Influence of Dose of [6RS]Leucovorin on Reduced Folate Pools and 5-Fluorouracil-mediated Thymidylate Synthase Inhibition in Human Colon Adenocarcinoma Xenografts


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ABSTRACT

Using preclinical models of human colon adenocarcinomas in immune-deprived mice, the influence of dose of [6RS]leucovorin ([6RS]LV, 20 to 1000 mg/m²) administered by 24-h i.v. infusion was determined on the following parameters: (a) plasma concentrations of the active [6S] and inactive [6R] isomers of [6RS]LV and the biologically active diastereoisomer of 5-methyltetrahydrofolate (5-CH₂-H₄PteGlu); (b) expansion of intratumor pools of 5,10-methylenetetrahydrofolates (CH₂-H₄PteGlu) and tetrahydrofolates (H₄PteGlu), that may influence the binding of 5-fluorodeoxyuridylate to thymidylate synthase; (c) the distribution of polyglutamate forms of CH₂-H₄PteGlu and H₄PteGlu; and (d) 5-fluorouracil (FUra)-mediated thymidylate synthase inhibition in Hx-ELCs, HxGCs, HxVRCs, and HxHC tumors. Folypolyglutamate synthetase activities were also determined in each line. Linear increases in plasma concentrations of [6RS]LV, [6RS]LV, and 5-CH₂-H₄PteGlu were determined over the complete range of [6RS]LV doses examined. However, in neoplastic tissues three patterns of biochemical modulation by [6RS]LV were evident. (a) In HxELCs; HxVRCs tumors, pools of CH₂-H₄PteGlu and H₄PteGlu were elevated in proportion to the dose of [6RS]LV between dose levels of 50 and 200 mg/m². Subsequent expansion of these pools continued that was disproportionate to the dose of [6RS]LV until no further increase was observed beyond 800 mg/m² [6RS]LV, at which point pools were maximally expanded by 4- to 4.5-fold. The extent of retardation of recovery of thymidylate synthase activity increased as the dose of [6RS]LV was increased in both tumors, when FUra (15 or 50 mg/kg), was administered by i.v. bolus injection 3 h into the 24-h infusion of [6RS]LV. This was related to the increase in predominance of CH₂-H₄PteGlu, with increasing dose of [6RS]LV. (b) For HxGC tumors, little expansion of CH₂-H₄PteGlu and H₄PteGlu pools (maximum, 137% of control) was detected at the highest dose levels of [6RS]LV, and no significant modulation of FUra-inhibited thymidylate synthase activity was detected, even at 1000 mg/m² [6RS]LV. CH₂-H₄PteGlu remained reduced or decreased as the dose of [6RS]LV was increased. (c) For line HxGC, pools of CH₂-H₄PteGlu and H₄PteGlu increased gradually from 169% of control at 20 mg/m² [6RS]LV to 233% of control at 1000 mg/m² [6RS]LV, and were intermediate between the expansion observed in HxGC in comparison to HxELCs and HxVRCs tumors. CH₂-H₄PteGlu were elevated at low dose levels of [6RS]LV. A greater extent of retardation of the recovery of thymidylate synthase following FUra administration was observed at a lower dose of [6RS]LV. No relationships was detected between the activity of thymidylate synthase and CH₂-H₄PteGlu species in tumors by [6RS]LV. The metabolic characteristics of modulation by [6RS]LV thus differed among the four colon xenograft lines providing a spectrum from little or no modulation to tumors in which pools could be markedly enhanced.

INTRODUCTION

Thymidylate synthase has proved to be a relatively effective target for cytotoxic drug action in the clinical management of adenocarcinoma of the colon. One of the most effective single agents used in the treatment of this disease is FUra, where the predominant mechanism of cytotoxicity in vivo appears directed at the thymidylate synthase locus. The combination of a reduced folate ([6RS]LV) with FUra has resulted in a substantial increase in the clinical activity of this agent. In several controlled, randomized clinical trials in colorectal adenocarcinoma, FUra alone has induced response rates of 7 to 15%, while FUra combined with [6RS]LV has elevated these rates to between 33 and 45% (1-4). These responses have been associated with a significant prolongation of time to disease progression (1, 2, 4) and overall survival (2, 4).

Several different doses of [6RS]LV have been used in these trials ranging from as low as 20 mg/m² (4) to 500 mg/m² (1, 3). In published studies of the concentration-time profiles of potentially biologically active reduced folate species [6S]LV, 5-CH₂-H₄PteGlu in plasma, a dose level of [6RS]LV of 500 mg/m² has been most frequently evaluated by both short-term (5) or prolonged (5-7) infusion. Thus, steady-state plasma levels of 1.2 to 24 µM for [6RS]LV and 1.3 to 17 µM for 5-CH₂-H₄PteGlu have been described. However, no definitive relationships between the dose of [6RS]LV used and the influence on response to FUra in the clinical disease have currently been established. In addition, no published data are available that relate the dose of [6RS]LV to expansion of reduced folate pools in these tumors and their influence on thymidylate synthase inhibition during [6RS]LV administration.

Using preclinical models of human colon tumors growing s.c. in immune-deprived mice, transient inhibition of thymidylate synthase has correlated with the insensitivity of these tumors to 5-fluoropyrimidines administered singly (8, 9). The active metabolite of FUra, FdUMP, forms a covalent ternary complex with thymidylate synthase and CH₂-H₄PteGlu, the cofactor used in the catalytic conversion of dUMP to dTMP. Due to the ordered mechanism of ligand binding and the unstable nature of this complex, the stability of the complex was determined to be dependent upon the concentration of the folate cofactor in studies using enzyme isolated from CCRF-CEM cells (10). Subsequently, it was determined in human colon xenografts that levels of CH₂-H₄PteGlu were suboptimal to allow maximal formation or stability of the ternary complex in these tumors (11, 12). The predominant species of CH₂-H₄PteGlu present in human colon adenocarcinoma xenografts were found to be polyglutamates (12). CH₂-H₄PteGlu, containing from 3 to 6 glutamate residues increased the binding affinity of [6H]-
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FdUMP to thymidylate synthase (7- to 10-fold reduction in the dissociation constant) in comparison to CH$_2$-H$_4$PteGlun, using thymidylate synthase purified from HxVRC$_5$ tumors (13). Consequently, when considering ways of expanding pools of CH$_2$-H$_4$PteGlun, to enhance the therapeutic activity of FUra, optimization to allow expansion of pools of these longer polyglutamate species is of importance.

$[6RS]$LV consists of a mixture of diastereoisomers of biologically active ($[6S]$) and inactive ($[6R]$) species. Since this is a stable reduced folate form, it has been convenient to use both clinically and experimentally to attempt to modulate the activity of FUra. Previously, we have determined using human colon adenocarcinoma xenografts that the preferred schedule for $[6RS]$LV administration in mice at a dose level of 500 mg/m$^2$ was by a prolonged (24 h) i.v. infusion, when compared to a shorter infusion period (4 h) or bolus injection (14). This was for several reasons: (a) elevated concentrations of reduced folates were maintained in mouse plasma for prolonged periods of time; (b) pools of CH$_2$-H$_4$PteGlun and H$_4$PteGlun remained elevated in tumors, only during the period of $[6RS]$LV infusion; and (c) predominance of longer chain length CH$_2$-H$_4$PteGlun species during infusion in comparison to the 4-h infusion schedule, which allowed the accumulation of shorter chain length forms. The 24-h infusion schedule was therefore selected for further evaluation of the relationship between dose of $[6RS]$-LV, and factors that may modulate the activity of FUra.

The objectives of the current investigation were therefore to examine (a) the influence of the dose of $[6RS]$LV on expansion of pools of CH$_2$-H$_4$PteGlun and H$_4$PteGlun in four human colon adenocarcinoma xenografts to determine at which point cellular metabolism may become saturated, and how this related to concentrations of reduced folates achieved in plasma; (b) modulation of FUra-mediated thymidylate synthase inhibition with respect to the dose of $[6RS]$LV in each tumor line; and (c) the influence of $[6RS]$LV on the distribution of polyglutamates of CH$_2$-H$_4$PteGlun and H$_4$PteGlun, and how FPGS activities may correlate with this distribution. The time point chosen for these studies was at the end of a 24-h infusion of $[6RS]$LV, when reduced folate levels in plasma were relatively constant, and at a maximum.

MATERIALS AND METHODS

Chemicals. [5-$^3$H]dUMP (specific activity, 20 Ci/mmole) and [6-$^3$H]-FdUMP (specific activity, 18 Ci/mmole) was obtained from Moravek Biochemicals, Brea, CA, and [3$^3$H]glutamic acid (specific activity, 25 Ci/mmol) was from New England Nuclear, Boston, MA. Glycine was obtained from Bio-Rad Laboratories, Richmond, CA. Lactobacillus casei thymidylate synthase (specific activity, 1.8 $\mu$mol/h/mg protein) was a gift from Dr. James Haggarty, National Cancer Institute, Bethesda, MD. Polyacrylamide and all other reagents for gel electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. H$^4$PteGlun and were electrophoresed on 9% polyacrylamide nondenaturing gels (28 cm). Equivalent amounts of reaction mixtures containing $[5-^3$H]dUMP over 3 min, 30°C, where the rate of reaction under the conditions used was determined to be independent of the glutamyl form of the cofactor (12). L. casei thymidylate synthase [purified further on columns of Sephadex G-100 and CM-Sephadex (17)]; final specific activity, 10 to 24 $\mu$mol/h/mg protein], heat-treated tumor extracts [containing 37 mm HCHO, 1% $\beta$-mercaptoethanol, and 10 mm sodium ascorbate in phosphate-buffered saline, pH 7.4] as the source of reduced folates, and a low concentration of [5-$^3$H]dUMP [243 nm] with high specific activity were used in reaction mixtures as described previously (12). Endogenous dUMP was initially removed by treatment of extracts with 5'-nucleotidase prior to assay as described (12). Data were analyzed statistically using the Dunnett's or Newman Keuls tests.

Distribution of Polyglutamates of CH$_2$-H$_4$PteGlun and H$_4$PteGlun. Determination of the influence of $[6RS]$-CHO-H$_4$PteGlun infusions on the predominance of individual CH$_2$-H$_4$PteGlun, and H$_4$PteGlun, species in tumors was based upon the technique described by Priest and Doig (13), where the distribution of these species had been characterized previously in untreated colon tumor xenografts (12). This method has been used for qualitative evaluation only, with quantitation of total pool size expansion by the $^3$H release assay described above. Thus, the distributions of polyglutamate species are relative and not quantitative in absolute terms. Ternary complexes were formed over 5 min at 30°C, using frozen tissue powders extracted rapidly at 2°C either in the absence of HCHO (to determine CH$_2$-H$_4$PteGlun species or in the presence of 37 mm HCHO (for evaluation of polyglutamate forms within the combined pools of CH$_2$-H$_4$PteGlun, and H$_4$PteGlun), as described previously (12). Ternary complexes were formed between [6-$^3$H]FdUMP (125 nm), excess L. casei thymidylate synthase, and CH$_2$-H$_4$PteGlun, and were electrophoresed on 9% polyacrylamide nondenaturing gels (28 cm). Equivalent amounts of reaction mixtures containing 10% glycerol were applied to each gel. Fluorograms were prepared as described previously (12), except that fixation of gels was in glacial acetic acid: methanol:water (10:30:60) for 1 h followed by treatment with En'hance for 1 h and agitation in an excess of cold water for 30 min prior to drying. Data were analyzed by scanning densitom-
etry of fluorograms to determine band intensities, where the relationship between peak height and dpm was linear ($r^2 = 0.944$) over the range examined.

Inhibition of Thymidylate Synthase. The effect of a 24-h i.v. infusion of [6RS]LV (20 to 1000 mg/m$^2$) on the inhibition of thymidylate synthase by FUra was examined in each of the four tumor lines. 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 [oAS]LV may be more readily determined. The doses of FUra used in mice bearing HxELC$_2$, HxGC$_3$, HxVRC$_5$, and HxHC$_3$ tumors were 50, 25, 15, and 10 mg/kg, respectively. FUra was administered i.v. by bolus injection 3 h into a 24-h infusion of [6RS]LV or saline, by means of a 3-way valve, and was flushed through the cannula with 0.1 ml saline (0.9%). At this time, plasma levels of reduced folates were approaching a steady state concentration, and intratumor pools of CH$_2$-H$_2$PteGlun and H$_2$PteGlun were elevated. At 4 and 21 h after FUra administration, tumors were excised (4 tumors pooled; 2 mice/time point), cytosoils were prepared, and endogenous nucleotides were removed by charcoal adsorption procedures, as described (19). The rate of release of $^3$H from [5-$^3$H]dUMP was subsequently determined in each sample and compared to the rate of release of $^3$H in control tumors, as reported previously (8, 19). For each tumor line, differences between FUra- and FUra+[6RS]LV-treated groups were analyzed statistically using a one-way analysis of variance and subsequently by the Dunnett’s test.

FPGS Activity. The activities of FPGS were determined in HxELC$_2$, HxGC$_3$, HxVRC$_5$, and HxHC$_3$ tumors using the method of McGuire et al. (20) with slight modification using CBA/CaJ mouse liver as a control. Tumors or livers were excised from mice and immediately placed on ice. Tissues were subsequently homogenized at 2°C in 3 mmol heparinized buffer containing 250 mM sucrose, 2 mM MgCl$_2$, 20 mM KCl, 100 mM $\beta$-mercaptoethanol, 50 $\mu$M aminopterin, 4 $\mu$M [H$^3$]glutamic acid (specific activity, 2.9 to 3.5 mCi/mmol), and 20 to 120 $\mu$ of cytosol (0.5 to 1.5 mg protein), at 2°C in a total volume of 250 $\mu$l. Incubations were at 37°C for 60 min, during which time the rate of reaction was linear. Reactions were terminated by the addition of an excess of nonradiolabeled glutamic acid (100 mM, 1 ml). Nonspecific radioactivity associated with background was determined in the absence of aminopterin as substrate. [H$^3$]Glutamic acid incorporated into polyglutamate species was separated from unreacted glutamic acid using DE52 columns (0.4 x 6 cm), as described (20). Protein concentrations were determined using the method of Bradford (21).

RESULTS

Plasma Levels of Reduced Folates in Mice. At the end of a 24-h infusion of [6RS]LV in mice at doses ranging from 50 to 1000 mg/m$^2$, concentrations of [6R]LV, [6S]LV, and 5-CH$_2$-H$_2$PteGlun achieved in plasma were determined (Fig. 1). The relationship between dose and concentration was linear for each reduced folate over the range of doses examined. In addition, the slopes of the dose-response relationships were similar among the three species. Concentrations of [6S]LV were intermediate between those of [6R]LV and 5-CH$_2$-H$_2$PteGlun, with [6R]LV achieving levels 2.7-fold higher than [6S]LV, which was 1.6-fold in excess of the concentrations of 5-CH$_2$-H$_2$PteGlun. Of interest was that for each incremental increase in dose of [6RS]LV by a factor of 2, there was a doubling of the concentration of each reduced folate species detected in plasma, even between doses of [6RS]LV of 500 and 1000 mg/m$^2$.

Expansion of Pools of CH$_2$-H$_2$PteGlun and H$_2$PteGlun in Tumors. In HxELC$_2$, HxVRC$_5$, HxGC$_3$, and HxHC$_3$ tumors, the effect of a 24-h infusion of [6RS]LV at doses ranging from 20 to 1000 mg/m$^2$ on these pools was examined at the end of the infusion period (Fig. 2).
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m². Between [6RS]LV doses of 50 and 100 mg/m², the greatest change in pool size expansion was observed, where this was essentially doubled. Between 100 and 800 mg/m², pools were increased by 21% only, which was not statistically significant (P = 0.67).

For HxHC, tumors, although a small upward trend in pool size expansion in response to [6RS]LV was observed, this was determined statistically to be nonsignificant. Pools were expanded maximally to 137% of control, at a dose level of [6RS]LV of 500 mg/m².

In line HxGC₃, pools of CH₂-H₄PteGlu₆ and H₄PteGlu₆ were expanded to a degree intermediate between that observed for HxHC in comparison to HxELC₂ or HxVRC₅ tumors. At a dose level of [6RS]LV of 20 mg/m², reduced folate pools were increased to 169% of control (P < 0.01). Thereafter, these pools continued to increase further (P = 0.01) to a maximum of 233% of control, achieved at 1000 mg/m² [6RS]LV.

Modulation of CH₂-H₄PteGlu₆ and H₄PteGlu₆ Species. For each of the four tumor lines, the influence of dose of [6RS]LV on the predominance of polyglutamate species was examined at the end of the 24-h infusion for both the CH₂-H₄PteGlu₆ pool and also the combined pools of CH₂-H₄PteGlu₆ and H₄PteGlu₆. Data for the CH₂-H₄PteGlu₆ pool have been presented in detail. Any similarities or differences in the distribution of individual species within the combined pools has been additionally described.

In HxELC₂ tumors, the penta- and hexaglutamate forms of CH₂-H₄PteGlu₆ predominated in untreated tumors. Subsequently, three dose levels of [6RS]LV were examined; 100, 400, and 1000 mg/m² (Fig. 3). At a dose level of 100 mg/m² [6RS]-LV, the tri-, tetra-, and pentaglutamate forms of CH₂-H₄PteGlu₆ were markedly elevated while CH₂-H₄PteGlu₆ decreased. As the dose of [6RS]LV was increased, the predominance of the three species also increased. At 400 mg/m² [6RS]-LV, the appearance of CH₂-H₄PteGlu₆ was observed, which was increased somewhat at 1000 mg/m² [6RS]LV. The greatest change in species between [6RS]LV dose levels of 400 and 1000 mg/m² was in the elevation in CH₂-H₄PteGlu₆ and CH₂-H₄PteGlu₆ for the combined pools (CH₂-H₄PteGlu₆ and H₄PteGlu₆), similar patterns of modulation of species were observed in relation to the dose of [6RS]LV (data not shown).

For line HxVRC₅, CH₂-H₄PteGlu₆ predominated in untreated tumors, with CH₂-H₄PteGlu₆ containing from 2 to 6 glutamate residues also present. At a dose level of 20 mg/m² [6RS]LV, CH₂-H₄PteGlu₆ decreased, whereas CH₂-H₄PteGlu₆ decreased (Fig. 3). At additional doses of 200 and 1000 mg/m² [6RS]LV examined, tri-, tetra-, and also diglutamate species increased with increasing dose of [6RS]LV (Fig. 3). For the combined pools of CH₂-H₄PteGlu₆ and H₄PteGlu₆, the tri-, tetra-, and pentaglutamates continued to increase with increasing dose of [6RS]LV, the hexaglutamate decreased, and the diglutamate was not detected at any dose of [6RS]LV (data not shown).

In HxGC₃ tumors (Fig. 3), CH₂-H₄PteGlu₆ and CH₂-H₄PteGlu₆ were elevated at a dose of 20 mg/m² [6RS]LV, with a slight increase in CH₂-H₄PteGlu₆. The tetraglutamate continued to increase up to a dose of 1000 mg/m² examined. CH₂-H₄PteGlu₆ was elevated substantially only at this highest dose of [6RS]LV used. CH₂-H₄PteGlu₆, a major species in untreated tumors, was no longer detected at 200 mg/m² [6RS]LV. For the combined pools of CH₂-H₄PteGlu₆ and H₄PteGlu₆, modulation by [6RS]LV was similar to that observed within the CH₂-H₄PteGlu₆ pool (data not shown).

For line HxHC, (Fig. 3), CH₂-H₄PteGlu₆ predominated in untreated tumors. At a dose level of 20 mg/m² [6RS]LV, tri-, tetra-, and pentaglutamates were elevated, whereas CH₂-H₄PteGlu₆ decreased. As the dose of [6RS]LV was increased to 250 and 1000 mg/m², CH₂-H₄PteGlu₆ and CH₂-H₄PteGlu₆ increased, whereas CH₂-H₄PteGlu₆ remained fairly stable (Fig. 3); in a separate experiment, CH₂-H₄PteGlu₆ appeared to decline (data not shown). CH₂-H₄PteGlu₆ was also decreased. Similar observations were made for the combined pools of CH₂-H₄PteGlu₆ and H₄PteGlu₆ (data not shown).

Thymidylate Synthase Inhibition. The effect of increasing the dose of [6RS]LV on the inhibition of thymidylate synthase induced by FUra was examined in each of the four tumor lines,
when FUra was administered by i.v. bolus injection at 3 h into the 24-h infusion of [6RS]LV (Fig. 4). Thymidylate synthase activity was subsequently determined in untreated tumors and at 4 and 21 h, after the administration of FUra. Doses of [6RS]LV used were 215, 430, and 644 mg/m² in studies utilizing HxELC₂, HxGC₃, and HxVRC₅ tumors and were 20, 250, and 1000 mg/m² for HxHC₁ tumors. Doses of FUra utilized were 50, 25, 15, and 10 mg/kg, respectively.

In HxELC₂ tumors (Fig. 4), the extent of thymidylate synthase inhibition at 4 h after FUra administration was similar (44 to 50% of control) among the four treatment groups (P = 0.402). By the end of the infusion of [6RS]LV, thymidylate synthase activity had returned to 94% of control in tumors receiving FUra alone. However, in the [6RS]LV-treated groups, as the dose of [6RS]LV was increased from 215 to 430 and 644 mg/m², FUra-mediated enzyme inhibition was also increased, where enzyme activity had recovered to 87% (P = 0.145), 65% (P < 0.001), and 55% (P < 0.001) of control, respectively.

In HxVRC₅ tumors, retardation of the recovery of thymidylate synthase following FUra administration was also related to the dose of [6RS]LV (Fig. 4). At 4 h after FUra, inhibition of thymidylate synthase was similar between the FUra alone and FUra-[6RS]LV-treated groups (20 to 24% of control; P = 0.154). By the end of the [6RS]LV infusion, thymidylate synthase had reached 64% of control in FUra-treated tumors. At dose levels of [6RS]LV of 215 and 430 mg/m², enzyme activity was depressed to between 48 and 52% of control (P < 0.001); and at a dose level of 644 mg/m², activity was further reduced to 35% of control (P < 0.001).

For line HxGC₃ (Fig. 4), FUra alone reduced thymidylate synthase activity to 43% of control, 4 h after drug administration. At this time, [6RS]LV potentiated the degree of enzyme inhibition to 31 to 35% of control (P < 0.003) at dose levels of 215 and 430 mg/m², whereas at a dose level of 644 mg/m², no increase in the degree of thymidylate synthase inhibition was observed (P = 0.142). By the end of the [6RS]LV infusion, enzyme activity had returned to 70% of control in tumors receiving FUra alone. Of interest was that the recovery of thymidylate synthase in [6RS]LV-FUra-treated tumors appeared inversely related to the dose of [6RS]LV. Hence, at 21 h after FUra, enzyme activity was 69, 64, and 51% of control in tumors treated with [6RS]LV dose levels of 644 (P = 0.264), 430 (P = 0.106), and 215 (P < 0.01) mg/m², respectively.

For line HxHC₁ (Fig. 4), at both 4 and 21 h after FUra administration, the degree of depression of thymidylate synthase activity was similar for tumors treated with FUra alone or FUra-[6RS]LV (20 to 1000 mg/m²). At 4 h after drug administration, thymidylate synthase activity was reduced to 8% (P < 0.001) and 55% (P < 0.001) of control, respectively. By the end of the [6RS]LV infusion, enzyme activity was at 20% of control in tumors receiving FUra alone and 16% of control in [6RS]LV-FUra-treated tumors (P = 0.142).

FPGS Activities. FPGS activities were determined in each of the four colon xenografts and also in livers derived from CBA/ CaJ mice (Table 1). Line HxELC₂ demonstrated the lowest activity (310 pmol/h/mg protein), and HxGC₃ and HxVRC₅ tumors were 3.3- to 4.2-fold higher (1015 to 1290 pmol/mg protein). Line HxHC₁ demonstrated an intermediate level of activity (786 pmol/h/mg protein). FPGS determined in mouse liver was 453 pmol/h/mg protein.

**DISCUSSION**

In this study, the concentrations of reduced folate species in plasma were linearly related to the dose of [6RS]LV. However, in neoplastic tissues, biochemical modulation in response to the dose of [6RS]LV differed among the four tumor lines. In HxELC₂ and HxVRC₅ tumors, the disproportionate expansion of CH₂-H₄PteGlu and H₄PteGlu pools beyond [6RS]LV doses of 100 to 200 mg/m² suggested that there may be saturation either in [6RS]LV uptake at the cellular level or in subsequent metabolism to other reduced folate species. At high dose levels of [6RS]LV, it is possible that saturation of the reduced folate transport system may occur. Alternatively, competition between the biologically inactive [6R] diastereoisomer and active [6S] isomer of [6RS]LV at the level of transport could proportionally reduce the concentration of [6S]LV inside the cell, as the dose of [6RS]LV is increased. The interference of [6R]LV with the metabolic processing of [6S]LV has yet to be proved, however (22). A more probable occurrence is saturation in the cellular metabolism of [6S]LV. In studies with the murine colon 38 tumor in mice, 5-CHO-H₄PteGlu appeared to readily enter neoplastic cells. However, due to apparently low levels of CH₂-H₄PteGlu synthetase, the enzyme responsible for the initial conversion of CH₂-H₄PteGlu to CH₃H₄PteGlu, inefficient metabolism of 5-CHO-H₄PteGlu was observed (23). The dose of [6RS]LV used in these studies was 400 mg/kg (approximately 1200 mg/m³). This constitutes a high dose level of [6RS]LV, and maximum expansion of pools of CH₂-H₄PteGlu and H₄PteGlu, (by 5- to 6-fold) may already have been achieved.

**Table 1: Activities of FPGS in human colon xenografts and CBA/CaJ mouse liver**

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<tr>
<th>Tissue</th>
<th>FPGS activity (pmol/h/mg protein)</th>
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<tbody>
<tr>
<td>HxGC₅</td>
<td>1290 ± 160*</td>
</tr>
<tr>
<td>HxVRC₅</td>
<td>1015 ± 203</td>
</tr>
<tr>
<td>HxHC₁</td>
<td>786 ± 31</td>
</tr>
<tr>
<td>HxELC₂</td>
<td>310 ± 52</td>
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<tr>
<td>Liver</td>
<td>453 ± 140</td>
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*Results represent the mean ± SD of 6 to 16 determinations from 2 to 4 separate experiments in neoplastic tissues and 23 determinations from 6 separate experiments in mouse liver.
The maximal degree of modulation of these pools in response to [6RS]-LV was similar to that observed in HxELC2 and HxVRC3 xenografts. In these human tumors, retardation of the recovery of thymidylate synthase also increased with increasing dose of [6RS]-LV.

In HxHC1 tumors, little effect of [6RS]-LV on either the degree or duration of thymidylate synthase inhibition was observed, even at the highest dose of [6RS]-LV used in the study (1000 mg/m²). This may be due to the lack of significant expansion of pools of CH2-H4PteGlu2 and H4PteGlu, in response to [6RS]-LV. However, a fairly high degree of thymidylate synthase inhibition (92%, 4 h after FUra) was observed at a low dose level of FUra (10 mg/kg), which would make small differences in enzyme inhibition difficult to detect. Of interest was the observation in HxGC3 tumors that an apparently greater degree of FUra-mediated thymidylate synthase inhibition was obtained with the lowest dose level of [6RS]-LV (215 mg/m²) examined. At present, the reason for this observation remains unclear. However, a report from the Mayo Clinic (4) has suggested that low-dose [6RS]-LV (20 mg/m²) might induce higher response rates (43%) than high-dose [6RS]-LV (200 mg/m²; 26%) in combination with FUra in patients with colorectal adenocarcinoma. Whether a subset of colon tumors might exist with this particular metabolic phenotype therefore requires further examination.

The overall expansion of the CH2-H3PteGlu pool will be determined by both increases and decreases in any particular polyglutamate species. Of note is that in all four tumor lines, CH2-H3PteGlu was decreased as the dose of [6RS]-LV increased. The reason for this consistent finding is unknown but may counter the effect of expanding the pools of lower polyglutamate forms of CH2-H3PteGlu particularly in HxGC3 and possibly in HxHC1 tumors. For example, in HxGC3 xenografts the total pool was elevated at a dose of 20 mg/m² [6RS]-LV. There was an increase in CH2-H3PteGlu in combination with FUra in patients with colorectal adenocarcinoma. Whether a subset of colon tumors might exist with this particular metabolic phenotype therefore requires further examination.

FPGS activities determined in the four colon xenograft lines differed by 4.2-fold. The activity determined for CBA/CaJ mouse liver was in the same range reported for others for the rat (320 pmol/h/mg protein). Line HxELC2 demonstrated the lowest activity of FPGS, whereas HxVRC3 and HxGC3 tumors contained the highest activities. The combined pools of CH2-H3PteGluu and H4PteGluu had been estimated to be 0.5, 2.7, and 1 μM, respectively, and for CH2-H3PteGluu approximately 0.2, 1.7, and 0.5 μM, respectively (12). However, the pattern of modulation of CH2-H3PteGluu, species in HxELC2 and HxVRC3 tumors was similar with increasing dose of [6RS]-LV. Thus, there was no relationship between the activity of FPGS and the pattern of modulation of CH2-H3PteGluu, species in tumors when [6RS]-LV was delivered by 24 h i.v. infusion. The increased distribution of CH2-H3PteGluu in high dose levels of [6RS]-LV in HxELC2 and HxVRC3 tumors are most likely responsible for the retardation of recovery of FUra-inhibited thymidylate synthase in both lines. In all tumors, CH2-H3PteGluu decreased during [6RS]-LV infusion. This may be due to its utilization in enzymic reactions without being as rapidly resynthesized, or alternatively, may be less efficient in competition for [6-3H]FdUMP binding due to the greater availability of other polyglutamate species.

The biochemical characteristics of these tumors are complex. However, of interest was that three patterns emerged concerning biochemical modulation by [6RS]-LV. HxELC2 and HxVRC3 tumors were similar, while HxGC3 and HxHC1 appeared to differ, providing a spectrum from little or no modulation to tumors in which pools could be markedly enhanced. HxELC2 and HxHC1 tumors had previously demonstrated some sensitivity to treatment with FUra or 5-fluoro-2'-deoxyuridine alone, which may relate, in part, to the low activities of thymidylate synthase in these tumors. However, due to the degree of expansion of CH2-H3PteGluu and H4PteGluu pools in HxELC2 and HxVRC3 tumors in response to [6RS]-LV and effects on FUra-induced thymidylate synthase inhibition, these tumors are the most likely to respond to 5-fluoropyrimidine-[6RS]-LV combinations, provided that thymidine salvage, thymidylate synthase levels, or concentrations of FdUMP do not limit the utility of the combination, and reduced folate pools are elevated sufficiently.

Previous studies from these laboratories have demonstrated schedule-dependent effects on expansion of pools of CH2-H3PteGluu and H4PteGluu, and the distribution of their polyglutamate species (26). Future studies must therefore determine the effect of dose and frequency of administration of [6RS]-LV on the cytotoxicity of FUra in the individual xenograft models. To maintain inhibition of thymidylate synthase and to maximize therapeutic activity, it will be necessary to use optimal doses of FUra and may be necessary to administer FUra-[6RS]-LV combinations over a period of 5 days, similar to the clinical application of the drug combination. Interpretation of data may also be aided by further analysis of the modulation of metabolic characteristics within individual tumors during this time period.

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REFERENCES


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