Cancer Chemother Pharmacol (1992) 30: 423–432

Cancer Chemotherapy and Pharmacology

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Factors that influence the therapeutic activity of 5-fluorouracil [6RS]leucovorin combinations in colon adenocarcinoma xenografts*

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Received 13 November 1991/Accepted 15 April 1992

Summary. The therapeutic activity of FUra alone or combined with [6RS]LV doses ranging from 50 to 1,000 mg/m^2 was examined in eight colon adenocarcinoma xenografts, of which five were established from adult neoplasms ($HxELC_2$, $HxGC_3$, $HxVRC_5$, $HxHC_1$, and $HxGC_3$/c1TK$^-$c3 selected for TK deficiency) and three were derived from adolescent tumors ($HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$). The growth-inhibitory effects of FUra were potentiated by higher doses of [6RS]LV (500–1,000 mg/m^2) in three lines ($HxGC_3$/c1TK$^-$c3, $HxSJC_3A$, and $HxSJC_3B$) and by a low dose of [6RS]LV in only one tumor ($HxVRC_5$). Expansion of pools of CH_2-H_4PteGlu$_n$+H_4PteGlu$_n$ ($\geq$2.4-fold) in response to higher doses of [6RS]LV was obtained in all lines except $HxHC_1$. Metabolism of [6RS]LV was high in $HxVRC_5$, with high levels of 5-CH_3-H_4PteGlu being detected, but not in $HxHC_1$, in which levels of 5-CH_3-H_4PteGlu and CH = H_4PteGlu+10-CHO-H_4PteGlu remained relatively low. In the adolescent tumors, levels of CH = H_4PteGlu+10-CHO-H_4PteGlu were consistently higher than those of 5-CH_3-H_4PteGlu following [6RS]LV administration, and in $HxSJC_3A$, in which pools of CH_2-H_4PteGlu$_n$+H_4PteGlu$_n$ were significantly expanded, 5-CH_3-H_4PteGlu concentrations were lower than those observed in the other two lines. The sensitivity of tumors to FUra ± [6RS]LV and the characteristics of [6S]LV metabolism did not correlate with the activity of CH = H_4PteGlu synthetase, the enzyme responsible for the initial cellular metabolism of [6S]LV to CH = H_4PteGlu. Thus, no single metabolic phenotype correlated with the [6RS]LV-induced expansion of CH_2-H_4PteGlu$_n$+H_4PteGlu$_n$ pools. Potentiation of the therapeutic efficacy of FUra by [6RS]LV was observed in $HxGC_3$/c1TK$^-$c3 xenografts but not in parent $HxGC_3$ tumors, demonstrating the influence of dThd salvage capability in the response to FUra-[6RS]LV combinations. Plasma dThd concentrations in CBA/CaJ mice were high (1.1 μM). The present data therefore demonstrate the importance of (1) higher doses of [6RS]LV, (2) expansion of pools of CH_2-H_4PteGlu$_n$+H_4PteGlu$_n$, and (3) dThd salvage capability in potentiation of the therapeutic efficacy of FUra in colon adenocarcinoma xenografts. The plasma levels of FUra achieved in mice are presented.

Introduction

We have been interested in elucidating the determinants of and the factors that influence the sensitivity of human colon adenocarcinomas to combinations of FUra and the reduced folate [6RS]LV. Clinically, rates of response to FUra have increased from between 7% and 15% for FUra alone to between 30% and 48% for FUra combined with [6RS]LV [5, 8, 23–25]. In some studies, these responses have also been associated with a significant prolongation of time to disease progression [5, 8, 25] and overall patient survival [8, 25]. Thus, in vivo, the predominant mechanism of FUra cytotoxicity appears to be directed at the thymidylate synthase locus.

We have previously reported that human colon adenocarcinomas maintained as xenografts in immune-deprived mice demonstrated levels of CH_2-H_4PteGlu$_n$ that were suboptimal for either maximal formation or maintenance

* Supported by NCI awards CA 32613, CA 23099, and CA 21765, by the American Lebanese Syrian Associated Charities, and by the Medical Research Council of Canada

Abbreviations: [6RS]LV, a mixture of the diastereoisomers of the biologically active [6S] and inactive [6R] forms of [6RS]leucovorin or 5-CHO-H_4PteGlu; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolate; 10-CHO-H_4PteGlu, 10-formyltetrahydrofolate; CH = H_4PteGlu, 5,10-methenyltetrahydrofolate; H_2PteGlu, dihydrofolate; PteGlu, folic acid; PABGlu, p-aminobenzoyl glutamic acid; CH_2-H_4PteGlu$_n$, 5,10-methylenetetrahydrofolate containing from 1 to 6 glutamate residues; H_4PteGlu$_n$, tetrahydrofolate containing from 1 to 6 glutamate residues; FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dThd, thymidine; Td_2, tumor volume-doubling time; HPLC, high-performance liquid chromatography; TK, thymidine kinase

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of the stability of the inhibitory ternary complex formed between the intracellular FUra metabolite, FdUMP, thymidylate synthase, and the reduced folate cofactor used in the normal catalytic reaction, CH_2-H_4PteGlu [12, 13, 16, 26]. We postulated that combining FUra with a reduced folate should increase the rates of response to FUra by virtue of elevating pools of CH_2-H_4PteGlu, particularly polyglutamate species (CH_2-H_4PteGlu$_{2-6}$), that increase the affinity of binding of FdUMP to thymidylate synthase [26].

Subsequently, using a series of four human colon adenocarcinoma xenograft lines, we determined that the metabolic characteristics of modulation of CH_2-H_4PteGlu$_n$ pools by [6RS]LV differed among the lines, providing a spectrum ranging from little or no modulation to marked enhancement of these pools [16]. Following 24-h i.v. infusion of varied doses of [6RS]LV (50–1,000 mg/m^2) in tumor-bearing mice, pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in $HxELC_2$ and $HxVRC_5$ tumors were elevated in proportion to [6RS]LV dose between 50 and 200 mg/m^2. Subsequent expansion of these pools continued, albeit disproportionately to the [6RS]LV dose used, reaching a maximal 4- to 4.5-fold enhancement at a [6RS]LV dose of 800 mg/m^2. In $HxGC_3$ tumors, CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ pools gradually increased to a maximal 2.3-fold elevation at 1,000 mg/m^2 [6RS]LV. In these three lines, CH_2-H_4PteGlu$_{2-5}$ pools were elevated as the dose of [6RS]LV was increased. However, in $HxHC_1$ tumors, little (maximum, 1.4-fold) expansion of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ pools was detected. Consequently, in the current study, we determined the growth-inhibitory properties of FUra applied either alone or in combination with varied doses of [6RS]LV (50–1,000 mg/m^2) in each of these four tumor lines.

Factors in addition to pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ that may influence tumor sensitivity to FUra were also examined, including the intratumor metabolism of [6RS]LV to other reduced folate species and the activity of CH = H_4PteGlu synthetase, the enzyme responsible for the initial conversion of [6S]LV to CH = H_4PteGlu within tumors. Since the capability of tumor cells to salvage dThd may significantly influence their degree of sensitivity to FUra ± [6RS]LV, levels of circulating dThd in the plasma of CBA/CaJ mice in comparison with humans were determined, as was the comparative in vivo sensitivity of $HxGC_3$ versus $HxGC_3$/c1TK$^-$c3 (selected for TK deficiency) to FUra ± [6RS]LV. The series of four tumor xenografts derived from adult tumors has since been expanded to include xenografts established from colon carcinomas in adolescents or young adults. The biochemical metabolism of [6RS]LV to CH_2-H_4PteGlu$_n$+H_4PteGlu$_n$, 10-CHO-H_4PteGlu+CH = H_4PteGlu, and 5-CH_3-H_4PteGlu and the in vivo sensitivity of tumors to FUra ± [6RS]LV is described. Finally, the plasma levels of FUra achievable in the CBA/CaJ mouse using these doses and schedules are reported.

Materials and methods

Immune deprivation of mice. Female CBA/CaJ mice (Jackson Laboratories, Bar Harbor, Me.) aged 4 weeks were immune-deprived by thymectomy and subjected, 3 weeks later to whole-body irradiation (950 cCy) using a ^{137}Cs source. Mice received 3x10^6 nucleated bone marrow cells within 6–8 h of irradiation [11].

Tumor lines. The characteristics of human colon adenocarcinoma xenografts derived from neoplasms of adults ($HxELC_2$, $HxGC_3$, $HxVRC_5$, and $HxHC_1$) have been described elsewhere [11, 12, 15, 16]. $HxGC_3$/c1TK$^-$c3, a TK-deficient variant of $HxGC_3$, was initially selected from the cloned GC_3 line established in tissue culture [27] and subsequently reestablished as a xenograft in mice. In addition, three tumors obtained from previously untreated adolescents or young adults were established as xenografts in immune-deprived mice. $HxSJC_2$, originally obtained from a 16-year-old girl, is a poorly to moderately well differentiated tumor exhibiting a volume-doubling time on the order of 15 days. $HxSJC_3$A and $HxSJC_3$B constitute two separate primary tumors derived from the same patient (a 26-year-old man). $HxSJC_3$A demonstrates poor to moderate differentiation, its volume-doubling time in mice being 10 days; $HxSJC_3$B exhibits slightly better differentiation, doubling in volume in 13 days. The histology of each of these three lines is similar to that of the original specimen from which it was derived. All tumors were maintained as xenografts by passage in the s.c. space of female CBA/CaJ immune-deprived mice.

Growth-inhibition studies. Mice bearing bilateral s.c. tumors received the agent when the tumors had reached a diameter of ≈ 0.5 cm. Tumor response was determined at 7-day intervals using digital calipers (Maxcal) interfaced with an IBM/PS2 microcomputer. Two perpendicular diameters were used to compute volumes [14]. Growth inhibition was calculated from the difference in days required for treated tumors to grow to 4 times their volume at the start of treatment as compared with vehicle-treated controls. Each treatment group comprised six tumor-bearing mice. Relative tumor volumes were calculated using the formula RTV = (V_x/V_o), where V_x represents the tumor volume on day X and V_o indicates the tumor volume at the initiation of treatment. To equate responses of tumor lines demonstrating different rates of growth, inhibition was normalized by expressing this as a function of Td_2. Tumor responses were graded as shown in the footnote to Table 1.

Formulation and administration of drugs. FUra (50 mg/ml), obtained from SoloPak Laboratories (Franklin Park, Ill.) as a pharmaceutical preparation, was diluted to a concentration of 3.75 mg/ml in sterile 0.9% saline. It was given to mice by i.v. bolus injection via the tail vein for three courses (days 1, 8, and 15) after randomization of the animals. The dose used was 75 mg/kg (≈225 mg/m^2) given in a volume of 0.1 ml/20 g body weight and constituted the maximum tolerated dose on that schedule.

[6RS]LV, purchased from Sigma Chemical Co. (St. Louis, Mo.), was given to mice by i.v. bolus injection at doses of 50, 500, 800, or 1,000 mg/m^2 on days 1–5, 8–12, and 15–19 immediately prior to FUra commencing on the 1st day of FUra injection. [6RS]LV was dissolved in 0.9% sterile saline at concentrations of 2–25 mg/ml, depending on the dose, and after additional filtration, it was injected at volumes of 0.17–0.27 ml/20 g body weight. The dose relationship between milligrams per kilogram and milligrams per square meter body-surface area was determined for individual mice as previously described [9, 15, 16]. Comparisons between [6RS]LV dosing schedules of i.v. bolus daily × 5 versus continuous infusion for 3 weeks were not attempted due to the limited period (5 days) over which unrestrained mice could tolerate continuous infusion without developing substantial toxicity and to the need for the administration of several courses of therapy.

Statistical analyses. The results of individual studies were evaluated by one-way analysis of variance using the number of days required to reach 4 times the original tumor volume as the dependent variable followed by the Newman-Keuls range test for differences between means [32]. Only tumors from mice that survived the entire study were included in the

analyses, and any tumor that failed to reach 4 times its original volume was assigned a default value for the maximal duration of the study. To compare the efficacy of various courses of treatment, data were collapsed across studies within a given tumor line. The percentage of tumors showing partial and/or complete regression were calculated for the individual tumor lines.

Expansion of pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$. The assay of extracts from $HxGC_3$/c1TK$^-$c3, $HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$ tumors was based on the catalytic release of tritium from [5-^{3}H]-dUMP (22 Ci/mmol; Moravek Biochemicals, Brea, Calif.) over 3 min at 30° C as described elsewhere [13, 16]. Prior to tumor excision and extraction, tumor-bearing mice were initially infused i. v. for 24 h with varied doses of [6RS]LV (20–1,000 mg/m^2) for comparison with data previously obtained in $HxELC_2$, $HxGC_3$, $HxVRC_5$, and $HxHC_1$ xenografts [13, 15, 16].

[6RS]LV metabolism. Metabolism of nonradiolabeled [6RS]LV to CH = H_4PteGlu+10-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu in $HxHC_1$, $HxVRC_5$, $HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$ tumors following 24-h i. v. infusions of [6RS]LV (50–1,000 mg/m^2) in tumor-bearing mice was determined according to the method of Duch et al. [6]. Briefly, tumors were excised, immediately placed in liquid N_2, and ground to a fine powder under N_2 using a pestle and mortar. Tissue (2.5 g) was extracted in 3 vol. (7.5 ml) of hot (90° C) 1% ascorbate/1% β-mercaptoethanol and heated at 95° C for 5 min. Following cooling on ice and flushing with N_2, samples were homogenized on ice using a polytron and were again flushed with N_2. Tubes were centrifuged at 20,000 *g* for 20 min at 4° C. Supernatants were retained and treated with rat plasma containing γ-glutamyl hydrolase (ratio of extract: plasma, 4 : 1) for 3 h at 37° C. Digestion of folylpolyglutamates was terminated by heating at 95° C for 5 min followed by cooling on ice. Samples were centrifuged at 20,000 *g* for 20 min at 4° C and were stored overnight at –70° C under argon. Extracts were subsequently thawed, adjusted to pH 4.5 with 1 M acetic acid, applied to a 1-cm column of Dowex 50W-X4 (BioRad, Rockville Center, N.Y.) for folate purification, and were then passed through C-18 Bond Elute (Analytichem International, Harbor City, Calif.) for concentration. After drying under N_2, the folates were resuspended in 275 µl 0.1% ascorbate/0.1% β-mercaptoethanol and analyzed by HPLC. At all stages, buffers had previously been flushed with N_2.

Monoglutamylfolates were separated by reverse-phase ion-pair HPLC methodology according to the method of Duch et al. [6] on a Waters µBondapak C_{18} 10-µm column (3.9 × 300 mm; number 27 324). Separation was effected using a Beckman System Gold HPLC (monitored at 284 nm) equipped with model 126 pumps, a model 168 Diode Array Detector and a model 507 refrigerated autosampler. HPLC solvents were maintained under helium, and 220 µl extract was analyzed in duplicate for each separate experiment. Retention times of PABGlu, 10-CHO-H_4PteGlu, H_4PteGlu, 5-CHO-H_4PteGlu, H_2PteGlu, PteGlu, and 5-CH_3-H_4PteGlu were 9.8, 12.5, 16.9, 19.7, 26.3, 32.3, and 35.9 min, respectively. Under these conditions, CH = H_4PteGlu was converted to 10-CHO-H_4PteGlu, and CH_2-H_4PteGlu was converted to H_4PteGlu. The fidelity and purity of each folate species was verified by UV scanning of the appropriate peaks using the diode-array detector, and the UV spectrum was compared with that of authentic standards. The recovery of 5-CH_3-H_4PteGlu, H_4PteGlu, 10-CHO-H_4PteGlu, PteGlu, H_2PteGlu, and 5-CHO-H_4PteGlu was determined to be 100%, 91%, 85%, 84%, 45%, and 26%, respectively, following extraction and analysis.

Activity of CH = H_4PteGlu synthetase. The activity of this enzyme in the eight colon-adenocarcinoma xenograft lines was determined in tumor extracts as described by Bertrand et al. [2].

Determination of plasma dThd. Extraction and analysis of dThd from the plasma of mice or human volunteers was based on the methods of Howell et al. [19] and Khym [21]. Protein was precipitated from heparinized blood samples (500 µl) using 50 µl ice-cold 4.4 M PCA. Following clarification and centrifugation, samples were neutralized with either Alamine 336 (0.5 M; Henkel Corp., Tucson, Ariz.) in Freon (Sigma Chemical Co., St. Louis, Mo.) or tri-*N*-octylamine (0.5 M; Sigma Chemical Co.) in Freon. Following vigorous mixing, cooling, and centrifugation, the upper aqueous layers were transferred to Eppendorf tubes and subsequently analyzed by reverse-phase HPLC. Individual human samples were initially lyophilized prior to analysis, whereas several mouse samples were pooled. dThd was eluted on a Waters µBondapak C_{18} 10-µm column (3.9 × 300 mm) in 0.1 mM ammonium acetate (pH 7.0) with an MeOH gradient of 4%–30% over 35 min; UV absorbance was monitored at 260 and 280 nm. The retention time for dThd under these conditions was 14.7 min, and the minimal detectable concentration was 40 nM. The recovery of dThd following extraction from plasma ranged between 82% and 86%.

Plasma levels of FUra. Non-tumor-bearing, immune-deprived female CBA/CaJ mice were injected i. v. with the 75-mg/kg FUra dose used in the in vivo growth-inhibition studies. At various intervals ranging from 1 to 90 min after drug administration, blood was drawn by cardiac puncture (four mice at each time point) into heparinized syringes. Following centrifugation at 2,000 *g* for 10 min at 2° C, FUra and FUra nucleosides were extracted from plasma samples using the method of Amicucci et al. [1]. Samples were analyzed by HPLC on the reverse-phase column described above using an isocratic mobile phase consisting of 2.5 mM ammonium acetate and 2.5% MeOH (pH 5.0). Peaks were monitored by UV absorbance at 265 nm for 35 min. Retention times for FUra, FUrd, and FdUrd were 5.4, 8.9, and 12.2 min, respectively, and the recovery was 89%, 83%, and 83%, respectively, following extraction. The limit of detectability was 1–1.5 µM.

Results

Growth-inhibition studies

The therapeutic activity of FUra combined with different doses of [6RS]LV (50–800 mg/m^2) in tumor xenografts established from adult colonic neoplasms ($HxELC_2$, $HxVRC_5$, $HxHC_1$, and $HxGC_3$) and in $HxGC_3$/c1TK$^-$c3 selected for TK deficiency is shown in Table 1. In $HxELC_2$ tumors, FUra alone induced slight growth inhibition that differed significantly from the control values ($P < 0.05$); this inhibition increased marginally, but not significantly, when the [6RS]LV dose was increased to 500–800 mg/m^2. The growth of control tumors and FUra/[6RS]LV (50 mg/m^2)-treated tumors was similar. Deaths (2/6) occurred in groups receiving 50 or 800 mg/m^2 [6RS]LV. In line $HxVRC_5$, sensitivity to FUra ± [6RS]LV ranged from ++ to ++++ (see Table 1), with all treatment groups showing significant differences from the control values ($P \leq 0.05$). The growth inhibition observed in mice receiving higher doses of [6RS]LV (500 or 800 mg/m^2) did not differ from that noted in those receiving FUra alone but appeared to be greater ($P < 0.05$) in animals treated with a lower dose of [6RS]LV (50 mg/m^2). Deaths (2/6) were observed in groups receiving FUra ± [6RS]LV (800 mg/m^2). In $HxHC_1$ tumors, growth delay was significant but identical (28 days) in both FUra- and FUra/[6RS]LV (500 mg/m^2)-treated groups. Toxicity was observed in mice receiving FUra alone.

The sensitivity of $HxGC_3$ tumors to FUra ± [6RS]LV combinations is shown in Table 1 and Fig. 1. In this line, growth inhibition was significant (28 days), albeit similar, in mice given FUra alone or in combination with [6RS]LV (500 mg/m^2). All mice survived treatment. Although we have previously shown that high doses of [6RS]LV (500–1,000 mg/m^2) result in 2.3- to 4.5-fold elevations in

Table 1. Sensitivity of colon adenocarcinoma xenografts to FUra ± [6RS]LV

Tumor	Dose of FUra[a] (mg/kg)	Dose of [6RS]LV[a] (mg/m²)	Vol (mm³)[b]	Time to 4x(days)[c]	GD[d] (days)	Td_2 saved[e]	Response (%)[f] PR	Response (%)[f] CR	Deaths (%)	Ranking[g]
$HxELC_2$	0	0	0.50 ± 0.06	42.1 ± 5.3	–	–	0	0	0	–
	75	0	0.32 ± 0.07	61.4 ± 4.9*	19.3	0.9	0	0	0	+
	75	50	0.46 ± 0.06	48.9 ± 3.0	6.7	0.3	0	0	33	±
	75	500	0.58 ± 0.06	66.2 ± 5.5*	24.1	1.1	0	0	0	+
	75	800	0.38 ± 0.05	68.5 ± 6.8*	26.3	1.3	0	0	33	+
$HxVRC_5$	0	0	0.58 ± 0.06	12.6 ± 1.3	–	–	0	0	0	–
	75	0	0.58 ± 0.08	25.1 ± 2.2*	12.5	2.0	0	0	33	++
	75	50	0.90 ± 0.21	37.1 ± 6.1*,**	24.5	3.9	0	0	0	++++
	75	500	0.46 ± 0.07	23.6 ± 2.5*	11.0	1.8	0	0	0	+→++
	75	800	0.53 ± 0.07	23.4 ± 1.4*	10.9	1.7	0	0	33	+→++
$HxHC_1$	0	0	0.16 ± 0.03	21.3 ± 6.0	–	–	0	0	0	–
	75	0	0.53 ± 0.10	48.8 ± 3.2*	27.5	2.6	50	25	33	++++
	75	500	0.36 ± 0.07	49.4 ± 5.4*	28.0	2.6	83	0	0	++++
$HxGC_3$	0	0	0.51 ± 0.07	19.8 ± 1.7	–	–	0	0	0	–
	75	0	0.46 ± 0.08	32.2 ± 5.1*	27.5	2.6	10	0	0	++→+++
	75	500	0.81 ± 0.13	30.5 ± 1.6*	28.0	2.6	0	0	0	++→+++
$HxGC_3$/c1TK⁻c3	0	0	0.25 ± 0.03	41.3 ± 4.1	–	–	0	0	0	–
	75	0	0.27 ± 0.04	72.3 ± 4.0*	31.0	1.5	40	20	0	+→++
	75	50	0.22 ± 0.05	80.5 ± 4.6*	39.2	1.9	100***	60	17	++++
	75	500	0.25 ± 0.05	80.5 ± 4.0*	39.2	1.9	100***	56	0	++++
	75	800	0.44 ± 0.11	114.2 ± 5.5*,**	72.9	3.5	71***	0	33	+++++
	15	0	–	70.2 ± 5.8	28.9	1.4	33	33	0	+→++
$HxSJC_3A$	0	0	0.29 ± 0.05	20.4 ± 2.2	–	–	0	0	0	–
	75	0	0.52 ± 0.08	35.4 ± 1.8	15.0	1.5	0	0	0	+→++
	75	50	0.36 ± 0.05	34.6 ± 2.9*	14.1	1.4	0	0	33	+→++
	75	500	0.27 ± 0.06	54.0 ± 9.7*,**	33.6	3.3	25	0	0	++++
	75	1000	0.46 ± 0.07	68.5 ± 7.8*,**	48.1	4.7	22	11	17	++++
$HxSJC_3B$	0	0	0.58 ± 0.11	25.9 ± 2.4	–	–	0	0	0	–
	75	0	0.62 ± 0.10	38.7 ± 3.5*	12.8	1.0	0	0	0	+
	75	50	1.01 ± 0.22	36.0 ± 1.6*	10.1	0.8	0	0	17	± →+
	75	500	0.83 ± 0.22	46.9 ± 4.2*,**	21.0	1.6	0	0	17	+→++
$HxSJC_2$	0	0	0.81 ± 0.15	29.4 ± 4.5	–	–	0	0	0	–
	75	0	1.00 ± 0.16	41.5 ± 2.9	12.1	0.8	0	0	17	± →+
	75	50	1.08 ± 0.22	32.7 ± 2.6	3.4	0.2	0	0	17	±
	75	500	0.58 ± 0.08	40.0 ± 4.4	10.6	0.7	0	0	17	± →+
	75	800	0.63 ± 0.12	37.5 ± 2.3	8.2	0.6	0	0	33	± →+

[a] FUra (75 mg/kg) was given i. v. on days 1, 8, and 15 following randomization of mice bearing established tumors to 6 mice per group. [6RS]LV was injected i. v. on days 1–5, 8–12, and 15–19, completing 3 courses of therapy. Alternatively, mice received FUra at 15 mg/kg i. v. daily x5 for 3 weeks

[b] Initial volume of tumors at the initiation of treatment; mean values ± SE

[c] Time taken for tumors to reach 4 times their pretreatment volume; mean values ± SE

[d] GD = growth delay calculated when tumors have reached 4 times their pretreatment volume

[e] Td_2 saved = number of volume doublings saved by treatment; GD/Td_2

[f] PR, CR = partial response (≥50% tumor regression) and complete response (≥90% tumor regression), respectively

[g] Ranking: –, no response; ±, slight response; +, growth inhibition = $1xTd_2$; ++, growth inhibition = $2\times Td_2$; +++, growth inhibition = $3xTd_2$; ++++, growth inhibition = $\geq 2\times Td_2$ plus volume regression of >50% or growth inhibition $>3\times Td_2$; +++++, growth inhibition $\geq 3\times Td_2$ plus volume regression of ≥50%

[h] Two courses of therapy

* Significantly different from control values ($P \leq 0.05$)

** Significantly different from values obtained for the group treated with FUra alone ($P < 0.05$)

*** Maximal T/C significantly different from values obtained for the group treated with FUra alone ($P < 0.01$)

pools of CH_2-$H_4PteGlu_n$ and $H_4PteGlu_n$ in $HxELC_2$, $HxVRC_5$, and $HxGC_3$ tumors [16], no substantial potentiation of FUra efficacy was demonstrated in the current study. Consequently, $HxGC_3$/c1TK⁻c3 was selected and subsequently evaluated for the effects of increasing doses of [6RS]LV on the potentiation of FUra activity in cells lacking capacity to salvage preformed dThd (Table 1, Fig. 1).

All FUra ± [6RS]LV treatment groups showed significant inhibition of the growth of $HxGC_3$/c1TK⁻c3 tumors ($P < 0.05$). Among the mice receiving FUra alone, only 40% showed partial responses (PRs) and 20% achieved complete responses (CRs). At [6RS]LV doses of 50 and 500 mg/m², 100% of the mice showed PRs and 56%–60% of the animals achieved CRs, with greater volume regression being observed at the higher dose. The growth inhibi-

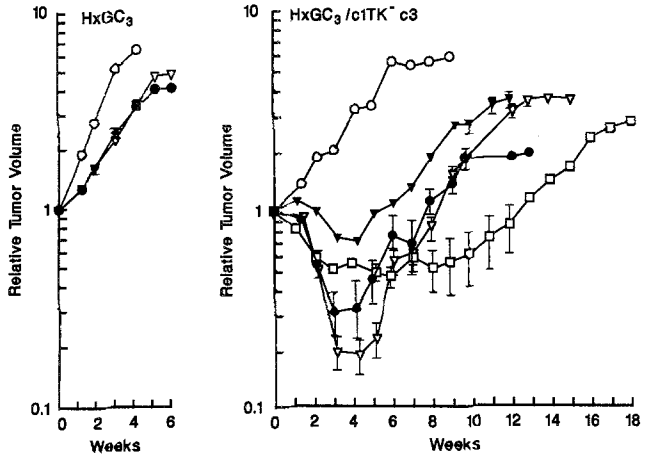

Fig. 1. In vivo evaluation of the therapeutic efficacy of FUra ± [6RS]LV in $HxGC_3$ (*left panel*) or $HxGC_3$/c1TK-c3 (*right panel*) xenografts. Mice bearing established tumors and randomized to 6 animals/group received FUra (75 mg/kg) i. v. on days 1, 8, and 15 and different doses of [6RS]LV i. v. on days 1–5, 8–12, and 15–19, representing 3 consecutive courses of treatment. Tumor volumes were determined weekly using Vernier caliper measurements, and the relative tumor volume for each treatment group was determined. $HxGC_3$: ○, control; ●, FUra; ▽, FUra + 500 mg/m^2 [6RS]LV. $HxGC_3$/c1TK-c3: ○, control; ▼, FUra; FUra + [6RS]LV doses of 50 (●), 500 (▽), 800 mg/m^2 (□)

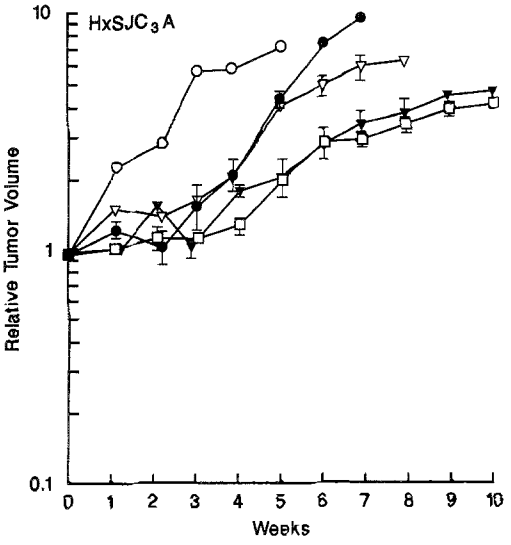

Fig. 2. In vivo evaluation of the therapeutic efficacy of FUra alone (●), or combined with [6RS]LV at doses of 50 (▽), 500 (▼), or 800 mg/m^2 (□) in $HxSJC_3A$ xenografts. Conditions were the same as those described in the legend to Fig. 1, except that only 2 courses of treatment could be given

tion observed in these two treatment groups was similar and was greater, albeit not significantly different from, that noted in tumors exposed to FUra alone. However, when FUra was combined with [6RS]LV (800 mg/m^2), 71% PR's were obtained, with a significantly enhanced potentiation of the growth-inhibitory activity of FUra (P <0.05). Thus, the efficacy of FUra increased with increasing doses of [6RS]LV. The maximum values were also significantly higher (P <0.01) in all FUra+[6RS]LV-treated groups as compared with those given FUra alone. In this experiment, FUra ± [6RS]LV (500 mg/m^2) appeared to be equitoxic (no deaths), whereas 1/6 and 2/6 mice died in the groups receiving 50 and 800 mg/m^2 [6RS]LV, respectively. The efficacy of an equimolar dose of FUra (15 mg/kg) given daily × 5 for 3 weeks did not significantly differ from that of FUra (75 mg/kg) given once a week for 3 weeks (Table 1).

Xenografts established from colonic tumors arising in adolescents ($HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$) were examined for their sensitivity to FUra ± [6RS]LV (Table 1). For $HxSJC_3A$ tumors (Table 1, Fig. 2), the sensitivity to FUra alone ranged from + to ++ (growth inhibition, 15 days), increasing significantly (P <0.05) to ++++ when higher doses of [6RS]LV (500 and 1,000 mg/m^2) were used (growth inhibition, 33.6 and 48.1 days, respectively). The growth delay observed in groups receiving FUra ± [6RS]LV (50 mg/m^2) was not significantly different. In mice treated with FUra+[6RS]LV (500 or 1,000 mg/m^2), growth inhibition was superior (P <0.05) compared to animals receiving the lower dose of [6RS]LV; 2/6 and 1/6 mice died in the groups receiving 50 and 1,000 mg/m^2 [6RS]LV, respectively. Since doses of FUra ± [6RS]LV (500 mg/m^2) were equitoxic in this study (no deaths), the therapeutic index of FUra was enhanced in the presence of [6RS]LV.

$HxSJC_3B$ tumors showed slight but significant sensitivity to FUra alone (+; 12.8 days growth inhibition; P <0.05) that was further increased significantly (21 days growth inhibition) in the presence of a high (500 mg/m^2; P <0.05) but not a low (50 mg/m^2) dose of [6RS]LV. The difference in the sensitivity of groups receiving [6RS]LV at 50 or 500 mg/m^2 was significant (P <0.05), with a similar degree of toxicity (1/6 deaths) occurring in both groups. $HxSJC_2$ tumors showed no significant response to FUra in either the absence or the presence of [6RS]LV (50–800 mg/m^2; Table 1). Deaths occurred in all treatment groups, with the toxicity being somewhat higher in mice receiving 800 mg/m^2 [6RS]LV.

The toxicity of each treatment regimen with regard to drug-induced deaths is shown in Table 1. Toxicity was somewhat variable between different experiments, although in general, more toxicity was observed in groups receiving 800 mg/m^2 [6RS]LV. Evaluation of the overall toxicity of the treatment regimens tested revealed that the mortality of mice receiving FUra alone amounted to 10.4% (5/48). When [6RS]LV was given concurrently at 50, 500, 800, and 1,000 mg/m^2, the percentage of mice lost due to toxicity was 19.4% (7/36), 4.2% (2/48), 33.3% (8/24), and 16.7% (1/16), respectively. Thus, only following a higher dose of [6RS]LV (800 mg/m^2) given in combination with FUra was toxicity consistently observed to increase somewhat; at lower [6RS]LV doses, toxicity was not consistent with dose. Insufficient numbers of mice were evaluated at 1,000 mg/m^2 [6RS]LV to obtain an accurate assessment of toxicity.

Expansion of CH_2-$H_4PteGlu_n$ and $H_4PteGlu_n$ pools in xenografts

We have previously shown that CH_2-$H_4PteGlu_n$ + $H_4PteGlu_n$ pools are maximally elevated in the parent $HxGC_3$

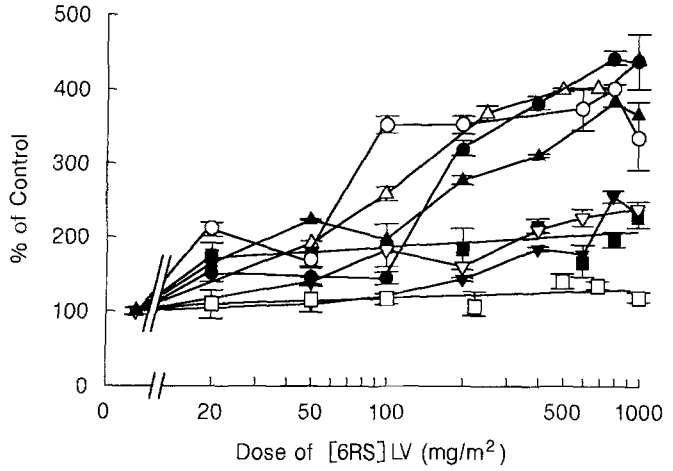

Fig. 3. Effect of 24-h i.v. infusions of varied doses of [6RS]LV (20–1,000 mg/m^2) on the size of pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in $HxELC_2$ (●), $HxVRC_5$ (○), $HxHC_1$ (□), $HxGC_3$ (■), $HxGC_3$/c1TK-c3 (▲), $HxSJC_3A$ (△), $HxSJC_3B$ (▼), and $HxSJC_2$ (▽) xenografts. Pools were assayed using a method based on the catalytic release of tritium from [5-^{3}H]-dUMP [13, 16]. Data represent mean values ± SD for 3 determinations per point

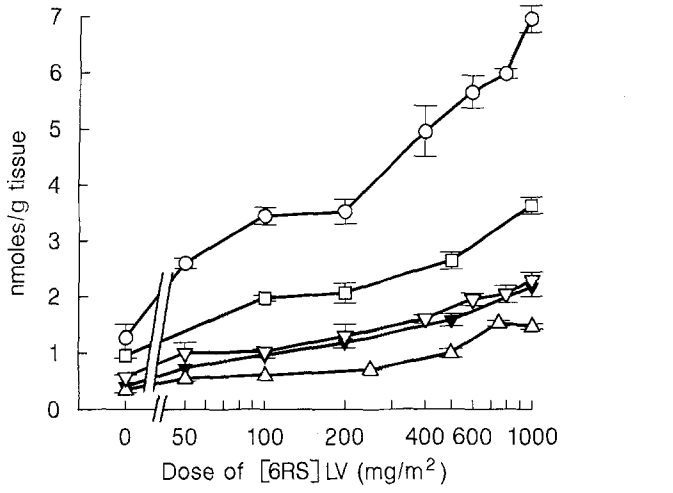

Fig. 4. Metabolism of [6RS]LV to CH = H_4PteGlu + 10-CHO-H_4PteGlu in xenografts. Tumor-bearing mice received different doses of [6RS]LV (50–1,000 mg/m^2) i.v. for 24 h. Excised tumors were subsequently extracted and reduced folate pools were analyzed according to the procedure of Duch et al. [6]. ○, $HxVRC_5$; □, $HxHC_1$; △, $HxSJC_3A$; ▼, $HxSJC_3B$; ▽, $HxSJC_2$. Data represent mean values ± SD for 2–4 determinations at each [6RS]LV concentration

xenograft to between 233% and 344% of control values during 24-h i.v. infusions of [6RS]LV (500–1,000 mg/m^2 [15, 16]) in tumor-bearing mice. To determine whether these effects would be similar in $HxGC_3$/c1TK-c3 tumors, tumor-bearing mice were infused i.v. for 24 h with varying doses of [6RS]LV (20–1,000 mg/m^2) prior to assay of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools. Slightly higher pool-size expansion was observed in $HxGC_3$/c1TK-c3 (Fig. 3). At 20 mg/m^2 [6RS]LV, pools were increased to 165% of control values; they continued to increase as the dose of [6RS]LV was increased, reaching a maximum of 368%–384% of control values at doses of 500–800 mg/m^2 [6RS]LV. Basal levels of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ were estimated to be 276 pmol/g (≈552 nM), lying within the same range previously reported for the parent $HxGC_3$ line (484 pmol/g; ≈968 nM [16]). The distribution of polyglutamates within the combined pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ were also similar in $HxGC_3$ and $HxGC_3$/c1TK-c3; penta- and hexaglutamate species predominated in untreated tumors, with the distribution shifting to shorter-chain-length forms as the dose of [6RS]LV was increased from 20 to 1,000 mg/m^2 (data not shown). The previously reported expansion of pools of CH_2-H_4PteGlu$_n$ and H_4PteGlu$_n$ in response to the dose of [6RS]LV used in $HxELC_2$, $HxHC_1$, and $HxVRC_5$ tumors [16] are also shown in Fig. 3 for purposes of comparison.

Among the tumors derived from adolescents (Fig. 3), some expansion of pools of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ was detected in $HxSJC_3B$ and $HxSJC_2$ tumors, with maximal elevations of 240%–257% of control values being detected at 800–1,000 mg/m^2 [6RS]LV; levels were expanded to between 182% and 220% of control values at 500 mg/m^2 [6RS]LV. However, in line $HxSJC_3A$, pools of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ were increased to 192% of control values at 50 mg/m^2 [6RS]LV, to 402% of control values at 500 mg/m^2 [6RS]LV, and to 442% of control levels at 1,000 mg/m^2 [6RS]LV, demonstrating greater pool-size expansion in this line than in the other two tumor lines.

Metabolism of [6RS]LV

Modulation of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools by [6RS]LV in colon adenocarcinoma xenografts varied from little, if any, modulation ($HxHC_1$) to marked (up to 4.5-fold) enhancement ($HxVRC_5$, $HxELC_2$, and $HxSJC_3A$). Consequently, the metabolism of [6RS]LV to 5-CH_3-H_4PteGlu and the combined pools of CH = H_4PteGlu and 10-CHO-H_4PteGlu were subsequently examined in five tumors following 24-h i.v. infusions of tumor-bearing mice with varied doses of [6RS]LV (50–1,000 mg/m^2). Levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu are shown in Fig. 4, and pools of 5-CH_3-H_4PteGlu are illustrated in Fig. 5.

In lines $HxHC_1$ and $HxVRC_5$ pools of CH = H_4PteGlu, 10-CHO-H_4PteGlu, 5-CH_3-H_4PteGlu, and 5-CHO-H_4PteGlu (LV) comprised 89%–97% of the total folate species measured. In $HxVRC_5$, levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu in untreated tumors were 0.6 nmol/g; these levels gradually increased to 469% of control values (3 nmol/g) following the administration of [6RS]LV at 1,000 mg/m^2. Pretreatment levels of 5-CH_3-H_4PteGlu were somewhat higher (0.96 nmol/g), and these pools increased substantially to 7 nmol/g as the dose of [6RS]LV was increased to 1,000 mg/m^2. Concentrations of tumor-associated [6RS]LV increased from 0.07 to 8.25 nmol/g between doses of 50 and 1,000 mg/m^2 [6RS]LV, whereas the maximal level of H_2PteGlu detected in tumors was 0.17 nmol/g (data not shown). The data therefore indicated considerable intracellular metabolism of [6S]LV within reduced folate pools in $HxVRC_5$ tumors.

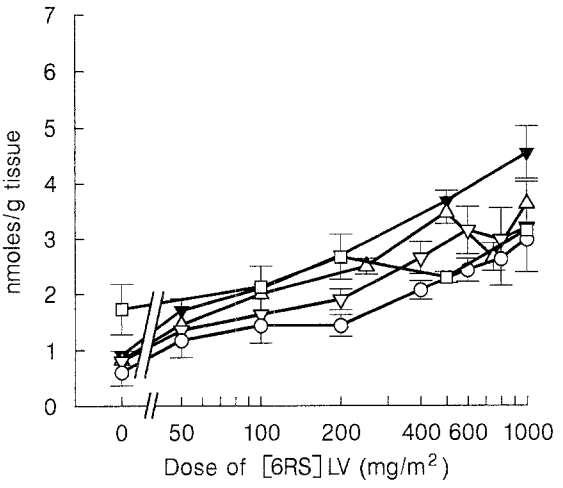

Fig. 5. Influence of 24-h i. v. infusions of [6RS]LV at doses ranging from 50 to 1,000 mg/m^2 on pools of 5-CH_3-H_4PteGlu in $HxVRC_5$ (○), $HxHC_1$ (□), $HxSJC_3A$ (△), $HxSJC_3B$ (▼), and $HxSJC_2$ (▽) tumors. Conditions were the same as those described in the legend to Fig. 4. Data represent mean values ± SD for 2–4 determinations at each [6RS]LV concentration

In line $HxHC_1$, basal levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu (1.73 nmol/g) were higher than those observed in $HxVRC_5$, and these pools increased to only 182% of control values (3.15 nmol/g) following the administration of 1,000 mg/m^2 [6RS]LV (Fig. 3). Initial levels of 5-CH_3-H_4PteGlu (1.28 nmol/g) increased maximally to 3.65 nmol/g following [6RS]LV administration, which corresponds to only 52% of the maximal elevation determined in $HxVRC_5$ tumors. Tumor-associated levels of [6RS]LV increased from 1.1 to 11.6 nmol/g, whereas the amount of H_2PteGlu detected in tumors reached 0.25 nmol/g (data not shown). The results indicated substantially lower concentrations of [6S]LV metabolites in $HxHC_1$ as compared with $HxVRC_5$ xenografts.

In contrast to $HxVRC_5$, in $HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$ tumors, levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu were higher than concentrations of 5-CH_3-H_4PteGlu in all three tumor lines (Figs. 4, 5). Basal levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu were similar among the different tumors (0.8–0.9 nmol/g) and increased by 400%–506% of control values to 3.2–4.6 nmol/g after the administration of 1,000 mg/m^2 [6RS]LV. Levels of 5-CH_3-H_4PteGlu in untreated tumors were 0.4–0.6 nmol/g; these values showed a similar increase to a maximum of 1.5–2.3 nmol/g following [6RS]LV administration. Tumor-associated concentrations of [6RS]LV amounted to 0.9 and 1.6 nmol/g, respectively, in $HxSJC_3A$ and $HxSJC_3B$ tumors after dosing of mice with 100 mg/m^2 [6RS]LV; these values increased to 5.2 and 10.9 nmol/g, respectively, following the administration of 1,000 mg/m^2 [6RS]LV. In line $HxSJC_2$, tumor-associated [6RS]LV was initially detected after a dose of 400 mg/m^2 [6RS]LV (2.8 nmol/g) and increased to 7.9 nmol/g at 1,000 mg/m^2 [6RS]LV (data not shown). Apart from the greater [6RS]LV-induced expansion of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools in $HxSJC_3A$ tumors, the only other detectable difference noted in the characteristics of [6RS]LV metabolism in line $HxSJC_3A$ was that 5-CH_3-H_4PteGlu levels were on average 65% of those determined in $HxSJC_3B$ and $HxSJC_2$ tumors.

Activity of CH = H_4PteGlu synthetase in tumors

To investigate whether CH = H_4PteGlu synthetase would influence the metabolism of [6RS]LV in tumors, we determined the activity of this enzyme in each of the eight colon adenocarcinoma xenografts studied (Table 2). Levels ranged from 0.33 units/mg in $HxGC_3$/c1TK$^-$c3 to 1.68 units/mg in $HxHC_1$. In tumors in which elevated levels of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ had been detected (3- to 5-fold elevations; $HxGC_3$, $HxGC_3$/c1TK$^-$c3, $HxELC_2$, $HxVRC_5$, and $HxSJC_3A$), the activity of CH = H_4PteGlu synthetase varied from 0.33 to 1.63 units/mg. In the other tumor lines, particularly $HxHC_1$, in which considerably less [6RS]LV metabolism was detected, CH = H_4PteGlu synthetase activity was in the high range (0.97–1.68 untis/mg). Levels of CH = H_4PteGlu synthetase did not correlate with the metabolism of [6S]LV to CH = H_4PteGlu + 10-CHO-H_4PteGlu in tumor cells or with the capacity of [6RS]LV to potentiate the therapeutic activity of FUra. CH = H_4PteGlu synthetase activity therefore did not appear to be a limiting factor in the metabolic conversion of [6S]LV in tumor cells. Enzyme activity also showed no correlation with the rate of tumor growth in this series of xerografts whose volume-doubling times ranged from 6.3 to 20.7 days (Table 2).

Plasma levels of FUra in mice

To determine the concentrations of FUra and FUra nucleosides that are achievable in the plasma of mice, we injected FUra i.v. at the dose of 75 mg/kg used in the growth-inhibition studies and evaluated the samples by HPLC from 1 to 90 min postinjection. The peak plasma level of FUra detected at 1 min was 1.1 mM (Fig. 6). Elimination of FUra was biphasic, with the $t_{1/2}\alpha$ value during the first 30 min being 4.6 min and the subsequent $t_{1/2}\beta$ value being 21.2 min. Within 5 and 30 min, plasma con-

Table 2. Activity of CH = H_4PteGlu synthetase in human colon adenocarcinoma xenografts

Tumor line	Td_2 (days)[c]	Enzyme activity (units/mg protein)[a]
$HxGC_3$/c1TK$^-$c3	20.7±2.0	0.329±0.173
$HxGC_3$	9.9±0.9	0.383±0.138
$HxELC_2$	21.1±2.7	0.466±0.089
$HxVRC_5$	6.3±0.7	0.831±0.487
$HxSJC_3B$	13.0±1.2	0.968±0.285
$HxSJC_2$	14.7±2.3	1.142±0.373
$HxSJC_3A$	10.2±1.1	1.630±0.400[b]
$HxHC_1$	10.7±3.0	1.677±1.247

Data represent mean values ± SD for 3 or 4 determinations in each tumor

[a] 1 unit represents 1 μmol CH = H_4PteGlu formed per minute

[b] Mean values ± average deviation for 2 determinations

[c] Td_2 = volume-doubling time; mean values ± SE for 14 tumors/group

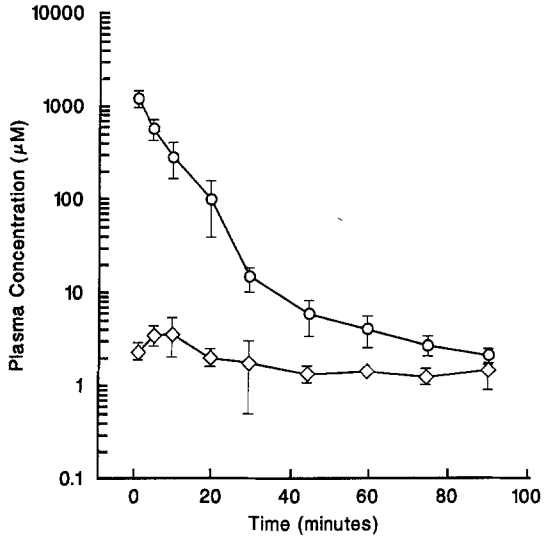

Fig. 6. Plasma concentrations of FUra (○) and FUrd (◇) in mice following i.v. bolus injection of FUra at a dose of 75 mg/kg. Compounds were extracted and analyzed as described in Materials and methods. Data represent mean values ± SD for 3–4 independent determinations carried out in individual mice at each time point

centrations of FUra were reduced to 555 and 14 μM, respectively, and at 90 min they amounted to 2 μM. FUrd was also detected in mouse plasma, but at considerably lower levels than was observed for the parent drug. Levels of FUrd accumulated, reaching a maximum of 3.5 μM at 10 min postinjection. The $t_{1/2}\alpha$ value was 13.2 min and the $t_{1/2}\beta$ value was 40.0 min. FUrd concentrations declined to between 1.2 and 1.4 μM at 75–90 min, bordering on the limits of detectability. Levels of FdUrd were sufficiently low that they could not be accurately determined.

Plasma levels of dThd

There was a 3.7-fold difference between the mean plasma dThd concentration determined in six mouse samples and that determined in blood obtained from four healthy volunteers. The mean plasma level in mice was higher (1.13 ± 0.53 μM; range, 0.59–2.0 μM) than that in the human volunteers (0.31 ± 0.25 μM; range, 0.04–0.64 μM).

Discussion

In the present investigation, we determined the therapeutic activity of FUra in the absence or presence of different doses of [6RS]LV in preclinical human colon-adenocarcinoma xenograft models that have been well characterized biochemically [12, 13, 16]. In several phase III randomized clinical trials in colorectal cancer patients, the therapeutic efficacy of FUra has clearly been potentiated by concurrent administration of [6RS]LV [5, 8, 23–25]. However, controversy has remained concerning (1) the optimal dose of [6RS]LV required to obtain maximal improvement in the therapeutic index of FUra and (2) the most appropriate schedule for administration of both the reduced folate and the 5-fluoropyrimidine, whether by daily bolus administration or by continuous infusion.

The dose of [6RS]LV used in clinical trials has varied from 20 to 500 mg/m^2. In the randomized study reported by Poon et al. [25], in which these two doses of [6RS]LV given in combination with FUra were compared in i.v. bolus daily × 5 regimens, a greater level of significance for increased tumor response rates, prolonged time to disease progression, and improved quality of life was obtained in the group receiving [6RS]LV (20 mg/m^2), although the median survival intervals (12 months), which were significantly increased in these patients as compared with those given FUra alone (7.7 months), were similar. However, in the study reported by the Gastrointestinal Tumor Study Group [24], a significant improvement in response (P <0.01) was obtained after treatment of patients with FUra + [6RS]LV (500 mg/m^2) but not following the administration of FUra combined with 25 mg/m^2 [6RS]LV when the folate was given weekly for periods ranging from 10 min to 2 h.

In our preclinical xenograft studies, higher doses of [6RS]LV (500–1,000 mg/m^2) were most frequently superior to the lower dose tested (50 mg/m^2). In $HxGC_3$/c1TK-c3 tumors, the administration of 500 mg/m^2 [6RS]LV together with FUra caused greater tumor-volume regression than did a dose of 50 mg/m^2, and 800 mg/m^2 [6RS]LV significantly increased tumor-growth inhibition as compared with the 50- or 500-mg/m^2 doses. In this experiment, FUra ± [6RS]LV (500 mg/m^2) appeared to be equitoxic. In $HxSJC_3A$ tumors, the administration of doses of 500 or 1,000 mg/m^2 [6RS]LV together with FUra was significantly superior to treatment either with 50 mg/m^2 [6RS]LV in combination with FUra or with FUra alone; doses of FUra ± [6RS]LV (500 mg/m^2) were also equitoxic in this study. Similarly, in line $HxSJC_3B$, treatment with [6RS]LV (500 mg/m^2) combined with FUra was superior to the use of either [6RS]LV (50 mg/m^2) together with FUra or of FUra alone, although 1/6 mice died in the [6RS]LV-treated group. Toxicity in the treatment groups was variable, although it was consistently slightly higher in groups receiving 800 mg/m^2 [6RS]LV. However, since both generally, and in the particular studies involving the $HxGC_3$/c1TK-c3 and $HxSJC_3A$ lines the toxicity of FUra ± [6RS]LV (500 mg/m^2) was equivalent, it may be concluded that the therapeutic index of FUra was improved at that dose of [6RS]LV. In addition, although the mortality of mice bearing $HxGC_3$/c1TK-c3 xenografts increased from 0 to 33% (overall increase, from 4% to 33%) when the dose of [6RS]LV was increased from 500 to 800 mg/m^2, the activity of FUra was increased significantly. In only one of four tumors, namely, $HxVRC_5$, was the activity of FUra potentiated to a greater extent by low as opposed to high doses of [6RS]LV. The data therefore suggest that [6RS]LV has greater potential for improving the therapeutic efficacy of FUra if higher doses of the folate are used. This rationale may also be based on the effect of the dose of [6RS]LV on the expansion of CH_2-$H_4PteGlu_n$ + $H_4PteGlu_n$ pools, as considerably greater elevations of these pools were obtained following 24-h i.v. infusions of 500–1,000 mg/m^2 [6RS]LV as compared with 50 mg/m^2 [16].

With regard to the administration schedule for the folate species, bolus daily x5 versus continuous-infusion regimens could not be directly compared in this study due to the necessity for repeated courses of therapy and to the inability to repeat 5-day infusions in mice. However, based on biochemical considerations, reduced folate species remain maximally elevated for the longest periods in both xenografts and plasma of mice during continuous exposure to [6RS]LV. This treatment modality should also facilitate the maximal formation and stability of the FdUMP/thymidylate synthase/CH_2-H_4PteGlu$_n$ complex. Exposure to FUra in mice appeared to be similar to that achieved in patients. Both the plasma levels of FUra detected in mice following bolus administration of 75 mg/kg and the $t_{1/2}$ value for elimination lay within the previously reported clinical ranges [4, 10, 22]. Following i. v. bolus injection of FUra (500 mg/m^2) in patients, plasma levels of FUra were on the order of 420 μM at 5 min postinjection and decreased to 5 μM at 90 min [4, 10]. Plasma $t_{1/2}\alpha$ values ranging from 4.6 to 12.9 min have also been reported [4, 10, 22]. For the administration of the 5-fluoropyrimidine, a bolus daily x5 schedule for 3 weeks as compared with a single dose of FUra given weekly for three courses yielded similar results for growth inhibition and tumor-volume regression in $HxGC_3$/c1TK$^-$c3. However, an expanded study may be necessary to address this question critically.

Since pools of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ were expanded 3.5- to 4.2-fold in $HxVRC_5$ xenografts during 24-h infusions of [6RS]LV (100–800 mg/m^2) but were not elevated significantly in the $HxHC_1$ line, the intratumor metabolism of [6RS]LV to other reduced folate species was examined. Indicative of the high level of incorporation of [6S]LV into the CH_2-H_4PteGlu$_n$ pool in $HxVRC_5$ tumors was the substantial accumulation of 5-CH_3-H_4PteGlu during [6RS]LV infusion, which was considerably higher than that of CH = H_4PteGlu + 10-CHO-H_4PteGlu. In line $HxHC_1$, however, the levels of 5-CH_3-H_4PteGlu and CH = H_4PteGlu + 10-CHO-H_4PteGlu remained comparably low. The data obtained in $HxHC_1$ tumors would therefore suggest either that [6S]LV is not readily incorporated into the pools of CH = H_4PteGlu and/or 10-CHO-H_4Pte-Glu or that its level of metabolism is low following such incorporation. The capacity for initial metabolism of [6S]LV to CH = H_4PteGlu would not appear to be limiting, since the observed activity of CH = H_4PteGlu synthetase was high. Alternatively, the transport of reduced folates may be deficient in this line.

Among the colon adenocarcinoma xenografts derived from adolescents, $HxSJC_3A$ was most sensitive to FUra ± [6RS]LV; treatment with [6RS]LV (500–1,000 mg/m^2) increased the Td_2 saved by treatment by 2–3-fold. The data correlated with the significantly greater expansion of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools in this line in response to [6RS]LV. Of particular interest was the finding that the characteristics of [6RS]LV metabolism in this line differed from those observed in either the $HxVRC_5$ or the $HxHC_1$ tumors. In $HxSJC_3A$, $HxSJC_3B$, and $HxSJC_2$ tumors, the levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu were considerably higher than those of 5-CH_3-H_4PteGlu. The activity of CH = H_4PteGlu synthetase also lay within the high range for these tumors, correlating with the higher levels of CH = H_4PteGlu + 10-CHO-H_4PteGlu achieved. Therefore, in $HxSJC_3B$ and $HxSJC_2$, it would appear that interconversion between the CH = H_4PteGlu, 10-CHO-H_4PteGlu, and H_4PteGlu pools may be rate-limiting or, alternatively, that pool sizes are tightly regulated to maintain the balance in favor of 10-CHO-H_4PteGlu. In $HxSJC_3A$, reduced levels of 5-CH_3-H_4PteGlu may be indicative of a reduced capacity to metabolize CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu by CH_2-H_4PteGlu reductase, resulting in the expansion of CH_2-H_4PteGlu + H_4PteGlu pools in response to [6RS]LV. Thus, there is no single metabolic phenotype in this series of human colon adenocarcinoma xenografts that correlates with or predicts the [6RS]LV-induced expansion of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools.

It is noteworthy that in tumors such as $HxELC_2$, $HxGC_3$, and $HxVRC_5$, significant expansion (2.3- to 4.5-fold) of CH_2-H_4PteGlu$_n$ + H_4PteGlu$_n$ pools was detected in response to higher doses of [6RS]LV. However, the anticipated significant potentiation of the therapeutic activity of FUra was not obtained. Since circulating levels of dThd in the mouse may be capable of salvaging neoplastic cells from the antithymidylate effect of FUra, the $HxGC_3$/c1TK$^-$c3 line was used to assess the contribution of salvage in the xenograft model. This enabled the direct comparison of the relationship between thymidylate synthase inhibition and growth inhibition induced by FUra ± [6RS]LV in the absence of a dThd salvage capability. Under these conditions, the influence of FUra + [6RS]LV was clearly greater in the TK-deficient line as compared with the parent xenograft.

It has previously been reported that serum levels of dThd in man (0.13–0.19 μM [7, 18, 19] may be lower than those in the mouse (0.5–1.0 μM [20, 28]. In our studies, the average plasma dThd concentration in the CBA/CaJ mouse lay within the previously reported range (1.1 μM) and that in man was slightly higher than the published values (0.3 μM). Consequently, the intrinsic resistance of colon adenocarcinomas to FUra may be attributable to the availability of dThd supplied by the host (either man or mouse). An interesting finding obtained in the human population from our study and in that of Howell et al. [19] involved a 15- to 17-fold spread in plasma dThd concentrations, with samples from several individuals containing ≤40 nM dThd. Consequently, certain patients may exhibit a reduced availability of dThd for the salvage of neoplastic cells from FUra-containing regimens. However, in patients in whom this is clearly not the case, dThd salvage capability may form a rationale for the introduction of a nucleoside transport inhibitor such as dipyridamole into FUra/[6RS]LV regimens, an approach that is currently being applied in clinical trials [3, 29]. Alternatively, the improved therapeutic benefit gained from the use of FUra combined with [6RS]LV could be clinically augmented by the addition of agents that may act at sites other than that of thymidylate synthase. Interferons may fall into this category, since interferon α2a has been reported to increase the rates of clinical response to FUra [30, 31] and to potentiate the cytotoxicity of FUra ± [6RS]LV in colon adenocarcinoma cells in culture [17].

In summary, potentiation of the therapeutic activity of FUra by [6RS]LV in colon adenocarcinoma xenografts correlates with (1) higher doses of [6RS]LV, (2) expansion of CH_2-$H_4PteGlu_n$ + $H_4PteGlu_n$ pools in tumors, and (3) a reduced capacity to salvage preformed dThd. Future studies in colorectal cancer should build upon the improved response rates obtained using FUra-[6RS]LV combinations. Since the salvage of dThd partially abrogates the efficacy of FUra ± [6RS]LV and because dThd levels in humans are lower than those in mice, the present xenograft model probably underestimates the potential of such combinations. This caveat should be borne in mind for the translation of these preclinical data into the design of therapeutic trials.

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