Modulation of Both Endogenous Folates and Thymidine Enhance the Therapeutic Efficacy of Thymidylate Synthase Inhibitors


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ABSTRACT

Plasma levels of folates and thymidine in mice are about 10-fold higher than in humans and may influence the therapeutic efficacy of thymidylate synthase (TS) inhibitors, such as 5-fluouracil (5FU) and the antifolates pemetrexed (MTA) and raltitrexed (RTX). Therefore, we tested their therapeutic efficacy in various murine tumor models, grown in mice on a normal and a folate-depleted diet, with high and low thymidine kinase (TK) levels. MTA and RTX were inactive against Colon-26-10 [doubling times gained by treatment; growth delay factor (GDF), 0.5 and 0.3, respectively], whereas 5FU was very active (GDF, >10; complete cures). Colon-26-10/F, grown in mice on a folate-depleted diet, was more sensitive to RTX and MTA (GDF, 2.1 and 1.3, respectively) but not to 5FU (GDF, 1.2); however, leucovorin reversed the effect leading to complete cures. Folate depletion did not reverse resistance of Colon-26A and Colon-26G (low TK) to MTA and RTX, whereas leucovorin only enhanced the 5FU effect in Colon-26A and Colon-26A/F. Folic acid at 15 mg/kg did not improve the therapeutic efficacy of MTA in folate-deficient mice. The folate-depleted diet decreased the reduced folates in Colon-26A/F and Colon-26-G/F tumors less (4–5-fold; P < 0.01) than in Colon-26-10/F tumors (8-fold; P < 0.001). Folate depletion increased TS levels 2–3-fold in all of the models and TK levels 6-fold (P < 0.01) in Colon-26/G/F, explaining the lack of activity of MTA and RTX in Colon-26/G/F. In contrast, TK-deficient FM3A/TK tumors were much more sensitive to RTX and MTA, and 5FU than parent FM3A tumors, which have comparable TS levels. The rate of thymidine phosphorylation varied considerably in all of the tumors without a clear relation to antitumor activity.

In conclusion, tumor folates may potentiate (5FU) or protect (antifolates). Murine tumor models should combine low folates and low thymidine rescue to optimize preclinical testing of antifolates.

INTRODUCTION

In vivo tumor models are an essential step in the preclinical evaluation of anticancer drugs to determine the pharmacokinetic and toxicity parameters (1). However, these models have some major disadvantages that can influence the sensitivity of drugs such as TS3 (2–5). TS is an essential enzyme in the DNA synthesis. It catalyzes the de novo pathway for synthesis of dTMP. 5,10-CH2-H4-folate serves as a methyl donor and is converted into dihydrofolate (6).

Murine models have 10-fold higher plasma levels of folates, predominantly 5-CH3-H4-folate, than human plasma (7, 8). This may abrogate the activity of folate-based TS inhibitors such as RTX (Tomudex, ZD1694) and MTA (LY231514; Ref. 9). More frequent and higher doses will be required to compete with natural folates at different levels, including the uptake into the cell via the reduced folate carrier and the linkage of polyglutamate side chains by the enzyme folypolyglutamate synthetase, which would enhance antifolate retention in the cell and, finally, the binding to the target enzyme TS (10).

High folate levels in mice can also mask the effect of LV (5-CHO-H4-folate) on 5FU treatment (4). 5FU is a precursor of a nucleotide-based inhibitor of TS. The 5FU metabolite, FdUMP, binds to TS, and together with 5,10-CH2-H4-folate, a ternary complex is formed (11). 5,10-CH2-H4-folate stabilizes this ternary complex, and when folate pools are limiting, they can be elevated by administration of LV (4). Subsequently, the inhibition of TS by the 5FU will be enhanced, and the antitumor activity will be increased. Depending on the tumor model (12), such an effect might be not observed in mice because of their high plasma folate levels. In other in vivo studies (13, 14), it has been shown that LV and TdR can abrogate both the antitumor effect and the toxicity of RTX. The effects of MTA in vitro can be reversed by TdR in combination with hypoxanthine (15). The latter is related to the other targets of MTA, dihydrofolate reductase and glycaminide ribonucleotide formyltransferase.

The 10-fold higher level of TdR in murine plasma compared with human plasma (3, 14, 16, 17) is another problem of in vivo models. TdR is converted into dTMP in a reaction catalyzed by TK. This can salvage dTMP depletion caused by inhibition of TS and may reduce the antitumor activity of 5FU and folate-based TS inhibitors (5, 10, 14).

Several approaches have been used to solve these two problems of murine models. The folate levels in mice can be reduced by feeding the mice a folate-deficient diet. The effects of a folate-deficient diet have been studied extensively in another rodent, the rat (18). Mice can also be kept on such a diet, efficiently reducing folate levels (7). Studies with C3H mice on the activity of 5FU and LV (4) and another antifolate drug, lometrexol (7, 19), showed that the intrinsic level of folates in mice on normal diet play an important role. The toxicity of lometrexol increased by three orders of magnitude in folate-deficient mice, mimicking some unexpected toxicity seen in humans.

A combination of folate-depleted diet and coadministration of FA has been tested to manipulate the therapeutic window of some antifolates. This approach allowed a precise control of the folate status of murine plasma and tissues. Studies with the antifolate TS inhibitor GW1843U89 (17) have shown that this concept works very well in a preclinical setting. Administration of FA allowed an increase of the antifolate dose; the antitumor activity improved, and the FA protected normal tissues against toxic side effects, whereas no protection of antitumor activity was observed up to a dose of 366 mg/kg FA (oral administration). A similar effect was observed with lometrexol and FA (19). Lometrexol had a limited therapeutic activity in folate-deficient mice with C3H mammary tumors compared with that of...
mice on a standard diet. When FA was coadministered p.o. to mice that were mildly folate-deficient, antitumor activity was observed, and toxicity was reduced. The range of lometrexol doses that allowed safe therapeutic use of this drug increased at higher folate intake, but excessively high folate intake reversed the antitumor activity of lometrexol.

The effect of high plasma TdR levels can be circumvented in two ways, either by using a tumor that is TK deficient (14, 20–22) or by feeding the mice a conjugate of polyethylene glycol-thymidine phosphorylase (5, 17). The TK− will prevent any interference of TdR with antitumor activity of a TS inhibitor, whereas systemic toxic side effects of the drug might be slightly reduced. The polyethylene glycol-thymidine phosphorylase will catabolize all of the TdR in the plasma into thymine and deoxyribose-1-phosphate, and thus plasma TdR levels will be decreased.

In this study, we used three variants of the murine colon tumor model Colon 26 to evaluate the influence of folates on the antitumor effect of 5FU, 5FU/LV, and the antifolates MTA and RTX. Balb/c mice were put on a folate-deficient diet, and we developed low folate variants of the tumors. One variant with a very low TK activity and a TK-deficient variant of the murine mammary tumor FM3A were used to evaluate the role of TdR levels. The antitumor activity of drugs was related to the effect of folate depletion on several relevant biochemical properties, such as TS activity, TK activity, and TP activity.

MATERIALS AND METHODS

Materials. 5FU was obtained from PCH Pharmacemie (Haarlem, the Netherlands). d-FU and FA were provided by the Pharmacy Department of the University Hospital Vrije Universiteit (Amsterdam, the Netherlands). These drugs were diluted to appropriate concentrations in 0.9% NaCl. RTX was from AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). MTA was generously supplied by Lilly Research Laboratories (Indianapolis, IN). These drugs were diluted in a 50 mM NaHCO₃ buffer. [6-3H]FdUMP (specific activity, 20 Ci/mmol) and [2,4-14C]TdR (specific activity, 59.2 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA). [5-3H]GMP (specific activity, 10.9 Ci/mmol) was from Amersham International (Buckinghamshire, United Kingdom). TS (3.2 units/mg protein) was purified from the Escherichia coli strain that overproduces Lactobacillus casei TS (23). The E. coli strain was a gift from D. V. Santi (University of California, San Francisco, CA). 5,10-CH₂-H₄-folate reductase (0.52 units/mg protein) and 10-formyltetrahydrofolate dehydrogenase (0.2 units/mg protein) were purified from pig (24, 25). Methylentetrahydrofolate synthetase (0.1 units/mg protein) was purified from rabbit liver. All of the other chemicals were from analytical grade and commercially available.

FA-deficient chow (0 mg of FA/100 kg) to which no FA was added and standard rodent chow (3.65 mg of FA/100 kg) were obtained from Hope Farms (Woerden, the Netherlands).

Animals. Female Balb/c and C3H/He mice (Harlan, Zeist, the Netherlands) 6–10 weeks of age (18–20 g) were used for dose-finding studies and antitumor activity experiments. The mice were housed six to eight/cage with normal sawdust bedding in a room with a controlled light-dark cycle (8 h light, 16 h dark). Water, normal chow, and folate-deficient chow were provided ad libitum. To establish a mouse model with low folate levels, mice were fed the same 5FU dose/schedule was tested in C3H/He mice. RTX and MTA were from analytical grade and commercially available.

In this study, we used three variants of the murine colon tumor model Colon 26 to evaluate the influence of folates on the antitumor activity experiments. The mice were housed six to eight/cage with normal sawdust bedding in a room with a controlled light-dark cycle (8 h light, 16 h dark). Water, normal chow, and folate-deficient chow were provided ad libitum. To establish a mouse model with low folate levels, mice were fed the same 5FU dose/schedule was tested in C3H/He mice. RTX and MTA were from analytical grade and commercially available.

Toxic side effects were evaluated by weight loss. Statistical analysis was performed with the Student t test for unpaired data. All of the animal experiments were approved by the Scientific and Animal Ethical Committees of the Vrije Universiteit.

Tissue Preparation. Blood was collected in hematocrit capillaries, smears for reticulocytes were made, and the remaining part was centrifuged immediately. Hematocrit was determined, and the plasma was collected and diluted with an equal volume of cold 50 mM Tris/HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA. This was stored at −70°C until analysis. Tumor and liver tissue were excised from the mice, washed with cold PBS, frozen in liquid nitrogen, and stored at −70°C. The frozen tissues were pulverized by means of a microdisemembrator (29).

Folate Analysis. The ternary complex assay used to measure reduced folates is based upon enzymatic cycling of reduced folates to 5,10-CH₂-H₄-folate followed by entrapment into a stable ternary complex with excess L. casei folate. The TK− ultimately will prevent any interference of TdR with antitumor activity of a TS inhibitor, whereas systemic toxic side effects of the drug might be slightly reduced. The polyethylene glycol-thymidine phosphorylase will catabolize all of the TdR in the plasma into thymine and deoxyribose-1-phosphate, and thus plasma TdR levels will be decreased.

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Dose-finding studies were performed with mice that did not bear tumors. The MTD and schedule for 5FU and LV in Balb/c were known from previous experiments (10 mg/kg 5FU, 2 × 50 mg/kg LV; q7d × 2; Refs. 12, 26), and the same 5FU dose/schedule was tested in C3H/He mice. RTX and MTA were given in 5 times daily schedule (4q × 5) at start doses of 10 mg/kg and 80 mg/kg, respectively. The RTX and MTA dose were escalated further by 1.3-fold steps. MTD studies in low folate mice were initiated with the standard 5FU dose and 10-fold reduced doses of MTA and RTX as compared with the MTD for normal mice. MTD studies on MTA with FA supplementation in mice on a low folate diet were initiated at 50 and 15 mg/kg, respectively. The FA dose was kept constant, whereas we increased the MTA dose by 1.3-fold until the MTD was reached. The MTD was defined as a weight loss of maximally 10% in mice without tumors. The doses are indicated further by xbars in the text, so SFU100 is 100 mg/kg.

For the antitumor activity experiments, tumors were excised from a previous passage in mice and cut into 1-mm³ pieces. The tumor pieces were implanted s.c. in the left and right flank with a 16-gauge trochar. Colon 26 variants were transplanted into female BALB/c mice. The following Colon 26 variants were used: Colon 26A was regarded as the parental tumor; Colon 26G was derived from Colon 26A by in vivo selection with gemcitabine and has a very low total TK activity (12% of Colon 26A; Ref. 27); and Colon 26-10 was derived from Colon 26A, a cell line C26-10 (12). FM3A and FM3A/TK− mammary carcinoma cells were a gift from Dr. J. Balzarini at the Rega Institute (Leuven, Belgium). The original FM3A/TK− cells were developed by Ayusawa et al. (28). Tumors were established by s.c. injection of 10⁶ mammary carcinoma cells into female C3H/He mice. The Colon 26 variants were also transplanted into low folate BALB/c mice. All of the variants (indicated as Colon 26.F/+) grew well in these mice and were subsequently maintained in low folate mice. At a tumor size of approximately 100 mm³, treatment was started according to the MTD of the drugs. Mice were randomized in treatment groups and a control group of six to eight animals, depending on the number of tumors. Weight and tumor volume were measured twice weekly. Antitumor activity was evaluated by the GDF, which indicated the gain of doubling time by treatment (12). Toxic side effects were evaluated by weight loss. Statistical analysis was performed with the Student t test for unpaired data. All of the animal experiments were approved by the Scientific and Animal Ethical Committees of the Vrije Universiteit.

Thymidine Analysis. Plasma TdR levels were determined by HPLC. Briefly, 75-μl samples of mouse plasma were diluted with 1.0 ml of water, and the plasma proteins were precipitated by the addition of 50 μl of ice-cold perchloric acid. After centrifugation, the obtained supernatant was treated with potassium carbonate to remove the perchlorate as the insoluble potassium salt. The sample was then purified by solid-phase extraction using columns packed with 100 mg of a high capacity reverse-phase polymeric sorbent. The column-bound TdR was eluted with methanol, and the eluates were evaporated to dryness in a vacuum centrifuge. The dried residues were reconstituted in 150 μl of water and stored frozen until analyzed by HPLC. The analysis was carried out using 250-nm Supelcosil C18 column using a mobile phase of 2% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1.25 ml/min. The retention time of the TdR peak was approximately 8.5 min, and it was measured at 267 nm using a 5-point standard curve ranging from 0.2 to 4 μM and corrected for sample processing losses. This correction factor was obtained by spiking samples with radiolabeled TdR and measuring the difference in activity after sample preparation.

Enzyme Assays. The enzyme activities of TS, TK, and TP in tumor tissues were determined according to previously published methods. For each assay,
the frozen pulverized tissue was suspended in the appropriate assay buffer, and an aliquot was taken for protein measurement.

TS activity was determined as the conversion rate of [5-3H]dUMP into dTMP, releasing tritiated water (31). The pulverized tissue was suspended in 0.2 M Tris/HCl (pH 7.4) buffer containing 20 mM mercaptoethanol, 100 mM NaF, and 15 mM CMP. The reaction mixture consisted of 25 µl of 10,000 g supernatant, 5 µl 6.5 mM of 5,10-CH2-H4-folate, 10 µl of Tris/HCl buffer, and 10 µl of [5-3H]dUMP (final concentration, 10 µM; specific activity, 50 mCi/mol) and was incubated for 30 min at 37°C. The reaction was stopped by addition of 50 µl of ice-cold 35% trichloroacetic acid and 250 µl of 10% neutral activated charcoal. After centrifugation, 150 µl of the resultant supernatant was used for liquid scintillation counting of radioactivity.

Total TK activity consisting of cytosolic TK1 and mitochondrial TK2 was measured as described by Ruiz van Haperen et al. (32). A 50 mM Tris/I 1 mM EDTA (pH 7.4) buffer was used, and the assay mixture consisted of 25 µl of 10,000 g supernatant and 25 µl of substrate solution [21.9 µM TdR, final specific activity, 1.8 Ci/mmol; 20 mM ATP; 10 mM MgCl2; 10 mM Tris/200 µM EDTA (pH 7.4)]. When only TK1 was determined, 10 mM dCTP was added to the substrate solution, as a specific inhibitor of TK2 (33). The mixture was incubated at 37°C for 30 min and stopped by heating the mixture at 95°C for 3 min, followed by the addition of 10 µl of 5 mM TdR/5 mM thymine. Substrate (TdR) and product (dTMP) were separated by TLC on polyethylene imine cellulose layers, with distilled water as eluent. The spots could be visualized under UV, marked, and cut out. Radioactivity was estimated by liquid scintillation counting.

TP activity was measured with TdR as a substrate by means of a nonradioactive assay (34). TdR phosphorylation in murine tissue may also be catalyzed by uridine phosphorylase. TP activity as measured here also represents the mor activity of TS inhibitors. There was a large difference in MTD for low folate mouse model, we observed that the food consumption of folate-deficient chow was stable (±2.3 gram/mouse/day). Weight of the mice was stable (±20 g), but they did not grow anymore, whereas mice of the same age on a normal diet did grow. The formation of RBCs in the bone marrow is highly dependent on folates, but we saw no significant changes in hematocrit or reticulocytes during 5 weeks on folate-deficient diet.

**Folate Pools.** In mice kept on a folate-deficient diet, a marked reduction in plasma folate levels (8–28-fold; Table 2) and in the colon tumor tissue (4–10-fold) was noted. Interestingly, the 5,10-CH2-H4-folate and H4-folate pools in parent Colon 26-10 tumors were 2.6-fold (P < 0.001) higher than the pools of Colon 26A and 26G (Table 2). Among the low folate variants, Colon 26-10/F had the highest 5,10-CH2-H4-folate and H4-folate pools. There appeared to be a shift in the folate pools, because the 5-CH3-H4-folate pools were significantly lower in Colon 26-10 tumors compared with the other two colon tumors. The decrease in 5,10-CH2-H4-folate and H4-folate levels of Colon 26-10/F was 8-fold compared with the parent Colon 26-10. The other colon tumors showed smaller changes, 5-fold and 4-fold for Colon 26A and 26G, respectively. The mammary tumor FM3A/TK had 1.6-fold higher 5,10-CH2-H4-folate and H4-folate pools (P < 0.01) than FM3A tumors.

**TdT Levels.** The TdT levels in plasma of Balb/c mice on a normal diet and that of Balb/c mice on a folate-depleted diet were comparable. TdT in the plasma sample of two Balb/c mice on a normal diet were 0.57 and 0.56 µM; in a Balb/C mouse on low folate diet, 0.64 µM TdT was measured in plasma. The other mouse strain C3H/He also had comparable TdT levels. Measurements on two plasma samples from different C3H/He mice showed 0.29 and 0.73 µM TdT.

**Antitumor Effects.** The doubling time of the tumors Colon 26A and 26G did not change under dietary folate conditions, but Colon

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**Table 1. MTD of TS inhibitors in C3H/He mice and Balb/c mice on normal and low folate diet**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Schedule</th>
<th>MTD (mg/kg)</th>
<th>Weight Loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFU</td>
<td>q7d x 2</td>
<td>100</td>
<td>5.0</td>
</tr>
<tr>
<td>LV (+ SFU)</td>
<td>q7d x 2</td>
<td>50 + 50 mg/kg + 100 mg/kg</td>
<td>4.6</td>
</tr>
<tr>
<td>RTX</td>
<td>q4d x 2</td>
<td>10</td>
<td>16%</td>
</tr>
<tr>
<td>MTA</td>
<td>q4d x 2</td>
<td>100</td>
<td>9%</td>
</tr>
<tr>
<td>MTA + FA</td>
<td>q4d x 2</td>
<td>80 mg/kg + 15 mg/kg</td>
<td>9.0%</td>
</tr>
</tbody>
</table>

* LV was administered in two doses 30 min before and together with 100 mg/kg SFU. The schedule of a drug was similar for all of the mice. The weight loss given here was observed during therapy in tumor-bearing mice. The weight loss of the MTD in nontumor-bearing mice was below 10%. All of the drugs were given i.p., except FA, which was administered orally.

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**RESULTS**

**Dose-Finding Studies.** Development of the low folate mouse model allowed us to study the impact of dietary folate on the antitumor activity of TS inhibitors. There was a large difference in MTD for the folate-based TS inhibitors (Table 1), which is not observed for SFU. Furthermore, a mouse strain-dependent difference in MTD was observed. The dose of MTA in low folate mice could be increased to nearly the level of normal diet mice, when 15 mg/kg FA was administered as a rescue agent. The weight loss observed at the MTD in nontumor-bearing mice was always below 10% (data not shown), but the weight loss indicated in Table 1 accounted for the weight loss in tumor-bearing mice during therapy. This weight loss was a good indication for toxicity of the treatment. During the development of the
26-10/F grew two times slower than Colon 26-10 (P < 0.001; Table 3). The antitumor activities of the various drugs in mice on a normal and folate-free diet are summarized in Table 3. Colon 26-10 tumors were much more sensitive to 5FU_{100} and 5FU_{100} + LV_{100} than Colon 26A or Colon 26G. In the folate-depleted mice, the low folate variant Colon 26-10/F was more resistant to 5FU_{100}, but the sensitivity to 5FU_{100} + LV_{100} was unchanged. The Colon 26-10 tumor was rather resistant to RTX_{0.2} and MTA_{100} compared with Colon 26A and Colon 26G. The tumor-doubling time of Colon 26-10 tumors treated with these drugs did not differ from control as expressed by the low GDF.

The decrease of folate levels in Colon 26A/F, Colon 26G/F, and Colon 26-10/F tumors as compared with their high folate variants were much more sensitive to 5FU_{100} and 5FU_{100} than Colon 26A/F, 2.8-fold (P < 0.01), respectively. The total TK activity of the FM3A/TK2 tumor was still measurable but much lower than in the parental FM3A tumor. The FM3A/TK− cell line, which was used for the initiation of the solid tumors, had no TK1 activity and relatively high TK2 activity, as compared with the parental FM3A cells (data not shown). The remaining activity of TK1 in FM3A/TK− tumors was 4-fold lower than that of the TK1 activity of FM3A tumors. This change was similar to the 4-fold difference in total TK activity of the two tumors. A possible explanation for the remaining TK activity is inflammation of murine cells with normal TK activity into the tumor tissue.

TS activity of the Colon 26 variants under normal diet conditions was comparable (Table 5). A 2–3-fold increase was observed for the Colon 26.../F variants. The mammary tumor FM3A had a slightly higher TS activity than the standard Colon 26 variants, whereas the TS activity of the FM3A/TK− was comparable with FM3A tumors.

TP activity of Colon 26 tumors appeared to be very different in the three variants (Table 6). TP activity was lowest in Colon 26-10 tumors and did not differ significantly from its low folate variant. Colon 26A/F had a 5-fold reduced TP activity as compared with Colon 26A and reached a level of activity similar to Colon 26-10/F. Colon 26G/F had the highest TP activity also compared with its parental tumor Colon 26G. The activity in Colon 26G and Colon 26/F was significantly higher (P = 0.00002) than in the other colon tumors. The FM3A tumors had a 6-fold higher TP activity than the FM3A/TK− (P = 0.02).

**DISCUSSION**

The influence of endogenous plasma and tumoral levels of both folates and thymidine metabolism on the antitumor activity of TS inhibitors in vivo was substantial. The adaptation of the mice to low folate diet and initial differences in folate status were associated with sensitivity to 5FU, MTA, or RTX in the colon tumors. The role of TdR was evident from the increased antitumor activity of 5FU, MTA, and RTX in TK-deficient FM3A mammary tumors compared with parental FM3A tumors.

The MTD for RTX in Balb/c mice (10 mg/kg) was in the same range as the dose that was used by Clarke et al. (14). However, for the various strains and for both RTX and MTA, large differences in MTD were observed (14, 35). MTA required a larger dose reduction for mice on a low folate diet than RTX required. In contrast to experimental head and neck tumors (5), the therapeutic index of RTX was not superior to that of 5FU and LV in nearly all of the subtypes of Colon 26. This is consistent with the results of clinical Phase II (breast) and Phase III (colon) trials (36).

One of the biochemical effects of a low folate diet was a 2–3-fold increase in TS activity. A similar increase in TS mRNA expression

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Antitumor activity in mice on normal and low folate diet</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>GDFa</td>
</tr>
<tr>
<td>Colon 26A</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>Colon 26A/F</td>
<td>2.8 ± 1.3</td>
</tr>
<tr>
<td>Colon 26G</td>
<td>3.4 ± 1.7</td>
</tr>
<tr>
<td>Colon 26G/F</td>
<td>4.0 ± 1.5</td>
</tr>
<tr>
<td>Colon 26-10</td>
<td>4.1 ± 2.6</td>
</tr>
<tr>
<td>Colon 26-10/F</td>
<td>8.0 ± 2.9</td>
</tr>
<tr>
<td>FM3A</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>FM3A/TK</td>
<td>3.3 ± 1.8</td>
</tr>
</tbody>
</table>

a TD, tumor-doubling time.

b GDF < 1, resistant; 1–2, moderately sensitive; > 2, sensitive. Values for Colon 26A are means from seven experiments, the others are from one experiment.

c Statistical t-test unpaired data GDF, TD of treatment versus control, P < 0.001.

d Statistical t-test unpaired data GDF, TD of treatment versus control, P < 0.05.

e GDF could not be detected, because most of the tumors went into complete remission (CR).

f Three cures of seven animals, corresponding to 7 of 11 tumors, that did not regrow after 42 days.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>TK activity in murine tumors</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>Total TK activity (pmol/h/mg/protein)</td>
</tr>
<tr>
<td>Normal</td>
<td>7313 ± 380</td>
</tr>
<tr>
<td>Colon 26-A</td>
<td>996 ± 442</td>
</tr>
<tr>
<td>Colon 26-G</td>
<td>5804 ± 1574</td>
</tr>
<tr>
<td>Colon 26-10</td>
<td>3648 ± 1674</td>
</tr>
<tr>
<td>FM3A</td>
<td>866 ± 28</td>
</tr>
<tr>
<td>FM3A/TK</td>
<td>2515 ± 5</td>
</tr>
<tr>
<td>Low folate</td>
<td>6458 ± 1295</td>
</tr>
</tbody>
</table>

a Values are means ± SD of at least three tumors.

b TK2 activity was calculated as total TK = TK1 + TK2 for each assay.

c Statistical differences between normal and low folate tumors (Student’s t-test), P < 0.001.

d Statistical differences between normal folate and MTA (Student’s t-test), P < 0.05.
and FdUMP-binding sites in murine breast tumors was already reported by Priest et al. (37). The fundamental process underlying these changes could well be at the level of DNA methylation. Folate depletion caused diminished DNA methylation at least at one site within the TS promoter (37).

Folate depletion was associated with a decrease of the total TK activity in Colon 26A/F and Colon 26-10/F. In Colon 26G/F, total TK activity increased, which may be attributable to the omission of the selection pressure by gemcitabine, which is a substrate for TK2 (33). These changes suggest that TK is differently regulated in this tumor when compared with the other Colon 26 subtypes.

Also, the total rate of TdR phosphorylase did not show a consistent pattern of changes under low folate conditions. TP is also known as a platelet-derived endothelial cell growth factor that plays a role in tumor vasculization and proliferation (38). Although there was a large variation in TP activity, the proliferation rate of the tumors, except Colon 26-10/F, was similar. Ackland and Peters (38) suggested that folate-based TS inhibitors may be more effective in tumors with a high TP because of increased degradation of TdR. We observed that Colon 26G with a high TP activity in this panel of the tumors was not sensitive to MTA or RTX, indicating that other factors are more dominant for achievement of the antitumor effect.

The role of TdR in antifolate resistance has already been recognized by Pinedo et al. (2). The TdR levels in plasma of Balb/c mice on normal diet and folate-depleted diet and of C3H/He mice were comparable, but large differences were observed in the activity of the enzymes TK, TP, and TS. Their role is rather complex, especially when a drug like 5FU is involved because the metabolism to the active nucleotide FdUMP may also proceed via TP and TK. However, the present data provide additional evidence that metabolism of 5FU is not mediated by this pathway (11) because the TK activity increased, which may be attributable to the omission of the selection pressure by gemcitabine, which is a substrate for TK2 (33).

The pattern of enzyme activities could not completely explain the difference in antitumor activity of the Colon 26 subtypes such as resistance to 5FU in Colon 26A and Colon 26G and complete remissions of Colon 26-10, all of which have comparable TS activity. The pool of 5,10-CH₂-H₄-folate and H₄-folate in the tumors appeared to be important for the antitumor activity, especially of 5FU. The other differences that have been described for Colon 26-10 and Colon 26A were the longer duration of TS inhibition (39) and retention of [³¹⁸F]5FU and its metabolites (40) in Colon 26-10. The activity of enzyme dihydropyrimidine dehydrogenase responsible for 5FU degradation and also related to 5FU sensitivity was comparable in Colon 26A and Colon 26-10 (40). The activity of the 5FU-activating enzyme orotate phosphoribosyl-transferase is high in the parental Colon 26A tumors (41), whereas synthesis of FdUMP catalyzed by uridine phosphorylase and uridine kinase was >10 higher than that of FdUMP catalyzed by TP and TK. Therefore, it is unlikely that TP is a major 5FU-activating enzyme in this tumor. The low total TK activity of Colon 26G compared with the other colon tumors did not increase the sensitivity to 5FU, RTX, or MTA, but the relatively low folate pools might account for the 5FU resistance. However, in Colon 26-10/F, the decreased TK activity together with reduced 5,10-CH₂-H₄-folate and H₄-folate pools were associated with increased sensitivity to RTX and MTA. The importance of TK activity in folate depletion was illustrated by Colon 26G/F, which has an increased TK activity and increased resistance to RTX and MTA as compared with Colon 26G. Also, the low TK activity of FM3A/TK⁻ was associated with an increased antitumor activity for all of the compounds. This was in agreement with a study showing that treatment with MTA (300 mg/kg) cured 4 of 10 mice bearing GC3/TK⁻ colon tumors, whereas only a growth inhibition was seen in mice bearing the parental GC3 tumors (19). In the same GC3/TK⁻ tumors, a repeated dose of 5FU (75 mg/kg) and LV (800 mg/kg) induced a significant tumor reduction but no cures (42). The 5FU activity against these TK⁻ tumors was not superior to that observed in parental tumors, but the activity of 5FU and LV was (42). This suggests a typical TS inhibition induced antitumor activity. RTX was curative in mice with LS178Y TK⁻/TK⁻ lymphoma (10). In our study, FM3A/TK⁻ tumors still had TK activity, which, together with the relatively high 5,10-CH₂-H₄-folate and H₄-folate levels compared with FM3A, might explain why the effects were not always optimal with all of the drugs. These relatively high folate levels may support 5FU activity. MTA activity is believed to be additionally mediated by inhibition of dihydrofolate reductase and glycaminid ribonucleotide formyltransferase (15, 21), which may contribute to its antitumor effect.

The role of a low folate diet in the antitumor activity of 5FU has been studied previously (4), showing that LV potentiated 5FU anti- tumor activity when mice were put on a low folate diet. This is in agreement with our data, which showed a decreased antitumor effect of 5FU on Colon 26-10/F, but in combination with LV all of the tumors regressed. This is due to the TS-dependent 5FU antitumor effect, because in folate-depleted tumors 5,10-CH₂-H₄-folate pools are too low to stabilize the FdUMP-TS-5,10-CH₂-H₄-folate complex. The limiting 5,10-CH₂-H₄-folate pools in the tumor will be restored by treatment with LV.

Table 5 TS activity in murine tumors measured at a substrate concentration of 10 μM dUMP

<table>
<thead>
<tr>
<th>Tumor</th>
<th>(nmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Colon 26-A</td>
<td>2324 ± 720</td>
</tr>
<tr>
<td>Colon 26-G</td>
<td>2552 ± 227*</td>
</tr>
<tr>
<td>Colon 26-10</td>
<td>2852 ± 339</td>
</tr>
<tr>
<td>FM3A</td>
<td>3163 ± 437</td>
</tr>
<tr>
<td>FM3A/TK⁻</td>
<td>3237 ± 398</td>
</tr>
<tr>
<td>Low folate</td>
<td></td>
</tr>
<tr>
<td>Colon 26-A/F</td>
<td>4524 ± 2294</td>
</tr>
<tr>
<td>Colon 26-G/F</td>
<td>8998 ± 2197*</td>
</tr>
<tr>
<td>Colon 26-10/F</td>
<td>6980 ± 443*</td>
</tr>
</tbody>
</table>

* Values are means ± SD of at least three tumors.

Table 6 TP activity in murine tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>(nmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Colon 26-A</td>
<td>187 ± 89</td>
</tr>
<tr>
<td>Colon 26-G</td>
<td>349 ± 33</td>
</tr>
<tr>
<td>Colon 26-10</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>FM3A</td>
<td>85 ± 34</td>
</tr>
<tr>
<td>FM3A/TK⁻</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Low folate</td>
<td></td>
</tr>
<tr>
<td>Colon 26-A/F</td>
<td>39 ± 12</td>
</tr>
<tr>
<td>Colon 26-G/F</td>
<td>608 ± 265</td>
</tr>
<tr>
<td>Colon 26-10/F</td>
<td>36 ± 7</td>
</tr>
</tbody>
</table>

* Values are means ± SD of at least three tumors.
reduction of antitumor activity was observed (43), but reduction of the FA dose and adaptation of the diet might improve the efficacy. The successful combination of GW1843U89 with FA to prevent gut toxicity was based on the properties of GW1843U89 (17), which is a noncompetitive inhibitor of TS and only competes with natural folates for transport and polyglutamylation. MTA is a competitive inhibitor of TS (15), and RTX is a mixed noncompetitive inhibitor of TS with a tendency toward competitive (13). Moreover, RTX uptake and polyglutamylation were reduced by LV (13), and the antiproliferative activity of MTA was prevented by LV (15). In vivo FA is metabolized into reduced folates, such as 5-CH₃-H₄-folate, which is also rendered noncompetitive by the natural folates, as has been shown for dideazahydrofolate by oral folic acid. Cancer Res., 43: 6297–6307, 1983.

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Modulation of Both Endogenous Folates and Thymidine Enhance the Therapeutic Efficacy of Thymidylate Synthase Inhibitors

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