ABSTRACT

[6RS]Leucovorin (5-formyltetrahydrofolate; 5-CHO-H4PteGlu) 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 [6RS]leucovorin on the concentration-time profile of reduced folates in plasma, size of intratumor pools of 5,10-methylene-tetrahydrofolates (CH2-H4PteGlu4) and tetrahydrofollate (H4PteGlu4), and the distribution of their polyglutamate species have been examined.

Bolus injection i.v., or 4-h or 24-h infusion of [6RS]leucovorin (500 mg/m2) yielded similar concentration profiles of the biologically active [6S] and inactive [6R] isomers of 5-CHO-H4PteGlu and 5-methyl-tetrahydrofollate (5-CH3-H4PteGlu) in mouse plasma to those previously reported in humans, but with more rapid elimination half-lives (t1/2 = 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 [6RS]leucovorin administration. In HxELC2 and HxGC2 tumors, pools of CH2-H4PteGlu4 and H4PteGlu4, were increased from 350% to 700% of control, but only during [6RS]leucovorin infusion. Intracellular levels subsequently declined rapidly, similar to the loss of reduced folates from plasma. Increasing the rate of [6RS]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 CH2-H4PteGlu4 and H4PteGlu4, suggesting saturation in the cellular metabolism of [6RS]leucovorin.

In HxGC2 tumors, CH2-H4PteGlu4 were elevated more rapidly than in line HxELC2, 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 CH2-H4PteGlu until the infusion was discontinued, when this species increased as the shorter chain length forms were declining. However, during the 24-h infusion of [6RS]leucovorin, CH2-H4PteGlu4 were elevated in tumors. Since these species have been reported to increase the binding affinity of [6S]5-fluorodeoxyuridine monophosphate (6-5FdUMP) to thymidylate synthase, and intratumor pools of CH2-H4PteGlu4 and H4PteGlu4 were elevated during the 24-h infusion of [6RS]leucovorin, this was considered to be the preferred schedule for administration. When FUra (6.25 to 25 mg/kg) was administered 3 h after a 24-h infusion of [6RS]leucovorin (500 mg/m2) 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 [6RS]leucovorin, (b) frequency of administration, (c) utility of 5-CH3-H4PteGlu, and (d) [6RS]-CHO-H4PteGlu influencing intratumor pools of CH2-H4PteGlu4 and H4PteGlu4, 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 CH2-H4PteGlu4 and CH2-H4PteGlu5, were suboptimal to allow maximal formation or stability of the covalent ternary complex formed between thymidylate synthase, CH2-H4PteGlu4, 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 CH2-H4PteGlu4, 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 [6RS]-leucovorin (7-11). In several independently conducted Phase III randomized clinical trials, FUra in combination with [6RS]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 [6RS]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/m2, 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 [6S]leucovorin, 5-CH3-H4PteGlu4, has also been reported for several clinical regimens (16-19). However, no data, either

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2 To whom requests for reprints should be addressed.
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$_3$PteGlu, or how [6RS]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 [6RS]leucovorin (500 mg/m$^3$) 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 [6RS]-leucovorin administration on the plasma concentration-time profiles of reduced folates in mice and, subsequently, on intracellular pools of CH$_2$H$_3$PteGlu, and H$_3$PteGlu, 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 [6RS]-leucovorin (500 mg/m$^3$) on FURA-induced thymidylate synthase inhibition.

**MATERIALS AND METHODS**

Chemicals. [5-'H]dUMP (specific activity, 20 to 22 Ci/mmol) and [6-'H]dUMP (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. Pteroylglutamates (for preparing CH$_2$H$_3$PteGlu; 6) were obtained either from Dr. Charles Baugh, University of South Alabama College of Medicine, Mobile, AL, or from American Radiochemistry, Inc., St. Louis, MO. Lactobacillus casei thymidylate synthase (specific activity, 1.8 to 8.2 $\mu$mol/h/mg; specific activity, 72.4 to 211 $\mu$mol/h/ml; 1 unit converts 1 $\mu$mol 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 kindly provided by Dr. John McGuire, Roswell Park Memorial Institute.

**Plasma Pharmacokinetics of [65]leucovorin, [6/f]leucovorin, and [6RS]-leucovorin in Tumor-bearing Mice.** Mice bearing HxELC$_2$ or HxGC$_1$ tumors were infused i.v. with [6RS]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$_3$PteGlu, and H$_3$PteGlu. Alternatively, pooled tumors were allowed to thaw to 2°C on ice prior to examination of the effect of [6RS]leucovorin on FURA-induced thymidylate synthase inhibition.

**Determination of Pool Size of CH$_2$H$_3$PteGlu and H$_3$PteGlu.** Due to the instability of CH$_2$H$_3$PteGlu, to heat treatment in the absence of excess HCHO (5, 24), endogenous concentrations of the combined pools of CH$_2$H$_3$PteGlu, and H$_3$PteGlu, were determined in the presence of excess HCHO that converted H$_3$PteGlu to CH$_2$H$_3$PteGlu. The assay was based on the catalytic release of $^3$H from [5-'H]dUMP over 3 min, where the rate of reaction was determined to be independent of the glutamylation form of the cofactor (5). Reactions were linear over 3 min under the conditions used. L. casei thymidylate synthase and tumor extracts (containing 1% $\beta$-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 [6R]10-CHO-H$_3$PteGlu 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$_3$PteGlu.** Determination of the predominance of CH$_2$H$_3$PteGlu polyglutamates in HxGC$_1$ and HxELC$_2$ tumors following [6RS]leucovorin administration was based upon the technique described by Priest and Doig (27), where the distribution of CH$_2$H$_3$PteGlu 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 CH2-H4PteGlu 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 Ethoxycell with 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 (r2 = 0.944) over the range examined. Each experiment was controlled internally by electrophoresis of untreated and [6RS]-leucovorin-treated samples on the same gel.

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 [6RS]-leucovorin (500 mg/m2) were examined. The data and computer-simulated plasma concentration-time profiles for [6S]-leucovorin and 5-CH3-H4PteGlu were shown in Fig. 1A and for [6R]-leucovorin in Fig. 1B for each of the 3 different administration regimens. The maximal plasma concentrations achieved and the half-time (t1/2) for elimination from plasma for each reduced folate and schedule are summarized in Table 1.

Bolus Administration. After i.v. bolus injection of [6RS]-leucovorin, plasma concentrations of the biologically active [6S] isomer of leucovorin were lower than for the inactive [6R] isomer (555 µM and 649 µM, respectively) when sampling commenced at 5 min. The [6S] isomer was rapidly eliminated, with a monoexponential t1/2 of 11.4 min (Table 1), and was undetectable at 2 h after injection. For [6R]leucovorin, elimination from plasma was more prolonged and biexponential (t1/2 = 18.2 min; t2/3 = 41.2 min). For the major metabolite of [6S]-leucovorin, 5-CH3-H4PteGlu, appearance in plasma was rapid, with the maximal concentration (34 µM) achieved at approximately 30 min following [6RS]leucovorin bolus administration. Its estimated elimination t1/2 from plasma (30.3 min) was intermediate between the values for the two enantiomers of [6RS]-leucovorin. At 100 min after injection, the ratios of the concentrations of [6R]leucovorin to [6S]leucovorin and of 5-CH3-H4PteGlu to [6S]leucovorin were estimated to be 11 and 8, respectively.

Four-h Infusion. During a 4-h infusion of [6RS]leucovorin, lower levels of all reduced folates were observed in plasma (Fig. 1; Table 1). At 3 h during the infusion, concentrations of [6S]-leucovorin, [6R]leucovorin, and 5-CH3-H4PteGlu 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 [6RS]leucovorin did not reliably predict [6S]-leucovorin and 5-CH3-H4PteGlu concentrations following the 4-h [6RS]leucovorin infusion (Fig. 1A), suggesting nonlinearity in disposition at differing dose rates. Thus, the t1/2 for elimination from plasma was estimated using only the postinfusion data (Fig. 1A, — — —). The data for [6R]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/m2 of [6RS]leucovorin was extended to 24 h, lower concentrations of [6S] (5.1 µM) and [6R] (16 µM) leucovorin and 5-CH3-H4PteGlu (3 µM) were determined. Concentrations of [6S]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 [6RS]leucovorin. The t1/2 values for elimination of [6S]leucovorin and [6R]leucovorin following the infusion were 15.6 and 35.3 min, respectively, consistent with data derived following the alternate two [6RS]leucovorin administration regimens. Although insufficient data were available to accurately estimate a t1/2 for elimination of 5-CH3-H4PteGlu from plasma following the 24-h [6RS]leucovorin infusion, the pattern of metabolite accumulation during the infusion was consistent with an elimination t1/2 of 35 min determined following a 4-h infusion.

For all [6RS]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 CH2-H4PteGlu and H4PteGlu, Pools in Neoplastic Tissues

In HxELC2 and HxGC3 tumors, modulation of the pools of CH2-H4PteGlu and H4PteGlu, as determined by the catalytic release of 3H from [5-3H]dUMP, followed the maintenance and disappearance of the potentially biologically active reduced
LEUCOVORIN METABOLISM IN COLON TUMORS

Fig. 1. Plasma concentration-time profiles of [6S]leucovorin (●) and 5-CH$_3$-H$_4$PteGlu (□) (A) and [6R]leucovorin (▲) (B) in mice following i.v. bolus administration (top), 4-h infusion (center), or 24-h infusion (bottom) of [6RS]leucovorin (500 mg/m$^2$). Plasma samples were analyzed and data evaluated according to procedures described in “Materials and Methods.” Data pertaining to [6S]leucovorin and 5-CH$_3$-H$_4$PteGlu 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

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Maximal plasma concentration (μM)*</th>
<th>$t_\text{a}$ (min)</th>
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<tr>
<td>i.v. bolus</td>
<td>[6S]leucovorin 555 ± 110$^a$ (4)$^c$</td>
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</tr>
<tr>
<td></td>
<td>[6R]leucovorin 649 ± 113 (4)</td>
<td>41.2$^d$</td>
</tr>
<tr>
<td>4-h infusion</td>
<td>5-CH$_3$-H$_4$PteGlu 34 ± 2 (2)</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td>[6S]leucovorin 11.4</td>
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<tr>
<td></td>
<td>[6R]leucovorin 649 ± 113</td>
<td></td>
</tr>
<tr>
<td>24-h infusion</td>
<td>5-CH$_3$-H$_4$PteGlu 7.7 ± 1.6 (8)</td>
<td>23.2$^c$</td>
</tr>
<tr>
<td></td>
<td>[6S]leucovorin 11.4</td>
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<td>[6R]leucovorin 649 ± 113</td>
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* Determined from derived data.

$^a$ Mean ± SD.

$^c$ Numbers in parentheses, number of data points.

$^d$ Terminal half-life estimated by fitting data to a two-compartment model. Initial $t_\text{a}$ = 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 ([6S]leucovorin and 5-CH$_3$-H$_4$PteGlu) from plasma. Following an i.v. bolus injection of [6RS]leucovorin, pools of CH$_3$-H$_4$PteGlu$_n$ and H$_4$PteGlu$_n$ 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 [6RS]leucovorin administration.

At the end of a 4-h infusion of [6RS]leucovorin in mice bearing HxELC$_2$ tumors, the combined pools of CH$_3$-H$_4$PteGlu$_n$ and H$_4$PteGlu$_n$ 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$_3$-H$_4$PteGlu$_n$ and H$_4$PteGlu$_n$ rapidly declined, following the elimination of reduced folates from plasma (Fig. 2).

During the 24-h infusion of [6RS]leucovorin in tumor-bearing mice (Fig. 2), intratumor pools of CH$_3$-H$_4$PteGlu$_n$ and H$_4$PteGlu$_n$ 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$_3$-H$_4$PteGlu Polyglutamates in Tumors

The influence of [6RS]leucovorin (500 mg/m$^2$) on the distribution of polyglutamate species of CH$_3$-H$_4$PteGlu and the combined pools of CH$_3$-H$_4$PteGlu and H$_4$PteGlu 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 [6RS]leucovorin administration. Consequently, data for the CH$_3$-H$_4$PteGlu pool alone have been presented.

HxGC$_3$ Tumors. In HxGC$_3$ tumors, there was a predominance of penta- and hexaglutamate species (Figs. 3A and 4A). Following bolus injection of [6RS]leucovorin (500 mg/m$^2$) to tumor-bearing mice, there was a rapid appearance of CH$_3$-H$_4$PteGlu.
and increased formation of CH₂-H₄PteGlu, within 2 h. Tetra-, penta-, and hexaglutamate species continued to predominate 8 h after initial administration of [6RS]leucovorin; a trace of CH₂-H₂PteGlu₂ was detected at 4 h.

At the end of a 4-h infusion of [6RS]leucovorin in mice bearing HxGC₃ tumors, the distribution of CH₂-H₂PteGlu₂ differed from that observed after i.v. bolus injection (Figs. 3B and 4B). It was evident in HxGC₃ tumors that, during the infusion, there was marked accumulation of CH₂-H₂PteGlu₂ and CH₂-H₄PteGlu₃, some increase in CH₂-H₄PteGlu₄, and a concomitant decrease in CH₂-H₄PteGlu₅ and rapid disappearance of CH₂-H₆PteGlu₆. By 1 h after the end of the infusion, CH₂-H₄PteGlu₂₃ predominated, and after a further 3 h, di- and triglutamate species were declining. CH₂-H₄PteGlu₂ could not be detected during and after [6RS]leucovorin infusion, although a small quantity of the hexaglutamate was detected within the combined pool of CH₂-H₄PteGlu and H₄PteGlu (data not shown). Thus, during the 4-h infusion of [6RS]leucovorin, accumulation of shorter polyglutamate chain length forms of CH₂-H₄PteGlu was detected in HxGC₃ tumors that was not observed following bolus administration of [6RS]leucovorin.

By the end of the 24-h infusion of [6RS]leucovorin, CH₂-H₄PteGlu₂₃ were the predominant species in line HxGC₃, while CH₂-H₄PteGlu₂ was not substantially elevated. CH₂-H₄PteGlu₂ was again not detectable in HxGC₃ following treatment of tumor-bearing mice with [6RS]leucovorin. For up to 6 h after the end of the infusion, CH₂-H₄PteGlu₂₃, predominated (Figs. 3C and 4C).

HxELC₂ Tumors. After i.v. administration of [6RS]leucovorin in mice, differences in the distribution of CH₂-H₄PteGlu were apparent in HxELC₂ tumors in comparison with line HxGC₃, with a shorter chain length form, either the mono- or diglutamate, accumulating to a greater extent than had been observed in line HxGC₃. After i.v. bolus injection of [6RS]leucovorin, this species was detected by 2 h postinjection, with little change in the pentaglutamate. Some elevation in the tri- and tetragnitamate species was also observed (Fig. 5A). As the shorter chain length form declined by 8 h after injection, CH₂-H₄PteGlu₂ and CH₂-H₄PteGlu₃ became elevated.

At the end of the 4-h infusion of [6RS]leucovorin (Fig. 5B), the predominant change observed in HxELC₂ tumors was similar to that observed in HxGC₃, in that accumulation of CH₂-H₄PteGlu₂ was evident, with some elevation in the tri- and tetraglutamates; CH₂-H₄PteGlu₃ was observed to decrease. Upon cessation of infusion, however, CH₂-H₄PteGlu₂ declined, with a concomitant increase in CH₂-H₄PteGlu₂, CH₂-H₄PteGlu₃, CH₂-H₄PteGlu₄, and CH₂-H₄PteGlu₅. By 4 h postinfusion, the tetra- and pentaglutamates were the predominant species.

With the 24-h infusion of [6RS]leucovorin (Fig. 5C), CH₂-H₄PteGlu₂ and CH₂-H₄PteGlu₃ were most markedly elevated by the end of the infusion in HxELC₂ tumors. CH₂-H₄PteGlu₂ was also elevated, but decreased as a percentage of the folate pool due to the large increases in CH₂-H₄PteGlu₂ and CH₂-H₄PteGlu₃. Some increase in CH₂-H₄PteGlu₃ was also detected at this time. CH₂-H₄PteGlu₂₃ also continued to predominate for at least 6 h postinfusion.

For all [6RS]leucovorin regimens, the detectability of CH₂-H₄PteGlu₂ appeared to decrease following treatment.

Fig. 2. The influence of [6RS]leucovorin (500 mg/m²) administered by i.v. bolus injection, 4 h or 24 h infusion, on the size of pools CH₂-H₄PteGlu and H₄PteGlu in HxGC₃ and HxELC₂ 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 CH₂-H₄PteGlu in HxGC₃ tumors following i.v. bolus administration (A), during and following a 4-h infusion (B), or 24 h infusion (C) of [6RS]leucovorin (500 mg/m²) in tumor-bearing mice. [6-³H]FdUMP-thymidylate synthase-CH₂-H₄PteGlu 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 En³Hance. Gels were further treated and dried, and fluorograms were prepared as described in "Materials and Methods."
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Analysis of [6-3H]FdUMP-Thymidylate Synthase-CH$_2$-H$_4$PteGlu Complexes under Denaturing Conditions

[6-3H]FdUMP ternary complexes, formed using tumor extracts prepared for analysis of the combined pool of CH$_2$-H$_4$PteGlu$^6$ and H$_4$PteGlu$^6$, by the $^3$H release assay, were analyzed by gel electrophoresis under denaturing conditions. For both HxELC$_2$ and HxGC$_3$ tumors, a single species with molecular weight between 36,000 and 43,000 daltons was detected prior to treatment with [6RS]leucovorin. In line HxGC$_3$ (Fig. 6), a broadening of this band was observed within 30 min of bolus administration of [6RS]leucovorin, although this was partially reversed by 4 h postinjection. Similarly, a broader species was observed at the end of the 24-h infusion of [6RS]leucovorin that decreased following treatment. Of interest was that, during and shortly after the 4-h infusion schedule, a distinct lower molecular weight species was detected. These results appeared to correlate with an increased predominance of shorter (4-h infusion) or longer (bolus or 24-h infusion) chain length forms of CH$_2$-H$_4$PteGlu following [6RS]leucovorin administration.

In HxELC$_2$ tumors (Fig. 7), the appearance of a lower molecular weight species was apparent at 1 and 2 h after bolus administration of [6RS]leucovorin, that correlated with the accumulation of a short chain length form of CH$_2$-H$_4$PteGlu$^6$ in tumors. This species was also apparent during and shortly after the 4-h [6RS]leucovorin infusion. In this study, a ternary complex that formed with a known standard of CH$_2$-H$_4$PteGlu$^6$ coelectrophoresed with ternary complexes formed using extracts from untreated tumors, while the ternary complex that formed with CH$_2$-H$_4$PteGlu$^6$ electrophoresed as a lower molecular weight species. During the 24-h infusion of [6RS]leucovorin in HxELC$_2$ tumors, a broadening of the single higher molecular weight species was observed on SDS gels.

Thymidylate Synthase Activity

The effect of a 24-h infusion of [6RS]leucovorin on the inhibition of thymidylate synthase by FUra was examined. 5-Fluorouracil was administered to tumor-bearing mice 3 h into...
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A. Bolus HxGC3

- 43,000

A. Bolus HxELC2

- 36,000

0 0.5 hr 1 hr 2 hr 4 hr 6 hr

B. 4 hr infusion

- 43,000

- 36,000

0 1 hr 2 hr 4 hr 5 hr 6 hr 8 hr 10 hr

B. 4 hr infusion

-43,000

-36,000

0 1 hr 2 hr 4 hr 6 hr 8 hr

B. 4 hr infusion

-43,000

-36,000

0 24 hr 26 hr 30 hr

C. 24 hr infusion

-43,000

-36,000

Fig. 6. Analysis of [6-3H]FdUMP-thymidylate synthase-CH2-H4PteGlu ternary complexes formed using CH2-H4PteGlu derived from the combined pool of CH2-H4PteGlu and H4PteGlu in HxGC3 tumors that had received [6RS]-leucovorin (500 mg/m2) by each of the 3 administration regimens. Electrophoresis was conducted under denaturing conditions in SDS prior to the preparation of fluorograms from gels.

In line HxGC3, in the presence of [6RS]leucovorin (500 mg/m2), thymidylate synthase inhibition was potentiated. Thymidylate synthase was inhibited by 71% 4 h after FUra (6.25 mg/kg); by the end of the saline infusion, enzyme activity had recovered to 60% of control (Fig. 8A). In the presence of [6RS]leucovorin infusion, at 4 h after FUra, enzyme activity was inhibited by 82% (P = 0.001), with some retardation in enzyme recovery. Twenty-one h after administration of FUra, inhibition was 47% in comparison with 40% in the FUra control group (P = 0.07). When a higher dose of FUra was used (25 mg/kg), less potentiation of thymidylate synthase inhibition of [6RS]leucovorin was observed 4 h after FUra (76.5% inhibition, FUra alone; 80% inhibition, FUra-leucovorin; P = 0.17), although recovery of enzyme activity was retarded by the end of the infusion (70% inhibition, FUra alone; 80% inhibition, FUra-leucovorin; P = 0.03; Fig. 8B).

In HxELC2 tumors, thymidylate synthase inhibition by FUra was also potentiated by [6RS]leucovorin. In three experiments, [6RS]leucovorin potentiated both the degree and duration of thymidylate synthase inhibition; in one experiment, no increased enzyme inhibition was observed 4 h after FUra, although enzyme recovery was retarded. A representative experiment is shown in Fig. 9. In this study, in the presence of [6RS]leucovorin, thymidylate synthase inhibition was increased by 15% 4 h after FUra (12.5 mg/kg), with reduced recovery at the end of the 24-h infusion of the folate. Potentiation of FUra-induced thymidylate synthase inhibition by [6RS]leucovorin was significant (P < 0.001) at both time points.

DISCUSSION

In patients with colon adenocarcinoma, [6RS]leucovorin has been utilized in different regimens with FUra, where the schedule for administration of the folate has ranged from i.v. bolus daily for 5 days (13, 15, 19) to continuous infusion (12). The objective of the current study was thus to attempt to select an optimal schedule for [6RS]leucovorin administration by examining the effects of scheduling of [6RS]leucovorin in preclinical models of human colon adenocarcinomas maintained in immune-deprived mice. Consequently, a dose level of [6RS]leucovorin frequently used clinically (500 mg/m2) was used to...
determine (a) the concentration-time profiles of [6S]leucovorin, [6R]leucovorin, and [6S]-5-CH3-H4PteGlu in the plasmas of mice for different regimens of [6RS]leucovorin (500 mg/m2) administration, (b) whether treatment with [6RS]leucovorin could expand the pools of CH2-H4PteGlu and H4PteGlu in human colon adenocarcinoma xenografts in vivo and how this related to the maintenance of reduced folates in plasma, (c) how the distribution of CH2-H4PteGlu polyglutamates was modulated in tumors, and (d) how the preferred schedule for [6RS]leucovorin administration in mice would influence FUra-mediated thymidylate synthase inhibition in tumors.

In mice, similar or slightly higher concentrations of [6S]-leucovorin (2-fold) and similar or slightly lower concentrations of 5-CH3-H4PteGlu (2-fold) were determined in plasma for short (16) or prolonged (17) [6RS]-leucovorin infusions in comparison with data derived in humans. For [6RS]-leucovorin (2-fold) and similar or slightly lower concentrations of 5-CH3-H4PteGlu (2-fold) were determined in plasma for short (16) or prolonged (17) [6RS]-leucovorin infusions in comparison with data derived in humans.

Fig. 8. Inhibition of thymidylate synthase by FUra in HxGC3 tumors in the absence (O) and presence (C) of [6RS]leucovorin (500 mg/m2). FUra at a dose level of 6.25 mg/kg (A) or 25 mg/kg (B) was injected i.v. 3 h into a 24-h i.v. infusion of [6RS]-leucovorin in tumor-bearing mice as described in "Materials and Methods." Thymidylate synthase activity was determined 4 and 21 h after FUra and compared with the activity at Time 0. Points, mean of 3 determinations; bars, SD.

Fig. 9. Inhibition of thymidylate synthase by FUra (12.5 mg/kg) in HxELC2 tumors in the absence (O) or presence (C) of [6RS]leucovorin (500 mg/m2) administered to tumor-bearing mice by 24-h i.v. infusion. Experimental conditions were as described in "Materials and Methods." Points, mean of 3 determinations at each time point; bars, SD.
growth. That thymidylate synthase inhibition may be modulated by \([6\alpha\overline{S}]\)leucovorin in vivo is of importance.

Solid tumors maintained under the in situ conditions of tumor formation (10), although this has not previously been evaluated in vitro. The predominance of CH\(_2\)-H\(_4\)PteGlu polyglutamate species observed in tumors during modulation by \([6\alpha\overline{S}]\)leucovorin must be influenced by several factors, including the balance between utilization of CH\(_2\)-H\(_4\)PteGlu\(_n\), enzymatic reactions and their formation, efficiency of polyglutamylation of CH\(_2\)-H\(_4\)PteGlu, and subsequent species, competition with other endogenous reduced folates for polyglutamate synthetase, competition between CH\(_2\)-H\(_4\)PteGlu\(_n\) species in the FdUMP binding assay, and the balance between the rate of formation and dissociation of ternary complexes (which will both be influenced by their relative concentrations). Analysis of ternary complexes on non-denaturing gels has therefore been used as a qualitative method to elucidate how CH\(_2\)-H\(_4\)PteGlu\(_n\) species may be modulated by \([6\alpha\overline{S}]\)leucovorin. Since these species continued to change with time both during and following \([6\alpha\overline{S}]\)leucovorin administration, quantitative changes in pool sizes were determined by an alternate method (\(^3\)H release), which appears independent of the glutamyl chain length of the cofactor (5). Of interest was that analysis of ternary complexes under SDS denaturing conditions confirmed the nature of the covalent linkage and revealed changes associated with the broadening of the single band on gels or the appearance of a distinct lower molecular weight species that correlated with the predominance of longer or shorter chain length forms of CH\(_2\)-H\(_4\)PteGlu\(_n\), respectively.

Previously, we have shown that CH\(_2\)-H\(_4\)PteGlu\(_{4-6}\) stabilized to the same degree ternary complexes (formed between \([6\alpha\overline{S}]\)-FdUMP, thymidylate synthase purified from a human colon adenocarcinoma xenograft, and CH\(_2\)-H\(_4\)PteGlu\(_n\)) at a >200-fold lower concentration than required for CH\(_2\)-H\(_4\)PteGlu\(_2\), with CH\(_2\)-H\(_4\)PteGlu\(_3\) being intermediate (6). Therefore, it would be optimal to increase the pools of CH\(_2\)-H\(_4\)PteGlu\(_3\) to maximize this interaction. The administration of \([6\alpha\overline{S}]\)leucovorin by prolonged infusion would thus appear to be the preferred schedule for delivery in mice, since pools of CH\(_2\)-H\(_4\)PteGlu\(_n\) and H\(_4\)PteGlu\(_n\) were elevated in neoplastic tissues for long periods of time, in comparison, for example, with these pools following i.v. bolus injection of \([6\alpha\overline{S}]\)leucovorin. In addition, CH\(_2\)-H\(_4\)PteGlu\(_3\), optimal for increasing the affinity of binding of FdUMP to thymidylate synthase (6), were elevated during this period. Consequently, the effect of \([6\alpha\overline{S}]\)leucovorin (500 mg/m\(^2\)), delivered by 24-h infusion, on Fura-induced thymidylate synthase inhibition was examined in HxELC\(_2\) and HxGC\(_3\) tumors. In both lines, \([6\alpha\overline{S}]\)leucovorin potentiated enzyme inhibition, and this was likely to be due to the generation of CH\(_2\)-H\(_4\)PteGlu polyglutamates. In cultured cell lines, \([6\alpha\overline{S}]\)leucovorin administration (20 \(\mu\)M) has been shown to retard the recovery of thymidylate synthase after Fura treatment (10), although this has not previously been evaluated in solid tumors maintained under the \(in\ situ\) conditions of tumor growth. That thymidylate synthase inhibition may be modulated by \([6\alpha\overline{S}]\)leucovorin \(in\ vivo\) is of importance.

From \(in\ vitro\) studies, 10 \(\mu\)M \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu was reported to be optimal in potentiating Fura cytotoxicity in cultured cells (10). Clinically, in excess of these concentrations, a level of 24 \(\mu\)M was achieved and maintained during short (2 h) infusions of \([6\alpha\overline{S}]\)leucovorin (500 mg/m\(^2\); 16), following bolus injection (\(\geq10\) \(\mu\)M for at least 1 h; 19), but not during a continuous infusion (1.2 to 4.5 \(\mu\)M 16, 17) of the reduced folate. However, in the latter instance, the sum of concentrations of \([6\alpha\overline{S}]\)leucovorin and the potentially biologically active \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu has reached 9.2 to 13.2 \(\mu\)M. Since \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu has been shown in murine S-180, human Hep-2, and human CCRF-CEM cells to be equally effective with \([6\alpha\overline{S}]\)leucovorin in potentiating the growth-inhibitory effects of Fura and FdUrd (10, 11), this may be a potential precursor of CH\(_2\)-H\(_4\)PteGlu in colon adenocarcinomas, although this has yet to be determined. Of interest, however, is that for each clinical regimen discussed, increased response rates to Fura-leucovorin have been obtained in comparison with Fura alone (12-16).

In the current study in mice, plasma concentrations of \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu (5.1 \(\mu\)M) and/or \(5\)-CH\(_3\)-H\(_4\)PteGlu (3.0 \(\mu\)M) achieved during 24-h infusion of \([6\alpha\overline{S}]\)leucovorin (500 mg/m\(^2\)) have been sufficient to elevate intratumor pools of CH\(_2\)-H\(_4\)PteGlu\(_n\), H\(_4\)PteGlu\(_n\), and H\(_3\)PteGlu\(_n\), and cause potentiation of Fura-induced thymidylate synthase inhibition. However, the influence of dose of \([6\alpha\overline{S}]\)leucovorin and the effects of daily administration of the reduced folate on Fura-induced thymidylate synthase inhibition, as well as the size and composition of CH\(_2\)-H\(_4\)PteGlu\(_n\), and H\(_3\)PteGlu\(_n\) pools, will be important in optimizing \([6\alpha\overline{S}]\)leucovorin regimens. Daily administration of the reduced folate may influence the efficacy of bolus delivery on enzyme inhibition due to the relatively transient effect of maintaining elevated pools of CH\(_2\)-H\(_4\)PteGlu\(_n\), and H\(_3\)PteGlu\(_n\) when given by this regimen. Whether continued administration of the reduced folate may be cumulative in its effects on reduced folate pools and thymidylate synthase inhibition must also be determined.

The optimal dose of the reduced folate will be influenced by tumor-specific metabolism. Of interest is a report (15) suggesting that low-dose \([6\alpha\overline{S}]\)leucovorin (20 mg/m\(^2\)) induces higher response rates (43%) than higher dose \([6\alpha\overline{S}]\)leucovorin (200 mg/m\(^2\); 26%) in combination with Fura, in comparison with Fura administered alone (10%). Certainly, differences in the metabolism of \([6\alpha\overline{S}]\)leucovorin have been observed between HxGC\(_3\) and HxELC\(_2\) colon adenocarcinoma xenografts with regard to the size of expansion of CH\(_2\)-H\(_4\)PteGlu\(_n\) and H\(_3\)PteGlu\(_n\) pools and the characteristics of polyglutamylation of CH\(_2\)-H\(_4\)PteGlu. The xenograft models described will be valuable tools in elucidating how differences in \([6\alpha\overline{S}]\)leucovorin metabolism further influence Fura-leucovorin interactions and potentiation of Fura cytotoxicity by \([6\alpha\overline{S}]\)leucovorin. Effects of dose, schedule, and duration of drug administration on tumor response \(in\ vivo\) may be comprehensively evaluated in these models. In addition, a thymidine kinase-deficient variant of HxGC\(_3\), that has been selected \(in\ vitro\) and also grows in immune-deprived mice, may be used to evaluate the influence of thymidine salvage on these regimens. The models will also be important in addressing questions that relate to the efficacy of 5-CH\(_3\)-H\(_4\)PteGlu in expanding intratumor pools of CH\(_2\)-H\(_4\)PteGlu\(_n\), and H\(_3\)PteGlu\(_n\), and in determining how the presence of \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu in plasma influences the subsequent intratumor metabolism of \([6\alpha\overline{S}]\)-CHO-H\(_4\)PteGlu.

REFERENCES


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LEUCOVORIN METABOLISM IN COLON TUMORS


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


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