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Thymidylate Synthetase Inhibition in Malignant Tumors and Normal Liver of Patients Given Intravenous 5-Fluorouracil¹

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ABSTRACT

Single surgical biopsies of solid tumor were obtained at 20 to 240 min after drug administration in 21 patients given first-dose bolus i.v. 5-fluorouracil (5-FUra), 500 mg/sq m, and assayed for 5-fluorodeoxyuridylylate (FdUMP), deoxyuridylylate (dUMP), total thymidylate synthetase (TS), and non-FdUMP-bound, free enzyme. Nineteen patients had cancer of gastrointestinal origin, 13 of these colorectal, and 2 patients had breast adenocarcinoma. In 9 patients, synchronous biopsies of surgically normal liver were obtained along with samples of hepatic tumors metastatic from gastrointestinal sites. Total TS averaged 4.18 pmol/g in the malignant tissues and 2.23 pmol/g in liver. FdUMP levels in the gastrointestinal tumors were higher than in normal liver, were highest at the earliest time interval studied, 20 to 30 min, and appeared to decrease exponentially through 120 min. TS inhibition averaged 70 to 80% in gastrointestinal tumor biopsies and less than 50% in normal liver. Levels of dUMP were low and varied little with time. Those gastrointestinal tumors with higher FdUMP:dUMP ratios showed significantly greater TS inhibition. Tumors of 3 patients who benefited from 5-FUra therapy (1 patient with colonic adenocarcinoma and the 2 patients with breast adenocarcinoma) showed greater TS inhibition than did tumors of remaining patients.

It is concluded that the apparent time course changes observed in FdUMP, dUMP, and TS in the grouped data are qualitatively similar to findings of murine studies *in vivo* and that the relationships between FdUMP:dUMP ratios and TS inhibition are consistent with established *in vitro* enzymic kinetics. Thus, biopsies of tumors at short time periods after 5-FUra administration may be usefully studied for biochemical parameters of TS inhibition, with the objectives of correlation of sensitivity to subsequent 5-FUra therapy and clarification of mechanisms of drug resistance.

INTRODUCTION

Patients with metastatic cancer of gastrointestinal origin have approximately one chance in 5 of showing an objective response to conventional 5-FUra³ treatment. Clinical evaluation of re-

sponse may take several months, which for most patients is potentially valuable time lost before a trial of investigational therapy can be instituted. Using explants of tumor biopsies, a variety of nonspecific cytotoxicity assays for prior determination of tumor sensitivity to 5-FUra has been attempted. None of these has achieved routine use, partly because such tissue is not fully representative of the tumor of origin. This is less of a problem with biochemical predictive tests that attempt to correlate enzyme or metabolite levels in a given tumor with clinical response to 5-FUra (21, 32). An additional advantage of biochemical methods is the potential for clarification of mechanisms of drug action and resistance. Knowledge of the individual biochemical characteristics of a given tumor may allow selective modulation of fluoropyrimidine metabolism in order to achieve a greater cytotoxic effect (2, 3, 5, 6, 11, 14, 26).

The mechanism of cytotoxic action of 5-FUra that is best understood is inhibition of TS, the final enzyme of the *de novo* pathway that converts dUMP to thymidylate by reductive methylation. Intensive studies in recent years have greatly detailed the chemistry and biochemical kinetics of inhibition of TS by FdUMP (9, 10, 13). FdUMP competes with dUMP for initial binding to TS, and then forms a "frozen" transition-state analogue of the normal reaction in the presence of CH₂FH₄. The latter combines with the TS-FdUMP binary complex to form a covalently-bound ternary complex, which slowly dissociates to starting materials. Equilibrium favors the ternary complex only in the presence of adequate levels of folates such as CH₂FH₄.

Recently, we have developed sensitive ligand-binding techniques, using [³H]FdUMP, for assay of tissue levels of TS_i, TS_{tot}, and FdUMP as the free nucleotide in small biopsies of tumors treated *in vivo* (18, 27, 29). Determination of TS_i by these methods in murine colon adenocarcinomas and in human tumor xenografts showed that tumor sensitivity to 5-FUra is apparently related to virtual ablation of enzyme activity (27, 29); however, detailed study was not made of other mechanisms of cytotoxicity of 5-FUra, such as incorporation of drug into RNA with alterations in its processing and function or incorporation of 5-FUra into DNA (13). These methods have made possible the present clinical study, in which we performed biochemical analyses of tumor biopsies taken from cancer patients given bolus i.v. 5-FUra.

To our knowledge, this is the first report of the intratumoral effects of an antimetabolite against its putative biochemical target in solid tumors in the clinical situation. This is largely attributable to the clinical difficulties involved in obtaining tumor

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³ The abbreviations used are: 5-FUra, 5-fluorouracil; TS, thymidylate synthetase; FdUMP, 5-fluorodeoxyuridylylate; dUMP, 2'-deoxyuridylylate; CH₂FH₄, L-(+)-5,10-methylenetetrahydrofolate; TS_{tot}, total thymidylate synthetase; TS_i, free non-5-

fluorodeoxyuridylylate-bound thymidylate synthetase; TS_o, ternary complex 5-fluorodeoxyuridylylate-bound thymidylate synthetase; FH₄, L-(+)-tetrahydrofolate; TS^{FP}, experimentally determined 5-[³H]fluorodeoxyuridylylate-binding sites. UG, University of Goteborg; USC, University of Southern California.

tissue following drug exposure. However, it is well established that regional hepatic infusion of 5-FUra or 5-fluorodeoxyuridine results in at least a doubling of the objective response rate over systemic drug administration (16); in such patients, biopsy of metastatic nodules is done readily during the laparotomy performed for catheterization of the hepatic artery or portal vein. The majority of patients in this report were in this category. In many of these, biopsy of surgically normal liver was also done at the time of tumor biopsy following bolus i.v. 5-FUra. Significant relationships between FdUMP:dUMP levels and percentage inhibition of TS are found in this study, along with significant differences in these parameters between tumor and normal liver. In addition, consideration of the time-to-biopsy interval establishes kinetic profiles for the biochemical parameters. These findings support our hypothesis that study of biochemical events related to TS inhibition in biopsies of solid tumors following 5-FUra administration may result in clinically useful information.

MATERIALS AND METHODS

Materials. [6-³H]FdUMP was from Moravsek Biochemicals, Brea, CA, and was periodically purified by DEAE-cellulose chromatography (19). [¹⁴C]Formaldehyde, from New England Nuclear, Boston, MA, was stored at 25° and purified at monthly intervals by chromatography using DEAE-cellulose (prepared in 0.1 N NaOH, washed with H₂O, then equilibrated with 1 M ammonium formate, pH 4.5, and washed with H₂O; the ¹⁴CH₂O elutes with H₂O, leaving impurities on the column). FH₄ was the racemic reagent obtained from Sigma Chemical Co., St. Louis, MO, and stored in 4- μ mol aliquots in glass ampuls under N₂ at -25° in the dark; [³H]-FdUMP-binding studies of TS from *Lactobacillus casei*, CCRF-CEM cells and human tumors with this preparation gave results that were comparable to those using enzymatically-synthesized FH₄ (19). All other materials were obtained as reported (29).

Tissue Handling. Biopsies of solid tissues were placed immediately on dry ice and were maintained at -25° or below until processed. In this state, levels of TS, FdUMP, and dUMP are stable for several days. However, because these parameters are labile in crude homogenates, TS assays were done within 6 hr of tissue disruption. The latter was achieved by addition of a 4- to 9-fold excess of 0.2 M Tris-HCl buffer, pH 7.4, at 4° (containing 20 mM 2-mercaptoethanol, 15 mM cytidylate, and 100 mM NaF), and sequential scissors-mincing, ground glass (Duell; Kontes Glass Co.) homogenization, and sonication as described (29). Cytosols were then prepared by centrifugation at 10,000 \times g for 15 min in an Eppendorf Micro 5414 centrifuge. Cytosols were sterilized by 0.22- μ m membrane (Millipore) filtration prior to TS assay, as were all buffers used in the TS assay.

Nucleotides were extracted from the crude sonicates by use of cold acetic acid (19). The resulting nucleotide preparations are stable at 4° and were therefore assayed in batches. Nucleic acids were determined by a modified Schmidt-Tannhauser procedure (29). DEAE column-separated FdUMP and dUMP were assayed by the [³H]FdUMP isotope-dilution method, and conversion to [¹⁴C]thymidylate, respectively (19).

Ascites specimens obtained by paracentesis were collected by gravity into 1-liter flasks, on wet ice, containing 5000 units heparin sulfate. Cell pellets were prepared at 4° by centrifugation at 2000 \times g for 10 min followed by one wash with 0.85% NaCl solution. None of the effusions studied was significantly contaminated with erythrocytes, grossly or by cytological analysis. Pellets were stored at -25° or below and then handled as solid tissues.

TS Assay. Measurement of cytosolic [³H]FdUMP-binding sites was done by the method reported for murine adenocarcinomas (29). TS_{tot} was assayed by allowing TS_b to dissociate to the TS_i state during a 3-hr 30° preincubation period, on 1:1 dilution of cytosol with pH 8.0 NH₄HCO₃ buffer (0.3 M, containing 100 mM NaF and 15 mM cytidylate). All assays were done in air-tight polypropylene tubes because of the

volatility of NH₄HCO₃ and 2-mercaptoethanol. TS_{tot} was then determined in the presence of 20 nM [³H]FdUMP and 250 μ M CH₂FH₄ for 20 min at 30°, by isolation of protein-bound radioactivity on addition of a 4-fold volume excess of 3% dextran-coated charcoal (0.2 N in HCl at 4°). TS_{pp} was found from results of cytosols diluted with the NH₄HCO₃ buffer but not subjected to the 3 hr pre-incubation. TS_i was calculated from TS_{tot} and TS_{pp} by assuming that 13% of any TS_b present becomes labeled with [³H]FdUMP during the 20-min assay (29), due to dissociation of endogenous unlabeled FdUMP. The results are reported as percentage inhibition of TS, which is $(1 - TS_i/TS_{tot}) \times 100$. The validity of the correction factor in cytosols of tumors from 2 patients was checked by determination of the rate of exchange of excess [³H]FdUMP into nonradiolabeled endogenous ternary complex TS_b enzyme over 3 hr. These rates for a breast adenocarcinoma and a gastric adenocarcinoma were experimentally indistinguishable from those reported for mammalian ternary complexes formed *in vitro* (29).

Patients. A total of 24 patients were entered into the study, at the UG, Ostra Hospital, Goteborg, Sweden (6 men and 8 women) and at hospitals affiliated with USC in Los Angeles, CA (4 men and 6 women). Median ages of the patients were 64 years (range 44 to 77) and 50 years (range 25 to 61) at UG and USC, respectively. The UG group included 9 patients undergoing laparotomy for the purpose of hepatic artery ligation and portal vein infusion therapy with 5-FUra for hepatic metastases from colorectal adenocarcinoma. All patients studied were undergoing a local procedure or surgery under general anesthesia that had been planned for approved medical purposes. All patients gave fully informed consent for the extra tissue to be taken for the biochemical assays of this study. Entry criteria for all patients included prior histological documentation of carcinoma, a minimum Karnofsky performance score of 60, an ability to tolerate bolus i.v. 5-FUra, 500 mg/sq m, based on hematological indices and liver function tests, no evidence of active infection, and no prior treatment with 5-FUra. Additional criteria for the USC patients included anticipated therapy with single-agent 5-FUra, 500 mg/sq m i.v. daily for 5 consecutive days every 4 weeks and measurable disease parameters for evaluation of response. The clinical justification for "single-agent" 5-FUra for the treatment of patients with breast cancer was partly based on the observation that combination chemotherapy given sequentially is at least as effective as concurrent drug administration (7). Partial response was defined as a 50% reduction in the product of perpendicular diameters [*i.e.*, a halving of surface area (28)] or greater, for at least 3 months, and complete response as resolution of all clinical and radiological evidence of disease. Disease stabilization was defined as less than a 26% increase in average tumor diameter for at least 6 months. All patients were given 5-FUra, 500 mg/sq m, as an i.v. bolus prior to tissue biopsy. In the UG patients, biopsies were taken at either 20 to 30, 50 to 60, or 115 to 125 min following 5-FUra injection; the timing of the biopsies from the USC patients varied from 60 to 240 min. Portions of biopsies were sent routinely for histopathological evaluation. The study was performed between September 1980, and February 1983. Analysis of the biochemical data for tests of statistical significance was done once, at the close of the study.

RESULTS

Table 1 summarizes the results of biochemical parameters of TS inhibition in the 22 patients in whom biopsies of solid tumor were obtained, 20 to 240 min after 5-FUra administration, 500 mg/sq m. Tumor tissue was histologically confirmed in 21 patients, but in 1 USC patient only normal liver was obtained on biopsy. The biopsies averaged 406 \pm 199 (S.D.) mg (UG) and 701 \pm 519 mg (USC) in size. The results are categorized by institution, histology, hepatic metastasis *versus* normal liver, and FdUMP:dUMP ratio in tumors. The biochemical data are presented on a wet weight basis, for easier conversion to intracellular concentration (13), and because correction for DNA content

Table 1

TS inhibition in malignant tumors and normal liver of patients given i.v. 5-FUra

Patients undergoing local procedures or surgery under general anesthesia for either routine diagnostic or therapeutic reasons were given a test dose of 5-FUra (500 mg/sq m) by i.v. injection, and intraoperative tissue biopsies were obtained for biochemical analyses.

	Time to biopsy (min)	TS (pmol/g)			Nucleotides		
		TS _i	TS _{tot}	% inhibition	FdUMP (pmol/g)	dUMP (nmol/g)	(FdUMP/dUMP) × 100
Summary by institution							
UG (14) ^a	52 ± 33 ^b	1.71 ± 1.36	5.88 ± 2.91	69 ± 22	452 ± 774	20.9 ± 13.4	1.61 ± 2.58
USC (8) ^c	186 ± 61	0.73 ± 1.24	4.19 ± 4.48	80 ± 11	58 ± 51	7.9 ± 4.8	0.97 ± 0.92
Classification of results by histology							
Breast adenocarcinoma (2)	183 ± 39	0.11 ± 0.08	5.71 ± 7.41	94 ± 6	62 ± 39	10.3 ± 9.7	0.76 ± 0.34
Colonic adenocarcinoma (13)	87 ± 64	1.17 ± 1.40	3.67 ± 3.02	73 ± 21	347 ± 694	16.0 ± 11.3	1.59 ± 2.60
Other gastrointestinal tumors (6) ^d	120 ± 97	2.14 ± 1.40	5.51 ± 3.10	65 ± 13	77 ± 115	13.6 ± 8.4	0.70 ± 0.66
All solid tumors (21)	106 ± 78	1.21 ± 1.34	4.18 ± 3.44	74 ± 19	238 ± 559	14.6 ± 10.4	1.26 ± 2.09
Malignant ascites (2) ^e	180 ± 0	0.61 ± 0.71	0.94 ± 0.44	47 ± 50	17 ± 11	2.1 ± 1.9	0.44 ± 0.18
Normal liver (10)	71 ± 52	1.26 ± 1.33	2.23 ± 2.83	34 ± 22	35 ± 45	24.2 ± 12.6	0.131 ± 0.182
Normal liver versus hepatic metastasis in paired biopsy specimens from 9 patients (UG)							
Normal liver	59 ± 38	1.38 ± 1.37	2.46 ± 2.94	36 ± 23	38 ± 47	26.3 ± 11.5	0.18 ± 0.21
Hepatic metastasis	61 ± 38	0.84 ± 1.07	3.49 ± 3.01	80 ± 13	240 ± 455	17.6 ± 12.4	1.03 ± 1.48
<i>p</i> ^f		0.017	0.215	0.0026	0.205	0.009	0.104
FdUMP:dUMP ratio as a determinant of TS inhibition in malignant gastrointestinal tissues^g, (FdUMP/dUMP) × 100							
<0.40 (8) ^h	113 ± 81	1.97 ± 1.51	5.90 ± 2.92	72 ± 19	24 ± 17	14.5 ± 7.4	0.20 ± 0.13
>0.40 (10)	126 ± 75	0.40 ± 0.63	2.98 ± 3.74	82 ± 9	78 ± 65	12.0 ± 12.2	0.92 ± 0.79
<i>p</i> ⁱ		0.50	0.015	0.070	0.210	0.040	0.50

^a Numbers in parentheses, number of patients. Data represent results of single biopsies except in 3 of the UG patients, in whom results of repeat biopsy were averaged.

^b Average ± S.D.

^c Excludes patients with ascites.

^d These include 2 gastric adenocarcinomas, a gallbladder adenocarcinoma, a pancreatic adenocarcinoma, a hepatocellular carcinoma, and a leiomyosarcoma.

^e Included a metastatic gastric and a metastatic ovarian adenocarcinoma.

^f Based on paired *t* test, liver versus hepatic metastasis.

^g All adenocarcinomas (leiomyosarcoma not included).

^h Numbers in parentheses, number of patients.

ⁱ Difference between means, comparison of groups.

did not reduce scatter in the data (Charts 1 to 3, and see analysis of heterogeneity, below) some of which was presumed to be due to variable mucin content. DNA averaged 4.85 ± 1.86 mg/g and 4.05 ± 2.38 mg/g in the tumors of UG and USC patients; RNA values were 2.47 ± 1.24 and 2.46 ± 2.17 mg/g, respectively. The percentage inhibition of TS was slightly greater in tumors of the USC patients, reflected in a lower average TS_i. FdUMP levels were nonetheless lower in these tumors, a result that is explained on the basis of the time-to-biopsy interval after 5-FUra administration. Correlation of the time interval after 5-FUra versus percentage inhibition of TS, FdUMP, or dUMP level in gastrointestinal malignancies and normal liver led to results interpretable in terms of pharmacokinetic profiles of these biochemical parameters, shown in Charts 1 to 3.

Classification of results by histology (Table 1) results in the observation that TS inhibition was greatest in the breast tumors, on either an absolute basis of TS_i or percentage of total enzyme.

FdUMP Levels. Chart 1 shows a semilogarithmic plot of FdUMP concentrations in the tumor specimens as a function of time. The rate of decrease in FdUMP through 120 min is exponential and is qualitatively similar to results in a human colon adenocarcinoma xenograft (27), and parallels plasma pharmacokinetics of parent 5-FUra (1). Thus, in the gastrointestinal tumors, it appears that activation of 5-FUra to FdUMP is rapid, reaching peak levels in less than 30 min, and that while subsequent catabolic loss also is a rapid event, this may be slower than in murine tumors (29). Greater scatter in the data occurs for the time points beyond 120 min, however. Exponential curve fitting to the best equation, for all data, resulted in quadratic expressions for both normal liver and the gastrointestinal tumors.

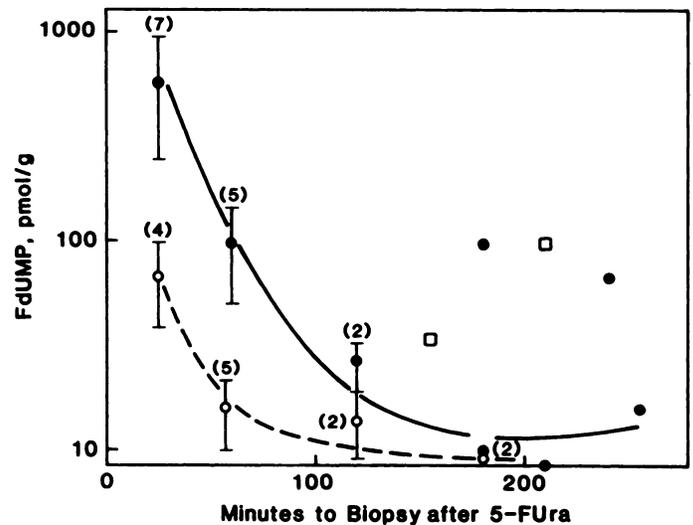


Chart 1. Levels of FdUMP in gastrointestinal-origin cancers (●), surgically normal liver (○), and breast adenocarcinoma (□) of patients given first-dose bolus i.v. 5-FUra, 500 mg/sq m, as a function of the time interval between drug administration and tissue biopsy. Points, mean ± S.D. (bars); the number of patients at each time interval is given in parentheses; points without parentheses, results of single patients. Average results were used in patients in whom multiple tumor biopsies were obtained.

On this basis, 50 and 26% of the statistical variation among FdUMP levels in normal liver and in gastrointestinal tumors, respectively, resulted from the effects of time alone. This analysis predicts a late, secondary rise in FdUMP, which we have observed in some animal tumors (29) and possibly reflects a similar

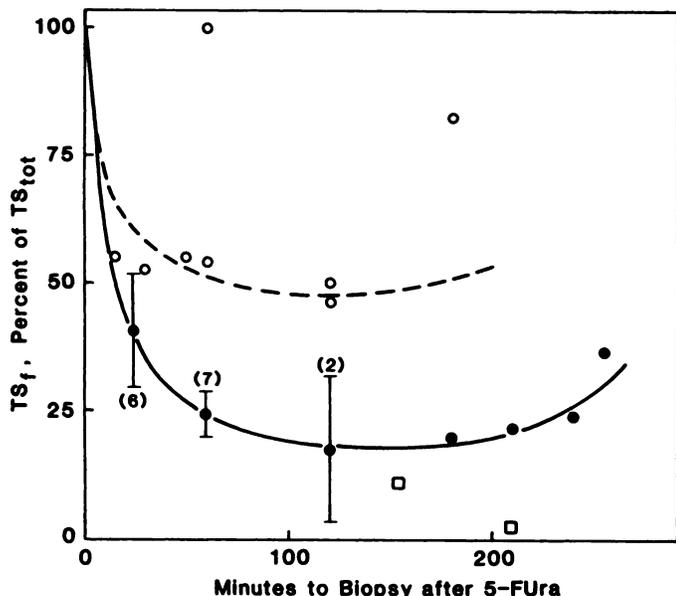


Chart 2. Levels of TS_i in human tissues as a function of time after 5-FUra administration. Symbols are the same as in Chart 1. Bars, S.D.

phenomenon seen occasionally in the plasma pharmacokinetics of 5-FUra (1). However, a more probable alternative explanation is that the timing of peak FdUMP formation may vary considerably with the tumor, as we have observed in human colon xenografts (27). Thus, the curvature of the solid line in Chart 1 is drawn to represent modal values at the later time points.

FdUMP levels at all time points were an order of magnitude greater in the gastrointestinal cancers than in the paired normal liver samples (see below). FdUMP levels in the 2 breast adenocarcinomas may have been somewhat high for their time intervals compared with results in the gastrointestinal tumors.

TS Inhibition. In Chart 2 are described the average time course changes in TS_i , as a percentage of TS_{tot} , present in the gastrointestinal tumors, breast cancers, and normal liver, following bolus i.v. 5-FUra. The assumption is made that TS_{tot} values represent pretreatment levels of active enzyme. In both normal liver and gastrointestinal tumors, three-fourths of average maximal TS inhibition occurred within the first 20 to 30 min following i.v. injection of the drug. This result is in agreement with the rapid inactivation of TS by FdUMP found in murine tumors *in vivo* (27, 29) or human enzymes *in vitro* (9, 10, 13). In both types of tissues, the percentage inhibition of TS apparently leveled off early and remained relatively constant through 120 min, although the TS inhibition averaged 30% greater in tumor than in liver. In agreement with the relatively high FdUMP levels, TS inhibition in the 2 breast cancers is seen to be greater than that found in the average gastrointestinal tumor.

dUMP Levels. Values of this nucleotide were consistent higher in the liver biopsies (Chart 3) despite the lower TS inhibition (and therefore less cause for increased dUMP levels) in normal liver than in tumors. A gradual decline in dUMP levels is evident in both normal and gastrointestinal tract tumors over the entire time course studied. The dashed lines represent hypothetical values at less than 20 min assuming base line dUMP values to be similar to those at the later time points. Preliminary studies in non-FUra-treated gastrointestinal tumors of 4 patients (data not shown) also suggested pretreatment dUMP values of 5 to 10 nmol/g.

Comparison of Surgically Normal Liver with Cancer Biopsies. Table 1 shows the average data for normal liver and paired hepatic tumors for the 9 patients in whom the essentially simultaneous biopsies were taken. The most striking difference between the 2 tissue types is in the percentage of TS inhibition, averaging in normal hepatic tissue less than half of the values in tumors. Although tumors had higher TS_{tot} levels, the greater TS inhibition in these tissues resulted in lower TS_i levels than in surgically normal liver. The highly significant difference in mean dUMP levels accounts in part for the difference in the degree of TS inhibition in the 2 tissues. Although FdUMP levels are not statistically significantly different between malignant and normal hepatic tissues, this is because of the wide variation in FdUMP levels in the tumors; the data of Chart 1 (differences between means) indicates that these differences are significant.

Results in Ascites. Levels in TS_{tot} , FdUMP, and dUMP were all low in the samples obtained by paracentesis (Table 1). Quantitation of the relative abundance of malignant cells and leukocytes was not done, but the microscopic impression was that the percentage of neoplastic cells was high. More patients were not added to the study because of the concern that free-floating malignant ascites cells may have a low growth fraction and instead accumulate by desquamation from peritoneal metastases (8), and because of the difficulties of pharmacokinetic interpretation.

Biochemical Variation due to Tissue Heterogeneity. In 3 of the UG patients, repeat biopsies of intraabdominal tumors were obtained, and in 2 of these, repeat samples of normal liver were also taken. The average sizes of the biopsies were 229 and 253 mg for tumor and liver, respectively. For each of the following parameters, the average S.D.s of the repeat biopsies in tumors were: FdUMP (7 pmol/g); dUMP (3.1 nmol/g); RNA (0.29 mg/g); and DNA (0.62 mg/g). The average S.D.s in the repeat liver biopsies were: FdUMP (12 pmol/g); dUMP (4.3 nmol/g), RNA (0.08 mg/g); and DNA (0.12 mg/g). For the nucleotides, these differences in repeat biopsies averaged 19% of the average values for all patients, and most of the small differences found on repeat biopsy could be attributed to the effects of time alone.

Correlation of Clinical Outcome with Parameters of TS Inhibition. The low response rate of gastrointestinal cancers to 5-FUra treatment necessitates *a priori* a larger group of patients than the present series. Of the 8 patients in the USC group who were followed prospectively and treated with single-agent 5-FUra, the only response occurred in a patient with breast ade-

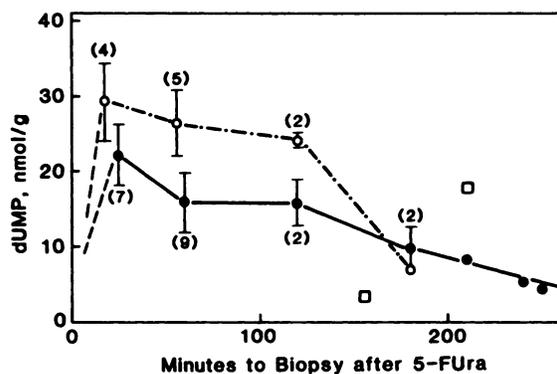


Chart 3. Levels of dUMP in patients given bolus i.v. 5-FUra as a function of the time-to-biopsy interval. Symbols are the same as in Charts 1 and 2. The earliest interval (---, 0 to 20 min) is assumed. Bars, S.D.

nocarcinoma. This patient showed complete resolution of a breast mass and regression of a biopsy-documented pulmonary metastasis within 3 months of 5-FUra treatment, with a response duration of at least 1 year; biochemical analysis of a biopsy of the primary tumor, at 210 min after 5-FUra, showed a low TS_{tot} (0.45 pmol/g) and nondetectable TS_i (≤ 0.05 pmol/g), and relatively high FdUMP and dUMP levels (Charts 2 and 3). The other patient with breast adenocarcinoma, with cervical and pulmonary metastases, was felt to show 6 months of disease stabilization as a result of 5-FUra. There were no responses observed among the patients with gastrointestinal origin tumor. One of these, however, a patient with hepatic metastases from colon adenocarcinoma, had a 9-month period of disease stabilization by 5-FUra, based on serial computerized tomography.

It is reasonable to assume that in the USC and UG patients in general, the average results of the biochemical parameters are more representative of nonresponding than of responding patients. Thus, a comparison of the biochemical results in tumors of the 3 patients who had some benefit from 5-FUra treatment with results in the remaining patients is of interest. Table 2 shows that these 3 patients had a relatively high percentage of TS inhibition and strikingly low absolute levels of TS_i . Both variables are highly significantly different from the results in all remaining patients, and percentage of TS inhibition is significantly greater than in the 5 remaining USC patients as well. Based on the time-to-biopsy interval, FdUMP levels in this group may also be seen to be relatively high. However, categorization of the biochemical parameters by histology (Table 1) also points up the difference between the results in the 2 patients with breast adenocarcinoma and those in remaining patients.

DISCUSSION

This study provides evidence that intracellular pharmacological events that result from antimetabolite chemotherapy may be directly and meaningfully determined in human tumors in the clinical setting. We have demonstrated the feasibility of obtaining portions of tumors at short time intervals following 5-FUra administration, added to the routine course of surgical management. The biochemical data that result are consistent with established pharmacokinetics of 5-FUra in plasma, and with known enzymic kinetics of TS inhibition by FdUMP. The findings that the biochemical data for gastrointestinal tumors and for biopsies of normal liver, as a group, show pharmacokinetic profiles gives assurance against 2 major concerns, that multiple serial biopsies

would be a minimal requirement for obtaining useful information on TS inhibition and that tumor heterogeneity would be prohibitive. TS inhibition clearly is rapid *in vitro* (9, 10, 13), in murine tumors *in vivo* (27, 29), and this is now demonstrated clinically. Once inhibited, regardless of changing metabolic conditions, TS bound as ternary complex can dissociate back to the nonbound, free state only at relatively slow rates (9, 13). This permits some flexibility in the intraoperative timing of the biopsy for determination of TS_i . The relative lack of metabolic heterogeneity, specifically shown by repeat biopsy in 3 patients, suggests that biopsies less than 200 mg may be adequate.

Our results in broad outline are consistent with findings in murine models *in vivo* (27, 29). In particular, the apparent time course of FdUMP levels (Chart 1) resembles the kinetics of FdUMP formation and loss found in human colon adenocarcinoma xenografts studied by serial biopsy (27). Levels of dUMP in gastrointestinal cancers of the present study may be somewhat higher than we found in murine tumors using the same methodologies (27, 28) but show little evidence of significant change with time and are relatively low compared to results obtained using less specific assay methodologies (20).

It is likely that average levels of TS_i after 5-FUra treatment in these tumors were not low enough to be cytotoxic. Reduction of TS_i to nearly undetectable levels (0.05 pmol/g) may be necessary to achieve the cytotoxic effect of "unbalanced growth." This is apparent in our murine studies (27, 29), where relatively fast proliferation kinetics occur, and therefore should be true of slowly growing human tumors in which the demand for DNA precursor synthesis must be relatively less.

The percentage inhibition of TS found in our samples is a function of the ratio of nucleotides, FdUMP:dUMP. The degree of initial TS inhibition is kinetically directly dependent on the initial FdUMP:dUMP ratio at dUMP levels above 4 μM for the human enzyme (13); the stability of ternary complex(s) formed depends greatly on the concentration of reduced folates such as CH_2FH_4 (9, 13). Low levels of reduced folates allow greater interference by dUMP in decreasing ternary complex formation with FdUMP (13); i.e., high dUMP levels and low CH_2FH_4 levels are synergistic in preventing TS inhibition by FdUMP. Inadequate concentrations of reduced folates may have been the basis for 5-FUra resistance in 3 murine adenocarcinomas studied by our methods (29), and this has been demonstrated convincingly in several tumor models *in vitro* (11, 14, 15, 30).

Evidence for the role of the FdUMP:dUMP ratio in influencing the percentage inhibition of TS in human tissues is given in Table

Table 2
Solid tumor patients benefiting from 5-FUra therapy versus remaining patients

There were 2 patients who showed disease stabilization and 1 patient who had a complete response to intermittent bolus 5-FUra treatment among 8 USC patients in whom the effects of 5-FUra on TS inhibition in solid tumor biopsies were studied and followed prospectively for response. Comparison is made of the results of biochemical analyses of tumor biopsies of these patients with those of remaining USC patients, and with the results of all remaining (USC plus UG) patients.

	Time to biopsy (min)	TS (pmol/g)			Nucleotides		
		TS_i	TS_{tot}	% inhibition	FdUMP (pmol/g)	dUMP (nmol/g)	(FdUMP/dUMP) \times 100
Complete response (1) ^a + apparent disease stabilization (2)	182 \pm 28 ^b	0.10 \pm 0.06	3.94 \pm 6.07	89 \pm 9	74 \pm 35	11.1 \pm 7.0	0.768 \pm 0.243
Remaining USC patients (5)	189 \pm 78	1.11 \pm 1.48	4.37 \pm 3.94	74 \pm 7	48 \pm 61	5.9 \pm 1.7	1.095 \pm 1.180
Remaining USC + UG patients (19)	102 \pm 75	1.39 \pm 1.47	4.36 \pm 3.27	70 \pm 20	226 \pm 499	15.9 \pm 13.4	1.006 \pm 1.384
		0.001	0.500	0.005	0.280	0.450	0.500

^a Numbers in parentheses, number of patients.

^b Average \pm S.D.

^c Based on differences between means; comparison with patients benefiting from 5-FUra therapy.

1. A direct comparison of results in hepatic metastases with paired specimens from surgically normal liver shows significantly greater inhibition in tumor, resulting in lower absolute levels of TS_t . Although dUMP levels may be expected to rise in response to effective TS inhibition (19, 20), the malignant tissues nonetheless showed significantly lower levels of dUMP than did normal liver. FdUMP levels and FdUMP:dUMP ratios were also consistently higher in tumors (Charts 1 and 3). Thus, the difference in TS inhibition between normal liver and hepatic cancers (Chart 2) is partly a result of the differences in their FdUMP:dUMP ratios. Table 1 also shows a comparison of average TS and nucleotide levels in all gastrointestinal cancers according to high or low FdUMP:dUMP ratios. In this instance, the main determinant of the ratio appears to be the FdUMP level. Tumors with high FdUMP:dUMP values had significantly lower TS_t levels.

An attempt was made in the present work to study levels of CH_2FH_4 by determination of [3H]FdUMP-binding sites in cytosols in the absence of added exogenous folate, analogous to the method of Houghton *et al.* (14). In some instances, however, TS_t^{pp} was greater in the absence of added folate, which suggested that increased labeling of endogenous TS_b enzyme had occurred, and in most cases TS_{tot} was lower because of the loss of endogenous reduced folates during the preincubation period. Also, 5-FUra treatment logically should alter the concentration of reduced folates. Thus, this approach may not be applicable to 5-FUra-treated tissues. In the latter, an additional restriction would be the requirement for k_s determination in order to make appropriate correction of [3H]FdUMP exchange labeling into TS_b enzyme in the absence of added excess CH_2FH_4 in the assay.

TS inhibition in tumors of the 3 patients who showed some benefit from 5-FUra therapy was significantly greater than in tumors of the remaining patients (Table 2). Since this included the 2 patients with breast adenocarcinoma, this result may reflect variation in TS inhibition with histology (Table 1). As a result, TS_t was very low in the cancers of these 3 patients, only 0.10 ± 0.06 pmol/g of tissue. In the one patient who was a complete responder to 5-FUra therapy, TS_{tot} also was low, in keeping with Washtien's postulate (31) that low TS levels in a tumor predict for 5-FUra sensitivity. This is reasonable, since FdUMP formation is generally in excess of TS binding sites, so that the degree of TS inhibition depends on the FdUMP:dUMP ratio. At given ratios, a constant percentage inhibition of TS will occur, regardless of TS_{tot} level (13); *i.e.*, all other factors being equal, tumors with higher TS_{tot} levels will have higher TS_t levels after FdUMP exposure. Moreover, these tumors will have higher levels of FdUMP-bound TS_b enzyme; the rate of dissociation of TS_b (ternary complex enzyme) back to the active TS_t state is a first-order process dependent only on the TS_b level (9, 10, 13). As a result, tumors with higher TS_b levels will show quicker reappearance of active TS_t enzyme. However, it has also been argued that colon tumors with higher TS levels may be more vulnerable to inhibition by 5-FUra treatment because of postulated greater metabolic demand and greater FdUMP formation (32). Our present data argue against this possibility, since tumors with high FdUMP:dUMP ratios actually had lower average TS_{tot} levels (Table 1). Should low TS levels correlate with 5-FUra sensitivity, this would provide an attractive explanation of the observation that patients whose colonic carcinomas respond to 5-FUra have an intrinsically longer natural history (17), or slower untreated growth, of their cancers.

The interest in having this kind of information in human tumors

is enormous, considering the extensive and active literature on plasma pharmacokinetics of 5-FUra. The site of dose-limiting toxicity of 5-FUra, bone marrow or gastrointestinal mucosa, varies with its schedule of administration; application of our methods to study of TS inhibition in these organs could clarify this phenomenon. The mechanism of action of 5-FUra, either TS inhibition, or incorporation of drug into RNA with consequent changes in RNA structure and function, or incorporation into DNA (13), or all of these, also may vary among different normal and neoplastic tissues with the schedule of administration of 5-FUra, or according to systemic biochemical factors such as the levels of deoxynucleosides and folates in the blood. Application of our methods could help to distinguish among the complex possibilities. Adaptation of our methods to the study of 5-FUra-containing drug combinations is also a logical extension of the present work. Folinic acid may improve the single-agent activity of 5-FUra (18) and logically should effect this action by increased TS inhibition, although other kinds of reduced folates should be more efficient (12, 15, 23, 25). Combination of 5-FUra with high-dose methotrexate plus folinic acid rescue is a clinically promising regimen (4, 22, 24) that also may be acting by an improved mechanism of TS inhibition. Study of tumor specimens, using our methods, in patients receiving such therapies could address these questions and point to ways by which more selective and profound TS inhibition in tumors may be obtained.

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