IN VITRO AND IN VIVO INHIBITION OF THYMIDYLATE SYNTHASE
OF HUMAN COLON CANCER BY 5-FLUOROURACIL.

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INTRODUCTION

Thymidylate synthase (TS) is a key enzyme in *de novo* synthesis of TMP (Fig. 1). TS catalyzes the conversion of dUMP to TMP for which 5,10-methylenetetrahydrofolate (CH_2THF) acts as the methyl-donor; the K_m for dUMP is about 1-5 μM (1-5). Inhibition of TS by FdUMP is one of the main mechanisms for the action of 5-fluorouracil (5FU). Without preincubation FdUMP acts as a potent competitive inhibitor of TS with a K_i of about 1 nM. The K_m/K_i ratio is about 1000. The inhibition of TS by FdUMP is mediated by the formation of a tight-binding covalent ternary complex of FdUMP with TS and CH_2THF . Retention of the inhibition of TS is mainly related to the FdUMP/dUMP ratio (6), the FdUMP binding to TS and the stabilization of the ternary complex by CH_2THF or one of its polyglutamates (6,7,9). In vitro, resistance against 5FU or its analog 5-fluoro-2'-deoxyuridine (FdUR) has been related to altered kinetics of TS with respect to K_m values for dUMP and FdUMP binding (2,5,8), disturbed folate pools (9), and the level of enzyme before treatment (6,8). Gene amplifi-

Fig. 1. Inhibition of TS by FdUMP (formed from 5FU), leading to a depletion of TMP, TDP and TTP; and accumulation of FdUMP and dUMP. DHF, dihydrofolate; 5-CHO-THF, 5-formyl-tetrahydrofolate.

cation of TS has been demonstrated for FUDR-resistant sub-cell lines (10, 11). Recently evidence for gene amplification has also been obtained in a patient with colon cancer who developed resistance against 5FU (12), while in breast cancer patients binding of FdUMP and the effect of CH_2THF decreased during development of resistance (13).

5FU is the standard treatment for patients with colorectal cancer, although the response rate is less than 20%. To establish a relationship between antitumor activity and TS we measured *in vitro* enzyme activity, inhibition by FdUMP and the binding of FdUMP to TS. *In vivo* inhibition of TS has been related to the response of murine tumors to 5FU (14) and more recently it has also been demonstrated that tumors of patients responding to 5FU showed greater inhibition than patients with progressive disease (15). We analyzed tumors of patients for FdUMP binding to TS after administration of 5FU.

MATERIALS AND METHODS

The sources of pyrimidine nucleosides and nucleotides and the synthesis of CH_2THF have been described previously (4,16). All other chemicals were of standard analytical quality. Biopsy specimens of primary colorectal tumors and adjacent healthy mucosa from untreated patients were obtained as soon as possible after surgical removal and immediately frozen and stored in liquid nitrogen. Under these conditions the enzyme was stable for at least 2 years.

For assay of TS and binding of FdUMP, tissues were pulverized using a micro-dismembrator as described (17). The powder was weighed, suspended in 4 volumes of assay buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM dithioerythrol, pH 7.4) and subsequently centrifuged for 10 min at 10,000g at 4°C. The supernatant was used for enzyme assays and measurement of FdUMP binding. TS was measured using the tritium release assay at 1 and 10 μM [$6\text{-}^3\text{H}$]-FdUMP as described (16). The assay was linear with time (up to 30 min) and protein. The amount of FdUMP binding was determined essentially as described (14,18). Shortly, 10,000g supernatant was incubated with [$6\text{-}^3\text{H}$]-FdUMP and CH_2THF (30 nM and 350 μM final concentrations, respectively) for 1 hr. The incubation was terminated by addition of 10% activated charcoal to bind free [$6\text{-}^3\text{H}$]-FdUMP, the suspension was centrifuged and radioactivity in the supernatant was determined.

For assay of FdUMP binding in 5FU treated patients, an i.v. injection of 5FU at 500 mg/m² was given 2-24 hr before surgery. Tissues (colon tumor, mucosa, liver and/or liver metastases) were chilled on ice immediately after surgical removal, dissected and frozen in liquid nitrogen. In these tissues TStot and TSf,app were determined. TStot represents the total amount of FdUMP binding, while TSf,app is the amount of binding which appears to be free and can be bound by [$6\text{-}^3\text{H}$]-FdUMP. They were determined essentially as described by Spears et al (14,15), but using an additional charcoal wash as suggested by others (18,19). Tissues were pulverized, suspended in "sonication" (0.2 M Tris-HCl, 20 mM mercaptoethanol, 100 mM NaF, 15 mM CMP, pH 7.2) buffer, centrifuged (at 4°C) for 15 min at 2500g (4000 rpm), and the supernatant was subsequently centrifuged at 12,000g for 10 min. One part (for TSf,app) was frozen at -70°C and the other part was used immediately for TStot. FdUMP was liberated from TS by a 4 hr incubation at 30°C of 50 μl 12,000g supernatant with 50 μl "dissociation" buffer (0.75 M NH_4CO_3 , 100 mM NaF, 20 mM mercaptoethanol, 15 mM CMP, pH 7.8) and 5 μl 1.6 mM dUMP (to facilitate dissociation, prevent reassociation of FdUMP and stabilization of TS). Nucleotides were then removed by addition of 100 μl 10% neutral charcoal, mixing and centrifugation. The frozen part of the 12,000 g supernatant was then thawed; to 50 μl super-

natant 50 μ l of "dissociation" buffer was added, immediately followed by 100 μ l 10% neutral charcoal. Measurement of $[6-^3\text{H}]\text{-FdUMP}$ binding was performed by addition of 50 μ l $\text{FdUMP-CH}_2\text{THF}$ (final concentrations in reaction mixture 34 nM and 267 μ M, respectively) to 50 μ l of the charcoal supernatant, followed by an incubation of 20 min at 30°C. The incubation was stopped by addition of 1 ml of 3% acid charcoal; after mixing and centrifugation 500 μ l of the supernatant was counted.

RESULTS

Untreated tissues

In both tumors and adjacent healthy mucosa from untreated patients we measured the activity of TS, its inhibition by FdUMP and the binding of FdUMP . The activity of TS and its inhibition by FdUMP were assayed at 1 and 10 μ M dUMP to detect possible variations in enzyme kinetics of TS (16). Optimal CH_2THF concentrations were used. FdUMP was added to the assay together with dUMP . Both at 1 and 10 μ M dUMP the assay was linear with time and protein. There was a large variation (18-513 nmol/hr per mg protein at 1 μ M dUMP) in the activity of TS in tumors, which was more pronounced (52-3018) at 10 μ M dUMP (Fig. 2). In mucosa the variation in the activity of TS, both at 1 and 10 μ M dUMP was less (8-10-fold). However, in all patients TS activity was higher in the tumor compared to mucosa, varying between 3-28-fold. The ratio of TS activity between 10 and 1 μ M dUMP in tumors varied between 2.9 and 5.9, the highest ratio was found at the highest activity. In mucosa this ratio varied between 2.2 and 4.8; however the distribution was different from that in tumors; within the same patient the highest tumor ratio and the lowest mucosa ratio was found. In tumors inhibition by 10 nM FdUMP varied between 70-90%, being higher at 1 μ M (Fig. 3). In healthy mucosa the inhibition by FdUMP (at 10 μ M between 10-80%) was less than in tumors.

Fig. 2. Activity of TS (assayed at 1 and 10 μ M dUMP) in primary tumors and adjacent healthy mucosa. Lines connect samples obtained from the same patient (A-C, F-H).

Fig. 3. Inhibition of TS (assayed at 1 and 10 μ M) by 10 nM FdUMP in tumors and mucosa.

Fig. 4. Amount of TS binding in tumor and mucosa samples from untreated patients assayed at optimal folate concentration in the absence of NaF.

FdUMP binding in tumor samples from untreated patients was determined in the absence and presence of optimal folate pools. The amount of FdUMP binding in tumors varied between 0.11 and 0.77, being less than the variation in TS activity (Fig. 4). Only in two patients (A and F) FdUMP binding could be detected in the absence of exogenous folate. In mucosa FdUMP binding to TS was at least 3-times lower than in tumors from the same patient; in those two mucosa samples where FdUMP binding was detectable it was lower than 0.1 pmol/mg protein. In the absence of folate no binding of FdUMP could be detected at all. Since it has been demonstrated that phosphatases can degrade FdUMP we added NaF and CMP to the binding assay and observed a significantly higher FdUMP binding. It was decided to add NaF and CMP to all binding assays, however the first samples could not be repeated.

FdUMP binding in tumors from 5FU treated patients

Several patients with primary tumors and liver metastases were treated with 5FU at 500 mg/m² in order to determine the FdUMP binding to TS. Tumors were surgically removed, immediately chilled on ice, dissected and frozen in liquid nitrogen. Tstot was determined after dissociation of FdUMP from the enzyme. We found a higher FdUMP binding when dissociation was performed in the presence of dUMP. The additional wash with neutral charcoal to remove endogenous nucleotides also resulted in higher values. The dissociation was complete after 3 hr, no difference was found between

TABLE 1. BINDING OF FDUMP TO TS IN TUMOR SAMPLES OF PATIENTS TREATED WITH 5FU AT 500 MG/M²

Patient	Tumor	Time# (hr-min)	TStot (fmol/mg protein)	TSf,app* (fmol/mg protein)
EB	colon ca.	2-30	90	n.d.
HB	colon ca.	2-05	172	26 (15)
GB	colon ca.	2-05	30	n.d.
AH	colon ca.	4-10	58	n.d.
GA	liver metas.@	5-05	130	30 (23)
CB	liver metas.	21-45	92	n.d.
CB	colon ca.	21-45	34	18 (53)
CvH	colon ca.	25-35	188	n.d.

#, the time of biopsy after 5FU administration; *, within parentheses the % of apparently free binding sites; @, this patient had been treated with 5FU before receiving this 5FU injection, for other patients this was the first 5FU treatment. ca., carcinoma; metas., metastasis; n.d., not detectable.

3 and 4 hr. The binding of FdUMP was linear with the amount of protein present in the assay. The binding of [6-³H]-FdUMP was not yet complete after 10 min, but reached a plateau after 20 min; under these conditions the FdUMP concentrations were optimal. Assays performed on tumor samples from the same patient were comparable. Tumor samples removed between 2 and 24 hr were studied (Table 1). In several samples obtained TSf,app was detectable, in the other patients TSf,app was not detectable. In one patient (CB) samples from both the primary tumor and the liver metastasis could be obtained, FdUMP binding was quite different. In several samples we also measured the activity of TS with the ³H-release assay, which is more sensitive. In samples from those patients with detectable TSf,app we could show inhibition of TS.

DISCUSSION

Analysis of the biochemical parameters that determine the antitumor activity of 5FU can be a useful tool for the treatment of colorectal cancer. Up to now, for patients with advanced colorectal cancer, 5FU is the only drug which shows some activity. Less than 20% of the patients respond to 5FU therapy, while most combinations with other drugs are not more active (5). Recently it has been shown that leucovorin can enhance the response rate of 5FU, both in patients (22) and in animal models (23). Although the metabolism of 5FU has been studied extensively *in vitro*, relatively little is known on its mechanism of action *in vivo* in patients. In this study it is demonstrated that in primary colorectal tumors TS shows a large variation in activity and inhibition by FdUMP. This was observed both by measurement of *in vitro* enzyme activity and *in vivo* binding of FdUMP to TS. It seems likely that this biochemical heterogeneity is related to variation in response. However, up to now not enough data are available from the patients described in this study to make such a correlation.

The activity of TS in tumors showed a larger variation than in healthy mucosa from the same patient. The relative small variation in mucosa from these patients might be related to the normal inter individual differences, since comparable differences have been observed in the activities of other enzymes involved in the activation of 5FU (17,21; Peters et al., manuscript in preparation). However, in tumors from these

patients the activities of these enzymes also show a relatively small variation, in contrast to that for TS. FdUMP binding to TS also showed a large variation, but the pattern was completely different from the TS activity. In the tumor with the lowest TS activity we even found the highest FdUMP binding. It has been demonstrated that in tumors a high phosphatase activity is present (12,24). In the first tumor samples we measured TS activity and FdUMP binding in the absence of NaF and CMP; in the later samples in which we added NaF and CMP to inhibit nucleotide breakdown and found a 3-10 fold higher FdUMP binding, but still we did not find a correlation between enzyme activity and FdUMP binding. Addition of NaF and CMP to the TS assay in tumor and mucosa samples led to contradictory results, in some samples TS activity was somewhat higher, but in the majority the activity was even lower. So, it is questionable whether NaF and CMP should be added to the TS assay, but in the binding assay its presence is essential.

The measurement of FdUMP binding in treated patients served several purposes. Firstly we wanted to determine the extent of *in vivo* binding of FdUMP, secondly to relate this binding in pretreated patients retrospectively with antitumor activity of 5FU, and thirdly to select prospectively those patients which might benefit from 5FU treatment in combination with leucovorin. For evaluation of the latter we do not have enough data. However, the *in vivo* data demonstrate that FdUMP binding to TS shows a large variation between the various patients, which is comparable to the *in vitro* data. In the samples obtained after 2 hr in most patients a complete binding was detected. For that reason the time-schedule was recently extended to 24 hr. The intention is to compare binding of FdUMP to TS in patients only treated with 5FU with those treated with leucovorin and 5FU, in order to select those patients in which resistance against 5FU can be circumvented by treatment with leucovorin. Preliminary data by others showed that this might be a valuable approach. In mice we recently demonstrated that pretreatment with 5FU followed by treatment with leucovorin-5FU enhanced the therapeutic efficacy in comparison with prolonged single agent therapy [23].

In conclusion, we demonstrated in tumors a large variation in TS activity and in the capacity to bind FdUMP to TS. It seems likely that this might be a criterium to select patients that might show a response to 5FU treatment. The analysis of the samples of 5FU-treated patients might even prove to have a better prognostic value.

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