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Sequential methotrexate/5-FU: FdUMP formation and TS inhibition in a transplantable rodent colon adenocarcinoma*

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Summary. Parameters for inhibition of thymidylate synthetase were studied after sequential methotrexate/5-fluorouracil (5-FU) administration in a dimethylhydrazine (DMH)-induced transplantable rat colon carcinoma. Tumor-bearing rats were treated with methotrexate (MTX) 40 mg/kg IP Bolus 5-FU, 100 mg/kg IP, was injected after 24 h. Micromethods for assay of 5-fluoro-2'-deoxyuridylate (FdUMP) and thymidylate synthetase (TS) were used to study the in vivo intracellular pharmacokinetics of 5-FU.

Formation of FdUMP was equally rapid in tumors regardless of MTX pretreatment, with peak values found at 30 min. Although MTX pretreatment did not increase peak FdUMP levels, it appeared to result in increased persistence of FdUMP, well in excess of available TS-binding sites, at 24 and 48 h. The combination therapy was less effective in terms of TS inhibition over the first 8 h after 5-FU administration, but may have been associated with improved TS inhibition at later time points.

Total levels of TS (TS_{tot}) steadily increased from a pre-5-FU treatment level of 18.8 pmol to more than 40 pmol/g at 24 h. MTX per se had no apparent effect on baseline TS_{tot} levels or on the 5-FU-mediated increases in TS_{tot}.

We conclude that MTX and 5-FU were antagonistic in terms of TS inhibition over the first 8 h after 5-FU in this DMH-induced rat colon carcinoma, but were possibly synergistic in increasing persistent levels of FdUMP and TS inhibition at later time points. The observation that 5-FU treatment can result in progressive increases in TS levels in some tumors suggests that this may be an important mechanism of 5-FU resistance.

Introduction

MTX and 5-FU are well-studied antimetabolites that have been used in cancer treatment for more than 20 years. An important cytostatic effect of 5-FU is mediated through formation of the metabolite, 5-fluoro-2'-deoxyuridylate (FdUMP), which together with methylenetetrahydrofolic acid (CH_2FH_4) binds to thymidylate synthetase (TS) and forms a stable ternary complex, resulting in significant enzyme inhibition [2, 12, 19, 23, 29]. Formation of 5-fluoro-2-uridinetriphosphate (FUTP) results in incorporation of the drug into RNA, and many studies have suggested the importance of incorporation of 5-FU into RNA or DNA as correlates of cytotoxicity [9]. 5-FU incorporation into RNA leads to inhibition of ribosomal maturation and alteration in post transcriptional modification of transfer RNA [11, 18]. There is a relationship between 5-FU dose and its incorporation into RNA, suggesting that this cytotoxic mechanism may predominate at high dose levels [14, 16]. This is in good agreement with the findings of Martin and Caricco, who have shown that thymidine, which should decrease toxicity by bypassing the block of thymidylate synthesis, leads to enhanced toxicity on co-administration, presumably by way of a decrease in TS activity with subsequent augmentation of the incorporation of 5-FU into RNA [8, 20].

The similar incorporation of 5-FU into DNA as the deoxyribonucleotide triphosphate (FdUTP) is perhaps less likely to occur, since FdUTP is rapidly hydrolyzed to FdUMP by dUTPase [22]. However, it has been proposed that FdUTP competes with dTTP and dUTP for incorporation into newly synthesized DNA; both erroneous bases, 5-FU and uracil, are excised by a glycosylase repair system [13, 34]. The repair system may be overwhelmed, resulting in significant levels of F-DNA and DNA strand breakage which can also result from incorporation of massive levels of dUTP [17].

Recent preclinical and clinical studies [1, 26] have suggested a therapeutic advantage for use of MTX and for high-dose MTX-folinic acid rescue in combination with 5-FU, based on the biochemical rationale that MTX pretreatment leads to increased levels of phosphoribosyl pyrophosphate (PRPP) by blockade of the purine anabolism and thus greater activation of 5-FU to nucleotides [7]. MTX is a folate analogue, which blocks the enzyme dihydrofolate reductase and thereby prevents regeneration of tetrahydrofolic acid from dihydrofolic acid. Thus, 5-FU and MTX may act at the same locus, preventing formation of thymidylate required for DNA formation and repair. There may be several interactions between 5-FU and MTX, some of which may be antagonistic. MTX-mediated

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Abbrevations: 5-FU, 5-fluorouracil; CH_2FH_4, 5,10-methylenepteroylmonoglutamatic acid; FdUMP, 5-fluoro-2-deoxyuridylate; TS, thymidylate synthetase; TS_f, free, non FdUMP-bound TS; TS_b, ternary complex bound TS enzyme; TS_{tot}, $TS_f + TS_b$; MTX, methotrexate

depletion of tetrahydrofolic acid could result in less effective TS inhibition by FdUMP binding in the ternary complex [4]. MTX forms pteroyl polyglutamates, which might replace CH_2FH_4 in the ternary complex and enhance FdUMP binding to TS [4]. MTX in vitro may increase TS activity, perhaps as a result of S-phase blockade, which could render blockade of thymidylate production more difficult [5]. Since TS activity is the only site of tetrahydrofolate oxidation in cells [12], 5-FU-mediated inhibition of TS should cause an elevation in FH_4 levels that could be antagonistic to MTX effects. 5-FU reduces the use of tetrahydrofolates and thus the production of dihydrofolates [7]. It has also been demonstrated that the sequence-dependent synergy observed in vitro between MTX and 5-FU is reduced in the presence of physiologic levels of purines [25].

Two randomized clinical trials have been conducted, by Browman et al [6] and by Coates et al. [10], to compare sequential MTX/5-FU and 5-FU/MTX and simultaneous administration. In both studies patients with squamous cell carcinoma of the head and neck were treated, and in the study of Coates et al. [10] some patients with gastrointestinal malignancies were also evaluated. In both studies oral leucovorin rescue was given. Browman et al. [6] failed to show any advantage of sequential administration of MTX/5-FU over simultaneous administration of the drugs, and in fact, the trend in their study favored simultaneous therapy. Nor could Coates et al. [10] show a statistically significant difference between MTX/5-FU and 5-FU/MTX, although there was a trend to higher tumor response rates in patients treated with MTX before 5-FU. However, as the authors themselves point out, this could be due to the short time interval (1 h) between MTX and 5-FU administration. Thus, the right schedule concerning dose and time may be crucial for otpimum cell kill with minimum host toxicity.

Methods have recently been developed by Spears et al. for determination of FdUMP and TS inhibition in vivo with capabilities for detection of less than 0.05 pmol of FdUMP and 0.005 pmol of TS [21, 31]. Application of these methods to the study of murine tumors [30, 31] and to evaluation of several human tissues [32] has suggested the importance of TS inhibition as a cytotoxic mechanism of 5-FU. The present study was designed to investigate the effects of MTX on TS inhibition by 5-FU by these methods.

Materials and methods

Reagents. Lactobacillus casei TS was purchased from the New England Enzyme Center, Boston, Mass, Ch_2FH_4 was freshly prepared for each experiment by addition of 4.0 μmol *dl*-L-tetrahydropteroylmonoglutamate (Sigma) to 50 μl 1 *M* ascorbate (pH 6.5), 2.5 μl 37% formaldehyde (w/v), and 1.95 ml of a buffer consisting of 50 m*M* potassium phosphate (pH 7.2), 20 m*M* 2-β-mercaptoethanol, and 2% (w/v) bovine serum albumin [21]. Methotrexate was obtained from Cyanamid Nordiska AB, Stockholm, Sweden, 5-FU was purchased from Roche AB, Stockholm, Sweden, and FdUMP was purchased from Sigma. 6[^{3}H]FdUMP (18 Ci/mmol) was obtained from Moravek Biochemicals, Brea, Calif. A stock solution of charcoal was prepared by adding BSA and T-70 Dextran (Pharmacia) to 10% of neutral charcoal (Sigma) as previously described [21]. This was diluted to 1 : 3 in 0.2 *M* HCl before use. All other chemicals used were of analytical grade and commercially available. FdUMP dilutions were made in 10 m*M* phosphate and TS dilutions were made in the buffer used for tissue homogenization (see below).

Experimental animals and tumor model. Male and female Wistar rats were used at 15–20 weeks of age (weight 100–180 g). They were maintained on chow and water ad libitum.

The tumor was a dimethylhydrazine (DMH)-induced colon carcinoma obtained from the Wallenberg Laboratory, Lund, Sweden, and showed intermediate grades of differentiation. It was passaged in the host of origin by SC transplantation. For our studies 1 ml medium (Parker 199) containing 1.0×10^6 cells was injected SC in the lumbar region. The experiments were performed at 2 weeks when the tumors reached 1–2 g in size. The rats received injections of MTX 40 mg/kg IP. At 24 h after MTX the rats were treated with 5-FU IP, 100 mg/kg. At each time point from 15 min to 48 h after 5-FU, three rats were sacrificed by cervical dislocation. The tumors were excised and immediately stored in liquid N_2 until assay. The same experiments were repeated without MTX pretreatment.

To study tumor heterogeneity, tumors were allowed to grow to 5 g in size. From each tumor four biopsies, each weighing 300 mg, were taken at 1 h after 5-FU, 100 mg/kg; three from the periphery and one from the grossly necrotic central regions of the tumors.

Preparations of homogenates. Tumors were thawed at 4 °C and placed in a four fold excess of a 0.2 *M* Tris-HCl buffer (pH 7.4) containing 20 m*M* 2-β mercaptoethanol, 15 ml cytidylate, and 100 mM NaF, disrupted by use of homogenizer and sonicated 3×20 s (100 W). Aliquots were taken for nucleotide extraction, and the rest of the crude sonicates were centrifuged (10000 *g* for 10 min) and stored at −80 °C until enzyme assay.

Nucleotide extraction was done according to Nazar et al. [24]: 300–500 μl of the crude sonicate was placed in a tenfold excess of 1 *M* acetic acid, freeze-thawed three times, and centrifuged. The supernatant was collected and the procedure was then repeated twice on the pellet. The three supernatants were pooled and lyophilized to dryness.

dUMP/FdUMP separation on DEAE-cellulose columns was done as previously described [28].

Biochemical assays

FdUMP, and TS as free [^{3}H]-FdUMP-titrable binding sites (TS_f) or as endogenous ternary complex-bound enzyme (TS_b) were measured by the methods developed by Moran et al. [21] and Spears et al [31].

TS assay. TS_f was measured by adding 50 μl cytosol to 6 pmol 6[^{3}H]-FdUMP and 50 nmol CH_2FH_4 in a total volume of 175 μl. After 20 min at 30 °C, 1.0 ml 3% ice-cold charcoal was added. The tubes were centrifuged at 4000 *g* for 20 min and 600 μl of the supernatant was removed for scintillation counting. Corrections were made for exchange labeling of cytosolic TS_b enzyme with [^{3}H]-FdUMP. TS_{tot} was determined after dissociation of the ternary complex at pH 8 for 2 h [12, 31] followed by addition of CH_2FH_4 and excess [^{3}H]FdUMP as in the TS_f assay [31, 32].

Because of the very high levels of FdUMP found in these studies, treatment of cytosols prior to assay with an equal volume of ice-cold 10% neutral charcoal was done, followed by centrifugation at 4000 *g* for 20 min to remove nucleotides.

FdUMP assay. After separation from dUMP on DEAE-cellulose the FdUMP fraction was lyophilized to dryness and dissolved in 5 m*M* potassium phosphate. Then 25 µl of the solution was added to 0.3 pmol [^{3}H]-FdUMP, 0.15 pmol TS, and 50 nmol CH_2FH_4 in a total volume of 125 µl. After 2 h incubation at 30 °C ice-cold charcoal was added and ternary complex-bound [^{3}H]-FdUMP was isolated in the supernatant after centrifugation at 4000 *g* for 20 min. Since unlabeled FdUMP causes isotope dilution in the ternary complex, FdUMP levels were calculated using a standard curve made with known amounts of FdUMP [21].

Results

Treatment of the cytosols with 10% neutral charcoal before TS assay was found to be useful for removal of cytosolic FdUMP present in large quantities during the first 2 h after 5-FU administration, which resulted in very significant isotope dilution without this purification step. As shown in Fig. 1, the charcoal treatment of the cytosols nearly doubled the TS_{tot} values for the first 2 h after 5-FU, even after correction of the results of non-charcoal-treated cytosols for isotope dilution effects of non-radiolabeled FdUMP. The effect on TS_f, however, was not detectable during this time period, because of the extremely low levels of TS_f.

Heterogeneity

The results of the heterogeneity experiments are outlined in Table 1. The coefficient of variation (SD/mean × 100) of TS_{tot} levels was less than 10% in each tumor, while the scatter between different tumors was 30%. The scatter of the percentage of TS inhibition was less than 5% in each tumor and 4% between different tumors. FdUMP values, showed a scatter of 47%–160% (117%±40%) in each tumor, and the scatter between different tumors was 125%. Hence, in large tumors with macroscopic evidence of necrosis there was a striking lack of scatter in the TS parameters, but scatter of the FdUMP values was pronounced. FdUMP values appeared to be lower in the central necrosis, 743±653 vs 1477±1845 in the periphery, but this difference was not statistically significant.

Table 1. The coefficient of variation[a] in parameters of TS inhibition in SC-transplanted DMH-induced colon carcinoma 1 h after IP administration of 5-FU, 100 mg/kg to Wistar rats

	TS_{tot}	TS inhibition (%)	FdUMP
Intratumoral scatter	8.8 ± 1.9	2.29 ± 2.36	117 ± 40
Intertumoral scatter	30	4	125

[a] (Standard deviation ÷ mean) × 100

FdUMP kinetics

The in vivo intratumoral pharmacokinetics of FdUMP formation and its loss are shown in Fig. 2. The results at each time point represent the average (±SD) values of tumors of three rats. Peak levels of FdUMP were found at 30 min after 5-FU administration and approached 5000 pmol/g in rats treated with 5-FU alone. Peak levels in tumors of the MTX-pretreated animals were slightly, but not statistically significantly, lower than in tumor exposed only to 5-FU. The time course over the first 8 h appeared to be unchanged as a result of MTX pretreatment. However, in contrast to the relatively low persistent levels of FdUMP after 5-FU alone, MTX pretreatment resulted in substantial FdUMP levels at 8 and 24 h, and relatively high levels (281±24 pmol/g) were still found 48 h after 5-FU administration (Fig. 2). Hence, MTX pretreatment resulted in persistence of relatively high levels of FdUMP throughout the observation time of 48 h after bolus 5-FU.

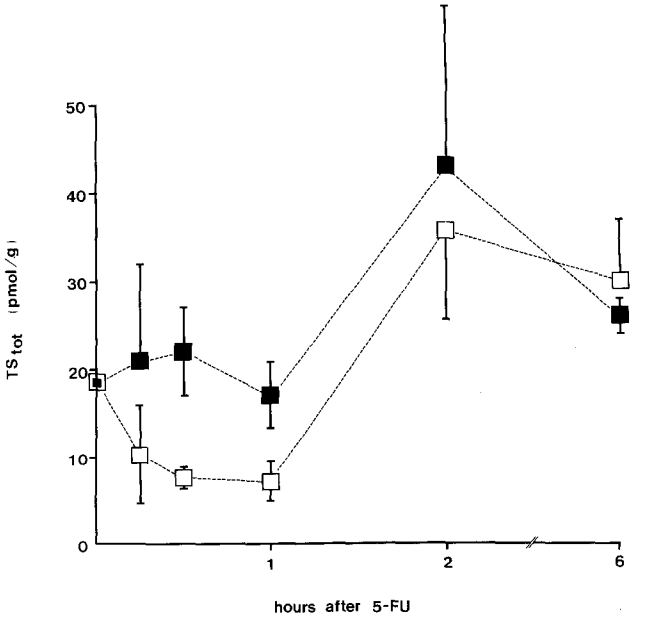

Fig. 1. TS_{tot} values with (■) and without (□) pretreatment of the cytosol with an equal volume of 10% neutral charcoal

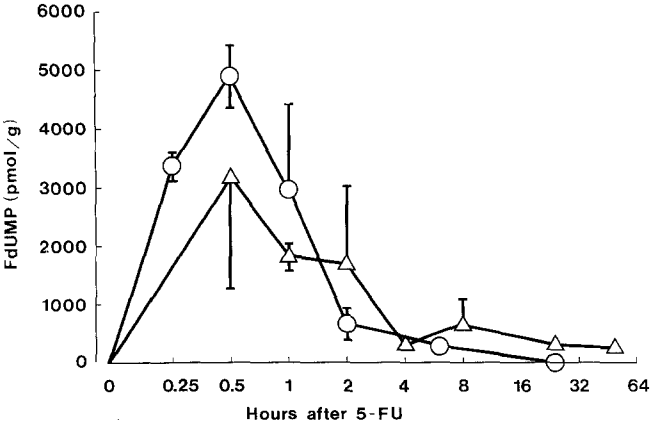

Fig. 2. Intracellular concentration of FdUMP in rat colon adenocarcinoma as a function of time after bolus 5-FU, 100 mg × kg^{-1} with (△) and without (○) IP injection of MTX, 40 mg/kg, 24 h before 5-FU administration

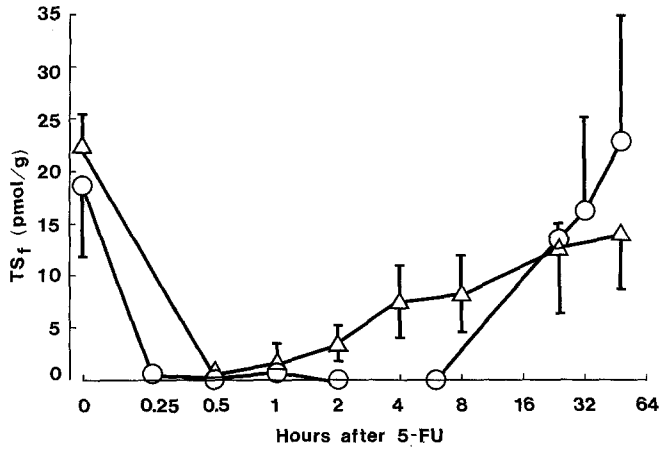

Fig. 3. In vivo kinetics of TS_f in transplantable rat coloncarcinomas following 100 mg 5-FU kg^{-1} with (△), and without (○) MTX pretreatment

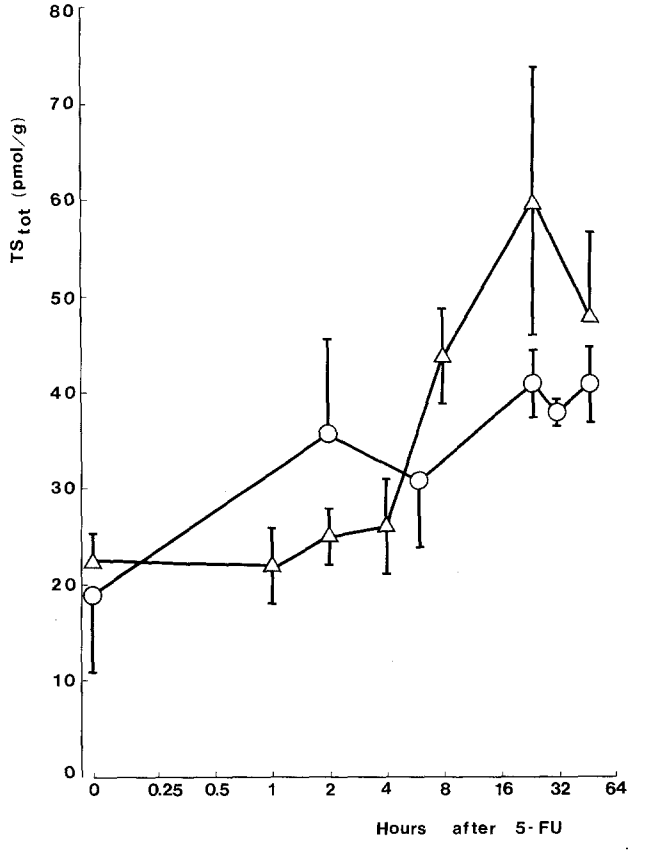

Fig. 4. TS_{tot} as a function of time after bolus 5-FU 100 mg/kg with (△) and without (○) IP injection of MTX, 40 mg/kg, 24 h before 5-FU administration

Kinetics of TS inhibition

The inhibition of TS and its recovery are shown in Figs. 3 and 4. Bolus 5-FU, 100 mg/kg, alone resulted in rapid decrease of TS_f levels, with less than 1 pmol/g at 30 min. At 6 h TS inhibition was still nearly complete. In contrast, TS_f activity in tumors pretreated with MTX, 40 mg/kg, 24 h before 5-FU, was not completely abolished in any animal, and the recovery of TS activity appeared to be more rapid, resulting in TS_f levels above 3 pmol/g at 2 h (Fig. 3). At 48 h in the tumors not exposed to MTX, TS_f levels approached pretreatment levels in spite of a continued inhibition of TS by more than 30%. This was explained by an increase in TS_{tot} levels (Fig. 4). The effect of MTX at 48 h appeared to be a slightly greater percentage inhibition of TS than was achieved by 5-FU alone, concomitant with continued high levels of FdUMP at this time. Pretreatment value of TS_{tot} averaged 18.8 ± 7.9 pmol/g if no drugs were given and 20.4 ± 3.0 pmol/g in tumors treated with MTX. After 24 h the level of TS_{tot} had increased to more than 40 pmol/g ($P < 0.05$) independently of MTX pretreatment. Hence, altogether MTX pretreatment resulted in persistence of significant FdUMP levels throughout the 48-h observation time. This appeared to prolong inhibition of TS, although this finding is uncertain since the pharmacokinetics were followed for only 48 h. However, the TS inhibition was less efficient in the first 8 h after bolus 5-FU, as a result of MTX pretreatment.

Discussion

To investigate the metabolic effects of heterogeneity, parameters of TS inhibition were initially estimated in relatively large tumors with macroscopic signs of necrosis, which resembles the clinical situation. TS values showed a remarkable lack of heterogeneity, and the variation between different tumors was also modest. FdUMP values, however varied significantly in different biopsies from the same tumor, and there was also a wide variation between different tumors. To at least partially circumvent this problem, the tumors were studied at 2 g in size, and the whole tumor was excised and processed.

The sequence of 5-FU followed by MTX has been reported to be antagonistic in cytotoxicity against both normal cells and tumor, but the reversed sequence is usually additive both in antitumor effect and in toxicity [7]. In this study MTX pretreatment appeared to change the pharmacokinetics of 5-FU in terms of at least doubling the period time of persistence of FdUMP at levels well above available binding sites on TS. At 48 h, the last time point observed, this was associated with a greater percentage inhibition of TS than was found in tumors treated only with 5-FU. The explanation to this could be increased incorporation of FUTP into RNA, since RNA does turn over and may thus act as slow-release deposit for FdUMP over a period of time [16].

As reported by Cadman et al., MTX blocks purine synthesis, and the PRPP not utilized then becomes available for the conversion of 5-FU to FUMP via the action of phosphoribosyltransferase [7]. This could result in increased incorporation of FUTP into RNA, as well as increased FdUMP levels, and the authors observed a fivefold increase in FdUMP formation after MTX exposure. Such an increase in FdUMP peak levels could not be demonstrated in this DMH-induced colon carcinoma.

It seems possible that in some instances, where PRPP pools are ratelimiting for FUMP formation, that one effect of MTX pretreatment would be a prolonged, but modest increase in PRPP pools, which could be responsible for our observations. The toxicity-sparing effects of allopurinol on the clinical tolerance of 5-FU, which are probably mediated by decreases in PRPP pools [15], are more pronounced with continuous infusion administration of 5-FU

than with bolus delivery, and would be compatible with this concept.

Hence, it is possible, as we and others have suggested [16, 33], that the effect of 5-FU incorporaiton into RNA is not only a disruption of RNA function, but also a prolongation of FdUMP-mediated blockade of TS.

In this study statistically significant prolonged TS inhibition was not clearly demonstrated. However, even if a significant percentage of TS was inhibited there was still enough TS_f to permit adequate rates of thymidylate formation, and as we have reported earlier, it appears that almost complete ablation of TS activity is required for 5-FU response [31, 32].

At time points up to 8 h, TS inhibition was actually poorer after MTX pretreatment. This could be a result of depletion of cofactors, since MTX blocks dihydrofolate reductase. While MTX can substitute for the $N^{5,10}$ methylene tetrahydrofolic acid it does not permit the same high-affinity binding [4, 12]. Thus, MTX would antagonize the formation of CH_2FH_4-FdUMP-TS ternary complex. Bonny et al. have reported that MTX treatment leads to increased TS levels in rat liver and human lymphoblasts [5]. This could lead to 5-FU resistance, as suggested by several reports of increased TS activity accompanying acquired resistance to 5-FU [3, 27]. Spears et al. reported progressive increases in TS_{tot} levels after bolus 5-FU in all of three murine colon adenocarcinomas investigated [30] that were 5-FU resistant. In our DMH-induced colon carcinoma we found a two- to threefold increase in TS_{tot} levels 48 h after bolus 5-FU independently of MTX pretreatment (Fig. 4), which occurred in a linear fashion over the observation period. This finding may equally be explained either by a synchronization of the cells into S-phase, or by new synthesis of the enzyme induced by TS inhibition.

The recently developed ultrasensitive assays for intracellular in vivo pharmacokinetics of FdUMP and TS provide a useful means of studying the effects of modulation of 5-FU biochemical effects with agents such as MTX. The present study demonstrates that MTX alters the pharmacokinetics of 5-FU in this tumor, resulting in persistence of FdUMP for at least twice as long as after bolus 5-FU alone. The cytotoxic effect of this remains obscure, since free TS enzyme can be present in spite of FdUMP levels in excess of binding sites. Further studies are needed to see whether the addition of different cofactors, e.g., leukovorin, to the schedule can abolish TS activity for a longer period.

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