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Cancer Res 1988;48:5894-5900.

[CANCER RESEARCH 48, 5894–5900, October 15, 1988]

Mechanisms of Innate Resistance to Thymidylate Synthase Inhibition after 5-Fluorouracil[1]

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ABSTRACT

Fifty-four patients with metastatic adenocarcinoma received i.v. bolus 5-fluorouracil, 500 mg/m², prior to surgical biopsy of tumor at 20–400 min, for analysis of biochemical parameters of resistance to thymidylate synthase (TS) inhibition. The majority of patients, 37, had colon or rectal adenocarcinoma, five had breast cancer, five had gastric primary disease, four had pancreatic adenocarcinoma, and three had hepatocellular adenocarcinoma. Fluorodeoxyuridylate (FdUMP) was assayed by isotope dilution of [³H]FdUMP binding to bacterial TS; free and total TS was determined by [³H]FdUMP binding; and deoxyuridylate (dUMP) was assayed by conversion to [¹⁴C]thymidylate. Free levels of TS were lower in breast cancers, 0.08 ± 0.06 pmol/g, than in other histologies (overall average, 1.41 ± 2.25), associated with significantly greater percentages of TS inhibition (88.6% *versus* 62.0% overall). Colorectal tumors showed significantly greater FdUMP levels than other gastrointestinal malignancies, associated with somewhat lower free TS values. Plots of FdUMP levels, or (FdUMP/dUMP) × 100 values *versus* percentages of TS inhibition suggested minima of 75 pmol/g and 0.10, respectively, for achieving maximal enzyme inhibition. Analyses of normal tissues showed: poor TS inhibition in liver and normal colonic mucosa, related to low FdUMP levels; and very high dUMP levels in bone marrow leukocytes suggestive of reactive increases in dUMP as an important mechanism of recovery in this tissue. Among the 30 of the 37 colorectal tumors that showed suboptimal (less than 85%) inhibition of TS, 16 (53%) showed FdUMP levels less than 75 pmol/g, 8 (27%) showed relatively high dUMP levels (over 35 nmol/g), and 16 (53%) showed poor efficiency of inhibition of TS, with the major overlap between these mechanisms of resistance being high dUMP and poor binding in 6 (20%). These data provide a strong rationale for administration of leucovorin to the majority of patients receiving 5-fluorouracil, since increased intratumoral reduced folates potentially can overcome multiple mechanisms of resistance including low FdUMP, high dUMP, and high total TS levels, in addition to that caused by isolated folate deficiency.

INTRODUCTION

Leucovorin addition to fluoropyrimidine therapy of patients with gastrointestinal and breast cancer has shown improved objective response rates in a number of studies (1–7). The moderate gains of leucovorin modulation have created enormous clinical and scientific interest, in part because the specific biochemical rationale of increased FdUMP[3]-mediated TS inhibition by expanded CH_2FH_4 pools offers a specific objective of treatment, one that is associated with a variety of exploitable variables.

We have developed and applied methods for assay of several key biochemical determinants of TS inhibition following fluoropyrimidine therapy *in vivo*. TS inhibition in blood leukocytes of patients receiving 5-FUra had been previously shown in 1967 by Roberts, Kessel, and Hall (8). Incorporation of tritiated deoxyuridine into DNA was also used by Eaglestein *et al.* (9) to show that TS inhibition in actinic keratases after systemic or topical 5-FUra was greater than in normal skin.

In a series of well-characterized murine adenocarcinomas, we found that complete TS inhibition occurred only in the 5-FUra-sensitive tumor 38 after i.p. 5-FUra (10). We suggested the basis for this difference was probably variation in the reduced folate cofactor pools. Tumor 38 also was unique in showing an acute drop in dUMP, and a late drop in total TS. Conversely, total TS can increase rapidly in resistant tumors after bolus 5-FUra exposure (10–12). Similar observations of TS inhibition were found in paired 5-FUra-sensitive and -resistant human colon xenografts serially biopsied after i.p. 5-FUra (13); in the resistant tumor, a relationship was observed between dose of 5-FUra, FdUMP formation, and TS inhibition. A series of colon carcinoma xenograft studies have shown significant variations in cytosolic folates, assayed by binding of exogenous FdUMP to endogenous TS, as a basis for variation in 5-FUra sensitivities (14, 15).

In patients receiving i.v. 5-FUra, 500 mg/m², we reported (16) highly variable FdUMP levels in tumors accompanied by profound but often incomplete TS inhibition. A breast cancer patient who was a complete responder to subsequent single-agent 5-FUra therapy showed very low intratumoral total TS, absent free TS, and high FdUMP levels. Prolonged recovery of intratumoral TS activity after leucovorin plus 5-FUra administration has also been suggested to correlate with clinical response in preliminary reports by the group at Roswell Park (17, 18). In addition, Allegra *et al.* (19) have recent evidence that the degree of TS inhibition in breast cancer biopsies at 24 h after FUra is improved by leucovorin, and shows significant correlations with objective response.

The present report expands on our earlier experience in performing pharmacodynamic analyses of the TS mechanism of cytotoxicity in single, post-drug surgical tumor biopsies of patients receiving test-dose i.v. bolus 5-FUra. The majority of patients had colorectal carcinoma, and most of these had hepatic metastases as the site of biopsy. The principal goal of these studies was to demonstrate that individual mechanisms of resistance of tumors to 5-FUra could be identified that potentially can be overcome by specific biochemical strategies.

MATERIALS AND METHODS

Patients. Fifty-four patients received i.v. 5-FUra, 500 mg/m² given as a 1–2-min bolus, prior to surgical biopsy of tumor as a part of these studes. Only post-5-FUra tissue specimens were studied. All subjects subsequent to our initial series (16) were patients at the University of Göteborg undergoing surgery, usually laporatomy, for routine medical purposes, such as hepatic infusion therapy. The primary site of origin of tumor was large bowel or rectum in 37, stomach in five, pancreas in four, and liver in three patients. In addition, five patients with metastatic breast cancer were studied. Biopsy sites were metastatic hepatic nodules

Received 1/20/88; revised 6/1/88; accepted 7/20/88.

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[1] This work was supported by NIH Grant CA39629 awarded to C. P. S., and by funding from the Swedish Research Society to B. G. G.

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[3] The abbreviations used are: FdUMP, 5-fluorodeoxyuridylate; 5-FUra, 5-fluorouracil; TS, thymidylate synthase; dUMP, 2′-deoxyuridylate; CH_2FH_4, l-5,10-methylenetetrahydrofolate; F-RNA, RNA containing incorporated 5-FUra; FdUrd, 5-fluorodeoxyuridine.

in 37, 27 of which were colorectal carcinoma, primary tumor in 14, and inguinal lymph nodes in two patients. There were 30 men and 24 women, median age, 61 years (range, 38–74).

Tissue Handling. Specimens, pared of fat and necrotic tissue, were frozen within 30 s of surgical removal in liquid N_2 or dry ice, stored below −80°C and shipped within 4 weeks in dry ice to Los Angeles for analysis. Tumors were biopsied by an incisional approach. Average biopsy size was 497 ± 297 mg. Homogenization was done by Polytron or ground-glass grinding followed by sonication. Nucleotides were extracted with acetic acid (22), but assays of 4000 × *g* supernatants of heat-treated (65°C for 20 min) sonicates have recently shown equal results without the potential interference from residual material after lyophilization. The nucleotide extracts were diluted with water to conductivities less than 1 mmho prior to DEAE-cellulose column chromatography. Complete separation of FdUMP from dUMP is necessary for accurate assay of these nucleotides, and FdUMP determination is needed for improved accuracy in the TS assays. Bone marrow leukocytes were obtained in five patients during laparotomy by anterior iliac crest aspiration of 5–10 cc material into a heparinized syringe, and 2000 × *g* centrifugation separation of buffy coat for analysis, with results reported on the basis of DNA content.

Assays. Materials for the assays were obtained as described (10, 22); commercial, racemic *d,l*-tetrahydrofolate (Sigma) was occasionally substituted for the enzymatically prepared *l*-form (22), with equivalent results. [6-^{3}H]FdUMP stocks (18–20 Ci/mmol, Moravek) were purified of radiolysis breakdown products at least monthly by DEAE-cellulose chromatography, and newly synthesized replacement stocks were obtained every 6–8 months. A detailed summary of methods for FUra/leucovorin pharmacodynamic analyses appears in Ref. 24.

Free and total (FdUMP-bound plus free) TS levels were assayed on the day of tissue processing by determination of [^{3}H]FdUMP binding sites by the dextran-coated charcoal method that corrects for [^{3}H]-FdUMP labeling of endogenous cytosolic FdUMP-bound enzyme (10, 24). Use of 5 pmol (200,000 dpm) of newly synthesized/purified [^{3}H]FdUMP in the TS assay gave backgrounds (no enzyme) of approximately 250 dpm, with an experimental SD of less than 30 dpm. Heat-treated cytosols (65°C for 20 min) gave backgrounds the same as cytosol-free blanks. Use of the 3-h preincubation prior to [^{3}H]FdUMP labeling, for total TS assay, did not increase backgrounds. Such low backgrounds and high precision of repeatability were occasionally necessary for accurate measurement of levels of apparent free TS when the percentage of enzyme inhibition was high and total enzyme low. Average dpm for total and apparent free TS levels were approximately 1500 and 300, respectively. Freshly dialyzed *Lactobacillus casei* TS as a standard was assayed at pH 7.4 because of the instability of bacterial enzyme at pH 8.0. Use of neutral charcoal pretreatment of cytosols to remove interfering nucleotides (12, 25) did not improve results, possibly because of damage to TS (15). Correction for isotope dilution in the TS assays (apparent free and total enzyme) by endogenous cytosolic FdUMP was made prior to the correction for [^{3}H]FdUMP labeling of cytosolic TS-FdUMP-CH_2FH_4 ternary complex. It should be noted that this correction factor, 13% labeling of TS_b enzyme at pH 8 and 20 min at 30°C, reduces to about 5% for pH 7.4 and 10-min conditions. However, pH 8 assays were used for free TS because of the need for total TS determinations for post-FUra specimens, in the absence of pretreatment biopsy data. The assays for tissue FdUMP and dUMP levels were done by the [^{3}H]FdUMP isotope dilution method and the [^{14}C]dTMP synthesis procedure, respectively (22). The weight equivalents of parent tissue present in each assay tube, done in replicates, averaged approximately 40 mg and 2 mg for the TS and nucleotide assays, respectively.

Statistics. Data were stored and analyzed on a VAXS 11/750 computer with an RS1 statistical program, and a VAXS 11/780 computer with BMDP statistics.

RESULTS

Although the sole biopsy site of 12 of the patients with gastrointestinal cancer was primary site disease, average values of all parameters studied showed no significant differences from those of hepatic metastases. For example, free TS was 1.68 ± 1.21 pmol/g in primary tumors *versus* 1.32 ± 2.40 pmol/g for the hepatic metastases. The results in primary and metastatic disease were therefore pooled, for comparison of histologies.

Resistance to the TS effect of 5-FUra was analyzed in four principal ways: (*A*) comparison among different histological tumor types, (*B*) comparison of gastrointestinal malignancies with sensitive and nonsensitive normal tissues, (*C*) kinetic analysis of variations in the results of malignant tumors and normal hepatic tissues as a whole, and (*D*) classification of mechanisms of resistance to TS inhibition in the colorectal specimens.

Resistance to TS Inhibition Based on Tumor Histology. Table 1 shows the averaged values for all specimens obtained from patients who received i.v. bolus 5-FUra test dose, 500 mg/m^2, as a first exposure to this agent, prior to intraoperative surgical biopsy. The classification is based on histology, grouped according to decreasing values of free TS, *i.e.* non-drug-inhibited enzyme.

The descending order of levels of free TS in Table 1 is essentially the same order as that for decreasing response rates in the literature to single-agent i.v. 5-FUra: breast < colon < gastric < pancreatic and liver cancer. Since total enzyme (FdUMP-bound enzyme plus free TS) showed no statistically significant differences between histological types, this sequence of free TS values was largely due to differences in percentages of TS inhibition. Statistical comparisons indicate that breast cancer specimens showed significantly greater percentages of TS inhibition than did colorectal cancers, while pancreatic cancer biopsies showed significantly lower percentages of TS inhibition.

FdUMP levels showed a general relationship to percentages of TS inhibition. Although colorectal cancers had somewhat higher overall average FdUMP levels than other histologies, Fig. 1 shows that breast cancers also had relatively high levels of FdUMP for their given time intervals. Inclusion of a number of colorectal patients biopsied at early time intervals partly accounts for the relatively high average FdUMP levels, FdUMP/dUMP ratios, and wide SDs of colorectal specimens as a whole. Gastric, pancreatic, and hepatic adenocarcinomas had significantly lower FdUMP levels than colorectal origin tumors (Table 1 and Fig. 1). Normalization of the data according to time, by taking values as percentages of geometric means for given times, did not improve discrimination between groups.

TS Inhibition in Normal Tissues. Normal hepatic tissue and colonic mucosa are naturally highly resistant to toxic effects of i.v. bolus 5-FUra. The low percentages of inhibition of TS, low FdUMP levels, and very low FdUMP/dUMP ratios in these tissues (Table 1) are consistent with the postulate that TS inhibition is an important correlate of 5-FUra cytotoxicity. Comparison of surgically normal liver with hepatic tumor nodules, obtained as nearly simultaneously biopsied, paired samples from 15 patients, is shown in Table 2. As with our earlier analysis in the first nine patients of this series (16), TS inhibition was highly significantly greater in malignant tissue than in liver, by either the paired *t* test or differences between means. Average FdUMP was 4-fold higher in tumor than liver, but because of the large SD, the *P* value was only 0.10. Levels of dUMP now show no significant difference, in contrast to the slightly higher values in liver previously observed. The findings in bone marrow leukocytes from five patients (Table 1) were notable. Assuming at least 4 mg DNA/g of marrow, the Table 1 values can be multiplied by about 4 for comparison with other tissues. FdUMP levels were thus relatively high, and dUMP

Table 1 *Pharmacodynamics of 5-fluorouracil: summary table*

Parameters of TS inhibition in single post-5-FUra biopsies of adenocarcinomas and normal tissues of patients given bolus i.v. 5-FUra, 500 mg/m². Results by histology: values ± SD.

		TS (pmol/g)			Nucleotides		
	Time (min)	Free	Total	% inhibition[a]	FdUMP (pmol/g)	dUMP (nmol/g)	FdUMP/dUMP[b] ratio × 100
Malignant tissues							
Breast (5)[c]	115 ± 68[d]	0.079 ± 0.062[e]	3.26 ± 4.51	88.6 ± 17.6[f]	85 ± 88	18.6 ± 10.3	0.620 ± 0.542
Colorectal (37)	102 ± 161	1.32 ± 2.40	4.22 ± 5.85	66.9 ± 27.9	400 ± 701	28.0 ± 21.0	1.32 ± 2.00
Gastric (5)	120 ± 73	2.18 ± 1.16	6.80 ± 3.80	62.5 ± 19.9	50 ± 57[e]	15.9 ± 8.1[e]	0.366 ± 0.605[f]
Pancreas (4)	43 ± 21	2.85 ± 2.10	4.68 ± 3.08	36.8 ± 15.0[e]	37 ± 46[e]	24.1 ± 9.6	0.291 ± 0.487[e]
Hepatoma (3)	128 ± 156	2.87 ± 3.44	5.88 ± 5.54	62.0 ± 22.6	24 ± 11[e]	19.1 ± 18.6	0.287 ± 0.259[e]
All Malignant Tissues (54)	100 ± 142	1.41 ± 2.25	4.44 ± 5.35	66.0 ± 27.3	299 ± 601	26.1 ± 20.2	1.07 ± 1.73
Normal tissues							
Normal Liver (19)	102 ± 105	1.98 ± 1.81	3.56 ± 3.63	36.1 ± 22.0	58 ± 107	27.2 ± 13.9	0.201 ± 0.398
Normal Mucosa (3)	363 ± 569	2.23 ± 0.91	3.59 ± 1.56	37 ± 17	13 ± 14	27.8 ± 20.0	0.050 ± 0.053
Bone Marrow (5)	78 ± 40	0.88 ± 0.54[g]	2.65 ± 1.39[g]	64.8 ± 17.2	123 ± 126[g]	246.5 ± 209[g]	0.060 ± 0.060

[a] Percentage inhibition of TS = [(total TS − free TS)/total TS] × 100. Values corrected for isotope dilution by cytosolic FdUMP and for 13% ternary complex dissociation during 20-min [³H]FdUMP labeling.
[b] Gives FdUMP as a percentage of dUMP molecules present.
[c] Numbers in parentheses, number of patients.
[d] Average values, ± SD.
[e] Difference from colorectal group, $P < 0.01$, based on difference between means.
[f] Difference from colorectal group, $P < 0.05$, based on difference between means.
[g] Units/mg DNA. To obtain approximate units for wet weight comparison, multiply by 4 mg DNA/g, a conservative estimate (10, 16).

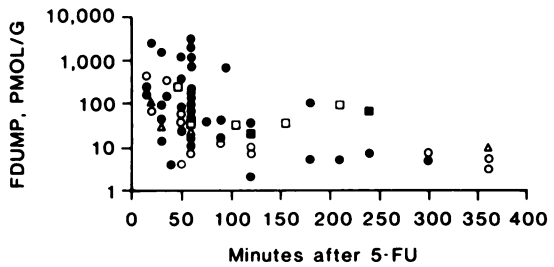

Fig. 1. Semilog plot of tissue levels of FdUMP, pmol/g wet weight, present in post-5-FUra biopsies, as a function of time between i.v. 5-FUra, 500 mg/m², and time of operative biopsy, for colorectal (●), gastric (■), pancreatic (▲), hepatocellular (△), and breast (□) carcinomas, and normal liver (○).

levels were enormous. Since such high dUMP levels would be expected to prevent the moderate degree of TS inhibition found, these possibly reflect reactive elevations in response to TS inhibition.

Kinetic Evaluation of TS Inhibition. The relative value of a given level of FdUMP, free TS, and dUMP may be interpreted according to the time courses of these parameters in group-averaged data (Figs. 1–3). Peak changes in all three parameters appear to have occurred by 60 min in gastrointestinal tumors, but may have occurred earlier in normal liver. As noted, events in bone marrow leukocytes may be faster still. The similar occurrence of maximal TS inhibition and peak FdUMP formation is compatible with the known rapid on-rates for TS-FdUMP-CH_2FH_4 ternary complex formation (26, 27).

The time course data were useful for establishing a time frame, 20–400 min, for analyzing the relationship between FdUMP and dUMP nucleotide levels and the degree of TS inhibition, Fig. 4, *A* and *B*. Both FdUMP levels and FdUMP/dUMP ratios during this time period showed determining effects on TS inhibition, consonant with *in vitro* kinetics (26–28). The finding of slightly greater TS inhibition for most samples than would be expected on an *in vitro* basis, however, is probably because the majority of FdUMP/dUMP ratios represent post-peak values (average time after 5-FUra, 100 ± 142 min for malignant tissues, Table 1). The slight improvement in clustering of values, Fig. 4*B*, by use of the dUMP data is expected on a kinetic basis, although the effect is small because of the relatively narrow range of dUMP levels found in most tissues.

The data of Fig. 4 suggest that, at an average of 100 min after 5-FUra, attainment of maximal TS inhibition requires the presence of at least 75 pmol/g of FdUMP. However, very high levels of FdUMP and more favorable FdUMP/dUMP ratios did not regularly achieve maximal inhibition of TS. For example, tumors of the seven colorectal carcinoma patients with FdUMP levels greater than 800 pmol/g averaged only 72 ± 14 percent inhibition of TS. By contrast, TS inhibition was excellent in four of the five breast cancer specimens, as noted above.

Mechanisms of Resistance to TS Inhibition in Colorectal Carcinomas. Among the 37 colorectal tumor biopsies, 30 showed less than 85% inhibition of TS. Fig. 5 shows the distribution of biochemical mechanisms of resistance to TS inhibition determined in the tumors of these 30 patients. A cut-off point for a low FdUMP level, 75 pmol/g, was obtained from Fig. 4. A high value of 35 nmol/g of dUMP was chosen more arbitrarily, but would appear to be a lower limit for reactive increases (Fig. 3). The result of this analysis shows that 16 (53%) of the 30

Table 2 *Thymidylate synthase inhibition in paired biopsies of normal liver versus hepatic metastasis in 15 patients given bolus i.v. 5-FUra, 500 mg/m²*

		TS (pmol/g)			Nucleotides		
	Time (min)	Free	Total	% inhibition	FdUMP (pmol/g)	dUMP (nmol/g)	FdUMP/dUMP × 100
Normal liver	79 ± 85[a]	1.32 ± 1.20	2.19 ± 2.32	37 ± 22	67 ± 110	27.2 ± 11.8	0.276 ± 0.469
Hepatic mets	81 ± 84	1.20 ± 1.45	3.65 ± 3.19	69 ± 27	296 ± 594	25.5 ± 22.4	0.863 ± 1.246
P value[b]		0.50	0.10	0.01	0.10	0.50	0.10

[a] Average values, ± SD.
[b] Paired *t* test.

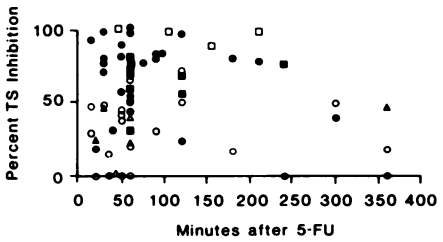

Fig. 2. Time of biopsy *versus* percentage inhibition of TS in single, post-5-FUra biopsies, based on a [6-^{3}H]FdUMP ligand binding method for total (free plus FdUMP-bound) enzyme that uses a preassay dissociation of cytosolic ternary complexes (10, 24). Symbols are the same as in Fig. 1.

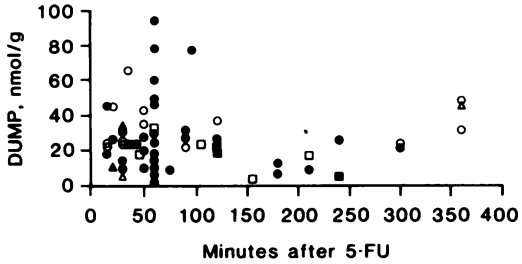

Fig. 3. Tissue levels of dUMP *versus* time of biopsy in single, post-5-FUra biopsies in patients receiving bolus i.v. 5-FUra, 500 mg/m^2. Symbols are the same as in Fig. 1.

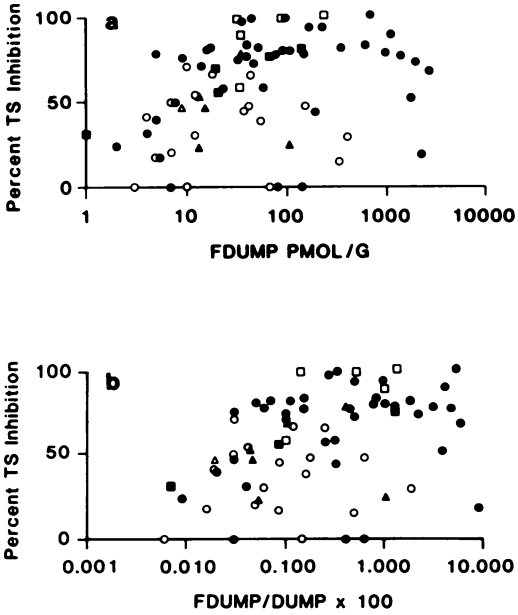

Fig. 4. Relationship between percentage inhibition of TS in single, post-5-FUra biopsies, 20–400 min after 5-FUra, as a function of tissue FdUMP level (*A*) or as a function of (FdUMP/dUMP) × 100 (*B*). Symbols are the same as for Fig. 1.

specimens with poor TS inhibition had low FdUMP levels, with 12 (40%) of these showing low FdUMP levels as a sole mechanism of resistance. In contrast, the eight tumors (27%) with high dUMP values all showed apparent overlap mechanisms: two (7%) also had low FdUMP levels, and the other six all had favorable ratios (0.1 or greater) of FdUMP/dUMP × 100 suggestive of some other factor(s) limiting TS inhibition such as CH_2FH_4 deficiency. The latter mechanism appeared to be an isolated factor in 8 (27%) patients, and overall was a factor in 16 (54%) of the entire group of 30 tumors that showed less than 85% inhibition.

Analyzed on the basis of absolute values, levels of free TS greater than 0.5 pmol/g were associated in some cases with

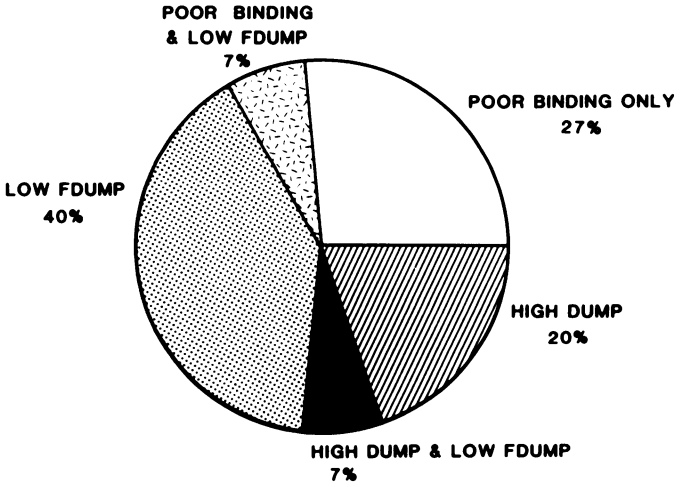

Fig. 5. Distribution of mechanisms of resistance to TS inhibition among 30 of the 37 colorectal carcinoma biopsies showing less than 85% TS inhibition, 20–400 min after i.v. bolus 5-FUra, 500 mg/m^2. The high dUMP group also showed poor binding. See text for discussion.

high levels of total enzyme. Colorectal tumors with free TS less than 0.5 pmol/g averaged significantly lower total TS (2.01 ± 0.13 pmol/g; $N = 19$) levels than tumors with free TS higher than 0.5 pmol/g (total TS = 5.73 ± 7.32; $N = 18$; $P = 0.05$). The adverse effect of higher total TS levels, however, in the group with free TS < 0.5 pmol/g was also due to poorer percentage inhibition of TS (51 ± 31 *versus* 81 ± 13% TS inhibition; $P = 0.0001$). Logically, high total TS levels may be expected to be accompanied by lower CH_2FH_4 levels. In support of this, a possible reciprocal relationship was found between total TS and levels of dUMP in the colorectal carcinomas, at 20–400 min (data not shown). Thus, colon tumors with low total TS levels tended to show high dUMP levels and *vice versa*. This apparent relationship did not obtain when all malignant tissues were included, however.

DISCUSSION

As the enzyme receptor for mediating the cytotoxic effects of 5-FUra, TS is an attractive target for analysis in tumors of patients in whom 5-FUra therapy is considered. Intraoperative 5-FUra administration has been used in many adjuvant trials and adds no apparent postoperative morbidity (29, 30). Surgical biopsies of tumor greater than 100 mg have shown surprisingly little heterogeneity between specimens in parameters related to TS inhibition (16, 31), an experience similar to that of hormone receptor analysis in primary breast cancer which uses similar assay methodology.[4] Levels of total TS in human tissues (Table 1) are also of the same approximate concentrations as estrogen receptors in hormone-positive breast cancers (assuming 50–150 mg protein/g wet weight). A major conceptual difference of the present study, of course is that the parameters of 5-FUra drug action show fairly rapid kinetics of change. The kinetics of grouped data obtained from single post-5-FUra biopsies of the patients in this report, Figs. 1–4, provides reference values for future interpretation and studies of more complete kinetics in the individual case.

A central goal of the present study was to show that intratumoral mechanisms of resistance to TS inhibition by fluoropyrimidine therapy can be identified in the clinical setting. Such

[4] C. P. Spears, A. A. Hayes, P. V. Danenberg, R. Frösing, and B. G. Gustavsson. Deoxyuridylate effects on thymidylate synthase-5-fluorodeoxyuridylate-folate ternary complex formation (submitted for publication).

a capability will help to determine early in a patient's course whether 5-FUra should be omitted altogether, or whether addition of a biochemical modulator be attempted for the purpose of improving TS inhibition by increased FdUMP, lowered dUMP, or increased CH_2FH_4 levels.

By several analytical approaches, a prominent mechanism of resistance of tissues to TS inhibition in our patients appeared to be poor FdUMP formation. As one goes down Table 1, the order of increasing values of free TS in the malignant tissues, breast < colorectal < gastric, pancreas, and liver, resembles the order of sensitivities of these tissues to 5-FUra treatment. FdUMP levels may have been relatively high in breast cancers for their given times, and were significantly higher in colorectal cancers than in other gastrointestinal malignancies. Percentages of TS inhibition were significantly lower in surgically normal liver than in paired hepatic metastases and average FdUMP levels were higher in tumor at all time points (Fig. 1), four times the levels found in normal liver. Plots of FdUMP levels or FdUMP/dUMP ratios *versus* percentages of TS inhibition for biopsies obtained 20–400 min after 5-FUra suggest a minimum value of FdUMP to be over 75 pmol/g, for attaining high degrees of TS inhibition, or a value of (FdUMP/dUMP ratio) × 100 greater than 0.1. Among the 30 colorectal tumors with less than 85% TS inhibition, 16 of these showed FdUMP levels below 75 pmol/g. However, two of the seven colorectal tumors which showed over 85% TS inhibition also had FdUMP levels below 75 pmol/g. The results in Fig. 4 are broadly suggestive that for given values of FdUMP and FdUMP/dUMP ratios, the efficiency of TS inhibition in malignant tissues maintains the sequence, breast > colorectal > other gastrointestinal tumors. The most likely explanation for this, of course, is variation in intracellular CH_2FH_4 level. A probably relevant clinical fact is that cancer cachexia rarely occurs in breast cancer patients but regularly does so in those with gastrointestinal cancer, especially pancreatic.

Although the Fig. 4 analysis of colorectal tumors suggests that inadequate CH_2FH_4 levels were present in only about one-half of the 37 tumors, it can be reasoned that leucovorin addition should be useful for overcoming multiple mechanisms of resistance to TS inhibition after 5-FUra. An optimal intracellular CH_2FH_4 concentration for producing maximal and prolonged TS-FdUMP-CH_2FH_4 ternary complex formation may be as high as 20 μM or more (15). Kinetic mapping of CH_2FH_4 concentrations required to overcome dUMP competition for initial TS binding (and dUMP consumption of CH_2FH_4) in model studies suggests a minimum of 0.5 μM for the monoglutamate.[4] A high CH_2FH_4 level can simultaneously overcome the mechanisms of relatively low FdUMP, low folate, high dUMP, and high total TS, to achieve low post-5-FUra free TS levels. The enhanced therapeutic value of leucovorin/5-FUra combination may be a consequence of naturally lower levels of CH_2FH_4 in tumors than in normal tissues.

Other mechanisms of tumor resistance to 5-FUra that were not evaluated in the present analysis were tissue levels of thymidine, which could abrogate any degree of TS inhibition, and decreased incorporation of drug into RNA and DNA. Recent literature supports the concept that TS inhibition is the lowest-dose, threshhold mechanism of cytotoxicity of 5-FUra in the majority of cell types, if extracellular thymidine is low, TS is not amplified, and levels of reduced folates are not limiting (28, 32–36). Nevertheless, the obvious potential importance of incorporation of 5-FUra into RNA and DNA, and the potential importance of interrelationships between the different mechanisms, has led us to develop an assay of 5-FUra incorporation into RNA of the nonradiolabeled drug in tissues *in vivo* (37), preliminary applications of which wiil be reported elsewhere. One example of a connecting link between RNA and TS mechanisms, for which we have presented evidence, is the possibility that F-RNA acts as a slow turnover storage compartment of drug that leads to persistent levels of FdUMP (37, 38). Other mechanisms whereby 5-FUra incorporation into RNA could be complementary to TS inhibition include cell cycle synergy (39), potential ternary complex formation between F-RNA, FH_4, and RNA methyl-group processing enzymes (23, 40), and conceivable F-RNA-mediated inhibition of TS gene application (TS-elevated FUra-resistant mutants are exceptionally rare, compared to FdUrd-resistant cell lines).

We have postulated that a relatively high degree of inhibition of TS is required for cytotoxicity (10). Biochemical reasoning may also be supportive. For example, it can be shown that TS with a specific activity of 0.24 nmol thymidylate production per min and a tissue concentration of 1 pmol/g could support a 15% growth fraction of a tumor with 4 mg/g DNA (10, 13), assuming adequate concentrations of substrates (dUMP and CH_2FH_4), no catabolic loss of thymidine, and an S-phase of about 30 h. Average growth fractions of human gastrointestinal tumors are probably lower than 15%, and overall average levels of free TS after 5-FUra are about 1.4 pmol/g (Table 1). On the other hand, it has occasionally been suggested that native TS levels are rate-limiting for *de novo* DNA synthesis (28). If this is true, which is possible on the basis of limiting tissue levels of CH_2FH_4 [we find dUMP levels nearly always in excess of either its k_m or K_s (28) in contrast to the situation for CH_2FH_4], one would have to postulate an important role for salvage pathway DNA synthesis to bypass the regular TS inhibition we have observed. In support of this, profound *in vitro* synergism can occur between 5-FUra or 5-fluorodeoxyuridine and the nucleoside transport inhibitor, dipyridamole (21, 41). The mechanism of synergy may either be blockage of thymidine transport and lowered dUMP levels (41) or increased FdUMP concentrations (21), but potentially this could also be due to decreased transport of uridine and UMP formation resulting in increased RNA effects as well. Human plasma thymidine levels may average about 0.1 μM (42), and logically, tissue thymidine levels should be elevated in areas of necrosis. Although 0.1 μM thymidine is too low to rescue most cell types, human CCRF-CEM cells can be rescued at this level (36).

Normal liver consistently showed approximately one-half the degree of TS inhibition as found in malignant tissues, as we reported previously (16). The lower FdUMP levels in liver may be due to more rapid catabolism, suggested by the data of Fig. 1. Relatively slower rates of FdUMP dephosphorylation in colon carcinoma than in the liver have been observed in tissue preparations (20). A report of higher FdUrd levels in liver than in tumor immediately after intraareterial hepatic 5-fluorodeoxyuridine (43) tends to support this interpretation, although colon carcinomas may also have higher levels of phosphoribosylpyrophosphate transferase than normal tissues (44, 45). In addition, despite an expectation of relatively favorable CH_2FH_4 levels in hepatic tissue because of its storage role in folate metabolism, relatively poor efficiency of FdUMP binding to TS in liver appeared to be the case (Fig. 4). The major storage form of hepatic folates is 5-methyl-FH_4, however, which binds FdUMP to TS poorly (14).[4] In preliminary studies, direct assays of hepatic CH_2FH_4 levels, however, have indicated high values (46). A likely explanation for the paradox of poor efficiency of TS inhibition in liver is that hepatic folates are predominately in hepatocytes which do little DNA synthesis, while TS activity

may be largely located in reticuloendothelial cells and other nonhepatocyte cell types. In support of the latter, the major dose-limiting toxicity of chronic intraarterial hepatic FdUrd is at the level of the biliary tree with sclerosing cholangitic changes. Other explanations that could be forwarded include variant enzymes (47) and intracellular compartmentalization of TS (48), which has been critically questioned (49).

Bone marrow stem cells were studied by assay of anterior iliac crest buffy-coat leukocytes in five patients. Average percentage of TS inhibition in marrow leukocytes was significantly greater than in normal liver, related in part to the high FdUMP levels found (for comparison, one can multiply Table 1 values, in mg DNA/g, by a factor of about 4 mg DNA/g or more). Average total TS values in leukocytes were probably the highest of any tissue type studied. In addition, dUMP values were extremely high. We have observed similarly high dUMP levels in human bladder mucosa exposed to continuous exposure intravesical 5-FUra (50), and like those, the high dUMP levels in bone marrow leukocytes may be a result of reactive increases following effective TS inhibition. The high TS levels and potentially rapid rise in dUMP in marrow leukocytes may offer an explanation for the lack of marrow toxicity observed with continuous infusion 5-FUra.

In conclusion, our results demonstrate that mechanisms of resistance to TS inhibition by fluoropyrimidine therapy can be identified in the clinical setting. Such information may be useful for prospective stratification of patients onto treatment arms in which biochemical strategies are tested. For the latter, more complete data in the individual patient may be desirable. We have recently broadened our protocols to include at least one predrug and several postdrug tumor biopsies in the patient with metastatic disease. Advantages of serial biopsies include improved interpretation of dUMP levels and lack of a need for total TS determination by use of the 3 h pH 8.0 preincubation method (10, 24); free TS levels may then be done at a more optimal, lower pH.

Our pharmacodynamic approach to solid tumors is currently being adapted to studies in patients with metastatic disease receiving leucovorin, particularly those in whom multiple serial biopsies can be obtained. A modification of the ligand-binding assay of CH_2FH_4 (24, 46, 51, 52),[4] which uses [^{3}H]FdUMP and bacterial TS, appears to be useful for this purpose. The importance of further investigations in this area is underscored by our very recent findings of positive correlations between free TS levels after FUra and clinical outcome, in the present study, as well as among patients with colon and breast cancer studied by serial biopsy (53), which results will be reported subsequently.

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