistical evaluation was performed using the Mann-Whitney U test by comparison of the TS levels mentioned in Tables 2 and 3 between the treatment groups specified.
When the patients who were treated with LV/5FU were included in the analysis (Table 4, groups D and E), the significance pattern hardly changed; however, when the patients who received LV/5FU as test drugs were included in the evaluation, the significance pattern was less clear (Table 4, groups F and G).

**DISCUSSION**

In this report, we demonstrate that bolus administration of 5FU resulted in inhibition of TS for a period of at least 45 hours, whereas coadministration of LV and 5FU resulted in an enhanced inhibition compared with 5FU alone. A relation between TS inhibition and the response to 5FU existed in patients studied more than 23 hours after drug administration.

Spears et al.\(^2\) described TS inhibition in human tumor samples, but measurements were limited to samples obtained between 20 and 240 minutes after 5FU administration. A large variation in TS activity, comparable to our data, was observed. Only with short-term (< 2 hours) sampling have pretreatment and posttreatment TS levels\(^2\) been reported. Inhibition of TS in our tissue samples could only be evaluated by comparison of inhibited TS and total TS. The FdUMP ligand binding assay lacked sensitivity to determine the very low TS levels in humans and, therefore, a number of samples were not assessable (Fig 1). For this reason, we also measured TS activity with the more sensitive catalytic assay, which is a parameter of the actual capacity to convert dUMP to dTMP. The higher total TS activity, with both assays in tumor samples from patients studied at 23 hours after 5FU, probably represents an adaptive reaction of the tumor cells to 5FU-induced cellular stress,\(^3\) resulting in increased TS synthesis, similar to that observed in experimental model systems after administration of 5FU.\(^3\) It is of interest that in several patients with a high, possibly upregulated level of TS in the tumor, treatment was not effective, whereas a low TS was related to a response.

Inhibition of TS appeared to be time-dependent. Maximal inhibition of TS was observed soon after treatment, consistent with data of other investigators.\(^\text{3,5,15,22,23,31}\) Clinical data on retention of TS inhibition at time points greater than 24 hours are not available. However, in murine tumors, we observed a long-term inhibition (up to 3 days) followed by a several-fold increase in TS activity.\(^13\) The long duration of TS inhibition might be related to the prolonged presence of high tissue concentrations of 5FU, even several days after treatment, exceeding the threshold required to maintain a sufficiently high concentration of FdUMP to obtain a maximal inhibition of TS.

Although it was not feasible to obtain sequential samples from the patients, it was evident that LV potentiated the TS inhibition in human colon cancer. In vitro, such a potentiating effect of LV on 5FU-induced inhibition of TS has been described repeatedly.\(^3\) To achieve this retention it was essential to pretreat cells with LV or add LV simultaneously to facilitate an optimal stabilization of the ternary complex from the moment 5FU is added. In murine models, this approach resulted in the most optimal schedule for administration of LV and 5FU,\(^3\) in which LV was administered 1 hour before and simultaneously with 5FU, which led to enhanced antitumor effect.\(^13,33\) Weekly administration of LV/5FU resulted in a more pronounced prolonged TS inhibition than 5FU alone\(^13\) or 5FU administered as a continuous infusion for 3 weeks.\(^34\) This information is of considerable clinical importance, because it suggests that repeated administration of LV/5FU results in a maximal TS inhibition.

In a number of patients, we observed a low total TS activity (both binding and catalytic) that was associated with a complete inhibition of TS; this correlated with a PR to 5FU therapy. This correlation was most pronounced when we compared samples obtained at 23 and 45 hours (Table 4). We observed a complete inhibition of TS in most patients within the first hours after treatment; in a number of these patients, subsequent treatment did not result in a response. It appears that the best predictive values could be obtained at 23 or 45 hours after administration of single-agent 5FU. In the initial studies reported by Spears et al.\(^22\) (maximum interval, 240 minutes), a
strong inhibition of TS (> 90%; also determined by the FdUMP ligand binding assay) was observed in three of 22 patients (one PR and two SDs). In the other patients (with PD), 70% inhibition was observed. However, there was a large variety between tumor types (colon, breast, ovarian, and gastric carcinoma). In tumor samples of patients to whom LV and 5FU was given, a complete inhibition of TS was observed. The latter findings are in agreement with our results, which show a more pronounced inhibition of TS in LV/5FU-treated patients. Swain et al. studied TS inhibition in patients with breast cancer before and after development of resistance to 5FU; a partial inhibition in biopsies obtained 23 hours after single-agent 5FU could be converted to a complete inhibition of TS after combined LV-5FU. Based on these and our data, it seems that one important predictive factor for response to 5FU or 5FU-containing therapy is the maintenance (≥ 23 hours) of inhibition. The absolute values of the inhibited enzyme required for response should be below a threshold of approximately 20 pmol/mg protein for the number of free binding sites and 30 fmol/h/mg protein for the residual TS activity. Moreover, it appears that the total activity of TS, measured with both assays, is the major determinant of prognosis; inhibition is inadequate in case of high values (> 200 for both assays), which result in poor response. The initial concentration of FdUMP is only limiting when it is below the level sufficient to saturate the enzyme; in all our patients, FdUMP levels were above saturating concentrations. In conclusion, it is clear from these data that 5FU administration to patients resulted in a pronounced inhibition of TS. The total TS activity and the TS inhibition correlated with response to 5FU. The duration of TS inhibition is enhanced by administration of LV. For future evaluation of TS, more parameters should be considered, such as the amount of TS protein and gene expression. Although these parameters do not give information on the actual events that will occur in the tumor after 5FU treatment, they appear to have predictive value, either concerning the heterogeneity of TS distribution (with an antibody) or concerning mutations of the gene that lead to either high TS levels or variations in enzymatic properties of TS. It is not yet clear whether measurement of pretreatment levels of TS is equivalent in predicting response to TS levels measured after 5FU treatment. Treatment of patients with advanced colorectal cancer is controversial. It would be useful to include such a test in each patient who undergoes surgery for colorectal cancer. In breast cancer, predictive tests have been developed for patients to be entered on adjuvant combination chemotherapy. Considering the fact that only one chemotherapeutic agent is being applied in adjuvant colorectal trials, a prospective study of the biochemical parameters in patients receiving adjuvant 5FU/leucovorin may help us to identify patients who will benefit of this treatment. In advanced colorectal cancer, the assay would guide us in the decision whether to treat the patient with 5FU or to offer experimental therapy.

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