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Mechanisms of Acquired Resistance to Modulation of 5-Fluorouracil by Leucovorin in HCT-8 Human Ileocecal Carcinoma Cells

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ABSTRACT. Repeated (10x) exposure of HCT-8 human ileocecal carcinoma cells to 5-fluorouracil (5-FU) for 2 or 72 hr, both incubations in the continuous presence of 20 μM leucovorin (LV), yielded two stable modulation-resistant sublines, FL2h and FL72h. Although LV potentiated growth inhibition by 5-FU 2-fold in parental HCT-8 cells, it did not potentiate the effect of 5-FU in the FL2h or FL72h sublines. LV modulation of 5-fluorodeoxyuridine (5-FdUrd) was also reduced (FL72h) or eliminated (FL2h). In the FL2h and FL72h sublines, the level of thymidylate synthase (TS) protein and TS activity in cell extracts, TS activity *in situ*, the rate of cellular uptake and metabolism of LV, and the level of 5-FU incorporation into total cellular RNA were similar to those in parental HCT-8 cells. However, LV significantly ($P < 0.01$) potentiated the inhibition of TS activity *in situ* in HCT-8 cells at 24 hr after a 2-hr treatment with either 5-FU or 5-FdUrd, but had no such activity in the FL2h and FL72h sublines ($P > 0.1$). Resistance to modulation of 5-FU by LV was associated with the inability of LV to increase the formation of intracellular TS–FdUMP–methylenetetrahydrofolate ternary complexes, and these complexes dissociated more rapidly ($T_{1/2} > 1.5$- to 3-fold faster) in the presence of different concentrations of 5,10-methylenetetrahydropteroylpentaglutamate. Thus, decreased stability of ternary complexes appears to be the mechanism of acquired resistance to the LV modulation of fluoropyrimidine cytotoxicity, possibly due to mutation(s) of TS in these two modulation-resistant HCT-8 sublines. BIOCHEM PHARMACOL 53;5:689–696, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 5-fluorouracil; leucovorin; thymidylate synthase; drug resistance; modulation

One of the major mechanisms of action of 5-FU† is the inhibition of TS. TS forms a covalent ternary complex with an active metabolite of 5-FU, FdUMP, in the presence of the folate cofactor 5,10-CH_2-H_4PteGlu$_n$ [1]. LV has been shown to potentiate the *in vitro* cytotoxicity of fluoropyrimidines [2, 3] and their clinical antitumor activity [4–7]. The level of intracellular folates as polyglutamates plays an important role in the growth inhibitory effect of 5-FU [8]. LV increases the intracellular level of 5,10-CH_2-H_4PteGlu$_n$ and thus stabilizes the TS ternary complex and prolongs the duration of TS inhibition [3]. The stability of the ternary complex increases with the increasing polyglutamate chain length of the 5,10-CH_2-H_4PteGlu$_n$ [9, 10]. Moreover, deficient polyglutamylation of 5,10-CH_2-H_4PteGlu may result in the loss of modulation of cytotoxicity of fluoropyrimidines by LV [11, 12].

Potential mechanisms of resistance to 5-FU include [13]: (1) increased TS activity; (2) decreased incorporation of 5-FU into RNA or DNA; (3) a decreased 5,10-CH_2H_4PteGlu$_n$ pool and/or level of polyglutamylation; (4) increased activity of the catabolic enzymes alkaline phosphatase or dihydropyrimidine dehydrogenase; (5) decreased activity of thymidine and uridine phosphorylases, thymidine and uridine kinases, and/or orotate phosphoribosyltransferase; (6) decreased binding affinity of TS for FdUMP; and (7) an increased intracellular dUMP pool. It is not known, however, which mechanisms are associated with the development of resistance to the modulation of 5-FU by LV.

In this paper, we report the mechanisms of resistance to LV modulation of 5-FU in two modulation-resistance HCT-8 sublines selected with IC_{50} concentrations of 5-FU for growth inhibition in the presence of 20 μM LV.

MATERIALS AND METHODS

Chemicals

5-FU, 5-FdUrd, dUrd, LV, H_4PteGlu and SRB were purchased from the Sigma Chemical Co. (St. Louis, MO).

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† *Abbreviations:* 5,10-CH_2-H_4PteGlu$_n$, 5,10-methylenetetrahydrofolate polyglutamates; 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$, combined pool of 5,10-methylenetetrahydrofolate and tetrahydrofolate polyglutamates; dUrd, 2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine monophosphate; 5-FdUrd, 5-fluoro-2′-deoxyuridine; 5-FU, 5-fluorouracil; LV, (6*R*,*S*)-5-formyltetrahydrofolate (leucovorin); IC_{50}, drug concentration required for 50% inhibition of cell growth; SRB, sulforhodamine B; TS, thymidylate synthase; and TMDES, 20 mM Tris–HCl, pH 7.5, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EDTA, 250 mM sucrose.

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[6-^{3}H]5-FU (16.7 Ci/mmol), (6S)-[3′,5′,7,9-^{3}H]LV (28 Ci/mmol), [5-^{3}H]dUMP (22 Ci/mmol), [6-^{3}H]FdUMP (24 Ci/mmol), and [5-^{3}H]dUrd (22 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Purified *Lactobacillus casei* TS was obtained from Dr. Priest (Medical University of South Carolina, Charleston, SC). (6S)-$H_4PteGlu_5$ was synthesized using the method described by Matthews *et al.* [14]. Sephadex G-25 fine was purchased from Pharmacia Biotechnology Inc. (Piscataway, NJ).

Cell Culture

Mycoplasma-free HCT-8 (ATCC CCL 244) human ileocecal adenocarcinoma cells were maintained in monolayer culture in RPMI 1640 medium containing 10% dialyzed horse serum and 1 mM sodium pyruvate. Cell growth inhibition was measured by the SRB assay as described [15]. In brief, cells were plated in 96-well plates at a density of 800 cells/well in 50 μL of medium, and drug treatment of cells was initiated 24 hr after plating. Growth inhibition was measured at 72 hr after the initiation of drug treatment. For enzyme assays in cell extracts, exponentially growing cells were harvested by trypsin treatment, washed twice with PBS, and frozen as cell pellets at −70°.

Development of HCT-8 Sublines Resistant to LV Modulation of 5-FU-Induced Growth Inhibition

HCT-8 cells (5×10^5) were seeded in 75-cm^2 flasks with 20 mL medium, and 24 hr after seeding, the cells were treated with either 160 μM 5-FU for 2 hr plus 20 μM LV for 72 hr or 4 μM 5-FU plus 20 μM LV for 72 hr. After the exposure, treated cells were rinsed twice with drug-free medium and allowed to grow until surviving cells reached confluence. This treatment was repeated 10 times, resulting in two HCT-8 sublines with resistance to growth inhibition by 5-FU/LV combinations. The above established FL2h and FL72h sublines were maintained in drug-free medium; resistance in each subline was stable for >40 passages (≈150 generations). The doubling time of HCT-8, FL2h, and FL72h was 18–20 hr.

LV Uptake and Accumulation

For uptake studies, cells were plated at 2×10^5 cells/well into 6-well plates in folate-free medium with 10% dialyzed horse serum at 37° for 24 hr. The medium was aspirated, and uptake was initiated by adding 0.5 mL of prewarmed (37°) folate-free RPMI 1640 medium containing 2 μM (6R,S)-LV and (6S)-[^{3}H]LV at 0.5 μCi/mL (18 nM). After incubation at 37° for various times (1–120 min), uptake was stopped by putting the plate on ice and washing the cells quickly with 5 mL of ice-cold PBS (pH 7.4). To remove cell surface bound [^{3}H]LV, cells were rinsed further with 5 mL of ice-cold saline, pH 3.0 (adjusted with acetic acid), for 1 min on ice and washed once with PBS. Cells in each well were solubilized with 0.25 mL of 1 N NaOH for 1 hr and neutralized with 0.25 mL of 1 N HCl. The cell lysates were transferred to 7-mL scintillation tubes and mixed with 5 mL of scintillation fluid. Radioactivity was quantitated in a Beckman LS 1701 scintillation counter. Cell number/well was determined by counting trypsinized cells in three representative wells with a ZBI Coulter counter. Non-specific uptake of [^{3}H]LV was determined by adding 1000-fold excess nonradiolabeled LV (2 mM) in the uptake medium at each time point and was subtracted from the total uptake.

5,10-$CH_2H_4PteGlu_n$/$H_4PteGlu_n$ Pool Size and Polyglutamate Chain Length Distribution

The method is based on the stoichiometric formation of a covalent ternary complex between 5,10-CH_2-$H_4PteGlu_n$, [6-^{3}H]FdUMP, and *L. casei* TS as described [16].

TS Assays

TS catalytic activity and FdUMP binding sites in cell extracts were measured as described by Zhang *et al.* [17]. TS activity *in situ* was measured by the release of [^{3}H]H_2O from [5-^{3}H]dUMP (anabolized from [5-^{3}H]dUrd added to the growth medium) as described by Yalowich and Kalman [18] with modifications as described before [19]. Inhibition of *in situ* TS activity by 5-FU and 5-FdUrd treatments in the absence and presence of LV was studied. Cells (2×10^5) were plated in a 25-cm^2 flask in 10 mL medium and allowed to attach for 24 hr. The cells were then treated for 2 hr with 200 μM 5-FU or 0.1 μM 5-FdUrd in the absence or continuous presence of 5, 20, or 80 μM LV. TS activity *in situ* was assayed 24 hr after drug removal.

Stability of FdUMP–TS–CH_2-$H_4PteGlu_n$ Ternary Complex

The method was adapted from Houghton *et al.* [20]. Cellular TS was extracted with TMDES buffer and filtered through Sephadex G-25 columns to remove endogenous nucleotides and free folates. A ternary complex in 900 μL was allowed to form between TS in filtered cell extracts, [6-^{3}H]FdUMP (0.1 μM), and (6R)-5,10-CH_2-$H_4PteGlu_5$ (0.5 μM) at 37° for 60 min. Aliquots (200 μL each) of the ternary complexes were then diluted with an equal volume of TMDES buffer (pre-warmed to 37°) containing 200 μM non-radiolabeled FdUMP and various concentrations of (6R)-5,10-CH_2-$H_4PteGlu_5$, giving final concentrations of (6R)-5,10-CH_2-$H_4PteGlu_5$ at 0.25, 0.5, 1, or 2 μM and non-radiolabeled FdUMP at 100 μM. After incubation at 37° for 0, 15, 30, 45, 60, and 75 min, 50 μL of the diluted mixture was removed, mixed with 10 μL of 6% SDS, and boiled immediately for 3 min to stabilize the ternary complexes. Ternary complexes were separated from free [6-^{3}H]FdUMP by centrifuging 25 μL of the boiled sample through a Sephadex G-25 mini-column (400 μL). Radioactivity in the filtrate was quantitated by scintillation

counting. Dissociation half-life ($T_{1/2}$) of the TS complex was calculated by the linear regression of data plotted as the percentage of ternary complex (logarithm) versus dissociation time, as described previously [9, 21].

Quantitation of TS Complex Formation in Intact Cells

The amount of intracellular TS complex in cells treated with 5-FU (2 hr) ± LV was quantitated at 24 hr after drug removal by immunoblot following nondenaturing PAGE. Human TS-specific rabbit antiserum was a gift from Dr. Edward Chu of the National Cancer Institute. Cell pellets (2×10^6 cells each) were extracted with 150 μL of TMDES buffer by freezing in dry ice/ethanol and thawing in a 37° water bath. Cell lysates were centrifuged at 8800 *g* for 10 min at 4°. An equal volume (90 μL) of supernatant of each sample was mixed with 30 μL loading buffer [60% glycerol, 0.04% (w/v) bromophenol blue, and 0.18 M Tris-HCl, pH 6.8]. Samples were resolved in nondenaturing discontinuous polyacrylamide slab gels (9% polyacrylamide for separating gel and 4.5% polyacrylamide for stacking gel) at 5 W for 18 hr in running buffer (0.025 M Tris, 0.192 M glycine, pH 8.3), the same condition described previously for analysis of 5,10-CH_2-H_4PteGlu$_n$ polyglutamate chain distribution in TS ternary complexes [16, 19]. Resolved proteins in the gel were electrophoretically transferred to nitrocellulose membranes in transfer buffer [0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, and 20% methanol] at 4° for 3 hr. The membranes were incubated in blocking buffer [PBS containing 5% (w/v) nonfat milk, 0.05% (v/v) Tween 20, and 0.1% thimerosal] at 37° for 1 hr or at 4° overnight. After washes in PBS–Tween 20 (0.01% Tween 20), the nitrocellulose blots were incubated in rabbit anti-human TS antiserum (1:3000 dilution in blocking buffer) for 1 hr at room temperature. After washes, the blots were then immersed in goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) for 1 hr at room temperature. After reaction with chemiluminol reagent (DuPont), the blots were exposed to X-ray films (DuPont) for 1–5 min. Relative levels of TS complex were quantitated by densitometric scanning.

Statistical Analysis

To analyze the statistical significance of cell growth inhibition and *in situ* TS activity inhibition in the presence of LV, four types of experiments were compared: 5-FU vs 5-FU/LV and 5-FdUrd vs 5-FdUrd/LV for inhibition of cell growth and TS activity, respectively. The *t*-test for means of paired samples was performed; for the data of IC_{50} in cell growth inhibition, $\log_{10}$ transformation was made to ensure normal distribution of data.

RESULTS

Modulation of 5-FU and 5-FdUrd Growth Inhibition by LV in Modulation-Resistant HCT-8 Sublines

The data in Table 1 indicate that a low but significant degree of modulation of 5-FU-induced growth inhibition by LV (2-fold) can be achieved in parental HCT-8, but the FL2h and FL72h sublines lost the susceptibility to modulation by LV during either 2- or 72-hr exposure to 5-FU. The loss of potentiation of 5-FU-induced growth inhibition by LV in FL2h and FL72h sublines is referred to as modulation resistance. Growth inhibition by 5-FdUrd in parental HCT-8 was potentiated in the continuous presence of LV by 5.7-fold for the 2-hr drug exposure and 2.6-fold for the 72-hr drug exposure (Table 2). In contrast, in FL2h, no potentiation of 5-FdUrd growth inhibition by LV was observed in either the 2- or the 72-hr drug exposure. The growth inhibition of 5-FdUrd in FL72h was modulated with LV by 3- and 2-fold in the 2- and the 72-hr drug exposure, respectively. When exposed to 5-FdUrd alone, FL2h was cross-resistant to the 72-hr exposure (2-fold), and FL72h was cross-resistant to both the 2-h exposure and the 72-hr exposure (5- and 6-fold, respectively).

LV Uptake and Intracellular Metabolism

The initial rates of uptake of 2 μM LV into HCT-8, FL2h, and FL72h were similar: 4.7 ± 0.3, 4.8 ± 0.7, and 4.9 ± 0.5 pmol/min/10^7 cells (N = 2), respectively (data not shown). LV accumulation at 120 min was 149, 163, and 138 pmol/

TABLE 1. Modulation of 5-FU-induced growth inhibition by LV

Cell line	IC_{50}* (μM)					
	5-FU, 2 hr	5-FU, 2 hr + LV	MF†	5-FU, 72 hr	5-FU, 72 hr + LV	MF†
HCT-8	238 ± 13 (1)‡	125 ± 6 (1)	2	5.4 ± 0.13 (1)	2.6 ± 0.13 (1)	2.0
FL2h	255 ± 15 (1.1)	247 ± 9 (2.0)§	1.1	6.5 ± 0.30 (1.2)	6.2 ± 0.24 (2.4)§	1.0
FL72h	320 ± 17 (1.3)	306 ± 12 (2.5)§	1.1	7.9 ± 0.26 (1.5)§	7.5 ± 0.18 (2.9)§	1.0

* IC_{50}: drug concentration required for 50% inhibition of cell growth; values are means ± SEM of 7 experiments, each with 6 replicates.

† MF; modulation factor by LV (20 μM, continuous exposure).

‡ Number in parentheses represents degree of drug resistance relative to HCT-8 cells.

§ Significant drug resistance relative to HCT-8 cells ($P < 0.01$).

TABLE 2. Modulation of 5-FdUrd-induced growth inhibition by LV

Cell line	IC_{50}* (nM) 5-FdUrd 2 hr	5-FdUrd 2 hr + LV	MF†	5-FdUrd 72 hr	5-FdUrd 72 hr + LV	MF†
HCT-8	80 ± 10 (1)‡	14 ± 2 (1)	5.7	1.8 ± 0.2 (1)	0.7 ± 0.3 (1)	2.6
FL2h	100 ± 10 (1.3)	90 ± 10 (6.4)§	1.1	3.4 ± 0.4 (1.9)§	5.4 ± 0.8 (7.7)§	0.63
FL72h	400 ± 40 (5.0)§	130 ± 10 (9.3)§	3.0	11 ± 1 (6)§	5.4 ± 0.7 (7.7)§	2

* IC_{50}: means ± SEM of 4 experiments, each with 6 replicates.
† MF; modulation factor by LV (20 μM, continuous exposure).
‡ Number in parentheses represents degree of drug resistance relative to HCT-8 cells.
§ Significant drug resistance relative to HCT-8 cells ($P < 0.01$).

10^7 cells in HCT-8, FL2h, and FL72h, respectively. To study the effect of LV on expanding the intracellular 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ pool, cells were cultured in medium containing 2.3 μM folic acid ± 20μM LV for 24 hr and then the combined 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ pools were measured in cell extracts. As shown in Table 3, 24-hr exposure to LV expanded the 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ pool to a similar level in all three cell lines.

Effect of LV on 5,10-CH_2-H_4PteGlu$_n$ Polyglutamate Chain Length Distribution and Intracellular TS Complex Formation

The distribution of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ polyglutamate chain lengths was measured in HCT-8, FL2h, and FL72h cells (Fig. 1A). In culture medium containing 2.3 μM folic acid, the predominant polyglutamates of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ contained five or six glutamate residues in all three cell lines. After exposure to 20 μM LV for 24 hr, a down-shift of chain lengths of polyglutamates was observed, with predominant polyglutamates of three and four glutamate residues. However, with or without addition of 20 μM LV to the culture medium, there was no change in the distribution of polyglutamate chain length of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ in FL2h and FL72h sublines as compared with that in parental HCT-8 cells as quantitated by densitometry (data not shown).

The effect of LV on the formation of intracellular TS complexes after 5-FU treatment was analyzed by immunoblot after resolution on nondenaturing PAGE (Fig. 1B). Free TS was the slowest in migration through the nondenaturing gel, as evidenced by the bands in untreated controls (Fig. 1B; lanes 1–3). After treatment with 5-FU alone (Fig. 1B; lanes 4–6) or 5-FU in combination with LV (Fig. 1B; lanes 7–9), the more rapid migration of the intracellular TS complexes from the two modulation-resistant sublines was similar to that from HCT-8 cells. The addition of 20 μM LV to 5-FU treatment decreased the electrophoretic migration of the intracellular TS complexes from all three cell lines, relative to 5-FU treatment alone. After treatment with 5-FU ± LV, free TS was decreased but total TS was increased in all three cell lines, predominantly in the form of TS complexes (compare intensity of signal in lanes 4–9 to that in 1–3). However, when the relative amount of TS ternary complexes formed in cells treated with 5-FU/LV was compared with that in cells treated with 5-FU alone, the presence of 20 μM LV in culture medium did not increase the formation of TS ternary complex in FL2h and FL72h sublines whereas LV increased the formation of TS complexes by 2-fold in parental HCT-8 cells (Fig. 1C).

TABLE 3. Combined pools of 5,10-CH_2H_4PteGlu$_n$/H_4PteGlu$_n$ in HCT-8 and its 5-FU/LV modulation-resistant sublines

Cell line	5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ (pmol/mg) Medium*	+20 μM LV†
HCT-8	53 ± 16‡	120 ± 32
FL2h	66 ± 20	122 ± 26
FL72h	47 ± 12	128 ± 20

* Cells were cultured in RPMI 1640 medium containing 2.3 μM folic acid.
† LV was present in the culture medium for 24 hr.
‡ Data represent means ± SD of 3 experiments.

TS Level and Activity in HCT-8 Sublines

FdUMP binding sites and TS catalytic activity were measured in modulation-resistant FL2h and FL72h and in parental HCT-8 cell extracts. The FdUMP binding sites, a measure of TS protein level, were 1.97 ± 0.17, 2.34 ± 0.35, and 1.95 ± 0.24 pmol/mg protein (mean ± SD) in HCT-8, FL2h, and FL72h cells, respectively. Both modulation-resistant sublines showed about 20% lower TS activity in cell extracts compared with parental HCT-8 cells; TS specific activities were 160 ± 32 pmol/min/mg in FL2h and 176 ± 45 pmol/min/mg in FL72h vs 216 ± 29 pmol/min/mg in parental HCT-8 cells.

Inhibition of TS Activity In Situ by 5-FU or 5-FdUrd in the Absence or Presence of LV

To study the effect of LV on the inhibition of TS activity *in situ* by 5-FU and 5-FdUrd, cells were treated for 2 hr with

FIG. 1. Effect of 20 μM LV on 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ polyglutamate chain length distribution (Fig. 1A) and on the formation of intracellular TS complexes at 24 hr after a 2-hr treatment with 200 μM 5-FU (Fig. 1, B and C) in HCT-8, FL2h, and FL72h cell lines. In Fig. 1A, cells were cultured in medium containing 2.3 μM folic acid (3 lanes on the left) or in medium with addition of 20 μM LV for 24 hr (3 lanes on the right), and the polyglutamate chain length distribution of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ was analyzed using nondenaturing PAGE as described in Materials and Methods. In Fig. 1B, free and complex TS in extracts of 1.1 × 10^6 cells were analyzed after nondenaturing PAGE by immunoblot using rabbit antiserum specific to human TS. Lanes 1, 2, and 3 (HCT-8, FL2h, and FL72h, respectively) are untreated controls; lanes 4, 5, and 6 (HCT-8, FL2h, and FL72h, respectively) are samples treated with 200 μM 5-FU for 2 hr and then cultured in drug-free medium for 24 hr; lanes 7, 8, and 9 (HCT-8, FL2h, and FL72h, respectively) are samples treated with 200 μM 5-FU + 20 μM LV for 2 hr and then cultured in the presence of 20 μM LV for 24 hr. Figure 1C shows the relative densitometric quantitation of intracellular TS complexes represented by Fig. 1B. For each separate experiment, the amount of intracellular TS complexes in HCT-8 cells treated with 5-FU alone was arbitrarily set to 1 unit. Data represent mean ± SD of three separate experiments.

200 μM 5-FU or 0.1 μM 5-FdUrd in the presence of 0, 5, 20, and 80 μM LV and then cultured in drug-free medium in the presence of 0, 5, 20, and 80 μM LV. At 24 hr after drug removal, TS activity *in situ* was measured (Fig. 2). Addition of 5–80 μM LV in cell culture significantly potentiated TS inhibition *in situ* in parental HCT-8 cells relative to 5-FU alone (Fig. 2A) or 5-FdUrd alone (Fig. 2B) ($P < 0.01$), but not in FL2h and FL72h cells ($P > 0.1$).

Stability of TS Ternary Complex

The effect of concentrations of exogenous (6*R*)-5,10-CH_2-H_4PteGlu$_5$ on the *in vitro* stability of ternary complex formed with [^{3}H]FdUMP and TS isolated from untreated cells of each cell line was studied (Fig. 3). When (6*R*)-5,10-CH_2-H_4PteGlu$_5$ was used as the folate cofactor, the [^{3}H]FdUMP ternary complexes formed with TS from both FL2h and FL72h sublines showed shorter dissociation half-lives than that with TS from parental HCT-8 cells. With increasing concentrations of (6*R*)-5,10-CH_2-H_4PteGlu$_5$ (0.25 to 2 μM), the increases in dissociation half-life of TS–FdUMP-CH_2-H_4PteGlu$_5$ complexes formed by TS from FL2h and FL72h sublines were very small (from 40 to 61 min and from 36 to 62 min, respectively) compared with the increases (from 69 to 238 min) in dissociation half-life of the complex formed by TS from parental HCT-8 cells.

DISCUSSION

In this report, we studied the mechanisms of acquired resistance to the modulation of 5-FU-induced growth inhibition by LV in human HCT-8 ileocecal adenocarcinoma sublines. The phenomenon of modulation resistance was demonstrated by growth inhibition assays in FL2h and FL72h sublines, in which the addition of 20 μM LV to culture medium did not potentiate the growth inhibitory potency of 5-FU (Table 1). Measurements of *in situ* TS activity were consistent with the growth inhibition studies in that the addition of 5–80 μM LV to either 5-FU or 5-FdUrd potentiated the inhibition of TS activity in HCT-8 but not in FL2h and FL72h cells (Fig. 2). However, while TS inhibition *in situ* was not modulated by LV at 24 hr after drug removal (Fig. 2), cell growth inhibition by 5-FdUrd was partially modulated by LV in the FL72h subline (Table 2). This apparent inconsistency may result from measuring *in situ* TS inhibition and growth inhibition at different times.

Using TS-specific immunoblots following nondenaturing gel electrophoresis (Fig. 1B), we were able to demonstrate free TS as well as intracellular TS complexes that migrate further than free TS and have a wide range of mobility in nondenaturing PAGE. The identification of the high mobility species containing TS as ternary complexes is based on two observations. First, these species only appear in 5-FU-treated cells. Second, the addition of 20 μM LV to 5-FU treatment decreased the migration of TS complexes in the gel; this is consistent with the downshift in 5,10-

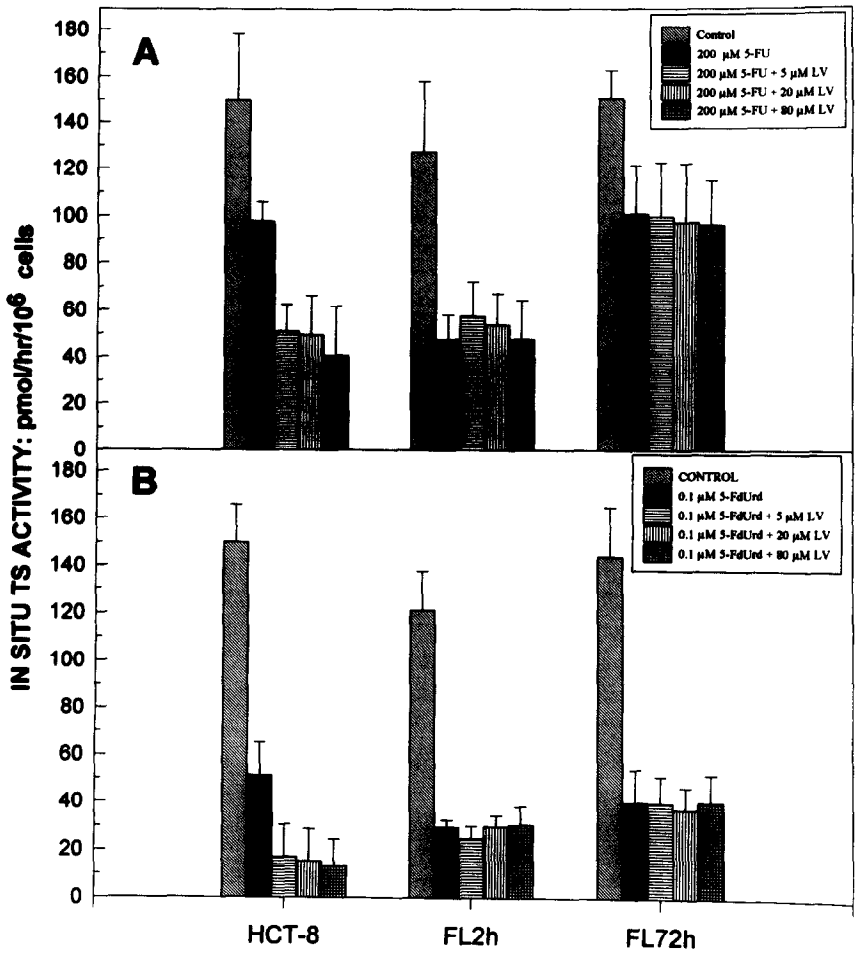

FIG. 2. Inhibition of TS activity *in situ* in HCT-8, FL2h, and FL72h cells treated with 200 μM 5-FU (Fig. 2A) or with 0.1 μM 5-FdUrd (Fig. 2B), with or without the addition of 5, 20, or 80 μM LV. Exponentially growing cells were treated with 5-FU or 5-FdUrd for 2 hr and then allowed to grow for an additional 24 hr in drug-free medium. TS activity *in situ* was assessed as described in Materials and Methods. Data represent an average ± SD of three separate experiments.

CH_2-H_4PteGlu$_n$ polyglutamate chain length observed following LV treatment (Fig. 1A), since the migration of TS ternary complexes in nondenaturing PAGE is directly dependent on the polyglutamate chain length [22]. Direct evidence for a lack of significant potentiation of ternary complex formation by LV in 5-FU-treated FL2h and FL72h sublines was demonstrated by this assay (Fig. 1 B and C), while addition of 20 μM LV to 5-FU increased the formation of intracellular ternary complex by 2-fold relative to 5-FU alone in the parental HCT-8 cell line. The appearance of more species of TS complexes (Fig. 1B) than species of 5,10-CH_2-H_4PteGlu$_n$ in each cell line (Fig. 1A) indicates that the intracellular TS ternary complexes after treatment with 5-FU ± LV are probably a mixture of monomeric (TS:FdUMP:CH_2-H_4PteGlu$_n$ = 1:1:1) and dimeric (TS:FdUMP:CH_2-H_4PteGlu$_n$ = 1:2:2) complexes [22]. Furthermore, an increase in total TS was observed in 5-FU ± LV-treated cells compared with untreated cells in all three cell lines, but the extent (>10-fold) of increase in total TS in HCT-8 cells induced by 5-FU treatment was higher than that (around 3-fold) reported in other cell lines [23].

Innate resistance to LV modulation of 5-FU cytotoxicity has been described in cultured cell lines [24, 25], but the mechanism of modulation resistance is unknown. Lack of LV modulation of 5-FU antitumor activity has also been observed in clinical trials of patients pretreated with 5-FU [26, 27] and in an *in vivo* study of human tumor xenografts [28]. Lack of folate pool expansion and poor polyglutamylation may be involved in loss of LV modulation of fluoropyrimidines [11, 12, 28]. In the present study, the HCT-8 cell line and the two modulation-resistant sublines showed similar rates of LV uptake, similar expansion of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ pools, and similar polyglutamate chain length distribution of 5,10-CH_2-H_4PteGlu$_n$ after LV treatment. We therefore exclude the involvement of LV uptake, LV metabolism, and polyglutamylation in the 5-FU/LV modulation resistance in FL2h and FL72h sublines.

Several other conditions may cause the lack of modulation of 5-FU by LV: (a) thymidine salvage sufficient to abrogate the effects of TS inhibition on cell growth; (b) insufficient intracellular FdUMP formation, rendering potentiation of TS inhibition unachievable even in the presence of sufficient folate cofactor; (c) poor binding affinity of TS for FdUMP and/or 5,10-CH_2-H_4PteGlu$_n$. Indirect evidence from studies in our laboratory and others [29, 30] indicates that thymidine salvage from non-dialyzed serum may render LV modulation of 5-FU ineffective. When the HCT-8 cell line was cultured in medium containing non-dialyzed horse serum, no modulation of 5-FU-induced growth inhibition by LV could be achieved, and the addition of thymidine did not protect cells from 5-FU cytotox-

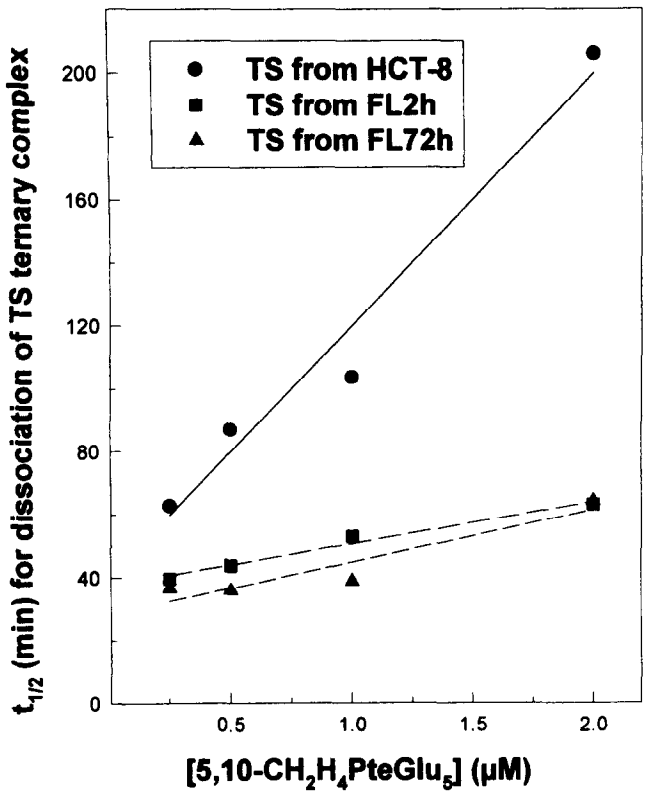

FIG. 3. Relationship between dissociation half-life ($T_{1/2}$) of TS–[^{3}H]FdUMP–CH_2-H_4PteGlu$_5$ complex and exogenous concentration of (6*R*)-5,10-CH_2-H_4PteGlu$_5$. The formation and dissociation of TS ternary complex and the calculation of dissociation $T_{1/2}$ are described in detail in Materials and Methods. The $T_{1/2}$ values are replotted versus concentrations of (6*R*)-5,10-CH_2-H_4PteGlu$_5$; each line is the linear regression of the data as shown above.

icity [29, 30]. In the present study, a low (2-fold) but significant enhancement of 5-FU cytotoxicity by LV was achieved in the HCT-8 cell line when it was cultured in dialyzed horse serum.

An alteration of 5-FU metabolism that abrogates TS as a target (e.g. poor FdUMP formation) might also negate LV modulation of 5-FU. Since inhibition of *in situ* TS activity by 5-FU alone was similarly achievable in parental HCT-8 and the two modulation-resistant sublines, poor FdUMP formation appears to be ruled out as the mechanism of 5-FU/LV modulation resistance in FL2h and FL72h sublines. No further analysis of 5-FU metabolism enzymes was performed. There was no change in the incorporation of 5-FU into DNA or total cellular RNA (data not shown) in the two modulation-resistant sublines as compared with the parental HCT-8 cells.

Another possible mechanism of 5-FU/LV modulation resistance is a quantitative or qualitative change in TS. HCT-8 cells and the modulation-resistant FL2h and FL72h sublines showed similar TS protein levels (based on [^{3}H]FdUMP binding sites) and *in situ* TS activities. Analysis of ternary complex stability revealed that the TS–FdUMP–CH_2-H_4PteGlu$_5$ complexes formed in the cell-free system by TS isolated from FL2h and FL72h sublines had significantly shorter half-lives in the presence of (6*R*)-5,10-CH_2-H_4PteGlu$_5$ than the complexes formed by TS isolated from HCT-8 cells (Fig. 3). Diminished half-life of the TS–FdUMP–CH_2-H_4PteGlu$_1$ complex and lower binding affinity of TS for nucleotides have been observed in a 5-FdUrd-resistant subline of CCRF-CEM human leukemic cells [31].

The development of acquired resistance to LV modulation of 5-FU may have important clinical implications. In the clinical treatment of colorectal cancer, the combination of 5-FU/LV was most successful in patients without prior chemotherapy [4–7], while patients resistant to 5-FU treatment did not respond to 5-FU/LV [26, 27]. Theoretically, tumor cells resistant to 5-FU through the mechanism of poor binding of TS to FdUMP and the folate cofactor may be selected by repeated clinical treatments, and these cells would also be resistant to the modulation of 5-FU by LV. The reduced binding affinity of TS to FdUMP and the folate cofactor could be caused by a point mutation in the TS gene, as exemplified in studies by Berger and Barbour, that showed poor binding affinity for both FdUMP and 5,10-CH_2-H_4PteGlu$_1$ in a naturally occurring TS variant from the HCT 116 cell line with a single amino acid substitution [32, 33]. The probable mutations of TS gene in FL2h and FL72h sublines are under study.

In conclusion, these studies demonstrated that acquired resistance to LV modulation of 5-FU in FL2h and FL72h sublines is associated with shorter half-lives of TS-FdUMP–CH_2-H_4PteGlu$_n$ complexes *in vitro*. This shorter half-life of ternary complex may be the direct cause for the lack of potentiation of intracellular complex formation and for the lack of potentiation of inhibition of *in situ* TS activity by LV after 5-FU treatment, even though the presence of 20 μM LV increased the intracellular combined pool of 5,10-CH_2-H_4PteGlu$_n$/H_4PteGlu$_n$ by 2- to 3-fold.

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