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# Modulation of Fluorouracil Antitumor Activity by Folic Acid in a Murine Model System

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**ABSTRACT.** The biochemical basis for modulation of fluorouracil (FU) activity by leucovorin is elevation of the metabolite methylenetetrahydrofolate, which stabilizes the inhibitory ternary complex formed between thymidylate synthase and the active metabolite of FU, 5-fluorodeoxyuridylate. Folic acid, because it can also potentially be metabolized to methylenetetrahydrofolate, was evaluated for its ability to potentiate FU antitumor activity in a dietary folic acid restricted murine model. The plasma pharmacokinetics and tissue distribution of folic acid and all stable metabolites thereof were determined in the model to establish administration schedules. FU was administered to mice implanted subcutaneously with a mammary adenocarcinoma 4 hr after folic acid administration, when the metabolites, methylenetetrahydrofolate and tetrahydrofolate, were elevated maximally in both plasma and tumor tissue. While FU alone suppressed growth 25%, folic acid in combination with FU increased growth suppression to over 70%. These results indicate that folic acid is a potent modulator of FU activity and could be considered as an alternative to leucovorin in the clinical setting. BIOCHEM PHARMACOL **58**;5:835–839, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** dietary folate depletion; folic acid; fluorouracil; mammary adenocarcinoma; mouse model

LV† in combination with FU has become an important therapy for the treatment of advanced colorectal and other types of cancer [1–3]. The biochemical basis for this FU/LV potentiation is associated with elevation of the active LV metabolite, $CH_2FH_4$, which stabilizes the inhibitory ternary complex formed between TS and the active metabolite of FU, FdUMP [4, 5]. Since folic acid potentially can also cause elevation of $CH_2FH_4$ [6, 7], the ability of this folate form to modulate FU antitumor activity was investigated. This study was undertaken in mice maintained on a low folic acid diet. Animals maintained on this diet exhibit plasma folate levels 10-fold lower than those of animals maintained on regular chow, which is richly supplemented with folic acid. This level is much closer to that found in humans. And, this diet improves substantially the modulatory response when LV is administered before FU [8].

## MATERIALS AND METHODS

### *Materials*

Folic acid was obtained from Ben Venue Laboratories. C3H mammary adenocarcinoma was obtained from Lilly Research Laboratories and was maintained in C3H mice (Charles River Co.) as described previously [8, 9]. Folic acid-deplete chow (No. 5831C-2) containing 0.01 ppm folic acid and 1% succinylsulfathiazole to deplete intestinal flora were purchased from Purina Mills. [$^3$H]FdUMP was purchased from Moravek Biochemicals. Sephadex G-25 was obtained from Pharmacia. NADPH, ATP, and all other reagents were purchased from the Sigma Chemical Co. TS (4 U/mg protein) was purified from an *Escherichia coli* strain that overproduces *Lactobacillus casei* TS [10]. The *E. coli* strain was a gift from D. Santi (University of California). 5,10-Methylenetetrahydrofolate reductase (0.52 U/mg protein) and 10-formyltetrahydrofolate dehydrogenase (0.2 U/mg protein) were purified from pig liver as described previously [11, 12].

### *Tumor System*

Mice were maintained on a folic acid-deplete diet as previously described and were monitored for weight gain or loss [8]. No difference in weight gain was detected between dietary deficient and control mice maintained on a standard diet over the time the experiments were conducted. Mouse mammary adenocarcinoma was excised from seed mice and implanted subcutaneously as described previously [8, 9]. Implanted tumors were allowed to grow for 10 days before initiation of pharmacokinetic experiments. Folic acid (45 mg/kg) and/or FU (10 mg/kg) were diluted in sterile saline and injected i.p. in a total volume of 0.3 mL/mouse. Tumor size was evaluated with graduated cali-

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† *Abbreviations*: 5-$CHOFH_4$ or LV, 5-formyltetrahydrofolate or leucovorin; FU, fluorouracil; $CH_2FH_4$, 5,10-methylenetetrahydrofolate; $FH_4$, tetrahydrofolate; 5-$CH_3FH_4$, 5-methyltetrahydrofolate; 10-$CHOFH_4$, 10-formyltetrahydrofolate; TS, thymidylate synthase; and FdUMP, fluorodeoxyuridine monophosphate.

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pers, and volume was calculated as length × width × height × 0.5 [8, 13]. Mice were killed in a $CO_2$ chamber to obtain blood, liver, and tumor samples.

### *Plasma and Tissue Preparation*

Whole blood (~600 μL) was collected by cardiac puncture and centrifuged immediately at 400 *g* for 5 min. The plasma obtained was diluted with an equal volume of cold 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA and stored at −70°. Liver and tumor tissue was excised from mice, washed with cold PBS, and stored at −70°. For folate analysis, tissues were homogenized in cold 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA, and centrifuged at 10,000 *g* for 10 min at 4° to remove cell debris. An aliquot of the supernatant was used to assess soluble protein by the method of Bradford [14]. The remainder of the supernatant was placed immediately in a boiling water bath for 5 min and centrifuged to remove precipitated protein. The resultant supernatants were used for folate estimation. Because $CH_2FH_4$ can potentially be dissociated to $FH_4$ and formaldehyde under these conditions, the sum of these folates is reported. Other reference folates were stable under these conditions, with routine recovery in the range of 70–95% [15].

### *Estimation of Reduced Folates*

The ternary complex assay is based upon enzymatic cycling of reduced folates to $CH_2FH_4$ followed by entrapment into a stable ternary complex with excess *L. casei* TS and [$^3$H]FdUMP. Methods have been described previously for estimation of the biologically active stereoisomers of $CH_2FH_4$, $FH_4$, 5-$CH_3FH_4$, and 10-$CHOFH_4$ using this approach [15]. Typically, reaction mixtures contained 20 mU TS and 125 nM [$^3$H]FdUMP (20 Ci/mmol) in 200 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA. Additional enzymes and cofactors were added as necessary to cycle each reduced folate to the $CH_2FH_4$ form. Ternary complex formation was allowed to proceed at 25° for 30 min. Addition of 1% SDS and boiling for 10 min were used to stop reactions. Aliquots (25 μL) were applied to Sephadex G-25 mini columns and eluted by centrifugation to separate tritiated complexes from free [$^3$H]FdUMP. Bound radioactivity was determined by scintillation counting. The practical limit of detection for $CH_2FH_4$ and other folates under these conditions was 7 fmol [15].

### *Estimation of Folate Polyglutamate Chain Length*

The polyglutamate chain length of the tumor $CH_2FH_4$ + $FH_4$ pool was determined by electrophoresis of ternary complexes followed by immunoblot detection with TS antibody as described previously [16]. Briefly, undenatured ternary complexes were electrophoresed on 7% polyacrylamide gels and transferred to Immobilon-P membranes. Membranes that had been preincubated with a blocking solution were incubated with the primary TS antibody, washed, and treated with anti-rabbit horseradish peroxidase-conjugated secondary antibody. Bands were visualized using the enhanced chemiluminescence (ECL) detection kit (Amersham).

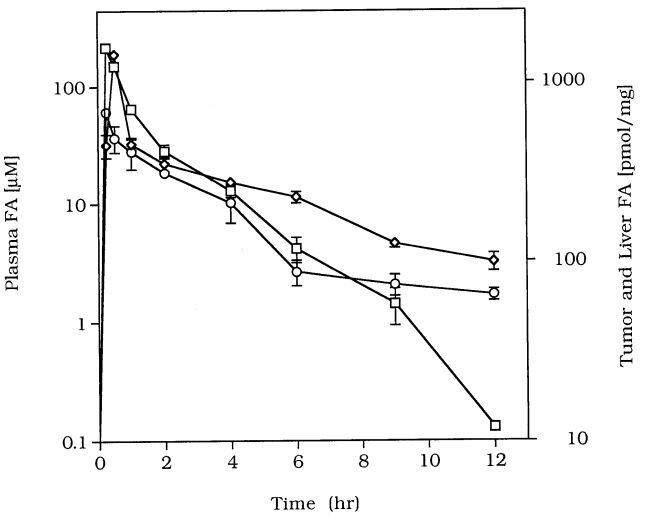


**FIG. 1. Accumulation of parent compound in mouse plasma and tissues after FA administration. FA was estimated by the ternary complex assay in plasma (□), liver (◇), and implanted tumor (○) after i.p. injection of 45 mg/kg of FA. Values represent the means ± SEM of duplicate analyses of tissues from three mice.**

### *Statistical Analysis*

All results are reported as the numerical mean and the associated SEM. Confidence levels for differences between values are based on Student's *t*-test.

## RESULTS

It can be seen in Fig. 1 that following folic acid administration to mice maintained on a folic acid-restricted diet, plasma levels achieved a peak above 200 μM very early. This peak was followed by a rapid decline with an elimination half-life of just over one-half hour (see Table 1). Thus, even though a relatively high plasma accumulation was obtained transiently, the overall AUC over the 12-hr period studied was relatively modest. Liver accumulation of folic acid was also transient and achieved a level of approximately 1400 pmol/mg protein. On the other hand, levels in a mouse mammary tumor implanted subcutaneously prior to folic acid administration achieved only about one-half the level that liver did and exhibited a concomitantly lower AUC. Tumor and liver both had elimination half-lives that were approximately three times longer than plasma.

Plasma as well as tumor and liver pharmacokinetic parameters for folic acid and all of its metabolites are shown in Table 1. Plasma $C_{max}$ and AUC values for the parent compound were substantially greater than for metabolites.

**TABLE 1. Pharmacokinetic parameters for folic acid and its metabolites in mouse plasma, tumor, and liver**

| Folate | $C_{max}$* | $AUC_{0-12\ hr}$* | Elimination $T_{1/2}$ (hr) | Time to peak (min) |
|---|---|---|---|---|
| Folic acid | | | | |
| Plasma | 214.0 ± 12.0 | 241.0 ± 6.0 | 0.6 ± 0.1 | 15 |
| Tumor | 676.0 ± 30.0 | 2,300.0 ± 302.0 | 1.8 ± 0.1 | 15 |
| Liver | 1,430.0 ± 42.0 | 3,400.0 ± 217.0 | 1.8 ± 0.1 | 30 |
| $CH_2FH_4$ + $FH_4$ | | | | |
| Plasma | 2.4 ± 0.3 | 12.6 ± 0.4 | 2.1 ± 0.3 | 240 |
| Tumor | 60.0 ± 11.0 | 580.0 ± 31.0 | 25.66† | 240 |
| Liver | 1,031.0 ± 64.0 | 6,479.0 ± 270.0 | 4.9 ± 0.4 | 120 |
| 5-$CH_3FH_4$ | | | | |
| Plasma | 2.1 ± 0.2 | 9.3 ± 0.5 | 2.0 ± 0.2 | 240 |
| Tumor | 100 ± 4 | 946.0 ± 14.0 | 23.1† | 240 |
| Liver | 389 ± 134 | 2,358.0 ± 99.0 | 3.9 ± 0.4 | 60 |
| 10-$CHOFH_4$ | | | | |
| Plasma | 0.7 ± 0.1 | 4.4 ± 0.4 | 2.1 ± 0.1 | 120 |
| Tumor | 25.0 ± 4.0 | 205.0 ± 9.0 | 8.4 ± 1.2 | 120 |
| Liver | 525.0 ± 124.0 | 3,084.0 ± 408.0 | 3.1 ± 0.1 | 120 |
| Total | | | | |
| Plasma | 239.0 ± 12.0 | 291.0 ± 6.4 | 1.1 ± 0.1 | 15 |
| Tumor | 825.0 ± 20.0 | 4,106.0 ± 191.0 | 2.9 ± 0.2 | 15 |
| Liver | 2,475.0 ± 42.0 | 16,027.0 ± 506.0 | 4.3 ± 0.7 | 30 |

Values represent the means ± SEM from three mice.

*AUC and $C_{max}$ values for plasma are expressed as μM · hr and μM, respectively. AUC and $C_{max}$ values for tumor and liver are expressed as pmol · hr/mg protein and pmol/mg protein, respectively.

†Value represents one determination.

Nevertheless, there was marked elevation of the FU modulatory pool $CH_2FH_4$ + $FH_4$. In all cases, $C_{max}$ and AUC values for metabolites exhibited a much greater differential between liver and tumor than did the parent compound, folic acid. Clearly, liver is more effective than tumor in metabolizing folic acid.

While elimination half-lives for metabolite pools that are being formed as well as eliminated represent complex functions, there appeared to be a substantially delayed loss of each reduced folate pool from tumor compared with liver. And, in all cases, elimination half-lives were substantially greater from tissues than observed in plasma. Further, the time required to achieve peak concentration was substantially longer for each metabolite pool than for the parent compound in both plasma and tissues. Interestingly, peak accumulation times were identical for plasma and tumor for all three reduced folate pools, whereas liver achieved peak levels at a somewhat shorter time than plasma for both the $CH_2FH_4$ + $FH_4$ pool and the 5-$CH_3FH_4$ pool. In fact, the latter pool exhibited a time to peak in liver that was only about one-half that of $CH_2FH_4$ + $FH_4$.

Because the $CH_2FH_4$ + $FH_4$ pool is most closely related mechanistically with FU activity, and pharmacokinetic parameters alone cannot describe entirely the temporal profile, the time course for accumulation of this pool in plasma, tumor, and liver is shown in Fig. 2. While tumor tissue elevation was some 18-fold less than the maximum accumulation in liver, the level that was achieved was 4-fold above basal levels. Further, this elevation was maintained in a relatively constant manner over the 4- to 12-hr time period evaluated. And, it is this elevation from basal levels that most likely provides stabilization of the inhibitory ternary complex formed between thymidylate synthase and the active metabolite of FU.

To examine the modulation of FU antitumor activity by folic acid, the impact on growth of a subcutaneously implanted mammary tumor was determined. A dietary folic acid restricted mouse model system was used that had been demonstrated previously to yield a greater responsiveness to LV modulation of FU activity [8]. Likewise, an FU dose of 10 mg/kg was chosen based on optimization for LV modulation in these earlier studies, and the folic acid dose was

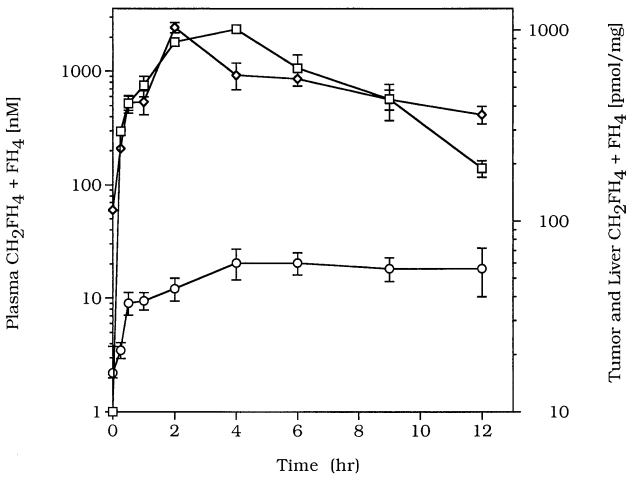


**FIG. 2. $CH_2FH_4$ + $FH_4$ accumulation in mouse plasma and tissues after FA administration. $CH_2FH_4$ + $FH_4$ was estimated by the ternary complex assay in plasma (□), liver (◇), and implanted tumor (○) after i.p. injection of 45 mg/kg of FA. Values represent means ± SEM from three mice.**

**TABLE 2. Folic acid potentiation of FU antitumor activity**

| FA (mg/kg) | FU (mg/kg) | Tumor growth ($mm^3$) |
|---|---|---|
| 0 | 0 | 920 ± 69 |
| 0 | 10 | 694 ± 55 |
| 45 | 10 | 259 ± 16 |
| 45 | 0 | 1829 ± 218 |

Mammary tumors were implanted s.c. into mice maintained on a folic acid deplete diet for 2 weeks. One week later, FU and/or FA were administered i.p. daily for 9 days. On day 10, tumor volume was estimated and subtracted from estimates at the initiation of drug treatment. Values represent the means ± SEM from five mice.

equivalent to the dose of active LV isomer. It can be seen in Table 2 that administration of 10 mg/kg FU alone inhibited tumor growth by approximately 25% ($P = 0.02$). When folic acid was administered 4 hr prior to FU, a time when maximum accumulation of $CH_2FH_4$ + $FH_4$ had been achieved, tumor growth suppression was over 70% ($P = 0.001$). Hence, in this model system, substantial modulation of FU antitumor activity was achieved with folic acid. Because of the potential that folic acid alone could enhance tumor growth, folic acid was also administered without the subsequent administration of FU. It can be seen that under these conditions folic acid stimulated tumor growth by approximately 100%.

An additional consideration in modulation of FU antitumor activity is the polyglutamate status of the folate pool. Hence, polyglutamate status of the $CH_2FH_4$ + $FH_4$ pool in tumor tissue was examined by the ternary complex assay. It can be seen in Fig. 3 that 4 hr after folic acid administration this pool existed almost totally in the monoglutamate form. Even the pre-existing pool, which was polyglutamylated to the 5- and 6-glutamate level prior to folic acid administration, apparently experienced a loss of γ-glutamates as a result of folic acid exposure. The $CH_2FH_4$ + $FH_4$ content in tumor prior to folic acid administration was 16 pmol/mg. And, at the 4-hr time point where polyglutamate status was evaluated, it had achieved a level of 60 pmol/mg. Hence, the pre-existing pool would potentially represent a readily detectable 25% of the total at this point in time.

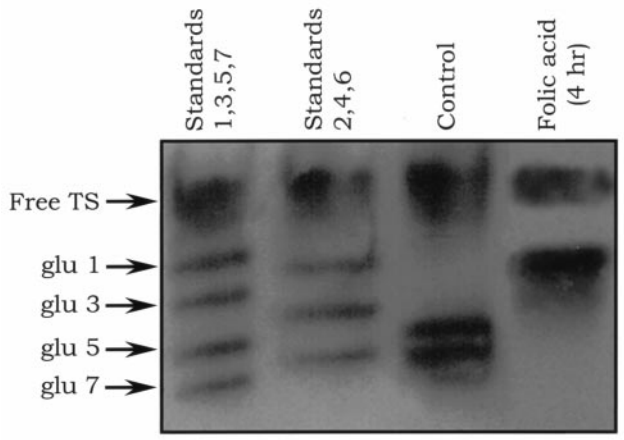


**FIG. 3. Tumor $CH_2FH_4$ + $FH_4$ polyglutamate status after FA administration. Reduced folates were extracted from tumors 4 hr after i.p. administration of 45 mg/kg of FA. Polyglutamate chain length was estimated by immunoblot analysis after electrophoretic separation of ternary complexes.**

## DISCUSSION

Folic acid, when administered to humans, yields the same plasma metabolites as the more traditional modulatory folate LV [7]. Because of difficulties in sampling, however, evaluation of folic acid and its metabolites in human tumor tissue has been hard to achieve. Hence, a mouse model system was used to examine tumor accumulation of folates after folic acid administration and to test the concept that folic acid can modulate FU activity.

Previous studies have shown that folic acid metabolites are not elevated as rapidly as LV metabolites in human plasma, but they remain elevated substantially longer [7]. Likewise, after folic acid administration to mice, there is a substantial elevation of the parent compound in plasma that declines with an elimination half-life of slightly more than 30 min. This relatively extended elimination half-life, compared with that of LV (15 min), contributes to the elevation of folic acid metabolites over a longer period than LV metabolites.

Some differences in the ratio of individual metabolites to each other occur when folic acid is compared with LV. For example, when the $CH_2FH_4$ + $FH_4$ pool was compared with the 5-$CH_3FH_4$ pool, a substantially greater relative elevation of the former was observed after folic acid compared with LV [9]. This same general behavior in relative accumulation of metabolites also has been observed in human plasma [7]. However, metabolite accumulation and persistence are delayed in humans compared with mice.

Of particular interest to FU modulation is the time course for accumulation of the $CH_2FH_4$ + $FH_4$ pool in tumor compared with plasma. In the mouse, peak accumulation occurred simultaneously in these two compartments. And, by analogy, peak accumulation of this modulatory pool in human tumor is likely to occur at or near the time of maximum accumulation in human plasma. While direct assessment would be preferable, the impracticality of systematic tumor sampling to evaluate folates in human tissues makes this problematic. Hence, until such time as direct assessment is accomplished, this model system suggests that the most appropriate time for FU administration is when human plasma $CH_2FH_4$ + $FH_4$ is elevated maximally.

It is of interest that folic acid, used in this study in the same model system and at the some dose as in the previous LV study, caused growth suppression to essentially the same extent as LV, the commonly used FU modulatory agent. That is, folic acid at 45 mg/kg caused 72% growth suppression, while LV at 90 mg/kg, but with only the [S] isomer active, caused 79% growth suppression. Hence, in this system folic acid is equivalent to LV as an FU modulating agent, most likely because folic acid can yield the same metabolites as LV, including the active modulatory metabolite $CH_2FH_4$. Of further interest, and potentially some

concern, is the fact that administration of folic acid alone, as was true for LV alone, caused the tumor growth rate to nearly double. Hence, the presence of folate metabolites in the absence of FU, which has a very short half-life, could be deleterious.

It has been shown that polyglutamate forms of $CH_2FH_4$ can stabilize the inhibitory ternary complex, TS–FdUMP–$CH_2FH_4$, better than monoglutamates *in vitro* [17]. However, monoglutamate forms are also effective stabilizing agents, albeit with somewhat lower efficiency. Results reported here indicate that the intratumor $CH_2FH_4$ + $FH_4$ pool was entirely in the monoglutamate form at a time when substantial modulation was obtained (4 hr). Thus, as was shown previously for LV in this same system, polyglutamylation is not essential to obtain effective modulation of FU [8].

The fact that polyglutamate forms of folate could not be detected at all 4 hr after folic acid administration is of interest. As was pointed out in previous studies with LV, the absence of detectable polyglutamates is not the result of new monoglutamates masking the pre-existing polyglutamate pool. This pool is a sufficiently large fraction of the post-administration total to be easily detectable. It is probable that the pre-existing pool was hydrolyzed to monoglutamate forms. Previous studies have shown that methotrexate can enhance glutamyl hydrolase activity in intraperitoneally growing ascites cells [18], but it is not clear that folates can exert the same effect.

It was one objective of this study to provide insight into the relative contribution of intratumor versus systemic metabolism of parent compound to yield active metabolites for modulation. However, because of the complex nature of the multiple metabolic sites in this *in vivo* system, and because of the potential linkages between folate metabolizing enzymes, evaluation of the precise contribution of each is a formidable problem. Even so, one seeming anomaly can be pointed out. It was anticipated that the general metabolic flow from folic acid would be: FA → $FH_4$ → $CH_2FH_4$ → 5-$CH_3FH_4$ and 10-$CHOFH_4$. Yet, the 5-$CH_3FH_4$ pool achieves $C_{max}$ in liver early and declines substantially while other metabolite pools continue to be elevated. The decline of this pool in liver no doubt results from both efflux into the circulatory system and metabolism. However, it is somewhat of an enigma why this pool would accumulate beyond the level that yields maximal efflux and metabolism, prior to other pools. There is a possibility that total folate capacity has been exceeded and, as other pools become elevated, the 5-$CH_3FH_4$ pool is effluxed or metabolized preferentially. Future studies will address this issue.

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