Relationship between Dose Rate of [6*RS*]Leucovorin Administration, Plasma Concentrations of Reduced Folates, and Pools of 5,10-Methylenetetrahydrofolates and Tetrahydrofolates in Human Colon Adenocarcinoma Xenografts[1]

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ABSTRACT

[6*RS*]Leucovorin (5-formyltetrahydrofolate; 5-CHO-H_4PteGlu) administered in different regimens in combination with 5-fluorouracil (FUra) has increased the response rates to FUra in patients with colon adenocarcinoma. Using preclinical models of human colon adenocarcinomas as xenografts in immune-deprived mice, the effect of the rate of administration of racemic [6*RS*]leucovorin on the concentration-time profile of reduced folates in plasma, size of intratumor pools of 5,10-methylenetetrahydrofolates (CH_2-H_4PteGlu$_n$) and tetrahydrofolates (H_4PteGlu$_n$), and the distribution of their polyglutamate species have been examined.

Bolus injection i.v., or 4-h or 24-h infusion of [6*RS*]leucovorin (500 mg/m^2) yielded similar concentration profiles of the biologically active [6*S*] and inactive [6*R*] isomers of 5-CHO-H_4-PteGlu and 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) in mouse plasma to those previously reported in humans, but with more rapid elimination half-lives ($t_{½}$ = 11 to 16 min, 23 to 41 min, and 30 to 35 min, respectively). Thus, reduced folates remained elevated in plasma during the period of [6*RS*]leucovorin administration. In $HxELC_2$ and $HxGC_3$ tumors, pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were increased from 350% to 700% of control, but only during [6*RS*]leucovorin infusion. Intracellular levels subsequently declined rapidly, similar to the loss of reduced folates from plasma. Increasing the rate of [6*RS*]leucovorin delivery by decreasing the time for administration from a 24-h to a 4-h infusion did not further increase the intratumor pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$, suggesting saturation in the cellular metabolism of [6*RS*]leucovorin.

In $HxGC_3$ tumors, CH_2-H_4PteGlu$_{4-5}$ were elevated more rapidly than in line $HxELC_2$, which accumulated predominantly a shorter chain length species following i.v. bolus injection. During the 4-h infusion schedule, di- and triglutamate species in particular accumulated in both tumors with no elevation in CH_2-H_4PteGlu$_5$ until the infusion was discontinued, when this species increased as the shorter chain length forms were declining. However, during the 24-h infusion of [6*RS*]leucovorin, CH_2-H_4PteGlu$_{3-5}$ were elevated in tumors. Since these species have been reported to increase the binding affinity of [6-^{3}H]5-fluorodeoxyuridine monophosphate ([6-^{3}H]FdUMP) to thymidylate synthase, and intratumor pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were elevated during the 24-h infusion of [6*RS*]leucovorin, this was considered to be the preferred schedule for administration. When FUra (6.25 to 25 mg/kg) was administered 3 h into a 24-h infusion of [6*RS*]leucovorin (500 mg/m^2) in tumor-bearing mice, potentiation of thymidylate synthase inhibition in comparison with FUra administered alone was observed. These studies raise important questions regarding the effect of (*a*) dose of [6*RS*]-leucovorin, (*b*) frequency of administration, (*c*) utility of 5-CH_3-H_4PteGlu, and (*d*) [6*R*]5-CHO-H_4PteGlu on influencing intratumor pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$, the inhibition of thymidylate synthase by FUra, and FUra cytotoxicity in colon tumors under the *in situ* conditions of tumor growth.

INTRODUCTION

5-Fluorouracil is one of the most effective single agents used in the treatment of adenocarcinoma of the colon. However, responses to the agent administered singly either in patients (1, 2) or to xenografted tumors in mice (3) have been transient. Previous studies from these laboratories suggested that, in human colon adenocarcinoma xenografts, concentrations of CH_2-H_4PteGlu$_n$[3] were suboptimal to allow maximal formation or stability of the covalent ternary complex formed between thymidylate synthase, CH_2-H_4PteGlu$_n$, and the metabolite of FUra, FdUMP (4–6). Data also suggested that it would be advantageous to administer a reduced folate with FUra *in vivo* to increase the pools of CH_2-H_4PteGlu$_n$ in colon tumors (4), in particular, concentrations of the longer polyglutamate chain length forms. These species have been shown to increase the affinity of binding of FdUMP to the enzyme at concentrations lower than required for binding in the presence of the monoglutamyl form (6).

Leucovorin, which is a mixture of diastereoisomers and a stable form of reduced folate, has subsequently been used in clinical trials in combination with FUra in the treatment of patients with colon adenocarcinoma. This strategy was based, in part, upon the xenograft studies and also upon *in vitro* studies using cultured cells that demonstrated a 1.5- to 4.6-fold potentiation of FUra- or FdUrd-induced cytotoxicity by [6*RS*]-leucovorin (7–11). In several independently conducted Phase III randomized clinical trials, FUra in combination with [6*RS*]leucovorin has shown significant increases in response rates over FUra administered alone (3- to 5-fold) in the treatment of colon adenocarcinomas (12–16). In the combination arms, a significant increase in time to disease progression (12, 13, 15), increase in patient survival (13, 15), and increased therapeutic index (15) over FUra administered alone have been reported. Of interest is that [6*RS*]leucovorin has been administered by i.v. bolus injection daily for 5 days (13, 15), by a short duration of infusion (2 h) weekly (14, 16), or by continuous infusion over 5 to 6 days (12) at dose levels of 20 to 500 mg/m^2, each of which has resulted in significant increases in response rates to FUra.

Plasma pharmacokinetics of the individual isomers of leucovorin and the major metabolite of the biologically active [6*S*]leucovorin, 5-CH_3-H_4PteGlu, has also been reported for several clinical regimens (16–19). However, no data, either

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[3] The abbreviations used are: CH_2-H_4PteGlu$_n$, 5,10-methylenetetrahydrofolates; 5-CHO-H_4PteGlu, 5-formyltetrahydrofolate ([6*RS*]leucovorin); FUra, 5-fluorouracil; H_4PteGlu$_n$, tetrahydrofolates; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolate; FdUMP, 5-fluorodeoxyuridine monophosphate; FdUrd, 5-fluorodeoxyuridine; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate.

clinical or preclinical, are available that determine how the plasma concentrations of these reduced folate forms and their maintenance relate to the elevation and maintenance of intratumor concentrations of CH_2-H_4PteGlu$_n$ or how [6*RS*]leucovorin influences the distribution of the polyglutamate species or the inhibition of thymidylate synthase by FUra. Consequently, we have examined these relationships in preclinical models of human colon adenocarcinomas maintained as xenografts in immune-deprived mice. A dose of [6*RS*]leucovorin (500 mg/m^2) was chosen to provide plasma concentrations similar to those used clinically (16–18) and was administered to mice i.v. by bolus injection, short infusion (4 h), and prolonged infusion (24 h). The effects of dose rate of [6*RS*]-leucovorin administration on the plasma concentration-time profiles of reduced folates in mice and, subsequently, on intracellular pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in two human colon xenograft lines were evaluated. In addition, the 24-h infusion schedule that was considered to be optimal was subsequently selected for examination of the effect of [6*RS*]-leucovorin (500 mg/m^2) on FUra-induced thymidylate synthase inhibition.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]dUMP (specific activity, 20 to 22 Ci/mmol) and [6-^{3}H]FdUMP (specific activity, 18 to 20 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA. Glycine was obtained from Eastman Kodak Co., Rochester, NY, and Tris (ultrapure) from Boehringer Mannheim Biochemicals, Indianapolis, IN. Polyacrylamide, sodium dodecyl sulfate, and all other reagents for gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. Pteroylpolyglutamates (for preparing CH_2-H_4PteGlu$_n$; 6) were obtained either from Dr. Charles Baugh, University of South Alabama College of Medicine, Mobile, AL, or from American Radiochemicals, Inc., St. Louis, MO. *Lactobacillus casei* thymidylate synthase (specific activity, 1.8 to 8.2 μmol/h/mg; specific activity, 72.4 to 211 μmol/h/ml; 1 unit converts 1 μmol of dUMP/h to dTMP) was purchased from Biopure, Boston, MA. Charcoal (activated, neutralized) was supplied by Sigma Chemical Co., St. Louis, MO. NCS tissue solubilizer and ACS scintillant were obtained from Amersham Corp., Arlington Heights, IL; PPO and POPOP were from Research Products International Corp., Elk Grove Village, IL; and En^{3}hance was from New England Nuclear, Boston, MA. Kodak X-Omat AR film was purchased from Eastman Kodak Co. HPLC-grade methanol was obtained from American Scientific (Muskegon, MI). μBondapak phenyl HPLC columns were purchased from Waters Chromatography Division, Milford, MA, and columns of bovine serum albumin bound to 10-μm spherical silica (Resolvosil) were from Rainin Instruments, Woburn, MA. [6*R*]10-CHO-H_4PteGlu was kindly provided by Dr. John McGuire, Roswell Park Memorial Institute. All other reagents were supplied by Sigma or were of reagent grade.

Tumor Lines. Human colon adenocarcinomas $HxELC_2$ and $HxGC_3$ were maintained as xenografts by passage in the s.c. space of female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, ME) immune-deprived by thymectomy, followed by potentially lethal whole-body irradiation and reconstitution with syngeneic bone marrow as described previously (6). Line $HxELC_2$ has shown some sensitivity to 5-fluoropyrimidines after treatment of tumor-bearing mice, whereas $HxGC_3$ tumors were intrinsically resistant to these agents *in vivo* (3). A 5-fold difference in thymidylate synthase activity has been determined in the 2 tumor lines, where the activity was 0.186 and 0.991 nmol/g of tissue/min, respectively, determined from the release of ^{3}H from [5-^{3}H]dUMP (20).

Plasma Pharmacokinetics of [6*S*]Leucovorin, [6*R*]Leucovorin, and [6*S*]5-CH_3-H_4PteGlu in Mice. One hundred seven non-tumor-bearing, immune-deprived mice received [6*RS*]leucovorin at a dose level of 500 mg/m^2 by i.v. bolus injection or by 4-h or 24-h infusion through a cannula introduced into the tail vein. For infusions, [6*RS*]leucovorin was delivered from a Harvard Model 975 infusion pump at a rate of 0.14 ml/h. The relationship between mg/kg and mg/m^2 was calculated for individual mice, as previously described (21). Blood samples were obtained at regular intervals for 360 min after bolus injection (n = 31), at 2 time points during and 4 time points following the 4-h infusion (n = 35), and at 7 time points during and 60 min following the 24-h infusion (n = 41). For each mouse, a single blood sample of 0.3 to 0.8 ml was obtained by cardiac puncture under metafane anesthesia. Samples were collected in heparinized syringes and placed on ice, and sodium ascorbate was added immediately to a final concentration of 1 mg/ml. Samples were centrifuged at 2000 × g for 10 min at 2°C. Plasma was removed and stored at −70°C prior to analysis.

A coupled HPLC achiral-chiral assay system was used to separate and quantitate the individual enantiomers of [6*RS*]leucovorin and 5-CH_3-H_4PteGlu using methotrexate as an internal standard. The enantiomeric mixture was quantitated on the achiral column and subsequently separated on a chiral system as previously described (22). Detection limits were 100 ng/ml (≈0.2 μM) for 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu. Retention times on the chiral column were 28 and 40 min for the [6*S*] and [6*R*] enantiomers of the parent compound and 58 min for the [6*S*]leucovorin metabolite, 5-CH_3-H_4PteGlu (22).

Pharmacokinetic parameters for a one- and two-compartment model were estimated using maximum likelihood with each observation weighted according to the inverse of the variance for the model estimate assuming a coefficient of variation of 10% (23). Model selection was based on comparative examination of residuals and the weighted sum of squares. Data for each of the enantiomers of [6*RS*]leucovorin for the bolus, 4-h, and 24-h infusions were analyzed separately. For the metabolite 5-CH_3-H_4PteGlu, pharmacokinetic parameters were determined using a parent-metabolite model. The independently estimated [6*S*]-leucovorin parameters were fixed as a driving function with the assumptions that 80% of the parent compound was converted to metabolite by a first order process. Varying the assumption of parent to metabolite conversion from 50 to 100% did not influence the estimate of metabolite plasma half-life.

Infusions of [6*RS*]Leucovorin in Tumor-bearing Mice. Mice bearing $HxELC_2$ or $HxGC_3$ tumors were infused i.v. with [6*RS*]leucovorin by bolus administration or by infusion for 4 h or 24 h. At various times during or after infusion, mice (2 per point) were killed. Tumors (2 per mouse) were rapidly excised, pooled (unless otherwise stated), and placed immediately in and subsequently stored in liquid nitrogen. Pooled tumors were ground to a fine powder under liquid nitrogen, and the extracted powders were used to examine the modulation of pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$. Alternatively, pooled tumors were allowed to thaw to 2°C on ice prior to examination of the effect of [6*RS*]leucovorin on FUra-induced thymidylate synthase inhibition.

Determination of Pool Size of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$. Due to the instability of CH_2-H_4PteGlu$_n$ to heat treatment in the absence of excess HCHO (5, 24), endogenous concentrations of the combined pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were determined in the presence of excess HCHO that converted H_4PteGlu$_n$ to CH_2-H_4PteGlu$_n$. The assay was based on the catalytic release of ^{3}H from [5-^{3}H]dUMP over 3 min, where the rate of reaction was determined to be independent of the glutamyl form of the cofactor (5). Reactions were linear over 3 min under the conditions used. *L. casei* thymidylate synthase and tumor extracts (containing 1% β-mercaptoethanol and 10 mM sodium ascorbate) as the source of reduced folates were used in reaction mixtures as described previously (5). Endogenous dUMP was removed by treatment of extracts with 5′-nucleotidase prior to assay. Batches of thymidylate synthase were examined for formylase activity (25) using [6*R*]10-CHO-H_4PteGlu and, where necessary, were further purified on columns of Sephadex G-100 and CM-Sephadex (26) that removed the contaminating enzyme.

Distribution of Polyglutamates of CH_2-H_4PteGlu. Determination of the predominance of CH_2-H_4PteGlu polyglutamates in $HxGC_3$ and $HxELC_2$ tumors following [6*RS*]leucovorin administration was based upon the technique described by Priest and Doig (27), where the distribution of CH_2-H_4PteGlu$_n$ had been previously characterized in untreated tumor xenografts (5). This method has been currently used as a qualitative technique only, with quantitation of pool size expansion

by the ^3H release assay described above. Ternary complexes formed among [6-^3H]FdUMP (125 nM), excess *L. casei* thymidylate synthase, and CH_2-H_4PteGlu$_n$ from tumor extracts were electrophoresed on 9% polyacrylamide nondenaturing gels (28 cm), and fluorograms were prepared. Equivalent volumes of reaction mixtures containing 10% glycerol (120 or 160 μl) were applied to each gel. A modified gel-processing procedure yielding improvement of sample reproducibility was used for some of the studies. The new procedure involved fixation for 1 h at room temperature in a mixture of glacial acetic acid (10%, v/v) and methanol (30%, v/v) in water, followed by treatment with En^{3}hance for 1 h with gentle agitation. Following impregnation, gels were agitated in an excess of cold water for 30 min (one change) and were subsequently dried. Data were analyzed by scanning densitometry of fluorograms to determine the intensity of bands. The relationship between peak height and dpm was linear ($r^2 = 0.944$) over the range examined. Each experiment was controlled internally by electrophoresis of untreated and [6*RS*]leucovorin-treated samples on the same gel.

SDS-Gel Analysis of [6-^{3}H]FdUMP-Thymidylate Synthase-CH_2-H_4PteGlu$_n$ Complexes. Tumor extracts (200 μl) prepared for the determination of size of pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were incubated at 37°C for 30 min with *L. casei* thymidylate synthase (1.8 units), [6-^{3}H]FdUMP (125 nM), and 25 mM Tris-HCl (pH 7.4) containing β-mercaptoethanol (1%) and sodium ascorbate (10 mM). Mixtures were subsequently heated at 100°C for 3 min with 50 μl of SDS sample buffer (5×) to denature covalent ternary complexes and were stored at −70°C until analyzed. Ninety or 180 μl of each sample were applied to 12% polyacrylamide-SDS gels (1.5 mm x 14 cm x 16 cm) with a 4% polyacrylamide-SDS stacking gel (4 cm). Ternary complexes were electrophoresed at 25 mA/gel according to the method of Laemmli (28). The method was used to qualitatively determine the appearance and disappearance of covalent ternary complexes during and following modulation by [6*RS*]leucovorin.

Inhibition of Thymidylate Synthase. The effect of a 24-h i.v. infusion of [6*RS*]leucovorin (500 mg/m^2) on the inhibition of thymidylate synthase by FUra in $HxELC_2$ and $HxGC_3$ tumors was examined. A suboptimal dose of FUra was selected that would cause incomplete inhibition of thymidylate synthase at the nadir, followed by some recovery by the end of the infusion, such that potentiation by [6*RS*]-leucovorin may be more readily determined. The dose of FUra used in mice bearing $HxELC_2$ tumors was 12.5 mg/kg and for $HxGC_3$ was 6.25 or 25 mg/kg. FUra was administered i.v. by bolus injections 3 h into a 24-h infusion of [6*RS*]leucovorin or saline, by means of a 3-way valve, and was flushed through the cannula with 0.1 ml of saline (0.9%). At this time, plasma levels of reduced folates were approaching a steady-state concentration, and intratumor pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were elevated. At 4 h and 21 h after FUra administration, tumors were excised (4 tumors pooled; 2 mice per time point), cytosols were prepared, and endogenous nucleotides were removed by charcoal adsorption procedures. The rate of release of ^3H from [5-^{3}H]-dUMP was subsequently determined in each sample and compared to the rate of release of ^3H in control tumors, as described (20, 29). Data were analyzed statistically using a one-way analysis of variance.

RESULTS

Plasma Concentration-Time Profiles for Reduced Folates

Concentrations of reduced folates achieved in plasma following bolus administration or 4-h or 24-h infusion of [6*RS*]-leucovorin (500 mg/m^2) were examined. The data and computer-simulated plasma concentration-time profiles for [6*S*]-leucovorin and 5-CH_3-H_4PteGlu are shown in Fig. 1*A* and for [6*R*]leucovorin in Fig. 1*B* for each of the 3 different administration regimens. The maximal plasma concentrations achieved and the half-time ($t_{½}$) for elimination from plasma for each reduced folate and schedule are summarized in Table 1.

Bolus Administration. After i.v. bolus injection of [6*RS*]-leucovorin, plasma concentrations of the biologically active [6*S*] isomer of leucovorin were lower than for the inactive [6*R*] isomer (555 μM and 649 μM, respectively) when sampling commenced at 5 min. The [6*S*] isomer was rapidly eliminated, with a monoexponential $t_{½}$ of 11.4 min (Table 1), and was undetectable at 2 h after injection. For [6*R*]leucovorin, elimination from plasma was more prolonged and biexponential ($t_{½\alpha}$ = 18.2 min; $t_{½\beta}$ = 41.2 min). For the major metabolite of [6*S*]-leucovorin, 5-CH_3-H_4PteGlu, appearance in plasma was rapid, with the maximal concentration (34 μM) achieved at approximately 30 min following [6*RS*]leucovorin bolus administration. Its estimated elimination $t_{½}$ from plasma (30.3 min) was intermediate between the values for the two enantiomers of [6*RS*]-leucovorin. At 100 min after injection, the ratios of the concentrations of [6*R*]leucovorin to [6*S*]leucovorin and of 5-CH_3-H_4PteGlu to [6*S*]leucovorin were estimated to be 11 and 8, respectively.

Four-h Infusion. During a 4-h infusion of [6*RS*]leucovorin, lower levels of all reduced folates were observed in plasma (Fig. 1; Table 1). At 3 h during the infusion, concentrations of [6*S*]-leucovorin, [6*R*]leucovorin, and 5-CH_3-H_4PteGlu were determined to be 55, 78, and 7.7 μM, respectively. Upon cessation of infusion, each was eliminated from the plasma, with half-lives of 8.7, 23.2, and 34.9 min, respectively. The pharmacokinetic parameters estimated from the bolus administration experiments with [6*RS*]leucovorin did not reliably predict [6*S*]-leucovorin and 5-CH_3-H_4PteGlu concentrations following the 4-h [6*RS*]leucovorin infusion (Fig. 1*A*), suggesting nonlinearity in disposition at differing dose rates. Thus, the $t_{½}$ for elimination from plasma was estimated using only the postinfusion data (Fig. 1*A*, – – – –). The data for [6*R*]leucovorin during and following the 4-h infusion were, however, adequately described by a linear pharmacokinetic model.

Twenty-four-h Infusion. When the duration of infusion of 500 mg/m^2 of [6*RS*]leucovorin was extended to 24 h, lower concentrations of [6*S*] (5.1 μM) and [6*R*] (16 μM) leucovorin and 5-CH_3-H_4PteGlu (3 μM) were determined. Concentrations of [6*S*]leucovorin approached maximum values within 1 to 3 h from the initiation of the infusion, but did show a tendency to accumulate during the infusion. Reduced folates remained elevated in plasma during the infusion and were again rapidly eliminated after discontinuing the infusion. A one-compartment linear pharmacokinetic model was used to describe the data for each reduced folate during and following the 24-h infusion of [6*RS*]leucovorin. The $t_{½}$ values for elimination of [6*S*]leucovorin and [6*R*]leucovorin following the infusion were 15.6 and 35.3 min, respectively, consistent with data derived following the alternate two [6*RS*]leucovorin administration regimens. Although insufficient data were available to accurately estimate a $t_{½}$ for elimination of 5-CH_3-H_4PteGlu from plasma following the 24-h [6*RS*]leucovorin infusion, the pattern of metabolite accumulation during the infusion was consistent with an elimination $t_{½}$ of 35 min determined following a 4-h infusion.

For all [6*RS*]leucovorin infusion regimens, where plasma concentrations of reduced folates had been determined in tumor-bearing mice, these were similar to those reported for the concentration-time profile study determined in non-tumor-bearing mice (data not shown).

Determination of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ Pools in Neoplastic Tissues

In $HxELC_2$ and $HxGC_3$ tumors, modulation of the pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$, as determined by the catalytic release of ^3H from [5-^{3}H]dUMP, followed the maintenance and disappearance of the potentially biologically active reduced

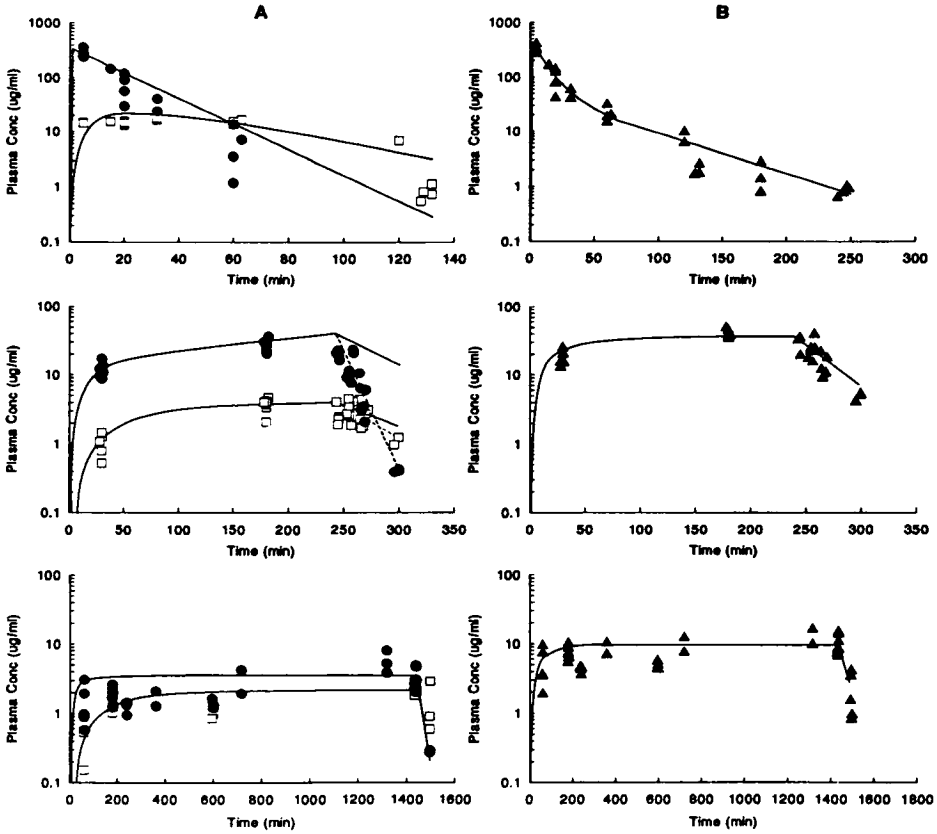

Fig. 1. Plasma concentration-time profiles of [6*S*]leucovorin (●) and 5-CH_3-H_4-PteGlu (□) (*A*) and [6*R*]leucovorin (▲) (*B*) in mice following i.v. bolus administration (*top*), 4-h infusion (*center*), or 24-h infusion (*bottom*) of [6*RS*]leucovorin (500 mg/m^2). Plasma samples were analyzed and data evaluated according to procedures described in "Materials and Methods." Data pertaining to [6*S*]leucovorin and 5-CH_3-H_4PteGlu for the 4-h infusion schedule were fit to the linear pharmacokinetic model (——) or, alternatively, data obtained following the infusion were analyzed by linear regression (– – – –).

Table 1 *Maximal plasma concentrations of reduced folates achieved in mice and $t_{1/2}$ for elimination following different schedules of [6RS]leucovorin administration*

Schedule	Maximal plasma concentration (μM)[a]			$t_{1/2}$ (min)		
	[6*S*]Leucovorin	[6*R*]Leucovorin	5-CH_3-H_4PteGlu	[6*S*]Leucovorin	[6*R*]Leucovorin	5-CH_3-H_4PteGlu
i.v. bolus	555 ± 110[b] (4)[c]	649 ± 113 (4)	34 ± 2 (2)	11.4	41.2[d]	30.3
4-h infusion	55 ± 10 (8)	78 ± 10 (8)	7.7 ± 1.6 (8)	8.7	23.2	34.9
24-h infusion	5.1 ± 3.1 (32)[e]	16 ± 7 (31)[e]	3.0 ± 1.0 (11)[e]	15.6	35.3	NE[f]

[a] Determined from derived data.
[b] Mean ± SD.
[c] Numbers in parentheses, number of data points.
[d] Terminal half-life estimated by fitting data to a two-compartment model. Initial $t_{1/2}$ = 18.2 min.
[e] Average value from all data derived between 3 and 24 h representing an average steady-state concentration.
[f] NE, not evaluable.

folates ([6*S*]leucovorin and 5-CH_3-H_4PteGlu) from plasma. Following an i.v. bolus injection of [6*RS*]leucovorin, pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were elevated in both tumor lines to 242% ($HxGC_3$) or 409% ($HxELC_2$) of control at 1 h after injection (Fig. 2), the earliest time point examined. After this time, intratumor reduced folate pools declined rapidly, approaching control levels by 6 h after [6*RS*]leucovorin administration.

At the end of a 4-h infusion of [6*RS*]leucovorin in mice bearing $HxELC_2$ tumors, the combined pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were elevated to 660% of control in tumors and were observed to return to basal levels at 4 h after the infusion had ceased (Fig. 2). In line $HxGC_3$, intratumor reduced folate pools increased to 253% of control 2 h into the 4-h infusion and were maintained at 221% of control at the end of infusion. However, as had been observed in $HxELC_2$ tumors following infusion, intratumor concentrations of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ rapidly declined, following the elimination of reduced folates from plasma (Fig. 2).

During the 24-h infusion of [6*RS*]leucovorin in tumor-bearing mice (Fig. 2), intratumor pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were elevated to 253% and 344% of control at 12 and 24 h, respectively, in $HxGC_3$ tumors, and to 677% and 702% of control at these times in line $HxELC_2$. However, these pools rapidly declined following the end of infusion, as plasma levels of reduced folates also decreased.

Distribution of CH_2-H_4PteGlu Polyglutamates in Tumors

The influence of [6*RS*]leucovorin (500 mg/m^2) on the distribution of polyglutamate species of CH_2-H_4PteGlu and the combined pools of CH_2-H_4PteGlu and H_4PteGlu was subsequently examined in both tumor lines for the 3 different rates of administration. Within a tumor line, qualitatively similar effects were observed for the two pools for each dose rate of [6*RS*]leucovorin administration. Consequently, data for the CH_2-H_4PteGlu$_n$ pool alone have been presented.

$HxGC_3$ Tumors. In $HxGC_3$ tumors, there was a predominance of penta- and hexaglutamate species (Figs. 3*A* and 4*A*). Following bolus injection of [6*RS*]leucovorin (500 mg/m^2) to tumor-bearing mice, there was a rapid appearance of CH_2-H_4PteGlu$_4$

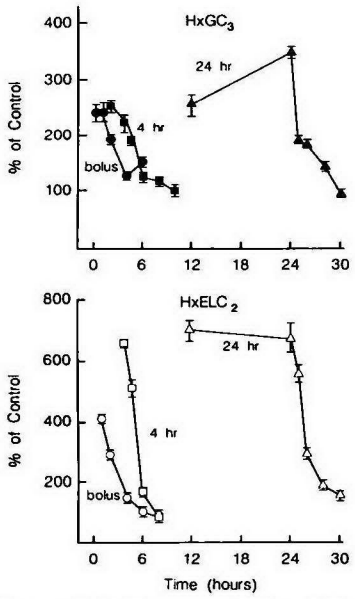

Fig. 2. The influence of [6*RS*]leucovorin (500 mg/m²) administered by i.v. bolus injection, 4 h or 24 h infusion, on the size of pools CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in $HxGC_3$ and $HxELC_2$ tumors. The assay was based on the release of ^3H from [5-^3H]dUMP as described in "Materials and Methods." *Points*, mean of 12 to 16 determinations on 4 individual tumors at each time point; *bars*, SE.

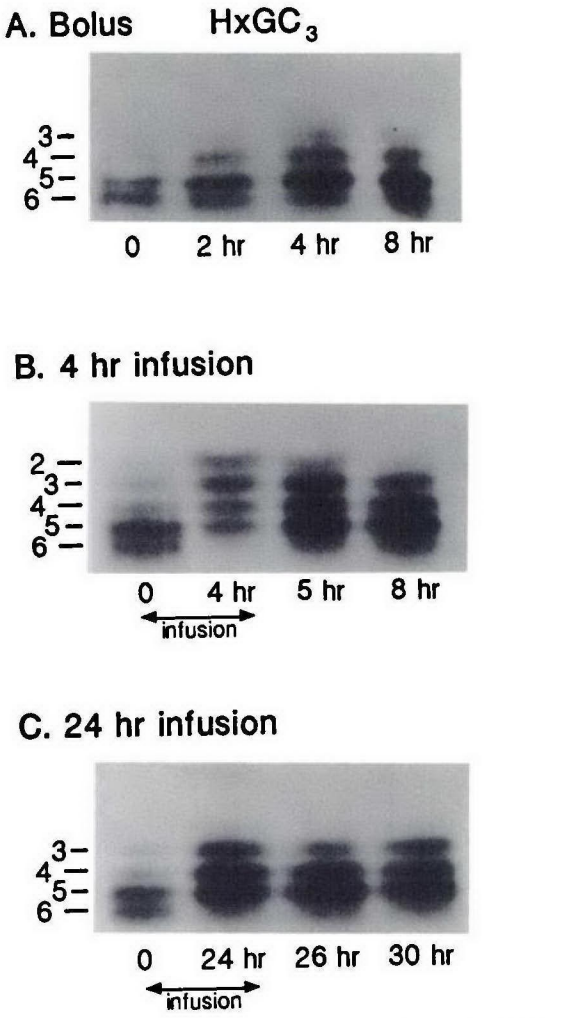

Fig. 3. The distribution of polyglutamate species of CH_2-H_4PteGlu in $HxGC_3$ tumors following i.v. bolus administration (*A*), during and following a 4-h infusion (*B*), or 24 h infusion (*C*) of [6*RS*]leucovorin (500 mg/m²) in tumor-bearing mice. [6-^3H]FdUMP-thymidylate synthase-CH_2-H_4PteGlu$_n$ complexes, formed using tumor extracts, were electrophoresed on 9% nondenaturing gels, which were subsequently fixed in 5% trichloroacetic acid (*A*) or 10% glacial acetic acid:30% methanol in water (*B* and *C*) prior to treatment with En^{3}Hance. Gels were further treated and dried, and fluorograms were prepared as described in "Materials and Methods."

and increased formation of CH_2-H_4PteGlu$_5$, within 2 h. Tetra-, penta-, and hexaglutamate species continued to predominate 8 h after initial administration of [6*RS*]leucovorin; a trace of CH_2-H_4PteGlu$_2$ was detected at 4 h.

At the end of a 4-h infusion of [6*RS*]leucovorin in mice bearing $HxGC_3$ tumors, the distribution of CH_2-H_4PteGlu$_n$ differed from that observed after i.v. bolus injection (Figs. 3*B* and 4*B*). It was evident in $HxGC_3$ tumors that, during the infusion, there was marked accumulation of CH_2-H_4PteGlu$_2$ and CH_2-H_4PteGlu$_3$, some increase in CH_2-H_4PteGlu$_4$, and a concomitant decrease in CH_2-H_4PteGlu$_5$ and rapid disappearance of CH_2-H_4PteGlu$_6$. By 1 h after the end of the infusion, CH_2-H_4PteGlu$_{3-5}$ predominated, and after a further 3 h, di- and triglutamate species were declining. CH_2-H_4PteGlu$_6$ could not be detected during and after [6*RS*]leucovorin infusion, although a small quantity of the hexaglutamate was detected within the combined pool of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ (data not shown). Thus, during the 4-h infusion of [6*RS*]leucovorin, accumulation of shorter polyglutamate chain length forms of CH_2-H_4PteGlu was detected in $HxGC_3$ tumors that was not observed following bolus administration of [6*RS*]leucovorin.

By the end of the 24-h infusion of [6*RS*]leucovorin, CH_2-H_4PteGlu$_{3-5}$ were the predominant species in line $HxGC_3$, while CH_2-H_4PteGlu$_2$ was not substantially elevated. CH_2-H_4PteGlu$_6$ was again not detectable in $HxGC_3$ following treatment of tumor-bearing mice with [6*RS*]leucovorin. For up to 6 h after the end of the infusion, CH_2-H_4PteGlu$_{3-5}$ predominated (Figs. 3*C* and 4*C*).

$HxELC_2$ Tumors. After i.v. administration of [6*RS*]leucovorin in mice, differences in the distribution of CH_2-H_4PteGlu$_n$ were apparent in $HxELC_2$ tumors in comparison with line $HxGC_3$, with a shorter chain length form, either the mono- or diglutamate, accumulating to a greater extent than had been observed in line $HxGC_3$. After i.v. bolus injection of [6*RS*]-leucovorin, this species was detected by 2 h postinjection, with little change in the pentaglutamate. Some elevation in the tri- and tetraglutamate species was also observed (Fig. 5*A*). As the shorter chain length form declined by 8 h after injection, CH_2-H_4PteGlu$_4$ and CH_2-H_4PteGlu$_5$ became elevated.

At the end of the 4-h infusion of [6*RS*]leucovorin (Fig. 5*B*), the predominant change observed in $HxELC_2$ tumors was similar to that observed in $HxGC_3$, in that accumulation of CH_2-H_4PteGlu$_2$ was evident, with some elevation in the tri- and tetraglutamates; CH_2-H_4PteGlu$_5$ was observed to decrease. Upon cessation of infusion, however, CH_2-H_4PteGlu$_2$ declined, with a concomitant increase in CH_2-H_4PteGlu$_3$, CH_2-H_4PteGlu$_4$, and CH_2-H_4PteGlu$_5$. By 4 h postinfusion, the tetra- and pentaglutamates were the predominant species.

With the 24-h infusion of [6*RS*]leucovorin (Fig. 5*C*), CH_2-H_4PteGlu$_3$ and CH_2-H_4PteGlu$_4$ were most markedly elevated by the end of the infusion in $HxELC_2$ tumors. CH_2-H_4PteGlu$_5$ was also elevated, but decreased as a percentage of the folate pool due to the large increases in CH_2-H_4PteGlu$_3$ and CH_2-H_4PteGlu$_4$. Some increase in CH_2-H_4PteGlu$_2$ was also detected at this time. CH_2-H_4PteGlu$_{3-5}$ also continued to predominate for at least 6 h postinfusion.

For all [6*RS*]leucovorin regimens, the detectability of CH_2-H_4PteGlu$_6$ appeared to decrease following treatment.

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