Cancer Chemother Pharmacol (1997) 40: 126–130

ORIGINAL ARTICLE

K. Raghunathan · John C. Schmitz · David G. Priest

Disposition of leucovorin and its metabolites in dietary folic acid-deplete mice – comparison between tumor, liver and plasma

Received: 24 July 1996 / Accepted: 29 October 1996

Abstract *Purpose*: A comprehensive pharmacokinetic study of leucovorin (5-formyltetrahydrofolate, 5-HCO-FH_4) and its metabolites was conducted in plasma, liver and implanted tumor tissue from mice maintained on a low folic acid diet. While it has been previously demonstrated that the antitumor activity of fluorouracil (FU) can be potentiated by 5-HCO-FH_4, the optimum time for administration of FU after 5-HCO-FH_4, to maximally elevate the active folate metabolite methylenetetrahydrofolate in tumor has not been established. Human plasma studies have defined the pharmacokinetics of circulating 5-HCO-FH_4 and its metabolites, but comparison with human tumor accumulation has not been practicable because of sampling difficulties. As an alternative, a mouse model system, based on low dietary folic acid, was used to evaluate plasma, liver and implanted tumor reduced folates after administration of 5-HCO-FH_4. *Methods*: Plasma and tissue samples were collected from folate-deplete mice over a 12-h period after intraperitoneal administration of 90 mg/kg [*R*, *S*] 5-HCO-FH_4. Reduced folates were evaluated using a ternary complex assay. *Results*: The time at which maximal accumulation of parent compound and all metabolites, except 5-methyltetrahydrofolate (5-CH_3FH_4), occurred in tumor was the same as in plasma. Alternatively, peak liver accumulation was delayed relative to plasma for all folates except 5-CH_3FH_4. *Conclusions*: The results suggest that mouse plasma accumulation of reduced folates, with the exception of 5-CH_3FH_4, can predict tumor accumulation. Hence, evaluation of human plasma folate accumulation may potentially provide a means to improve the timing of the administration of FU relative to 5-HCO-FH_4 to achieve a superior therapeutic outcome.

K. Raghunathan · J.C. Schmitz · D.G. Priest (✉)
Department of Biochemistry and Molecular Biology,
Medical University of South Carolina, 171 Ashley Avenue,
Charleston, South Carolina 29425-2211, USA
Tel. (803)-792-4321; Fax (803)-792-4322

Key words Reduced folates · Pharmacokinetics · Leucovorin · C3H mice · C3H mammary adenocarcinoma

Abbreviations *FU* 5-fluorouracil · *5-FdUMP* 5-fluorodeoxyuridine monophosphate · *5-HCO-FH_4* 5-formyltetrahydrofolate or leucovorin · *CH_2FH_4* 5,10-methylenetetrahydrofolate · *FH_4* tetrahydrofolate, *5-CH_3FH_4* 5-methyltetrahydrofolate · *10-HCO-FH_4* 10-formyltetrahydrofolate · *CH^+FH_4* 5,10-methenyltetrahydrofolate · *TS* thymidylate synthase · *MS* methenyltetrahydrofolate synthetase

Introduction

Leucovorin (5-HCO-FH_4) is currently in widespread use to potentiate FU antitumor activity against several types of advanced solid tumors [2, 11, 23]. This potentiation has been attributed to accumulation of the metabolite, CH_2FH_4, which stabilizes the inhibitory ternary complex formed between TS (EC 2.1.1.45) and the active metabolite of FU, 5-FdUMP [3, 5]. For this reason 5-HCO-FH_4 administration often precedes FU to permit folate metabolite accumulation. However, the time interval used, which has generally been based on accumulation of plasma folates, has varied widely [7–9, 12, 21]. Tumor accumulation of the active metabolite, CH_2FH_4, is the most direct determinant of activity, but it has been difficult to obtain more than very limited evaluation of human tumor CH_2FH_4 levels for comparison with plasma because it is difficult to obtain sufficient human tumor tissue sample [4, 22, 23]. To obtain sufficient tissue, an animal model is the only practicable alternative. Recently, we have established a dietary folic acid-deplete mouse model system and have shown that while there is only a marginal effect of the diet on tumor growth, the model is more sensitive to 5-HCO-FH_4 potentiation of FU antitumor activity [16]. This system was used to determine the pharmacokinetics

of 5-HCO-FH_4 and its metabolites in plasma and to compare these results with accumulation in an implanted mouse mammary tumor and liver.

Materials and methods

Materials

Leucovorin ([*R*,*S*]5-HCO-FH_4) was obtained from Ben Venue Laboratories, Ohio. C3H mammary adenocarcinoma was obtained from Lilly Research Laboratories (Indianapolis, Ind.) and was maintained in C3H mice (Charles River Co. Wilmington, Mass.) as subcutaneous (s.c.) axillary implants with a passage time between 14 and 20 days. Folic acid-deplete chow (#5831C-2) containing 0.01 ppm folic acid with 1% succinylsulfathiazole to deplete intestinal bacteria, and control chow (#5001) containing 5.9 ppm folic acid, were purchased from Purina Mills (Richmond, Ind.). 5-[^{3}H]FdUMP was purchased from Moravek Biochemicals (Brea, Calif.). Sephadex G-25 was obtained from Pharmacia (Piscataway, N.J.). NADPH, ATP and all other reagents were purchased from Sigma Chemical Co. (St. Louis, Mϕ.). TS (4 U/mg protein) was purified from an *Escherichia coli* strain that overproduces *Lactobacillus casei* TS [14]. The *E. coli* strain was a gift from D. Santi (University of California, San Francisco). 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 [10, 17]. 5,10-Methenyltetrahydrofolate synthetase (0.1 U/mg protein) was purified from rabbit liver largely as described previously [19]. Previous preparations contain low but interfering levels of [*S*]5-HCO-FH_4 bound to the purified enzyme. This [*S*]5-HCO-FH_4 has been removed by incubation of purified MS and ATP (0.5 m*M*) for 15 min at room temperature to convert enzyme bound [*S*]5-HCO-FH_4 to 10-HCO-FH_4. The resultant 10-HCO-FH_4 was separated from MS by Sephadex G-25 column chromatography. Purified MS was stored in 20 m*M* potassium phosphate (pH 7.2), 5 m*M* mercaptoethanol, 0.1% Tween-20 and 0.5 m*M* ATP. ATP does not interfere with detection of [*S*]5-HCO-FH_4 because it is present in excess during assay.

Animal experiments

C3H mice were maintained on the folic acid-deplete diet for 14 days. Animal weight was monitored and found to be no different from the control group (average weight 22 g). Mouse mammary adenocarcinoma was excised from seed mice, cut into pieces small enough to fit into a 13-gauge trocar, and implanted s.c. into the mice. Implanted tumors were allowed to grow for 10 days before initiation of pharmacokinetic experiments. [*R*,*S*]5-HCO-FH_4 was diluted in sterile saline and injected intraperitoneally (i.p.) (300 µl per mouse) at a dose of 90 mg/kg. To obtain blood, liver and tumor samples, animals were sacrificed in a CO_2 chamber.

Tissue preparation

Whole blood ($\sim$ 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 m*M* Tris-HCl buffer (pH 7.4) containing 50 m*M* sodium ascorbate and 1 m*M* EDTA and stored at −70 °C. Liver and tumor were excised from mice and washed with cold phosphate-buffered saline (PBS) and stored at −70 °C. For folate analysis, these tissues were each homogenized in cold 50 m*M* Tris-HCl buffer (pH 7.4) containing 50 m*M* sodium ascorbate and 1 m*M* EDTA and centrifuged at 10 000 *g* for 10 min at 4 °C to remove cell debris. An aliquot of the supernatant was used for soluble protein determination by the method of Bradford [1]. The remainder of the supernatant was immediately placed in a boiling water bath for 5 min and centrifuged to remove precipitated proteins. The resultant supernatant was used for folate estimation. Because CH_2FH_4 can potentially become dissociated to FH_4 and formaldehyde under these conditions, the sum of these folates is reported. Other reference folates are stable under these conditions with routine recovery in the range 70–95% [15].

Estimation of reduced folates

The ternary complex assay used is based upon enzymatic cycling of reduced folates (monoglutamates and polyglutamates) to CH_2FH_4 followed by entrapment into a stable ternary complex with excess *Lactobacillus casei* TS and 5-[^{3}H]FdUMP. Methods have been described previously for estimation of $CH_2FH_4 + FH_4$, 5-CH_3FH_4, 10-HCO-FH_4, and 5-HCO-FH_4 using this approach [15]. Typically, reaction mixtures contained 20 mU TS and 125 n*M* 5-[^{3}H]FdUMP (20 Ci/mmol) in 200 µl 50 m*M* Tris-HCl buffer (pH 7.4) containing 50 m*M* sodium ascorbate and 1 m*M* EDTA. Additional enzymes and cofactors were added as necessary for cycling each reduced folate to CH_2FH_4. Ternary complex formation was allowed to proceed at 25 °C 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 minicolumns and eluted by centrifugation to separate tritiated complexes from free 5-[^{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]. The area under the concentration-time curve ($AUC_{0\text{-}12h}$) for each folate in plasma, liver and tumor was calculated using the linear trapezoidal method [18].

Methenyltetrahydrofolate synthetase assay

A novel assay for determination of MS activity in crude tissue extracts was developed. This assay is based on enzymatic conversion of [*S*]5-HCO-FH_4 to CH^+FH_4 with subsequent hydrolysis to 10-HCO-FH_4. The latter folate was estimated by the ternary complex assay. Liver ($\sim$ 3 mg/ml) and tumor ($\sim$ 30 mg/ml) were homogenized in 50 m*M* Tris-HCl buffer (pH 7.4) containing 50 m*M* sodium ascorbate and 1 m*M* EDTA, and centrifuged to remove cell debris. For analysis, reaction mixtures, which contained 5–20 µl of tissue extract supernatant, 10 µ*M* [*R*,*S*]5-HCO-FH_4, 0.5 m*M* ATP and 10 m*M* magnesium acetate in the homogenization buffer in a final volume of 0.5 ml, were incubated at 37 °C for 5, 10 and 15 min and terminated by boiling for 5 min. One unit of MS activity corresponds to 1 µmol 10-HCO-FH_4 formed per min. Soluble protein in tissue supernatants was determined by the method of Bradford [1].

Results

To determine the disposition of 5-HCO-FH_4 and its metabolites, reduced folates were monitored over a 12-h period in plasma, liver and implanted tumor. Mice were maintained on a folic acid-deplete diet to permit more extensive elevation of reduced folates and to mimic more closely levels typically observed in humans. Figure 1 shows the behavior of the total folate pool (the sum of [*S*]5-HCO-FH_4 and its metabolites) in plasma and each tissue after i.p. injection of [*R*,*S*]5-HCO-FH_4. The corresponding AUC values are shown in Table 1. Plasma folates were extensively elevated over the first few minutes (from 6 pmol/ml to 135 nmol/ml) followed by a rapid depletion. Tumor reduced folates followed the same general profile, in that they achieved a peak concentration at the same time as plasma folates. Total tumor folates became elevated from basal levels of

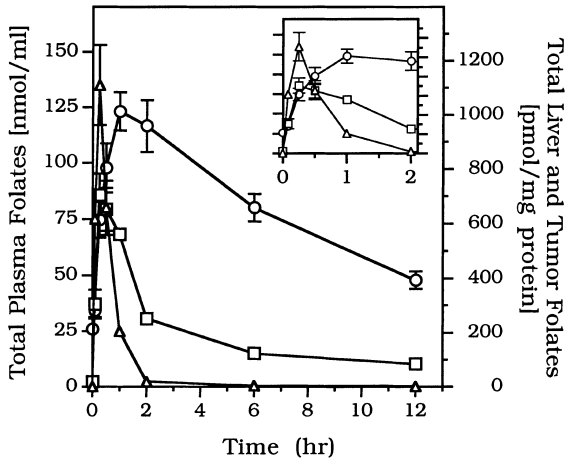


Fig. 1 Total reduced folate accumulation in mouse tissues after [*R,S*]5-HCO-FH_4 administration. Individual reduced folates were estimated by the ternary complex assay in plasma (△), liver (○) and implanted tumor (□) from folic acid-deplete mice after i.p. injection of 90 mg/kg [*R,S*]5-HCO-FH_4 and summed to obtain total folate estimates. The inset shows an expanded time axis for clarity of the early results. Values are the means ± SE of duplicate analyses of plasma and tissues from four mice

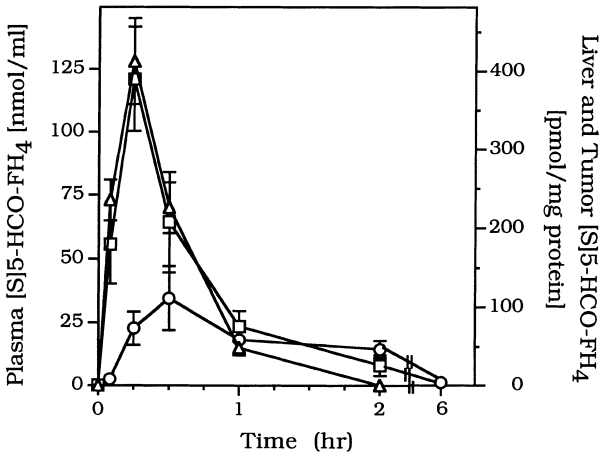


Fig. 2 [*S*]5-HCO-FH_4 accumulation in mouse plasma and tissues after [*R,S*]5-HCO-FH_4 administration. [S]5-HCO-FH_4 was estimated by the ternary complex assay in plasma (△), liver (○) and implanted tumor (□) from folic acid-deplete mice after i.p. injection of 90 mg/kg [*R,S*]5-HCO-FH_4. Values are the means ± SE of duplicate analyses of tissues from four mice

Table 1 AUC values for [*S*]5-HCO-FH_4 and its metabolites in mouse plasma, tumor and liver (values are the means ± SE from four mice)

Folate	$AUC_{0\text{-}12\ h}$		
	Plasma (nmol · h/ml)	Tumor (pmol · h/mg)	Liver (pmol · h/mg)
Total	92 ± 7	2356 ± 71	8102 ± 531
[*S*]5-HCO-FH_4	74 ± 6	338 ± 58	247 ± 47
IO-HCO-FH_4	4 ± 1	418 ± 31	1395 ± 289
$CH_2FH_4 + FH_4$	8 ± 1	547 ± 57	4587 ±123
5-CH_3FH_4	9 ± 1	1021 ± 58	1749 ± 263

18 pmol/mg protein to 705 pmol/mg. The loss of folate from tumors was somewhat slower than from plasma. The total folate pool in liver achieved a peak concentration (from 213 to 1013 pmol/mg) about 45 min later than in plasma and tumor, and remained elevated much longer. The AUC for liver total folate was nearly four times greater than for tumor. However, at peak, the total increase in folate above basal levels was five-fold in liver while tumor folates were elevated 28-fold.

The behavior of the biologically active form of [*R,S*]5-HCO-FH_4, [*S*]5-HCO-FH_4, is shown in Fig. 2 with the corresponding AUC values shown in Table 1. It can be seen that the kinetic patterns for plasma and tumor were essentially superimposable, while the peak concentration in liver was attained substantially later. AUC for tumor [*S*]5-HCO-FH_4 was approximately 40% greater than for liver because the peak accumulation was higher. For comparison purposes, AUC values for liver and tumor [*S*]5-HCO-FH_4 accounted for only a small fraction of total folate AUC in each tissue (3% and 14%, respectively) while plasma folate consisted primarily of [*S*]5-HCO-FH_4 (80% of total plasma AUC).

More extensive accumulation of [*S*]5-HCO-FH_4 in tumor compared to liver could potentially be explained by slower metabolism. Hence, the activity of MS, the only enzyme known to metabolize [*S*]5-HCO-FH_4, was determined [19]. Because of the relatively low activity of this enzyme in crude tumor extracts [13], it was necessary to develop a more sensitive assay than those previously available. This assay was based upon detection of the final product, 10-HCO-FH_4, by the ternary complex assay method, after enzymatic conversion of [*S*]5-HCO-FH_4 to CH^+FH_4, and subsequent hydrolysis to 10-HCO-FH_4. Using this approach, it was determined that liver contained a substantially higher level of activity than tumor (520 ± 178 vs 203 ± 27 μU/mg protein, $n = 4$).

The behavior of the leucovorin metabolites, 10-HCO-FH_4, $CH_2FH_4 + FH_4$ and 5-CH_3FH_4, in plasma, liver and tumor is shown in Fig. 3 with the corresponding AUC values shown in Table 1. As was true for the parent compound, the kinetic behavior of tumor 10-HCO-FH_4 was very similar to the profile in plasma. On the other hand, liver 10-HCO-FH_4 achieved a peak concentration somewhat later than plasma or tumor, and there was a three-fold greater AUC for this folate in liver than in tumor. The combined $CH_2FH_4 + FH_4$ pool (Fig. 3B) also followed the same general kinetic pattern

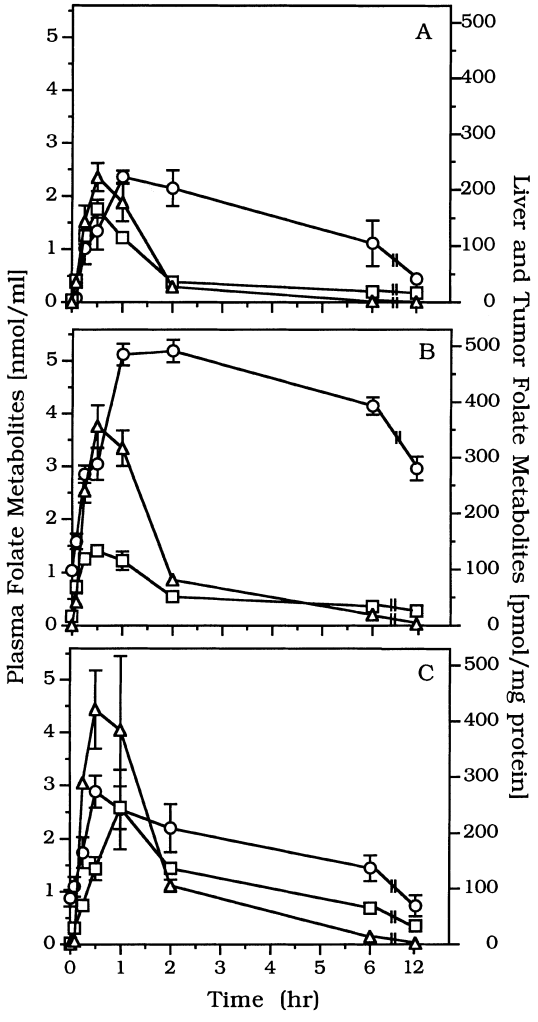


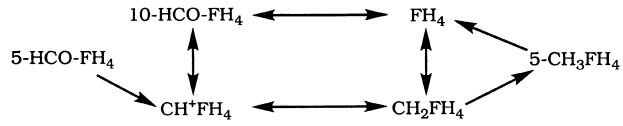
Fig. 3A–C Reduced folate metabolite accumulation in mouse tissues after [*R*,*S*]5-HCO-FH_4 administration. 10-HCO-FH_4 (**A**), CH_2FH_4+ FH_4 (**B**) and 5-CH_3FH_4 (**C**) were estimated by the ternary complex assay in plasma (△), liver (○) and implanted tumor (□) from folic acid-deplete mice after i.p. injection of 90 mg/kg [*R*,*S*]5-HCO-FH_4. Values are the means ± SE of duplicate analyses of tissues from four mice

in tumor as in plasma. On the other hand, the peak $CH_2FH_4 + FH_4$ accumulation in liver, like [*S*]5-HCO-FH_4 and 10-HCO-FH_4, occurred considerably later than in tumor. Further, there was an eight-fold greater AUC for this pool in liver than in tumor. Compared with total folate AUC, the $CH_2FH_4 + FH_4$ AUC accounted for more than half of the total folate in the liver but only 23% of tumor folate. In contrast to other metabolites, tumor accumulation and depletion patterns for 5-CH_3FH_4 did not mimic the behavior in plasma (Fig. 3C). However, the time of peak accumulation of this pool in liver was close to the peak time in plasma. Hence, tumor 10-HCO-FH_4 and $CH_2FH_4 + FH_4$ closely followed the pattern for these folates in plasma, but peak accumulation occurred later in liver. On the other hand, liver 5-CH_3FH_4 paralleled the plasma profile but peak accumulation occurred somewhat later in tumor.

Discussion

While the disposition of 5-HCO-FH_4 and its metabolites have been extensively studied in human plasma [15, 20], little direct evidence is available to establish the relationship between plasma pharmacokinetics and tissue accumulation [4, 22, 23]. Because of the difficulty in obtaining human tissue samples to adequately conduct pharmacokinetic studies, animal models have been sought as an alternative [6, 13, 24]. However, high basal tissue and plasma folate levels present in animals maintained on typical laboratory diets, which have extensive folic acid supplementation, have made assessment of folate increases in response to 5-HCO-FH_4 difficult to interpret. The dietary folic acid-deplete mouse model used in this study was able to overcome this difficulty.

The general schematic representation of folate metabolite interconversions that are expected to occur after 5-HCO-FH_4 administration shown below reflects the complexity of the overall system at the biochemical level.



Scheme 1 Scheme of folate metabolite interconversions

In addition, differences in uptake from plasma of the parent compound as well as metabolites formed elsewhere, make precise modeling exceptionally difficult. Hence, the current study was undertaken to evaluate empirically the relationship between plasma and tissue accumulation of each metabolite. One goal was to establish the time of peak accumulation of therapeutically critical metabolites in tumor for comparison with peak accumulation in more readily accessible plasma samples. Results show that the parent compound, and every metabolite except 5-CH_3FH_4, achieved a peak concentration at the same time in tumor as in plasma. On the other hand, peak accumulation in liver lagged well behind plasma, again, except for the 5-CH_3FH_4 pool. It is unclear why 5-CH_3FH_4, which is the primary circulating folate under basal conditions, behaves differently from the other pools.

The basis for the more extensive elevation of metabolites in liver versus tumor is likely related to more active metabolism of the parent compound in liver. This is supported directly by the observation that cell-free extracts of liver contain over twice as much MS activity as tumor extracts. This lesser ability to metabolize 5-HCO-FH_4 in tumor may suggest that MS activity is a rate-limiting step in the elevation of CH_2FH_4. If so, tumor MS level may be a critical determinant of therapeutic response. However, it should be pointed out that

CH_2FH_4 is present in the circulatory system after 5-HCO-FH_4 administration in this mouse system and in humans [15], which could also provide a source for tumor elevation. The relative contribution of CH_2FH_4 derived metabolically within the tumor itself, and that taken up from plasma, is currently unknown.

In summary, the dietary folic acid-deplete mouse model used in this study is particularly suitable for pharmacokinetic evaluation of 5-HCO-FH_4 metabolite disposition because plasma levels more closely approximate those of human plasma [15] and because a greater degree of elevation can be obtained. This system showed that there is excellent temporal agreement between plasma and tumor peak accumulation of reduced folate metabolites that are critical to modulation of FU. Under the premise that it is likely that this relationship will also be true for human cancer patients, it is proposed that the optimal time for administration of FU following 5-HCO-FH_4 can be predicted with a high degree of accuracy from evaluation of appropriate metabolites in plasma samples. Future studies with this system will attempt to provide additional evidence to support this proposal.

Acknowledgement This research is supported by an American Cancer Society Grant, DHP-51E.

References

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248
2. Buroker TR, O'Connell MJ, Wieand S, Krook JE, Gersfner JB, Mailliard JA, Schaefer PL, Levitt R, Kardinal CG, Gesme DH Jr (1994) Randomized comparison of two schedules of fluorouracil and leucovorin in the treatment of advanced colorectal cancer. J Clin Oncol 12: 14
3. Danenberg PV, Danenberg KD (1978) Effect of 5,10-methylenetetrahydrofolate on dissociation of 5-fluoro-2′deoxyuridylate from thymidylate synthase: evidence for an ordered mechanism. Biochemistry 17: 4018
4. Dohden K, Ohmura K, Watanabe Y (1993) Ternary complex formation and reduced folate in surgical specimens of human adenocarcinoma tissues. Cancer 71: 471
5. Houghton JA, Schmidt C, Houghton PJ (1982) The effect of derivatives of folic acid on the fluorodeoxyuridylate-thymidylate synthetase covalent complex in human colon xenografts. Eur J Cancer Clin Oncol 18: 347
6. Houghton JA, Williams LG, de Graaf SSN, Cheshire PJ, Rodman JH, Maneval DC, Wainer IW, Jaduad P, Houghton PJ (1990) Relationship between dose rate of [6RS]leucovorin administration, plasma concentrations of reduced folates, and pools of 5,10-methylenetetrahydrofolates and tetrahydrofolates in human colon adenocarcinoma xenografts. Cancer Res 50: 3493
7. Laufman LR, Bukowski RB, Collier MA, Sullivan BA, Mckinnis RA, Clendennin NJ, Guaspari A, Brenckman WD Jr (1993) A randomized, double-blind trial of fluorouracil plus placebo versus fluorouracil plus oral leucovorin in patients with metastatic colorectal cancer. J Clin Oncol 11: 1888
8. Leichman CG, Fleming TR, Muggia FM, Tangen CM, Ardalan B, Doroshow JH, Meyers FJ, Holcombe RF, Weiss GR, Mangalik A, Macdonald JS (1995) Phase II study of fluorouracil and its modulation in advanced colorectal cancer: a southwest oncology group study. J Clin Oncol 13: 1303
9. Levin RD, Gordon JH (1993) Fluorodeoxyuridine with continuous leucovorin infusion. A phase II clinical trial with metastatic colorectal cancer. Cancer 72: 2895
10. Mathews RG (1986) Methylenetetrahydrofolate reductase from pig liver. Methods Enzymol 122: 372
11. Mini E, Trave F, Rustum YM, Bertino JR (1990) Enhancement of the antitumor effects of 5-fluorouracil by folinic acid. Pharmacol Ther 47: 1
12. Morgan RJ Jr, Speyer J, Doroshow JH, Margolin K, Raschko J, Sorich J, Akman S, Leong L, Somlo G, Vaslilev S, Ahn C, Johnson D, Beller U (1995) Modulation of 5-fluorouracil with high-dose leucovorin calcium: activity in ovarian cancer and correlation with CA-125 levels. Gynecol Oncol 58: 70
13. Mullin RJ, Keith BR, Duch DS (1988) Distribution and metabolism of calcium leucovorin in normal and tumor tissue. Adv Exp Med Biol 244: 25
14. Pinter K, Davisson VJ, Santi DV (1988) Cloning, sequencing, and expression of the *Lactobacillus casei* thymidylate synthase gene. DNA 7: 235
15. Priest DG, Schmitz JC, Bunni MA, Stuart RK (1991) Pharmacokinetics of leucovorin metabolites in human plasma as a function of dose administered orally and intravenously. J Natl Cancer Inst 83: 1806
16. Raghunathan K, Schmitz JC, Priest DG (1996) The impact of schedule on leucovorin potentiation of fluorouracil antitumor activity in dietary folic acid deplete mice. Biochemical Pharmacol (in press)
17. Rios-Orlandi EM, Zarkadas CG, MacKenzie RE (1986) Formyltetrahydrofolate dehydrogenase-hydrolase from pig liver: simultaneous assay of the activities. Biochim Biophys Acta 871: 24–35
18. Rowland M, Tozer TN (1989) (eds) Clinical pharmacokinetics. Concepts and applications. Lea & Febiger, Philadelphia
19. Stover P, Huang T, Schirch V, Maras B, Valiante S, Barra D (1993) Purification and properties of rabbit liver 5-10-methenyltetrahydrofolate synthetase. Adv Exp Med Biol 338: 723
20. Straw JA, Szapary D, Wynn WT (1984) Pharmacokinetics of the diastereoisomers of leucovorin after intravenous and oral administration to normal subjects. Cancer Res 44: 3114
21. Tetef M, Doroshow J, Akman S, Coluzzi P, Leong L, Margolin K, Morgan RJ Jr, Raschko J, Shibata S, Somlo G, Ahn C (1995) 5-fluorouracil and high-dose calcium leucovorin for hepatocellular carcinoma: a phase II trial. Cancer Invest 13: 460
22. Tominaga T, Toi M, Shirasaka T (1993) Enhanced inhibition of thymidylate synthase by 5-fluorouracil and [6S]leucovorin combination therapy for breast cancer. Anticancer Res 3: 2425
23. Trave F, Rustum YM, Petrelli NJ, Herrera L, Mittelman A, Frank C, Creacen PJ (1988) Plasma and tumor tissue pharmacology of intravenous leucovorin calcium in combination with fluorouracil in patients with advanced colorectal carcinoma. J Clin Oncol 6: 1184
24. Wright JE, Pardo MA, Sayeed-Shah U, Alperin W, Rosowsky A (1995) Leucovorin and folic acid regimens for selective expansion of murine 5,10-methylenetetrahydrofolate pools. Biochem Pharmacol 49: 677