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LEUCOVORIN AND FOLIC ACID REGIMENS FOR SELECTIVE EXPANSION OF MURINE 5,10-METHYLENETETRAHYDROFOLATE POOLS

JOEL E. WRIGHT,*†‡ MARIA PARDO,* UMER SAYEED-SHAH,* WENDY ALPERIN* and ANDRE ROSOWSKY*†

*Dana-Farber Cancer Institute and †Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, U.S.A.

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Abstract—Mice bearing subcutaneously implanted EMT6 mammary adenocarcinoma were treated with leucovorin or folic acid by continuous subcutaneous infusion or bolus intraperitoneal injection. (6*R*)-5,10-Methylenetetrahydrofolate pools in cytosolic extracts of the tumor, marrow, and gut were measured by analysis of the ternary complex with thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP C-methyltransferase, EC 2.1.1.45) and 5-fluoro-2′-deoxyuridylate, and the polyglutamate distribution in the (6*R*)-5,10-methylenetetrahydrofolate pool was analyzed by native gel electrophoresis. Bolus intraperitoneal administration of either leucovorin or folic acid caused dose-dependent expansion of the (6*R*)-5,10-methylenetetrahydrofolate pool in the tumor, but not in the marrow or gut. For example, the AUC (0–5 hr) in the tumor increased from a baseline value of 8.2 to 20 nmol/mg protein·hr after a bolus dose of 1.5 mmol/kg of leucovorin or folic acid, whereas the increase in marrow and gut was 2- to 4-fold lower. Continuous subcutaneous infusion at the same total dosage over 3 days gave AUC (0–96 hr) values of 134 nmol/mg protein·hr for controls as compared with 347 nmol/mg protein·hr for the leucovorin group and 254 nmol/mg protein·hr for the folic acid group. In contrast to bolus treatment, the increase in (6*R*)-5,10-methylenetetrahydrofolate in the marrow and small intestine with both leucovorin and folic acid infusion was similar to the increase in the tumor. Thus, intraperitoneal bolus injection was tumor selective, but subcutaneous continuous infusion was not. Longer-chain polyglutamates of (6*R*)-5,10-methylenetetrahydrofolate in the tumor after bolus treatment with 0.375 and 0.75 mmol/kg of leucovorin or folic acid increased relative to controls. At higher doses of 1.5 and 2.25 mmol/kg, an increase was observed only in the mono/diglutamate fraction. In marrow, on the other hand, the mono/diglutamate fraction, but not the longer-chain polyglutamates, increased at all doses. In the constant infusion regimen, longer-chain polyglutamates increased in all three tissues, though in gut and marrow the mono/diglutamate fraction increased more than in tumor. Leucovorin and folic acid were converted to (6*R*)-5,10-methylenetetrahydrofolate more efficiently but less selectively during a 3-day subcutaneous infusion than after an intraperitoneal bolus. Longer-chain polyglutamates were selectively increased in tumor by both regimens of leucovorin administration.

Key words: 5,10-methylenetetrahydrofolate; leucovorin; folic acid; polyglutamylation; EMT6 mammary adenocarcinoma; drug modulation

Preclinical [1–9] and clinical [10–13] studies have demonstrated that LV§ can enhance anticancer activity when administered prior to, and during, fluoropyrimidine therapy. Mechanistic studies have shown that LV is metabolized *in vivo* to CH_2THF, the one-carbon source for TS-catalyzed synthesis of dTMP from dUMP. The enzymatic pathway from LV to CH_2THF involves ring closure by 5-formyltetrahydrofolate cyclodehydrase (EC 6.3.3.2) to form 5,10-methylenetetrahydrofolate, and reduction of the latter by 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) [14]. Part of the 5,10-methenyl compound also undergoes ring opening to 10-formyltetrahydrofolate, which, in turn, supplies carbons 2 and 8 of the purine nucleus [14].

The cytotoxicity of fluoropyrimidine anticancer agents depends upon their intracellular metabolism, which yields a number of mono-, di- and triphosphorylated nucleotide analogs [15]. The triphosphates may form cytotoxic lesions by misincorporation into DNA and RNA. The molecular target of the monophosphate analog, FdUMP, is the active site of TS, where alkylation of dUMP by CH_2THF yields dTMP [15]. Cycling cells in tumors and other rapidly proliferating tissues depend upon this unique *de novo* step to support DNA synthesis [16]. Alkylation of FdUMP by CH_2THF also takes place in the active site of TS; however, instead of releasing a product, the enzyme, inhibitor, and cofactor form a ternary covalent

‡ Corresponding author: Dr. J. E. Wright, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Tel. (617) 632–3123; FAX (617) 632–2410.

§ Abbreviations: LV, leucovorin; CH_2THF, (6*R*)-5,10-methylene-5,6,7,8-tetrahydrofolate (irrespective of Glu chain length); $CH_2H_4PteGlu_n$, (6*R*)-5,10-methylene-5,6,7,8-tetrahydrofolates (specific Glu_{1-7} species); FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridylic acid; DHFR, dihydrofolate reductase, 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3; and TS, thymidylate synthase, 5,10-methylenetetrahydrofolate: dUMP C-methyltransferase, EC 2.1.1.45.

complex whose dissociation is nearly, though not completely, irreversible [17]. The cytotoxicity of FdUMP is enhanced by high concentrations of cytosolic CH_2THF, which result in greater affinity of the nucleotide for TS and, therefore, an increase in the stability of the ternary complex [1–9].

Efficient competition by FdUMP for TS causes rapid expansion of the endogenous dUMP pool to levels several orders of magnitude higher than normal [18, 19]. The affinities of dUMP and FdUMP for TS are similar; thus, quantitative inhibition of TS by binary complexation with excess FdUMP may be competitively overcome at high dUMP levels [20]. In view of this, stabilization of the complex via formation of a covalent bond between CH_2THF and FdUMP is essential for long-lasting dTMP depletion.

Modulation of the antitumor activity of fluoropyrimidines has been observed in the laboratory [21] and tested in the clinic [22] not only with LV but also with folic acid. However, a significant difference between LV and folic acid is that the latter is converted to CH_2THF by DHFR (EC 1.5.1.3) and serine hydroxymethyltransferase (EC 2.1.2.1) rather than by 5-formyltetrahydrofolate cyclodehydrase and 5,10-methylenetetrahydrofolate reductase [14]. These metabolic differences and the recognition that, in contrast to folic acid, only the (6*S*)-diastereomer of LV can participate as a substrate in the folate pathway suggested the possibility that folic acid might be an advantageous, less costly [22] alternative to LV.

A useful *in vivo* model used earlier in our laboratory to study fluoropyrimidine modulation is the EMT6 mammary tumor in BALB/c mice. With this system, we showed that $CH_2H_4PteGlu_n$ pools could be selectively expanded by giving LV as a series of 8 bolus i.p. injections over 48 hr [23]. Other investigators working with mice found that for a 2-hr infusion the conversion of LV to 5-methyltetrahydrofolate is independent of dose, whereas a 2-day continuous infusion gives a dose-dependent increase of the metabolite [24]. In a study with humans, modulation of FU by bolus LV was improved when the dose was divided [25]. In another study, also with human subjects, a 2-fold greater AUC for 5-methyltetrahydrofolate was achieved when the latter was given by intravenous than by oral administration [26]. These studies indicated that the effect on CH_2THF pools might depend on whether LV was administered as a constant infusion (low, sustained levels of LV) or a bolus (high, transient peak levels of LV). We wished to see if these effects were tissue selective and whether they also applied when folic acid was used as the modulator. Therefore, we monitored the CH_2THF AUC in EMT6 tumor, bone marrow, and gut of BALB/c mice after administering LV or folic acid as a single i.p. bolus dose or a 3-day continuous s.c. infusion.

The stability of the ternary complex between FdUMP, CH_2THF and TS increases with increasing γ-polyglutamate chain length [27]. Therefore, the efficacy and selectivity of fluoropyrimidine modulation by LV *in vivo* may be expected to vary according to the relative ability of a particular tumor to form long-chain γ-polyglutamates in comparison with non-tumor tissues. Accordingly, the time-dependent distribution of $CH_2H_4PteGlu_n$ species in tumor, marrow, and gut during and after continuous s.c. infusion and their dose-dependent distribution after i.p. bolus injection of LV were also analyzed.

MATERIALS AND METHODS

Reagents. Magnesium (6*R*)-N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate tetrahydrate was purchased from SAPEC S.A., Lugano, Switzerland, for use as an analytical standard. (6*R*)-N^5,N^{10}-Methylene-5,6,7,8-tetrahydropteroyl di- through heptaglutamic acids were synthesized as analytical standards by the method of Priest and Doig [28], starting with the corresponding folic acid oligoglutamates (Dr. B. Schircks Laboratories, Jona, Switzerland). Disodium LV was prepared by passage of 1 g of calcium LV containing 0.8 g of NaCl (Lederle Parenterals, Carolina, Puerto Rico) through a 20 mm i.d. × 240 mm long column of Dowex 50W-X8, 20–50 mesh, Na^+ form. The product was lyophilized for 48 hr, and the resulting solid was characterized by elemental analysis ($Na_2C_{20}H_{21}N_7O_7 \cdot 3H_2O \cdot 6.5NaCl$, mol. wt 951.13). Disodium folate was prepared from folic acid (Sigma Chemical Co., St. Louis, MO) and 2 equivalents of NaOH. [^{3}H]FdUMP was obtained from Moravek Biochemicals, Brea, CA, and its purity was checked every 3 months by HPLC. Recombinant *Lactobacillus casei* TS was extracted from a 1-L fermentation of χ 2913 *Escherichia coli* transfected with the pkPTS-3 plasmid [29]. This vector expresses TS as 30% of its total cellular protein under control of the *lac* promoter. The extracted TS was purified to SDS electrophoretic homogeneity [30] by phosphocellulose and hydroxylapatite column chromatography. The *E. coli* TS vector and purification procedure were provided by Drs. P. J. Green and D. V. Santi, University of California, San Francisco.

Tumor transplants. Suspensions of 2×10^6 trypan blue excluding EMT6 tumor cells were implanted subcutaneously in the right flanks of female BALB/c mice weighing approximately 25 g. After 18 days, the orthogonal diameters of the tumors were approximately 6 mm × 6 mm by external caliper measurement. Treatment began at this time.

Treatment. For bolus studies, EMT6 tumor-bearing mice received LV (48 mice) or folic acid (48 mice) by i.p. injection at 0.38, 0.75, 1.50 or 2.25 mmol/kg. Two mice from each group were killed 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 hr after injection. Their tumors, the bone marrow from both tibias and fibulas, and 15-cm lengths of small intestine (including duodenum) were harvested and frozen at −85°. For determination of the distribution of individual $CH_2H_4PteGlu_n$ species, the tissues harvested 2 hr after injection were used. Eight tumor-bearing controls received 0.9% sodium chloride as an i.p. bolus prior to determination of baseline levels of total and individual $CH_2H_4PteGlu_n$ species in their tumors, marrow and gut at the time of injection (4 mice) or 5 hr later (4 mice).

For infusion studies, EMT6 tumor bearing mice received LV (10 mice) or folic acid (10 mice) as the disodium salts at a daily dose rate of 0.5 mmol/

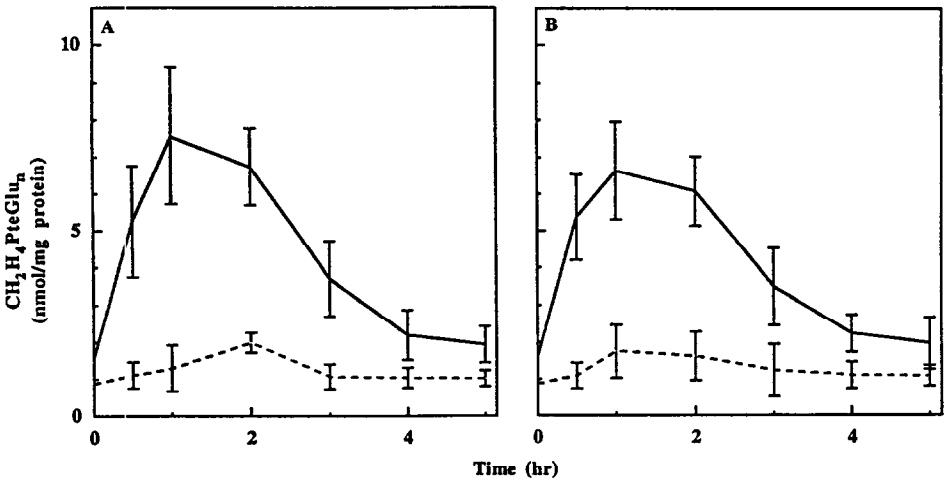

Fig. 1. Tumor (solid lines) and marrow (dotted lines) levels of total CH_2THF versus time after i.p. bolus injection of 1.5 mmol/kg of LV (A) or folic acid (B). Gut levels (not shown) were similar to marrow. Bars: range of 2 replicates.

kg·day from subcutaneously implanted Alzet model 1003D osmotic pumps (Alza Corp., Palo Alto, CA). Controls were treated by sham surgery (4 mice) or 0.9% sodium chloride (4 mice) by continuous 3-day infusion. Sham controls were killed immediately. Two mice from each treated group were killed after 24, 48 and 72 hr. Pumps were removed from the remaining mice at 72 hr, and two from each group were killed at 78 and 96 hr for tissue harvest as described above. Saline-treated controls were all killed at 96 hr.

Tissue assays. For determination of total CH_2THF derived from LV or folic acid, 10- to 20-mg samples of tumor, marrow or gut were extracted in a Dounce homogenizer with 0.5 mL of 50 mM Tris base, 50 mM sodium ascorbate, 1 mM EDTA and 250 mM sucrose adjusted to pH 7.5 with HCl (extraction buffer), as described previously [28].

Extracts were analyzed for CH_2THF content by ternary complex assay [31] with limiting quantities of tumor, bone marrow or gut samples, and several concentrations of the CH_2THF standards were incubated at 22° with excess [^{3}H]FdUMP and a *large excess* of *L. casei* TS for 45 min. The total volume was 100 μL. After boiling with 1% SDS to stabilize the complex, an aliquot was counted to determine total radioactivity, and an equal volume was chromatographed on a Sephadex G25–150 spin column to remove non-bound radioactivity. The fraction bound at each concentration of standard CH_2THF was then plotted. From this calibration curve, CH_2THF concentrations in the analytical samples were calculated. Values were expressed in proportion to the protein content of an equal aliquot, determined by the Bradford assay [32]. Four repetitions of each assay were performed on different days.

Tissue CH_2THF levels at all time points after injection or infusion of LV or folic acid were calculated. These values were plotted as a function of time, and the area under the curve (AUC) was calculated by the trapezoidal method. Comparisons between tumor, gut and marrow CH_2THF pools over 0–5 hr for bolus and 0–96 hr for infusion regimens were analyzed, and their significance was determined in PROC GLM (SAS Institute, Carey, NC) with time expressed as a cubic polynomial.

For measurement of individual $CH_2H_4PteGlu_n$ species derived from LV, 200- to 300-mg samples of tumor or 500- to 600-mg samples of gut and a 0.30-mL portion of Tris-ascorbate extraction buffer (adjusted to pH 8.5 in order to inhibit polyglutamate hydrolase activity) were frozen in the cavity of a liquid nitrogen-cooled Bessmer pulverizer (Spectrum Industries, Houston, TX), and the mixture was crushed to a fine powder. The work was performed in a glove bag filled with dry nitrogen to keep frost out of the sample. The powder was thawed in a microcentrifuge tube at 0–4° and immediately centrifuged for 1 min in an Ependorff model 5415C apparatus. The supernatant was dispensed in equal volumes into six conical 1.5-mL freezing tubes (Scientific Plastics, Ocala, FL), which were quickly frozen on dry ice and stored at −85°.

Tissue extracts from LV-treated mice were incubated with excess [^{3}H]FdUMP and TS to form ternary complexes of their total $CH_2H_4PteGlu_n$. However, no SDS was added, and the complexes were not boiled or passed through the Sephadex spin columns. Instead, they were separated according to glutamyl chain length by native gel electrophoresis on vertical 1.5 mm thick × 140 mm wide × 320 mm long polyacrylamide slabs according to a published procedure [28]. Autoradiograms prepared from the gels were quantitated by scanning densitometry on a model GS 300 instrument (Hoefer Scientific Instruments, San Francisco, CA). The densitometer was interfaced to a Macintosh IIvx computer via a Hoefer Datalogger analog-to-digital converter, and the tracings were integrated with the aid of a Hoefer GS370 storage and analysis program. Because the di- and heptaglutamate tracings were difficult to

Table 1. Tissue CH_2THF AUC (0–5 hr) after i.p. bolus leucovorin and folic acid

Dose (mmol/kg)	CH_2THF (nmol/mg protein·hr*)		
	Tumor	Gut	Marrow
Controls†	8.2 ± 1.0	5.2 ± 0.2	4.3 ± 0.3
Leucovorin‡			
0.38	10.4–12.2	5.8	4.4–4.6
0.75	13.2–15.8	6.2–6.4	4.5–4.6
1.50	17.3–22.3	6.2–6.9	4.8–6.4
2.25	25.2–29.4	6.6–7.4	7.0–7.7
Folic acid‡			
0.38	10.0–11.6	5.5	4.5
0.75	12.7–15.8	5.5	3.9–5.1
1.50	16.5–22.3	5.2–6.1	3.9–6.6
2.25	23.8–29.4	6.7–7.8	8.3–10.2

* Trapezoidal areas for 0–5 hr data.
† Mean ± SD of four experiments.
‡ Range of two experiments.

distinguish from those of the respective mono- and hexaglutamates, their area integrals were combined. From these integrals, individual mole fractions were calculated and multiplied by total $CH_2H_4PteGlu_n$ to obtain the n = 1 + 2, 3, 4, 5 and 6 + 7 tissue pools, expressed in nmol/mg protein.

RESULTS

Tumor CH_2THF pools increased in a dose-dependent fashion after bolus i.p. administration of either LV or folic acid. A typical example of the time course for this increase is shown in Fig. 1 for the 1.5 mmol/kg dose. Following LV administration, the level of CH_2THF (panel A) peaked at 1–2 hr and returned to baseline over the next 3–4 hr. The maximum increase over baseline was approximately 7-fold. Comparable results were obtained with folic acid (panel B). At a lower dose of 0.375 mmol/kg, the increase in CH_2THF was 70–80%, and at the higher 2.25 mmol/kg dose it was 3- to 4-fold. Expansion of marrow $CH_2H_4PteGlu_n$ was much less pronounced (<2-fold), and results in the gut (data not shown) were similar to marrow. For the 1.5 mmol/kg bolus doses of either leucovorin or folic acid, differences in tumor CH_2THF levels versus those of gut or marrow were highly significant ($P < 0.0001$).

Calculated AUC (0–5 hr) values for CH_2THF in the tumor, marrow, and gut are presented in Table 1 for animals treated with 0.38 to 2.25 mmol/kg of either LV or folic acid by bolus i.p. injection. A dose-related increase in the calculated tumor AUC (0–5 hr) was observed with both drugs, and the results for the two drugs did not differ substantially. The maximum increase, at the 2.25 mmol/kg dose, was between 3- and 4-fold. In the marrow and gut, the change in AUC (0–5 hr) was 2- to 4-fold less than in tumor, depending on the dose.

Infusion of LV at a dose rate of 0.5 mmol/kg·day for 3 days caused rapid expansion of CH_2THF pools in tumor, gut, and marrow during the first 24 hr, followed by a slower increase over the next 48 hr and a rapid return to baseline when the Alzet pumps were removed at 72 hr. The time course for this expansion is shown in Fig. 2A for tumor and marrow. Folic acid (Fig. 2B) given at the same dose rate as LV appeared to expand both the tumor and marrow CH_2THF pool more slowly during the first 48 hr than during the last 24 hr of infusion. Corresponding profiles for gut (not shown) were qualitatively similar to those for marrow, except that CH_2THF levels were approximately 25% greater for gut at each time point during infusion of either drug. Differences in tumor versus gut or marrow CH_2THF levels were not significant for the leucovorin ($P = 0.69$) or folic acid ($P = 0.84$) infusions.

The AUC (0–96 hr) for CH_2THF in the tumor (Table 2) was 37% higher for LV than for an equimolar dose of folic acid. In mice treated with LV, the AUC (0–96 hr) in the tumor was 10% greater than in gut and 37% higher than in marrow. During folic acid infusion, however, the AUC (0–96 hr) for gut exceeded that for tumor by 28%, whereas marrow and tumor AUC (0–96 hr) values were similar. Infusion of 1.5 mmol/kg of either drug over 3 days gave AUC (0–96 hr) values for all three tissues that were more than an order of magnitude greater than the AUC (0–5 hr) after equimolar bolus doses (Table 1).

The separation of radioactive ternary complexes of CH_2THF, [^{3}H]FdUMP, and TS by native polyacrylamide gel electrophoresis and autoradiography is shown in Fig. 3. Scanning densitometry resolved most of the bands, but the integrals for Glu_1 and Glu_2 had to be combined. This was also the case for Glu_6 and Glu_7. The distribution of individual $CH_2H_4PteGlu_n$ species in tumor, marrow, and gut 2 hr after an i.p. bolus injection of 0.38 to 2.25 mmol/kg of LV is given in Fig. 4. In tumor (panel A), at the lower doses (<1.5 mmol/kg), the $CH_2H_4PteGlu_n$ pool expansion was associated with higher amounts of long-chain polyglutamates (N = 5–7); however, at the higher doses the increase was mainly in mono/diglutamate species, while the hexa/heptaglutamate species began to decline. Gut levels (panel B) also followed this pattern, but at the highest dose a smaller increase in Glu_{1+2} was seen. In marrow (panel C), a dose-dependent increase was observed in the mono/diglutamate species, but not in the longer-chain species even at the highest dose tested.

The distribution of individual $CH_2H_4PteGlu_n$ species in tumor during LV infusion is shown in Fig. 5. Increases in all species (N = 1–7) during infusion (panels A–C) were fairly uniform. Gut and marrow levels of all species increased, but expansion of the mono/diglutamate pool was much more pronounced. At the beginning of infusion, all three tissues had a preponderance of the Glu_{5-7} species (panels A–C, 0 hr). Post-infusion pools in the tumor (panel D) appeared to be slightly enriched in the mono/diglutamate. Post-infusion gut and marrow (panels E and F) contained slightly more of the Glu_{1-3} species than at the beginning of infusion.

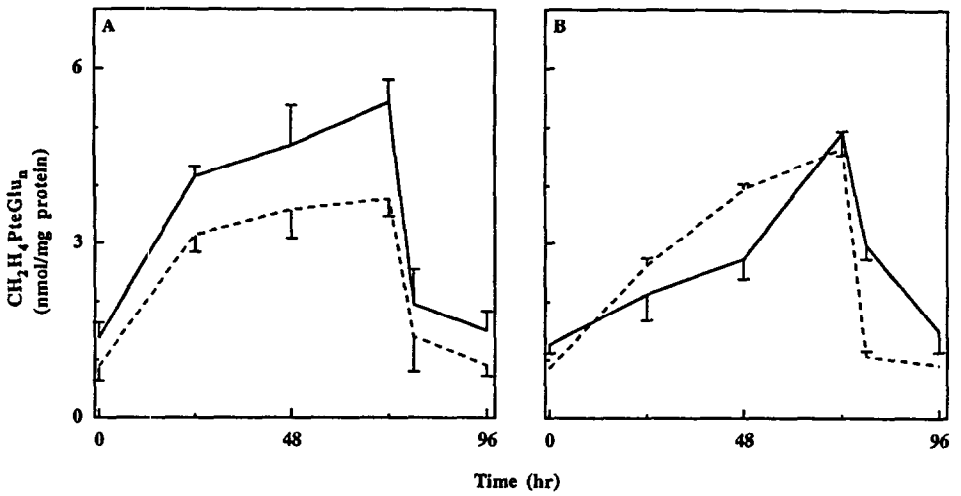

Fig. 2. Tumor (solid lines) and marrow (dotted lines) levels of total CH_2THF versus time during and after a 72-hr continuous infusion of 0.5 mmol/kg·day of LV (A) or folic acid (B). Gut levels (not shown) were similar to marrow. Truncated bars: one-half of the range of 2 replicates.

Table 2. Tissue CH_2THF AUC (0–96 hr) for 3-day continuous s.c. infusions of 0.50 mmol/kg·day of leucovorin and folic acid

Group	CH_2THF (nmol/mg protein·hr*)		
	Tumor	Gut	Marrow
Controls†	134 ± 31	118 ± 31	85 ± 1
Leucovorin‡	308–386	288–341	250–257
Folic acid‡	232–276	269–284	247–269

* Trapezoidal areas for 0–96 hr data.
† Mean ± SD of four experiments.
‡ Range of two experiments.

DISCUSSION

From the foregoing results we conclude that single i.p. bolus doses of LV may selectively expand tumor CH_2THF concentrations in a dose-dependent manner, as shown in Fig. 1. This is also evident from tumor/gut AUC (0–5 hr) ratios (Table 1), which increased from 1.9 to 4.0 over the 0.375 to 2.25 mmol/kg range. Similarly, tumor/marrow AUC (0–5 hr) increased from 2.5 to 3.7. Folic acid was likewise tumor-selective in this arm of the study, giving tumor/gut AUC (0–5 hr) ratios of 2.0–3.5 across the four doses tested. The tumor/marrow AUC (0–5 hr) ratio similarly rose from 2.5 to 3.6 over the 0.38 to 1.5 mmol/kg dose range, but declined when the dose was 2.25 mmol/kg. These data are qualitatively consistent with our earlier work [23]. In that study, LV given as an i.p. bolus every 4 hr for 2 days at

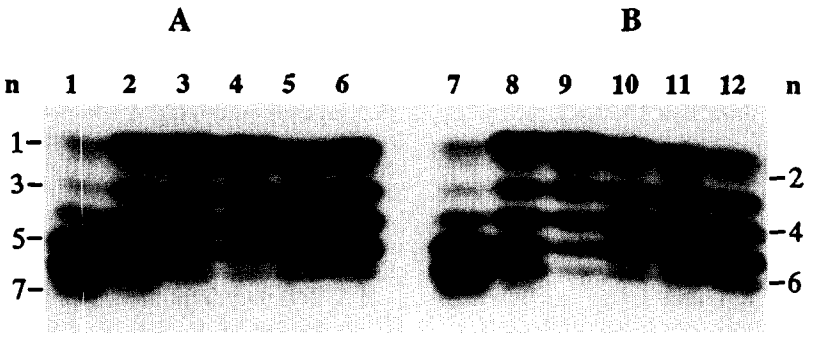

Fig. 3. Native polyacrylamide gel electrophoresis of $CH_2H_4PteGlu_n$ from BALB/c mouse gut after complexation with excess *L. casei* TS and [6-^{3}H]FdUMP. A and B are replicates with mice treated by continuous infusion of 0.5 mmol/kg·day of LV on different days. Times of harvest during infusion: Lanes 1 and 7, 0 hr; 2 and 8, 24 hr; 3 and 9, 48 hr; 4 and 10, 72 hr. Post-infusion: Lanes 5 and 11, 78 hr; 6 and 12, 96 hr.

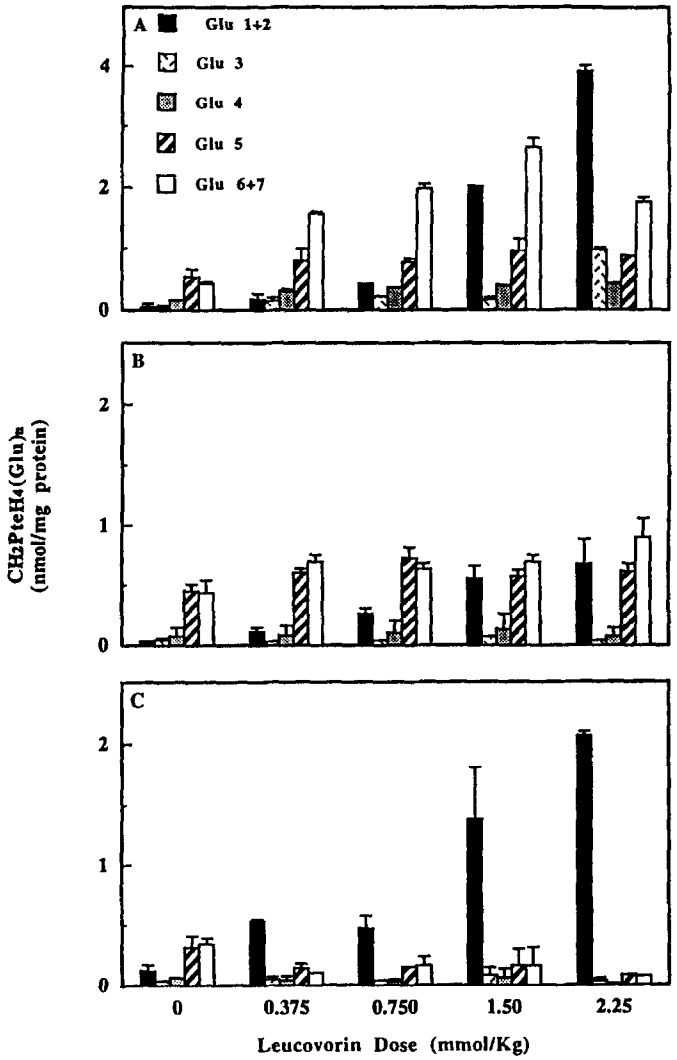

Fig. 4. Distribution of $CH_2H_4PteGlu_n$ species in tumor (A), gut (B) and marrow (C) versus i.p. bolus dose of LV. Tissues were harvested 2 hr after injection. Bars above: one-half of the range of 2 replicates.

total doses of 0.75, 1.5, and 3.0 mmol/kg produced increases in tumor/marrow CH_2THF ratio of 4.2, 7.4, and 3.1, respectively, in tissues harvested 2 hr after the last injection. Those results and the present AUC (0–5 hr) data show that LV given by the i.p. bolus route should be capable of modulating fluoropyrimidine therapy in the murine EMT6 mammary carcinoma model. Continuous 3-day s.c. infusion of LV was less selective than i.p. bolus administration, both in terms of total CH_2THF tissue levels (Fig. 2) and in terms of AUC (0–96 hr) values (Table 2). Marrow CH_2THF levels and AUC (0–96 hr) were only slightly lower than in the tumor.

Folic acid has also been studied as a possible modulator of fluoropyrimidine antitumor activity in cultured cells [21], but was more toxic and less therapeutically effective than FU alone in a clinical trial against metastatic colon cancer [22]. Since only (6*S*)-LV is a substrate for enzymes of the folate cycle, the maximum possible yield of CH_2THF from (6*R*,6*S*)-LV is only one-half that from folic acid. Our tumor AUC (0–5 hr) for CH_2THF in mice treated with 0.75 mmol/kg of folic acid had a range of 12.7 to 15.8 nmol/mg protein·hr, whereas for the animals receiving 1.5 mmol/kg of LV, the *mechanistically* equivalent dose, the range was 17.3 to 22.3 nmol/mg protein·hr (Table 1). The results were similar when doses of 0.38 mmol/kg of folic acid and 0.75 mmol/kg of LV were compared.

According to a recent study in another laboratory [33], the AUC for combined tetrahydrofolate and CH_2THF in the plasma of human volunteers receiving 25 or 125 mg/m^2 of oral or intravenous folic acid was twice as high as in those receiving 50 or 250 mg/m^2 of LV. If plasma and tumor CH_2THF levels are commensurate, we should expect folic acid to be better as a potential modulator of fluoropyrimidine antitumor activity than LV. In our murine model, the AUC for total tissue CH_2THF was not greater when folic acid was given rather than LV. However,

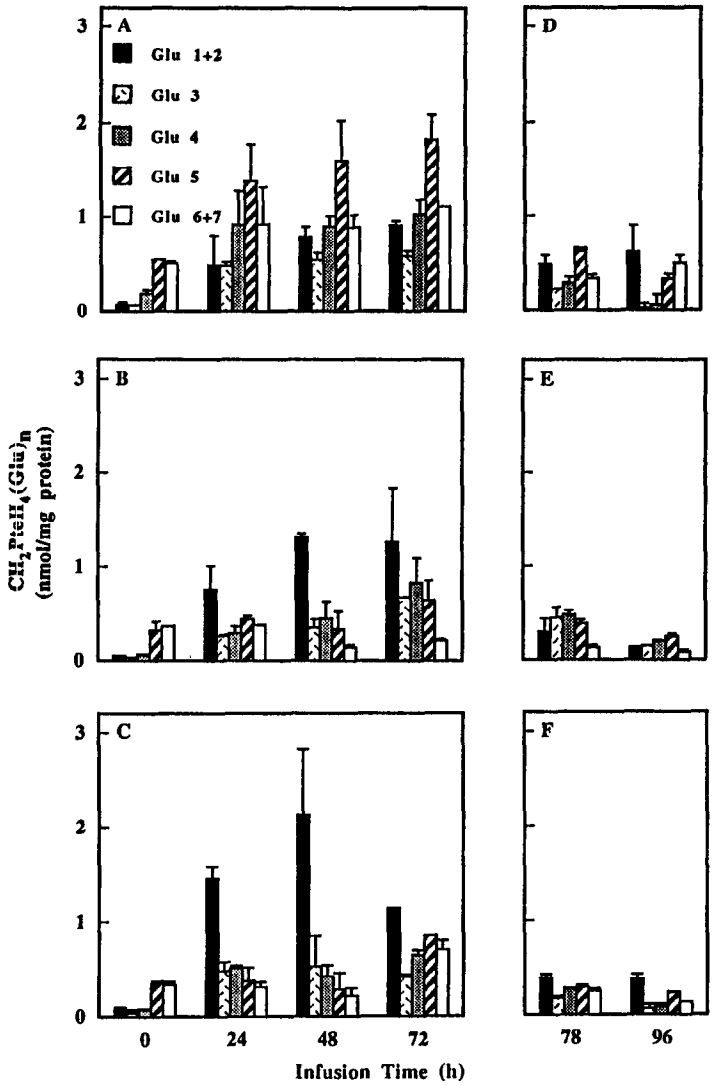

Fig. 5. Distribution of $CH_2H_4PteGlu_n$ species in tumor (A,D), gut (B,E) and marrow (C,F) versus time during (A–C) and after (D–F) a 72-hr continuous infusion of 0.5 mmol/kg·day of LV. Bars above: one-half of the range of 2 replicates.

the two studies are not strictly comparable. Our measurements were done only with cell extracts, and represent mono- and polyglutamates, whereas in the human study only plasma levels were measured and these presumably consisted of non-polyglutamylated species. We did not measure the concentration of unchanged LV or folic acid in the tissues or the levels of reduced folate metabolites in the plasma.

The CH_2THF levels we observed in mouse tissues after an i.p. bolus of LV or folic acid were much less persistent than the plasma reduced folate levels in humans after bolus i.v. injection of these compounds [33]. More rapid tissue clearance of CH_2THF in mice and the lack of a clear difference between tissues levels of CH_2THF after LV and folic acid treatment appear to limit the predictive ability of our model where modulation by these agents is concerned. Recognition that pharmacologic agents are more rapidly eliminated by mice contributes to the general concept that maximum tolerated doses of chemotherapeutic agents are about 12-fold greater for that species than for humans [34].

Analysis of $CH_2H_4PteGlu_n$ polyglutamate distributions in tumor, gut, and marrow were performed after i.p. bolus (Fig. 4) and s.c. infusion (Fig. 5) of LV. This analysis provided some insight into the differential metabolism of LV as a function of regimen. Bolus administration of LV resulted in a dose-dependent increase of the longer-chain species in tumor over the dose range 0.38 to 1.5 mmol/kg (Fig. 4A). Increases in the mono/diglutamate species were also seen, but to a lesser extent. Only at the highest dose of LV, 2.25 mmol/kg, did the mono/diglutamate species become predominant as the hexa/heptaglutamate species began to decline. An

expected therapeutic consequence of $CH_2H_4PteGlu_n$ chain-lengthening is that lower concentrations of such species are sufficient to block TS activity in the presence of FdUMP. Binding studies have shown that a 40-fold greater excess of the monoglutamate than of the pentaglutamate is needed to half-saturate TS in the presence of FdUMP [35].

The distribution of $CH_2H_4PteGlu_n$ chain-lengths in the gut (Fig. 4B) after bolus treatment with LV was qualitatively similar to that of tumor, although the increases were of a smaller magnitude (note the scale differences between panels A and B). Increases in Glu_{1+2} were not as pronounced as those in tumor, but were dose dependent. The method we used did not distinguish between proliferating and nonproliferating cells. Other investigators have shown that crypt cells comprise <20% of the epithelium in mouse intestine, but express as much as 4-fold higher folypolyglutamate synthetase activity than the less proliferative columnar cells [36]. We were not able to analyze $CH_2H_4PteGlu_n$ chain lengths in the crypt cells, because gut had to be frozen with pH 8.5 buffer immediately after excision to avoid rapid breakdown of $CH_2H_4PteGlu_n$. Thus, the reported technique for fractionation of gut epithelial tissue could not be applied.

In the marrow we observed a dose-dependent increase in mono/diglutamates (Fig. 4C), with no concomitant increase in longer-chain species after bolus LV administration. The lack of conversion of leucovorin to $CH_2H_4PteGlu_n$ polyglutamates in murine bone marrow is in accord with an earlier report showing that purified myeloid precursor cells from human bone marrow possessed a very limited capacity for polyglutamylation of the antifolate methotrexate [37]. There was a distinct difference between the response of the marrow and the tumor, the latter of which featured more prominent expansion of the longer-chain polyglutamates at all doses. This suggested that modulation with bolus LV may afford a form of fluoropyrimidine potentiation in which hematopoiesis is spared. Several studies have shown that, in tissues where shorter $CH_2H_4PteGlu_n$ chain lengths predominate, both the affinity of FdUMP for TS and the rate and persistence of TS inhibition are diminished [17, 38, 39].

Continuous s.c. infusion of LV or folic acid at a dose rate of 0.5 mmol/kg·day for 3 days produced a mean 2.6-fold increase in the tumor CH_2THF AUC (0–96 hr) with LV and a 1.9-fold increase with folic acid (differences not significant, $P = 0.70$). In gut, the corresponding increases were 2.7-fold with LV and 2.3-fold with folic acid, and in marrow they were 3.0-fold with either precursor. Time-dependent changes in tumor and marrow pools are shown in Fig. 2. In view of the tumor/gut and tumor/marrow CH_2THF ratios, it was clear that neither precursor was selective in this regimen. On the other hand, an increase in the total CH_2THF pool size *per se* may not be as important as a shift in polyglutamate distribution toward longer-chain species where the efficacy of modulation is concerned.

While all the individual $CH_2H_4PteGlu_n$ species in the tumor increased in a time-dependent manner throughout the 3-day infusion of LV (Fig. 5), the largest change occurred with the longer-chain polyglutamates, especially the Glu_5 species. In contrast, while the total CH_2THF pool in gut and marrow also expanded, the overall change was smaller than in tumor and the mono/diglutamates were predominant during each day of the infusion. Thus, despite the fact that tumor/marrow and tumor/gut ratios of total CH_2THF were smaller during infusion than after a bolus injection, there was a differential change in polyglutamate distribution, which supported the concept of giving a constant infusion of LV in conjunction with FU treatment [13].

In summary, bolus i.p. injection and constant s.c. infusion in the EMT6 model each seemed to have its own potential advantages. The bolus regimen expanded the total CH_2THF pool and produced a favorable shift in the tumor polyglutamylation profile toward longer-chain species. However, infusion of LV or folic acid did provide bigger increases in the AUC values for CH_2THF than the bolus regimens. Total CH_2THF pools increased much more in tumor than in gut or marrow. But polyglutamylation profiles were shifted in favor of the Glu_{1+2} species only in the marrow. The infusion regimen was much less tumor-selective in terms of the total CH_2THF pool, but still resulted in a preferential increase in long-chain polyglutamates in the tumor as compared with the marrow and gut. Comparing the two precursors, our data for the EMT6 tumor system show that folic acid did not provide a higher yield of CH_2THF than LV, whether given by bolus or infusion, despite the fact that only one of the diasteriomers of LV is metabolized.

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