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ENHANCEMENT OF ANTINEOPLASTIC ACTIVITY OF 5-FLUOROURACIL IN MICE BEARING COLON 38 TUMOR BY (6*R*)5,10-DIDEAZATETRAHYDROFOLIC ACID

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Abstract—(6*R*)5,10-Dideazatetrahydrofolic acid (DDATHF, Lometrexol), a potent antitumor drug *in vivo* and *in vitro*, is an inhibitor of the two folate-dependent enzymes in the *de novo* purine biosynthesis pathway: glycinamide ribonucleotide (GAR) and amino imidazole carboxamide (AICAR) transformylases. A single dose of DDATHF (50 mg/kg, i.p.) in C57/BL6 mice caused a prolonged depletion of purine nucleotides (ATP and GTP) in colon 38 tumor and only a temporary effect in liver. GAR transformylase activity was higher in colon 38 tumor than in liver, but a kinetic analysis on the purified enzyme showed no differences in K_i values for DDATHF or K_m values for the folate substrate. As a consequence of *de novo* purine synthesis inhibition, there was a 2- to 3-fold elevation of 5-phosphoribosyl-1-pyrophosphate pools in colon 38 tumor between 4 and 12 hr after DDATHF administration. When DDATHF (50 mg/kg) was administered 4 or 8 hr prior to 5-fluorouracil (5-FU; 85 mg/kg, i.p., weekly), these biochemical effects significantly increased the antitumor activity of 5-FU, with a modest increase in toxicity. Lower doses of DDATHF (25 and 37.5 mg/kg) when combined with 5-FU also resulted in an improved antitumor activity without additional toxicity. The two different schedules of administration for DDATHF, 4 and 8 hr prior to 5-FU, showed no differences in antitumor activity or toxicity.

Key words: chemotherapy; 5-fluorouracil; deazafolate; biochemical modulation; colon cancer; murine

DDATHF† is a potent tetrahydrofolic acid analog whose targets differ from the classical antifolate site of action, dihydrofolate reductase [1]. DDATHF is an inhibitor of cell growth *in vitro*, and an effective antitumor agent *in vivo* on murine models and human xenografts [2]. Presently, it is under clinical investigation for both solid tumors and leukemias. DDATHF has been shown to interfere with *de novo* purine synthesis by inhibition of the two folate-dependent enzymes along that pathway, GAR and AICAR transformylases. It has been shown in the past that a reduction in purine synthesis results in the elevation of the PRPP pool, the first element in the formation of the purine structure, because of decreased utilization [3, 4]. MTX, a classical antifolate has been shown to elevate the concentration of PRPP because of its secondary effect on purine synthesis. Several studies both *in vitro* and *in vivo* have exploited this observation combining with different modalities MTX and 5-FU and obtaining a synergistic combination effective in a variety of tumor models [5–10]. The main metabolic pathway for the activation of 5-FU to FUMP is through the orotate phosphoribosyltransferase reaction that requires PRPP. The potentiation of 5-FU activity has been attributed to the enhanced formation of its ribonucleotides and subsequent incorporation into RNA [11]. Two randomized clinical trials administering MTX at different intervals before 5-FU have shown a consistent advantage over a classical 5-FU therapy in terms of response rate for the treatment of gastrointestinal malignancies with a modest increase in host toxicity [12, 13]. Because DDATHF directly affects *de novo* purine synthesis, without involving the interconversion of reduced folates, it has a definite advantage over MTX. In this report, we explore the capacity of DDATHF to alter *de novo* purine synthesis and PRPP pools *in vivo*, and we describe the effect of DDATHF as a biochemical modulator of 5-FU cytotoxic activity in mice bearing colon 38 tumor.

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† Abbreviations: DDATHF, (6*R*)5,10-dideazatetrahydrofolic acid; GAR, glycinamide ribonucleotide; AICAR, aminoimidazole carboxamide; PRPP, 5-phosphoribosyl-1-pyrophosphate; MTX, methotrexate; 5-FU, 5-fluorouracil; FUMP, 5-fluorouridine monophosphate; MTD, maximum tolerated dose; and TCA, trichloroacetic acid.

MATERIALS AND METHODS

Drugs and chemicals. 5-FU was purchased from the Sigma Chemical Co. (St. Louis, MO). DDATHF (Lometrexol) was provided by Dr. Chuan Shih, Lilly Research Laboratories (Indianapolis, IN) [14]. [6-^{3}H]5-FU was purchased from Moravek Biochemicals (Brea, CA). [^{3}H]DDATHF was prepared by catalytic reduction of diethyl-2-acetyl-5,10-dideaza-9,10-didehydrofolate in glacial acetic acid using tritium

gas in the presence of Adams catalyst [15]. Orotidylate pyrophosphorylase and orotidylate decarboxylase were purchased from Sigma. All other chemicals were purchased from either Sigma or Schwarz-Mann (Cleveland, OH).

Animal experiments. Two- to three-month-old female C57/BL6 mice were purchased from NCI (Bethesda, MD) and inoculated subcutaneously in each flank with 1 mm^3 fragments of colon 38 tumor. Treatment was started when the tumors were 50–250 mg, determined by caliper measurement according to the formula [16]:

$$\text{tumor wt. (mg)} = \text{length (mm)} \times \text{width}^2\ (\text{mm}^2)/2$$

Groups of six mice, each containing a similar tumor size distribution, were treated on a weekly basis, and tumor size was determined once or twice a week [17]. Both DDATHF and 5-FU were dissolved in 0.85% NaCl solution and administered i.p. in a volume of 0.1 mL/10 g of body weight on a weekly basis. 5-FU was administered at 85 mg/kg, the MTD in C57/BL6 mice, on a weekly schedule. DDATHF was administered at 25, 37.5 and 50 mg/kg 4 or 8 hr prior to 5-FU injections.

Differences in tumor size and weight among groups were evaluated by ANOVA, followed by Bonferroni *t*-tests. Toxicity was evaluated by changes in body weight and expressed as a percentage of the initial body weight of the animal.

PRPP assay. Mice bearing colon 38 tumor received a single injection of DDATHF at different concentrations. At various times from 4 to 24 hr, animals were killed and liver, gut and tumor were removed and immersed rapidly in liquid nitrogen. The tissues were pulverized in a mortar under liquid nitrogen, and PRPP was extracted in 200 mM Tris–HCl, pH 7.4, containing 0.5 mM 2,3-diphosphoglyceric acid, 33 mM NaF and 2 mM EDTA. PRPP was assayed by the release of $^{14}CO_2$ from [^{14}C]orotic acid in the presence of orotidylate pyrophosphorylase and orotidylate decarboxylase [10]. The assay was linear over a concentration range of 50–1000 pmol of PRPP.

Differences among groups were evaluated by ANOVA, followed by Fisher PLSD and the Scheffer F-test.

Extract preparation for GAR transformylase. Normal tissues and tumors were removed and frozen in liquid nitrogen. Tissue samples were homogenized in 5 vol. of extraction buffer consisting of 50 mM sodium phosphate, pH 6.8, containing 25% glycerol with 1 mM PMSF, and the extract was centrifuged at 37,000 *g* for 1 hr at 4°.

GAR transformylase assay and purification. The extract was applied to a 10-formyl-5,8-dideazafolate-Sepharose column [18, 19], washed with 50 mM potassium phosphate with 25% glycerol, pH 6.8, in the presence of 1 mM PMSF, and subsequently washed with 2 M NaCl, 50 mM potassium phosphate, pH 7.4, and 25% glycerol.

GAR transformylase was eluted with 2 M urea, 50 mM sodium phosphate, pH 7.4, and 25% glycerol, dialyzed against 25 mM sodium phosphate, pH 7.4, and 25% glycerol and concentrated using dry Sephadex G200.

Enzyme activity was determined by a spectrophotometric assay with 10-formyl-5,8-dideazafolate as formyl donor. A solution of 0.25 mM GAR and 0.01 mM 10-formyl-5,8-dideazafolate in 50 mM potassium phosphate with 25% glycerol, pH 7.5, was incubated for 10 min at 37°. After enzyme addition the assay was monitored by following absorbance at 295 nm. Activity was found to be linear with respect to time and enzyme. K_m and K_i values were determined by the Lineweaver–Burk and Dixon methods.

[^{3}H]*5-FU incorporation into RNA.* C57/BL6 mice bearing advanced colon 38 tumor were injected with DDATHF (50 mg/kg) 4 hr prior to [6-^{3}H]5-FU (85 mg/kg, 25 μCi/mouse). Two hours after 5-FU administration, the animals were killed by cervical dislocation. Tissues of mice injected with [^{3}H]5-FU were immediately removed and frozen in liquid nitrogen. Frozen samples were weighed and homogenized in 2 vol. of ice-cold 15% TCA. The pellet was washed twice with TCA at 0° and dissolved in 2 vol. of 1 N NaOH by incubating overnight at 37° [17]. The solubilized tissues were neutralized with HCl before radioactivity determination.

Analysis of DDATHF polyglutamates. Mice were injected with 50 mg/kg of DDATHF spiked with 25 μCi/mouse of [^{3}H]DDATHF. At indicated times, the mice were killed and the tissues removed and placed in liquid nitrogen. Frozen tissues were homogenized in 50 mM sodium phosphate, pH 6.0, containing 200 mM 2-mercaptoethanol and boiled for 5 min. The homogenate was then centrifuged at 2000 *g* for 10 min.

DDATHF polyglutamate analysis utilized HPLC reverse-phase ion-pair chromatography. The 5 μm C18 Ultrasphere column (4.6 mm × 25 cm, Rainin, Woburn, MA) was eluted with a linear gradient from 20% methanol:acetonitrile (2:1) to 40% methanol:acetonitrile (2:1) in 100 mM ammonium phosphate and 1.5 mM tetrabutylammonium bromide, pH 6.5, at 1 mL/min. Sufficient amounts of chemically synthesized DDATHF polyglutamates (glu3, glu5 and glu7) were added to the radiolabeled samples to provide an internal standard [20]. The column effluent was collected in 1-mL fractions. Radioactivity was determined by scintillation counting after the addition of scintillation fluid (Optifluor, Packard Instrument Co., Downers Grove, IL).

Extraction and separation of ribonucleotides. Normal tissues and tumors were rapidly removed and frozen in liquid nitrogen. Tissue samples were homogenized in 2 vol. of ice-cold 15% TCA, and the acid-soluble fraction was neutralized by Trioctylamine-Freon extraction and stored at −20° until analysis.

Separation of nucleotides was performed on a Whatman Partisil-10-SAX anion-exchange column (Whatman, Woburn, MA), using 0.4 M ammonium phosphate isocratic elution at a flow rate of 1.5 mL/min [21]. Eluted nucleotide triphosphates were monitored with an Altex (Berkeley, CA) model 153 detector set at 254 nm, and the retention peak areas were determined with a Shimadzu C-RIA Chromatopac Integrator (Kyoto, Japan).

RESULTS

DDATHF has been shown, *in vitro*, to inhibit *de*

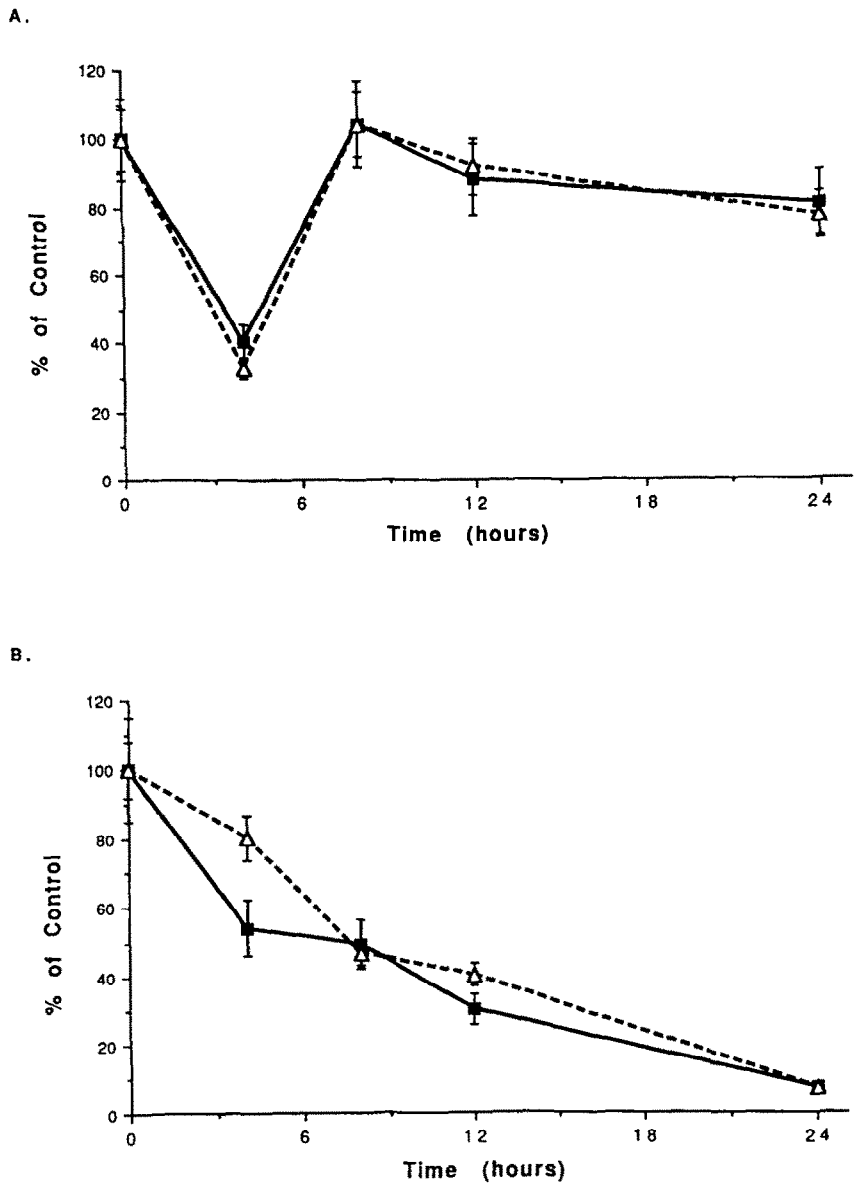

Fig. 1. Effects of DDATHF on purine nucleotide pools in mice bearing colon 38 tumor. A single dose (50 mg/kg) of DDATHF was administered i.p. to mice bearing colon 38 tumor. The animals were killed at the indicated intervals, and the tissues were immediately removed and placed in liquid nitrogen. The tissues were processed and analyzed for their ATP (■) and GTP (Δ) content, as described in Materials and Methods. Data are the means ± SD of at least three experiments conducted in duplicate. In liver (panel A), the concentrations of ATP and GTP were 909 ± 157 and 114 ± 20 μM, respectively. In tumor (panel B), the ATP and GTP concentrations were 449 ± 95 and 34 ± 14 μM, respectively.

novo purine synthesis, causing a rapid depletion of ATP and GTP pools [21].

Our data show that a 50 mg/kg dose of DDATHF causes a temporary depletion of both ATP and GTP levels in the liver of C57/BL6 mice 4–6 hr after administration (Fig. 1A), with recovery to normal levels within 8 hr. In contrast, we observed a 30–40% reduction in the purine nucleotide levels in colon 38 tumors after 4 hr and a continued depletion of the purine pools for 24 hr after the administration of DDATHF (Fig. 1B).

We also observed a very selective elevation of PRPP pools in the tumors as compared with normal tissues. DDATHF (50 mg/kg, i.p.) caused a 50–60% elevation of PRPP pools in colon 38 tumors between 4 and 12 hr after the antifolate administration. The only normal tissue that showed a significant increase in PRPP was the liver, which had a 15% increase in PRPP levels 4 hr after DDATHF administration. We did not observe any significant variation in the gut, which is one of the major target organs for toxicity (Fig. 2).

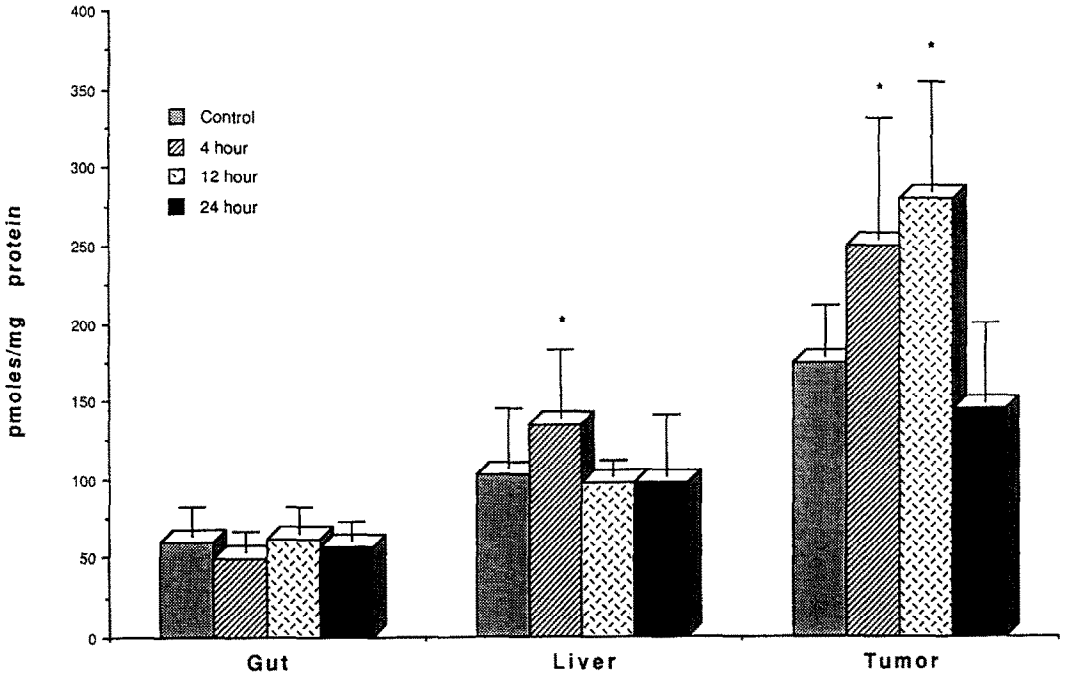

Fig. 2. Concentration of PRPP in tissues of mice after administration of DDATHF (50 mg/kg). Mice bearing colon 38 tumor were treated i.p. with a single dose of DDATHF. The animals were killed at the indicated time, and the tissues were removed rapidly. The determination of PRPP was conducted as described in Materials and Methods. Results are the means ± SD of 12–40 mice per each time point.
Key: (*) statistically significant at 95%, according to both Fisher PLSD and Scheffer F-test.

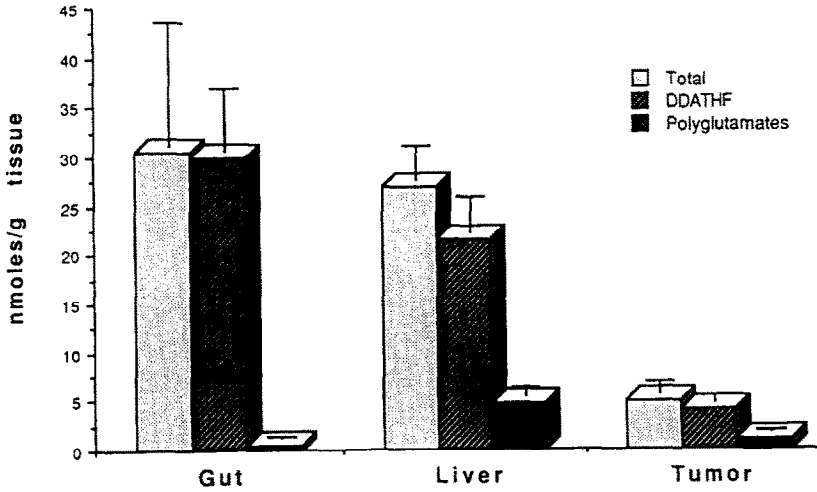

Fig. 3. Accumulation of DDATHF and its polyglutamates 4 hr after the administration of the antifolate. Three mice were treated with a 50 mg/kg dose of DDATHF (25 μCi/mouse). The animals were killed 4 hr later, and the tissues were removed and analyzed as described in Materials and Methods. Values are means ± SD.

To understand the reason for these tissue specific effects, we studied the distribution and metabolism of DDATHF in liver, gut and tumor. Surprisingly, incorporation of radiolabeled DDATHF was highest in the gut, whereas the tumor accumulated only 10–15% of the antifolate (Fig. 3). Differences in the metabolism of DDATHF to polyglutamates, however, may explain the tissue specificity of the biochemical effects. In colon 38 tumor, more than 25% of DDATHF accumulated in the pharmacologically active polyglutamate forms, whereas in normal gut only 1–2% of the drug present

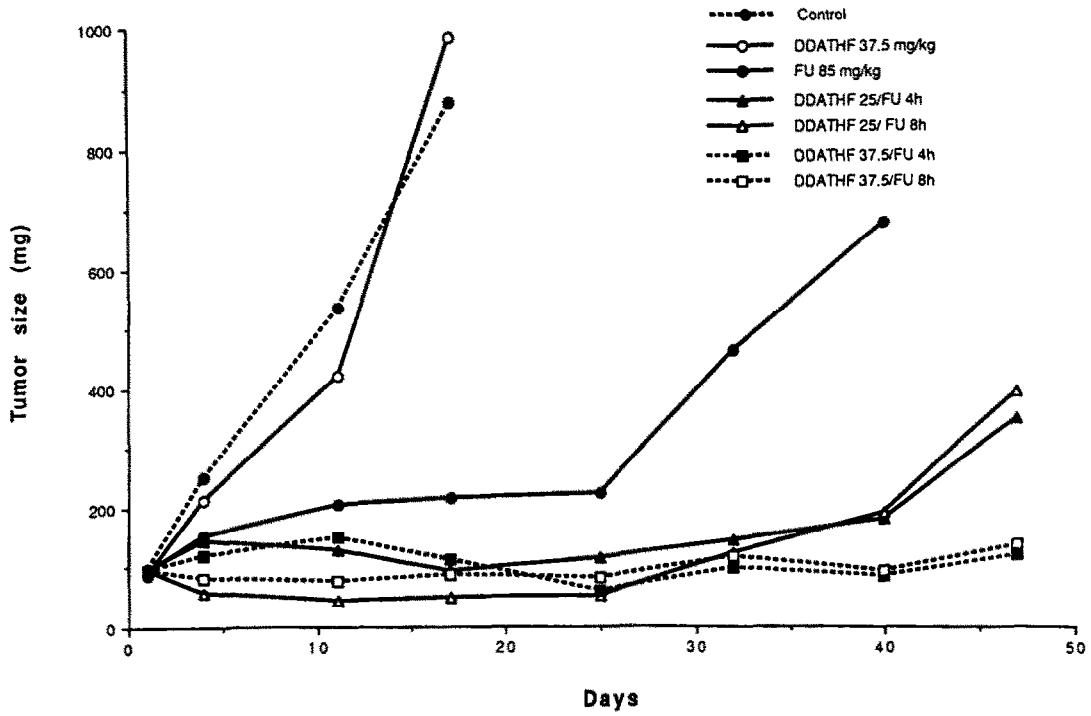

Fig. 4. Antitumor activity of the combination DDATHF/5-FU on colon 38 tumors in C57/BL6 mice. C57/BL6 mice bearing colon 38 tumors were randomized into groups of six mice each. DDATHF was administered on a weekly basis at 25 or 37.5 mg/kg, 4 or 8 hr prior to 5-FU (85 mg/kg). The tumor size and mouse weights were measured twice a week, as described in Materials and Methods.

had been metabolized to polyglutamates. In the liver, 15–20% of the total radioactivity was present as polyglutamates. The polyglutamates present were distributed in a similar pattern in all three tissues, with DDATHF (glu5) as the most prevalent metabolite (data not shown).

GAR transformylase was purified from crude extracts of liver and colon 38 tumor and was characterized. Colon 38 tumor showed higher GAR transformylase activity in the crude extract (0.0026 μmol/min/mg protein) than did the liver, which exhibited an activity of only 0.0002 μmol/min/mg. However, the kinetic parameters of enzyme from tumor and liver were almost identical, with a K_m for the substrate 10-formyl-5,8-deazafolate of 1.7 μM for the enzymes purified from both tissues and a K_i for DDATHF glu5 of 0.0045 μM in liver and 0.0066 μM in colon 38 tumor.

Low doses of DDATHF (25 and 37.5 mg/kg), administered 4 or 8 hr before 5-FU (85 mg/kg) on a weekly basis, inhibited tumor growth and potentiated the effect of 5-FU when given as a single agent. No differences in antitumor activity or in toxicity were apparent when the modulator DDATHF was administered 4 or 8 hr prior to 5-FU. The effect was dose dependent (Fig. 4). In the case of DDATHF at 37.5 mg/kg, the size of the tumor mass remained unchanged during the period of treatment, compared with 5-FU alone for 40 days from the beginning of the treatment, with P values of 0.018 and 0.023, respectively, for the 4- and 8-hr interval. The lower dose of DDATHF (25 mg/kg) also showed a statistically significant inhibition of tumor growth (P values of 0.041 and 0.047 for the 4- and the 8-hr interval) compared with 5-FU alone. The same doses of DDATHF (25 and 37.5 mg/kg) administered 24 hr prior to 5-FU did not enhance significantly the tumor growth inhibition of 5-FU. DDATHF used as a single agent at these doses, given on a weekly basis, did not have any antiproliferative effect compared with a control group. The addition of low doses of DDATHF to 5-FU did not increase significantly the toxicity (weight loss) of the regimens compared with 5-FU alone. A 50 mg/kg dose of DDATHF administered 4 hr before 5-FU (85 mg/kg) also resulted in the potentiation of the antitumor effect but coincided with an elevation of the cumulative toxicity, with 40% toxic deaths and 25% weight loss in this group compared with only 10% ($P = 0.048$) for the group treated with 5-FU alone (data not shown). The concurrent administration of DDATHF and 5-FU did not result in any therapeutic advantage but led to an increased host toxicity (data not shown).

The modulatory effect of DDATHF, with its inhibition of *de novo* purine synthesis and selective expansion of PRPP pools in colon 38 tumor, resulted in the formation of higher levels of 5-FU nucleotides in tumor and liver but not in gut (Fig. 5A). These differences in nucleotide levels were reflected in changes in 5-FU incorporation into RNA, which were also significantly higher in colon 38 tumor and liver (Fig. 5B).

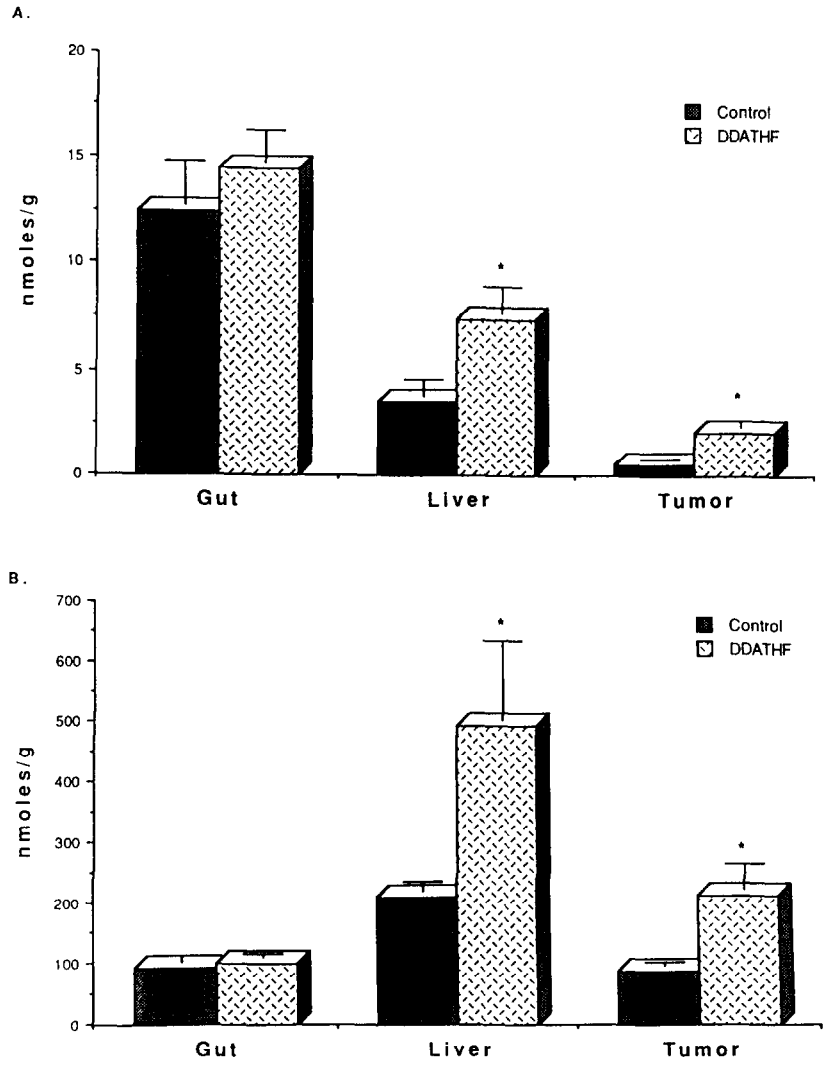

Fig. 5. Effect of DDATHF on 5-FU incorporation into RNA of normal tissues and colon 38 tumor *in vivo*. C57/BL6 mice bearing advanced colon 38 tumor were injected with DDATHF (50 mg/kg) 4 hr prior to [6-3H]5-FU (85 mg/kg, 25 μCi/mouse). Two hours after 5-FU administration, the animals were killed and normal and neoplastic tissues were removed rapidly. The tissues samples were then processed as described in Materials and Methods. Values are the means ± SD of three animals per group. In panel A is reported the formation of 5-FU nucleotides and in panel B their incorporation into RNA.
Key: (*) statistically significant at 95%, according to both Fisher PLSD and Scheffer F-test.

DISCUSSION

Modulation of the levels of PRPP has been utilized in the past as a way to improve the antiproliferative activity of 5-FU MTX, because of its inhibitory activity on *de novo* purine synthesis, secondary to an effect on the formation of dTMP, has been shown to increase PRPP concentration. Several authors have described *in vitro* and *in vivo* systems that exploit this fact to increase the incorporation of 5-FU into RNA and augment its cytotoxic effect [5–10].

This approach has been utilized in several clinical trials mostly for the treatment of gastrointestinal malignancies. The results of the combination MTX–5-FU have been positive overall, with an increased response rate compared with regimens with 5-FU alone. These results have been particularly encouraging in the treatment of colorectal stomach cancers [12]. The critical factor appears to be the interval of administration between the two agents. In the most recent study, which uses "leucovorin rescue" to minimize the toxic effect of the combination, a 24 hr interval was reported to be superior to a 1-hr interval [13].

Recently, a new class of antifolates whose primary effect is the inhibition of *de novo* purine synthesis has been introduced. DDATHF is a close analog of tetrahydrofolic acid in which nitrogens at the 5 and 10 positions have been replaced by carbon. These

substitutions render it impossible for DDATHF to participate in one-carbon transfer reactions or the interconversions of folate metabolism. DDATHF exerts its inhibitory activity on *de novo* purine synthesis by inhibiting GAR and AICAR transformylases. Despite its similarity to tetrahydrofolic acid, DDATHF inhibits *de novo* purine synthesis directly and does not cause any alteration in the main reduced folate pools.

In this report, we have described the effect of DDATHF on PRPP pools in mice bearing colon 38 tumor. DDATHF at a non-therapeutic dose of 50 mg/kg caused a depletion in tissue purine pools, which correlated with an increase in PRPP concentration. In the case of colon 38 tumor, this effect was sustained for more than 12 hr and in liver and colon 38 tumor it resulted in an increased incorporation of 5-FU into RNA.

Our results indicate that despite a more than 24 hr reduction in the purine levels (ATP and GTP), the tissue concentration of PRPP in tumors returns to the control value 12–16 hr after the drug administration. This apparent lack of correlation could be ascribed to a feedback inhibition on the activity of PRPP synthetase or to an imbalance in the levels of the precursors of PRPP, ATP and ribose-5-phospate, that could lead to a reduction of the synthetic process.

The combination of DDATHF and 5-FU exhibited an improved antitumor effect compared with 5-FU alone, without a significant increase in toxicity. The lack of additional toxicity suggests that tissue specific effects at the purine nucleotide level were translated into therapeutic advantage. The antitumor effect was achieved using low non-therapeutic doses of DDATHF (25 and 37.5 mg/kg) with a standard MTD dose of 5-FU for this murine model (85 mg/kg).

Such results suggest that DDATHF would be a better modulator of 5-FU activity than MTX. MTX is primarily an inhibitor of dihydrofolate reductase, and its effect on *de novo* purine synthesis is modest and secondary to its conversion to polyglutamate forms. DDATHF appears to be a specific inhibitor of purine biosynthesis and is, therefore, more effective at lower concentrations than MTX, which may allow it to avoid affecting other targets and causing toxicity to the host.

Furthermore DDATHF has been shown to be a better substrate than MTX for folypolyglutamate synthase, the enzyme necessary for polyglutamate formation [20]. This characteristic may account for the apparent selectivity of the DDATHF/5-FU combination. Previous reports have shown that, at least in murine models responsive to antifolate chemotherapy, there is a significant difference in the capacities of tumor and normal proliferative tissues to accumulate and retain polyglutamates of DDATHF [22]. Normal proliferative tissues are usually the site of limiting host toxicity. Our data reveal that the gut, which is one of the major targets for both 5-FU and antifolate toxicity, was affected minimally by DDATHF modulation and did not show any increased accumulation of 5-FU nucleotides into RNA. It is difficult to explain the different inhibitory effects of DDATHF on *de novo* purine synthesis in the liver and in colon 38 tumor, given the similar kinetic characteristics of GAR transformylase from each tissue and the fact that accumulation of DDATHF and its polyglutamates was significantly superior in the liver. One possible explanation could be the different size of the reduced folate pools that compete with DDATHF for binding to its target enzymes.

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REFERENCES

1. Beardsley GP, Moroson BA, Taylor EC and Moran RG, A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate, is a potent inhibitor of *de novo* purine synthesis. *J Biol Chem* **264**: 328–333, 1989.
2. Beardsley GP, Taylor EC, Grindey GB and Moran RG, Deaza derivatives of tetrahydrofolic acid: A new class of folate antimetabolite. In: *The Chemistry and Biology of Pteridines* (Eds. Whitehead VM and Cooper BA), pp. 953–957. Walter de Gruyter, New York, 1986.
3. Fox IH and Kelley WN, Human phosphoribosylpyrophosphate synthetase. Kinetic mechanism and end product inhibition. *J Biol Chem* **247**: 2126–2131, 1972.
4. Becker MA, Raivio KO and Seegmiller JE, Synthesis of phosphoribosylpyrophosphate in mammalian cells. *Adv Enzymol Relat Areas Mol Biol* **49**: 281–307, 1979.
5. Bertino JR, Sawicki WL, Lindquist CA and Gupte VS, Schedule-dependent antitumor effects of methotrexate and 5-fluorouracil. *Cancer Res* **37**: 327–328, 1977.
6. Cadman E, Heimer R and Davis L, Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: Explanation for drug synergism. *Science* **205**: 1135–1137, 1979.
7. Benz C, Schoenberg M, Choti M and Cadman E, Schedule-dependent cytoxicity of methotrexate and 5-FU in human colon and breast tumor cell lines. *J Clin Invest* **66**: 1162–1165, 1980.
8. Benz C and Cadman E, Modulation of 5-fluorouracil metabolism and cytoxicity by antimetabolite pretreatment in human colorectal adenocarcinoma HCT-8. *Cancer Res* **41**: 994–999, 1981.
9. Cadman E, Heimer R and Benz C, The influence of methotrexate pretreatment on 5-fluorouracil metabolism in L1210 cells. *J Biol Chem* **256**: 1695–1704, 1981.
10. Houghton JA, Tice AJ and Houghton PJ, The selectivity of action of methotrexate in combination with 5-fluorouracil in xenografts of human colon adenocarcinomas. *Mol Pharmacol* **22**: 771–778, 1982.
11. Kufe DW and Major PP, 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *J Biol Chem* **256**: 9802–9805, 1981.
12. Nordic Gastrointestinal Tumor Adjuvant Treatment Group, Superiority of sequential methotrexate, fluorouracil and leucovorin to fluorouracil alone in advanced symptomatic colorectal carcinoma. *J Clin Oncol* **7**: 1437–1446, 1989.
13. Marsh JC, Bertino JR, Katz KH, Davis CA, Durivage HJ, Rome LS, Richards F, Capizzi RL, Farber LR, Pasquale DN, Stuart R, Koletsky AJ, Makuch R and O'Hollaren K, The influence of drug interval on the effect of methotrexate and fluorouracil in the treatment of advanced colorectal cancer. *J Clin Oncol* **9**: 371–380, 1991.
14. Barnett CJ and Wilson TM, Asymmetric synthesis and absolute configuration of 5,10-dideaza-5,6,7,8-tetrahydropteroic acid and 5,10-dideaza-5,6,7,8-tetra-

hydrofolic acid (DDATHF). In: *The Chemistry and Biology of Pteridines* (Eds. Curtius HC, Ghisla S and Blau N), pp. 102–107. Walter de Gruyter, Berlin, 1989.
15. Cross AD, Morimoto H, Williams PG and Beardsley GP, Catalytic reduction in the synthesis of tritiated 6(*R*) and 6(*S*)-5,10-dideaza-5,6,7,8-tetrahydrofolate. *J Labelled Compd Radiopharm* **29**: 753–763, 1991.
16. Martin DS, Stolfi RL, Sawyer RC, Spiegelman S, Casper ES and Young CW, Therapeutic utility of utilizing low doses of *N*-(phosphonacetyl)-L-aspartic acid in combination with 5-fluorouracil: A murine study with clinical relevance. *Cancer Res* **43**: 2317–2321, 1983.
17. Darnowski JW and Handschumacher RE, Tissue specific enhancement of uridine utilization and 5-fluorouracil therapy in mice by benzylacyclouridine. *Cancer Res* **45**: 5364–5368, 1985.
18. Daubner SC and Benkovic SJ, Characterization of mammalian phosphoribosylglycineamide formyltransferase from transformed cells. *Cancer Res* **45**: 4990–4997, 1985.
19. Rode W, Scanlon KJ, Hynes J and Bertino JR, Purification of mammalian tumor (L1210) thymidylate synthetase by affinity chromatography on stable biospecific adsorbent. Stabilization of the enzyme with neutral detergents. *J Biol Chem* **254**: 11538–11543, 1979.
20. Pizzorno G, Sokoloski JA, Cashmore AR, Moroson BA, Cross AD and Beardsley PG, Intracellular metabolism of 5,10-dideazatetrahydrofolic acid in human leukemia cell lines. *Mol Pharmacol* **39**: 85–89, 1991.
21. Pizzorno G, Moroson BA, Cashmore AR and Beardsley GP, (6*R*)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid effects on nucleotide metabolism in CCRF-CEM human T-lymphoblast leukemia cells. *Cancer Res* **51**: 2291–2295, 1991.
22. Rumberger BG, Barrueco JR and Sirotnak FM, Differing specificities for 4-aminofolate analogues of folylpolyglutamyl synthetase from tumors and proliferative intestinal epithelium of the mouse with significance for selective antitumor action. *Cancer Res* **50**: 4639–4643, 1990.