ABSTRACT

Mechanisms of resistance to 5-fluorouracil (FUra) were compared between a cell line resistant to a short-term exposure (4 h) to this agent (HCT-8/FU4hR) and a cell line resistant to a prolonged exposure (7 days) to the fluoropyrimidine (HCT-8/FU7dR). The two cell lines were obtained by repeatedly exposing $10^9$ cells to a constant concentration of FUra (100 $\mu$M for 4 h or 15 $\mu$M for 7 days), able to produce 3–4 logs of cell kill. HCT-8/FU4hR cells were still sensitive to FUra given as a 7-day exposure, suggesting different mechanisms of resistance. In addition, HCT-8/FU7dR cells were cross-resistant to fluorodeoxyuridine and, to a lesser degree, methotrexate; while HCT-8/FU4hR cells were not. Both HCT-8/FU4hR and HCT-8/FU7dR cells were similar to parental HCT-8 cells with regard to uptake of FUra as well as the pattern of FUra-metabolizing and FUra target enzymes. Although neither in situ thymidylate synthase (TS) activity nor the degree of its inhibition by FUra showed any evidence of alteration in HCT-8/FU7dR cells, a rapid recovery of TS activity after drug removal was evident in this cell line. The addition of as much as 100 $\mu$M leucovorin did not completely inhibit the recovery of thymidylate synthase after FUra exposure. No differences were detected in the kinetic properties ($K_m$ for 2'-deoxyuridylate and 5,10-methylene tetrahydrofolate, concentration producing 50% inhibition for fluorodeoxyuridylate) or TS from HCT-8/FU7dR cells as compared to parental HCT-8 TS. Baseline levels of 5,10-methylene tetrahydrofolate were decreased in HCT-8/FU7dR cells, and analysis of the chain-length distribution of the polyglutamylated form of the folate cofactor showed that in this cell line the defect in 5,10-methylene tetrahydrofolate levels is accompanied by, and possibly due to, a defect in the polyglutamyltransferase of this cofactor. In contrast, HCT-8/FU4hR cells were similar to the parental cell line with regard to both the degree of in situ TS inhibition by FUra and duration of inhibition after FUra removal. Labeling studies with $[^{3}H]$FUra (4 h exposure, 100 $\mu$M) showed that the incorporation of the fluorouracil into RNA is significantly decreased in HCT-8/FU4hR cells as compared to parental HCT-8 cells. The mechanisms of resistance found in these cell lines indicate that the mechanism of cell kill by FUra differs depending on the dose schedule used: short-term exposure to high concentrations of FUra kills cells by an RNA effect, while prolonged exposure to low doses is cytotoxic via inhibition of thymidylate synthase and, consequently, DNA synthesis.

INTRODUCTION

FUra* has been used for 3 decades for the treatment of human solid tumors (1). Of particular note is its role in the treatment of colorectal cancer, in which this drug has been used either alone or in combination with modulating noncytotoxic agents, e.g., LV, leucovorin, rather than being included in polychemotherapeutic regimens (2–5). This disease, therefore, provides a unique opportunity to study FUra sensitivity and resistance.

In the last several years, the addition of modulating agents to FUra have improved the outcome of treatment of colon cancer [both in the advanced stage (4) and in the adjuvant setting (5)]. However, an objective clinical response is obtained in <40% of patients with advanced disease, and these responses are inevitably followed by the development of resistance (4). The outcome of FUra therapy may be improved if the biochemical (and molecular) basis for FUra resistance in tumors is identified and strategies are designed to overcome the development of resistance. The mechanisms of resistance to FUra operating in the clinical setting may be complex. FUra metabolism may involve different activation pathways (orotate phosphoribosyltransferase, thymidine phosphorylase and thymidine kinase, uridine phosphorylase and uridine kinase) and at least 3 cellular targets [thymidylate synthetase, RNA, DNA (1)]. Indeed, defective drug uptake and alterations in every step of FUra metabolism as well as mutations or increased levels of the target enzyme TS have been described in FUra-resistant experimental tumors (1, 6–15).

Both experimental and clinical data suggest that pulse FUra and continuous infusion FUra might have different mechanisms of cytoxicity. In the clinic, the maximum tolerated dose and dose-limiting toxicity of FUra depend upon the schedule of administration (16). Moreover, the slopes of the dose-response curve to FUra, both in experimental systems (17, 18) and in the clinic (19), are different depending on the schedule of drug administration. Also, from a theoretical point of view, the S-phase specificity of one mode of FUra cytotoxicity, i.e., TS inhibition, compared to the less tight dependence on cell cycle phase of the other mechanism of FUra action, i.e., FUra incorporation into RNA, is consistent with different mechanisms of action (and resistance), depending on the duration of exposure to this drug. In general, this complexity has not been taken into account in the previous studies of FUra resistance. Most investigators have studied resistant clones developed either by increasing the concentration of drug stepwise or by a single exposure to high doses with or without previous exposure to chemical mutagens. Such approaches usually lead to development of highly resistant clones but involve many disadvantages. The multistep procedure often produces unstable phenotypes, thus requiring the continuous presence of the drug for maintenance of resistance. Moreover, this procedure, as well as the one-step selection method, does not resemble the clinical situation in that patients receive repeated courses of the same dose of chemotherapy and resistance would be expected to be low grade (20). Therefore, the mechanisms of resistance operating under conditions of low (constant) selection pressure (as in the...
SCHEDULE-DEPENDENT MECHANISM OF RESISTANCE TO FUra

clinics may be different from the mechanisms of resistance described previously, and the relevance of some of these observations to the clinical use of FUra is probably limited. Also, while LV is now widely used in combination with FUra for the treatment of advanced colorectal cancer (4, 21, 22), only recently has it its intracellular metabolism and the intracellular pools of its main (to FUra therapy) derivative, CH₂FH₄, been correlated with sensitivity or resistance to fluoropyrimidines (23). Moreover, despite the current controversies about the optimal schedule of FUra administration (1), no attempts have been made to investigate the correlation between the schedule of FUra administration and the mechanism(s) of resistance.

In this paper, two different sublines resistant to FUra are described. Both were selected from a clone of wild-type HCT-8 cells by repeated exposures to a constant concentration of FUra either administered for 4 h (high dose) or for 7 days (low dose) to mimic the dose schedules used in the clinic (pulse versus continuous infusion). The two resistant cell lines obtained displayed different mechanisms of FUra resistance. The pulse FUra selection led to a decreased incorporation of this analogue into RNA, while continuous exposure to the fluoropyrimidine resulted in a resistant subline that was found to recover from TS inhibition more rapidly than the parental line. In both cell lines, new mechanisms of FUra resistance were found: impaired polyglutamylation of the thymidylate synthase cofactor CH₂FH₄ in the 7-day resistant subline (HCT-8/7dR) and decreased incorporation of FUra into RNA in the high-dose pulse resistant subline (HCT/4hR).

MATERIALS AND METHODS

Chemicals. FUra andFdUrd were purchased from Sigma Chemical Co. (St. Louis, MO). MTX (dissolved in saline) and LV were obtained in clinical form from Lederle (Pearl River, NY). 2′-[5-H]deoxyuridine, 2′-[5-H]deoxyuridylate, [6-3H]FdUDP, [6-3H]FUra, [6-14C]FUra, [6-14C]fluorouridine, [6-14C]FdUrd, and [methyl-3H]thymidine were purchased from Moravek Biochemicals (Brea, CA). *Lactobacillus casei* thymidylate synthase (activated, neutralized) was supplied by Sigma. Glycine was obtained from Eastman Kodak Co. (Rochester, NY) and Tris (ultrapure) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Polyacrylamide and all other reagents for gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Pteroyl polyglutamate standards with 2–7 residues were obtained from Dr. B. Schirks Laboratories (Jona, Switzerland) and were reduced to the corresponding tetrahydrofolate forms with human recombinant dihydrofolate reductase and NADPH (24). En'Hance was from New England Nuclear (Boston, MA) and Kodak X-O-mat AR film was purchased from Eastman Kodak. Media and sera for cell culture were obtained from Grand Island Biological Co. (Grand Island, NY), and plasticware was obtained from Corning Glass Works (Corning, NY).

Development of Resistant Cell Lines. The human colon adenocarcinoma cell line HCT-8 (25) was grown as a monolayer as previously described (20). Under these conditions, the doubling time was 18 h and the cloning efficiency was about 30%. Resistant cell lines were selected with a low selection pressure technique as previously described for antifolates (20). Starting from a clone of the parental cell line, we obtained resistance to FUra with either 4-h or 7-day exposure to FUra, repeated 6 times using the same drug concentration from the beginning to the end of the selection period. Cells were passaged (2 × 10⁵ cells/flask) and rechallenged with drug as soon as the cultures approached 100% of confluence. The initial selection concentration was the highest that allowed regrowth of at least a few clones, starting from 2 × 10⁵ cells/25-cm² flask (approximately 4 logs of cell kill). A progressive reduction in the time (days) at which confluence of the cultures at each successive passage was reached allowed the development of resistance to be monitored. The resulting FUra-resistant sublines were designated HCT-8/FU4hR, for the one obtained with 4-h exposure to FUra, and HCT-8/FU7dR, for the one obtained with 7-day exposure to FUra, and were maintained as described for the parental cell line. These experiments were repeated 3 times with 4 replicates for each point.

Cytotoxicity Assays. In order to quantitate the degree of resistance to FUra and cross-resistance of the cell lines to other drugs, a monolayer clonogenic assay was used (26). Cultures were trypsinized for 3 min, and an essentially monolayer suspension was obtained by passing the trypsinized cells through a 25-gauge needle. The cloning efficiency of the resistant sublines was similar to that of the parental sensitive line; thus, 5–10 × 10⁵ cells in 5 ml of medium containing 10% horse serum were dispensed into sterile 60-mm Petri dishes and incubated at 37°C and 100% humidity with 5% CO₂. Eighteen h later, when the cells were attached to the bottom of the Petri dish but had not yet divided, 0.1 ml of an appropriate dilution of drug in a 0.9% NaCl solution was added to each dish. Control dishes received the same volume of saline. After incubation as indicated, the medium was decanted, the cells were washed twice with 5 ml of saline, and 5 ml of fresh medium was added. Clonal growth was determined after staining with orcein. Colonies containing >200 cells were scored at ×10 magnification using a dissecting microscope. Under these growth conditions, >95% of the colonies from both sensitive and resistant cells contained >200 cells. The sensitivity of both parental and resistant cells to the antineoplastic agents was determined after 4- and 7-day exposures to the drugs. Each experimental point was determined in triplicate with 4 replicates controls; experiments were repeated at least twice.

FUra Uptake. Exponentially growing cells (10⁵ cells/well) were cultured 18–24 h before carrying out the assay in 24-well plates. To determine uptake, a modification of a method previously described for MTX was used (27). Briefly, old medium was discarded, and 2 ml of prewarmed (37°C) complete tissue culture medium containing 100 μM [6-3H]FUra (specific activity, 25 cpm/pmol) was added. The plates were incubated at 37°C and, at appropriate time points (shown in Fig. 2), triplicate plates were cooled to 0°C and washed three times with 3 ml of ice-cold PBS. The cells were removed by incubation with 1 ml of 1 N NaOH at 37°C for 45 min. A 0.5-ml aliquot was neutralized with an equal volume of 1 N HCl, and after 15 ml of Ecolume scintillation fluid was added, the content of radioactivity was measured in a Beckman model 5801 scintillation counter. Triplclicates cultures were collected by 10-min trypsinization for all cell counts. All experiments were repeated at least twice.

Assay of Enzymes of Pyrimidine Metabolism. Enzyme activities were assayed as previously described using cytosols from logarithmically growing cells (26). Assays were performed at 2 enzyme concentrations and several time points to ensure that rates were derived from <25% substrate conversion, and the rate was linear for at least 2 time points (10 and 20 min). Activity is expressed as nmol of product formed/mg of protein/h. Thymidine kinase activity was also measured using a modification of the method of Taylor et al. (28). A reaction mixture consisting of 5 μM MgCl₂, 1.0 mM ATP, 2.0 μM [methyl-3H]thymidine (82.40 Ci/mmol), and 0.2 μM Tris-HCl (pH 7.6) in a final volume of 100 μl was incubated with enzyme at 37°C for 15 min. At the end of this period, 50 μl from each tube was spotted on DE81 filter discs (24 mm; Whatman), washed with 1 ml ammonium formate (4 changes), and rinsed with ethanol before the filters were dried in a microwave oven at high power for 2 min. Protein concentration was estimated by the Bio-Rad method (29).

TS Catalytic Assay. Cytosols for TS activity measurements and kinetics studies were prepared according to a different procedure, which produces a higher TS yield and enzyme stability. Exponentially growing cells in 225-cm² tissue culture flasks were cooled on ice for 5 min, washed twice with 20 ml of ice-cold PBS, detached by 5- to 10-min trypsinization, and followed by addition of 15 ml of complete tissue culture medium. Keeping the cells on ice and using ice-cold reagents at all steps, washed twice with 5 ml of saline, and 5 ml of fresh medium was added. Clonal growth was determined after staining with orcein. Colonies containing >200 cells were scored at ×10 magnification using a dissecting microscope. Under these growth conditions, >95% of the colonies from both sensitive and resistant cells contained >200 cells. The sensitivity of both parental and resistant cells to the antineoplastic agents was determined after 4- and 7-day exposures to the drugs. Each experimental point was determined in triplicate with 4 replicates controls; experiments were repeated at least twice.

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glycerol and 0.1% Triton X-100, 4 μl/ml pepstatin, and 1 μl/ml leupeptin) for further processing.

No detectable loss of TS activity resulted from storage at −70°C for up to 1 month. Cell disruption was obtained with six 20-s bursts using a Vibraloc sonicator (Sonic and Materials, Inc., Danbury, CT) equipped with a microtip. No intact cells could be seen by light microscopy. Cell homogenates were then clarified by centrifugation at 12,000 × g and 4°C for 30 min, and aliquots of the supernatants were immediately used as the enzyme source in the following assay. Quantitation of TS activity was based upon the release of tritium from [5-3H]dUMP as [3H]H2O (30). The assay was performed in a total volume of 50 μl containing 50 mM 2'-[5-3H]deoxyuridylate (specific activity, 0.1 Ci/μmol), 250 μM 5,10-methylene-tetrahydrofolate, and enzyme buffered with 50 mM Tris-HCl (pH 7.4), 5 mM formaldehyde, 50 mM NaF, 10 mM Na ascorbate, and 150 mM β-mercaptoethanol. After 5 min preincubation at 37°C, the reaction was started by the addition of 5 or 10 μl of cell extract and carried out at 37°C for 15 or 30 min before being stopped by addition of 200 μl of a charcoal suspension (100 mg/ml) in 2% TCA. The samples were vortexed, and the charcoal was removed immediately by centrifugation at 10,000 × g for 10 min. A 100-μl aliquot of the supernatant was then assayed for [3H]H2O radioactivity by counting in a Beckman model 5801 liquid scintillation spectrometer after adding 5 ml of Ecolume scintillation cocktail. All assays were performed at least 3 times, and each point in the assay was done in triplicate. Enzyme activity is expressed in nmol of tritium released/h/mg of protein. All enzyme assays were linear with respect to time and enzyme concentrations at the substrate concentrations used. In order to measure TS activity in samples of cells previously exposed to FUra, it was necessary to remove any FuUMP bound to TS prior to incubation with [5-3H]dUMP (31). This was accomplished by incubating 50-μl aliquots of the 12,000 × g supernatants with 50 μl of dissociation buffer containing 0.6 M ammonium bicarbonate (pH 8.0), 80 μM dUMP, 100 mM β-mercaptoethanol, 100 mM NaF, and 15 mM CMP for 4 h at 37°C. At the end of the dissociation procedure, dUMP, free FuUMP, and cellular reduced folates were removed by adding 100 μl of an ice-cold 10% charcoal suspension, followed by centrifugation at 15,000 × g for 20 min. Twenty-μl aliquots of the supernatants were then used as the enzyme source in the assay described above for total TS activity determination. Another set of reactions was carried out omitting the ternary complex dissociation by immediately adding 50 μl of dissociation buffer and 100 μl of the 10% charcoal suspension to the 12,000 × g extract. This allowed quantitation of TS activity remaining after FuUMP exposure (i.e., free TS).

Enzyme Kinetics and Inhibition Studies. Cytosols for kinetic studies were prepared as described for the TS catalytic assay, without any further purification. Some experiments were performed on desalted cytosols, but results were similar to those obtained when crude extracts were used as the enzyme source. Apparent Km values for both dUMP and CH2FH4 were determined by varying the concentration of either cofactor, at this concentration, also during exposure to FUra as well as during reincubation in drug-free medium following FUra removal. After background subtraction (i.e., medium without cells), the counts were fitted to a straight line by linear regression, and the percentage of inhibition was calculated by comparing the slope of treated cultures with that of controls (without drug). Slopes in all assays fitted the linear regression model with a correlation coefficient of at least 0.9.

Assay of Intracellular Follates. A modification of the radioenzymatic binding assay described by Bunnin et al. (35) was used for intracellular CH2FH4 and FH4 quantitation. This method is based upon the entrapment of endogenous reduced folates, after conversion to CH2FH4, by TS and [3H]FdUMP to form a stable ternary complex (36). The amount of [3H]FdUMP bound to the protein is a function of the available levels of CH2FH4. Cells were washed twice with ice-cold PBS, resuspended in drug-free medium, and incubated for another 8, 24, or 48 h. After this period, medium containing 2'-[5-3H]deoxyuridine was added, and the assay was carried out as described for inhibition studies. When effects of LV on stability of TS inhibition were investigated, cells were plated in complete medium containing 100 μM LV; cultures were provided with the folate cofactor, at this concentration, also during exposure to FUra as well as during reincubation in drug-free medium following FUra removal. After background subtraction (i.e., medium without cells), the counts were fitted to a straight line by linear regression, and the percentage of inhibition was calculated by comparing the slope of treated cultures with that of controls (without drug). Slopes in all assays fitted the linear regression model with a correlation coefficient of at least 0.9.

Analysis of the CH2FH4 and FH4 Glutamate Chain Length Distribution. Determination of the predominant glutamate chain length of CH2FH4 and FH4 was based upon the electrophoretic separation of ternary complexes formed by L. casei TS and [3H]FdUMP reacting with intracellular CH2FH4. Folate extraction and ternary complex formation were performed according to the method of Priest and Doig (37). Separation of ternary complexes with different chain lengths of the folate cofactor was then obtained by polyacrylamide gel electrophoresis. Samples were electrophoresed for 18 h at 4°C, 8 mA, on a 0.75-cm thick polyacrylamide gel (9% in the resolving gel; 4.5% in the stacking gel) using 0.025 M Tris-HCl and 0.192 M glycine (pH 8.5) as the running buffer. The gels were then sequentially soaked in 5% TCA (30 min, 4°C), Enhance (30 min, room temperature), and distilled water.

In Situ TS Activity. Inhibition of thymidylate synthase activity by FUra in intact cells was measured by the in situ assay of Yawlich and Kalman (32) as modified by Rodenhuis et al. (33) and Pizzorno et al. (34). The assay is based upon the release of tritium from 2'-[5-3H]deoxyuridine, because it is methylated by thymidylate synthase after cellular uptake and phosphorylation to deoxyuridylate. The cells were cultured in 24-well plates 24 h before carrying out the assay, at a density of 50 × 10⁶ cells/ml in complete tissue culture medium. Duplicate wells for each condition received 100 μl of an appropriate dilution of FUra. Controls received the same volume of PBS. After a 4-h period of incubation with drug, 1 ml of complete medium containing 2'-[5-3H]deoxyuridine was added to achieve a final concentration of 1 μCi/ml, and 100-μl samples were taken at 0, 30, 60, 90, and 120 min. These were added to 200 μl of a 10% activated charcoal suspension in 2% TCA, vortexed, and centrifuged for 5 min at 10,000 × g. One hundred μl of the supernatant was added to 5 ml of Ecolume scintillation cocktail and counted in a Beckman model 5801 liquid scintillation counter. In recovery experiments, at the end of the 4-h drug exposure, cells were washed twice with PBS, resuspended in drug-free medium, and incubated for another 8, 24, or 48 h. After this time period, medium containing 2'-[5-3H]deoxyuridine was added, and the assay was carried out as described for inhibition studies. When effects of LV on stability of TS inhibition were investigated, cells were plated in complete medium containing 100 μM LV; cultures were provided with the folate cofactor, at this concentration, also during exposure to FUra as well as during reincubation in drug-free medium following FUra removal. After background subtraction (i.e., medium without cells), the counts were fitted to a straight line by linear regression, and the percentage of inhibition was calculated by comparing the slope of treated cultures with that of controls (without drug). Slopes in all assays fitted the linear regression model with a correlation coefficient of at least 0.9.

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SCHEDULE-DEPENDENT MECHANISM OF RESISTANCE TO FUra

RESULTS

Development of Resistance

The concentrations of FUra used in the selection of resistance were 1000 \(\mu M\) (4-h exposure) and 15 \(\mu M\) (7-day exposure), i.e., approximately 3- and 7-fold the ED50 values measured by inhibition of HCT-8 colony growth. These experimental conditions represent the highest drug concentrations that allowed minimal clonogenic survival (5-20 clones), thus reflecting approximately 4 logs of cell kill. The time to reach confluence after the first drug treatment illustrates this high degree of cell kill: 33 and 35 days (Fig. 1). In general, no surviving colonies were observed when slightly higher concentrations of FUra were used (2 mm for 4-h exposure and 20 \(\mu M\) for 7-day exposure). Because 2 \(\times\) 10^5 cells were exposed to drug, these findings confirm what we previously reported in this cell line using MTX and trimetrexate, that it is possible to eradicate 5 logs of cells in culture with only one treatment cycle (20). At the end of the selection process (6 cycles), a low degree of resistance was obtained in the HCT-8/FU4hR cells (ED50 for a 4-h exposure, 100 \(\pm\) 4 versus 30 \(\pm\) 2 \(\mu M\), mean \(\pm\) SE, in the sensitive parental cell line). In contrast, a higher level of resistance was achieved in HCT-8/FU7dR cells with the 7-day exposure (ED50, 20 \(\pm\) 1.0 versus 2.0 \(\pm\) 0.4 \(\mu M\)) in the sensitive HCT-8 cells. Also, HCT-8/FU7dR cells were cross-resistant to FdUrd (ED50 for a 7-day exposure, 46.0 \(\pm\) 2.5 versus 1.6 \(\pm\) 0.3 nm) and, surprisingly, MTX (ED50 for 7-day exposure, 55.0 \(\pm\) 7.5 versus 16.0 \(\pm\) 3.7 nm), while HCT-8/FU4hR cells showed a normal sensitivity to both the nucleoside and the antifolate. More importantly, HCT-8/FU4hR cells displayed full sensitivity to FUra when challenged with a 7-day exposure of this drug (ED50, 2.2 \(\pm\) 0.5 versus 2.0 \(\pm\) 0.4 \(\mu M\)).

FUra Uptake

Transport of FUra into sensitive and resistant HCT-8 cells, at an extracellular concentration of 100 \(\mu M\), was characterized by an initially rapid uptake followed by a continued slow accumulation before reaching a plateau at 15 min. In the initial phase of rapid influx, the slopes were similar for both sensitive and resistant HCT-8 cells. Also, the intracellular concentrations reached in the plateau phase were approximately equivalent for the 3 cell lines (42.15 \(\pm\) 0.65, 39.95 \(\pm\) 0.96, and 44.81 \(\pm\) 1.37 pmol/10^5 cells in HCT-8, HCT-8/FU7dR, and HCT-8/FU4hR cells, respectively).

Activities of FUra-metabolizing Enzymes

The activities of thymidine kinase, uridine kinase, thymidine phosphorylase, uridine phosphorylase, and orotate phosphoribosyl transferase, measured using saturating concentrations of substrates (50 \(\mu M\)), were similar in the three cell lines (Table 1). Since HCT-8/FU7dR cells were cross-resistant to fluorodeoxyuridine, thymidine kinase, an enzyme often involved in determining resistance to this nucleoside, was studied in more detail. The activity of this enzyme, measured with either thymidine or fluorodeoxyuridine as substrates, was similar in the three cell lines (Table 1). Also, saturation curves obtained with increasing concentrations of thymidine were identical, resulting in similar \(K_m\) values (6.5 \(\pm\) 1.1, 5.3 \(\pm\) 2.3, and 5.5 \(\pm\) 1.1 \(\mu M\) in

Table 1 Activity of enzymes of pyrimidine metabolism

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HCT-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase*</td>
<td>3.3 (\pm) 1.9</td>
</tr>
<tr>
<td>Thymidine kinase*</td>
<td>6.6 (\pm) 2.7</td>
</tr>
<tr>
<td>Uridine kinase</td>
<td>31.9 (\pm) 9.8</td>
</tr>
<tr>
<td>Orotate phosphoribosyl</td>
<td>10.5 (\pm) 3.7</td>
</tr>
<tr>
<td>Thymidine phosphorylase</td>
<td>4.8 (\pm) 0.2</td>
</tr>
<tr>
<td>Uridine phosphorylase</td>
<td>3.8 (\pm) 2.5</td>
</tr>
</tbody>
</table>

*Mean \(\pm\) SD of at least 2 experiments, each done in duplicate.

Table 1 was cited from the source: Cancer Res, 1988, 48(5): 1858-61.
TS Activity

Two different assays were used to evaluate the catalytic activity and the level of expression of this enzyme. Neither the catalytic nor the FdUMP-binding assay demonstrated significant differences in baseline TS levels between the three cell lines (Table 2).

Kinetics of Thymidylate Synthase Inhibition by FUra

Although no changes in basal activity of TS and FUra-metabolizing enzymes were detected, alterations in either the actual degree of TS inhibition achieved after FUra treatment or the stability of this inhibition over time or an increase (induction of this enzyme activity) could still be responsible for the FUra resistance of these cell lines. These possibilities were examined as follows.

Determination of Free and Bound TS. Cells were exposed to FUra for 4 h (30 or 100 μM) and either analyzed immediately or resuspended in drug-free medium for 24 or 48 h. TS activity in cell extracts was assayed with or without previous dissociation of the ternary complexes in order to quantitate free, bound, and total TS. After a 4-h exposure to either 30 or 100 μM FUra, free TS levels were similar in sensitive and resistant HCT-8 cells (approximately 25 and 18% of total intracellular TS, with 30 and 100 μM, respectively) (Table 3). However, after FUra removal, a marked increase in free TS was observed in HCT-8/FU7dR cells resulting in an almost complete release of the inhibitor FdUMP after 24 h (30 μM FUra) or 48 h (100 μM FUra) (Table 3). No recovery was observed in HCT-8 and HCT-8/FU4hR cells, and at least 65% of the total intracellular TS was still bound by FdUMP 48 h after the end of FUra exposure (Table 3).

In Situ TS Activity. When the in situ assay for thymidylate synthesis was used to follow the time course of TS inhibition

Table 2 Thymidylate synthase levels in sensitive and resistant HCT-8 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FdUMP-binding assay (pmol/mg protein)</th>
<th>Catalytic assay (nmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-8</td>
<td>1.87 ± 0.53</td>
<td>24.4 ± 6.90</td>
</tr>
<tr>
<td>HCT-8/FU7dR</td>
<td>1.17 ± 0.03</td>
<td>17.2 ± 3.00</td>
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<tr>
<td>HCT-8/FU4hR</td>
<td>1.27 ± 0.25</td>
<td>15.5 ± 5.80</td>
</tr>
</tbody>
</table>

Table 3 Free FdUMP-binding sites in sensitive and resistant HCT-8 cells after FUra treatment

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FUra (μM)</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-8</td>
<td>30</td>
<td>19.0 ± 4.5</td>
<td>37.0 ± 4.0</td>
<td>27.0 ± 3.0</td>
</tr>
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<td>25.7 ± 3.2</td>
<td>32.3 ± 2.3</td>
<td>35.6 ± 4.3</td>
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<tr>
<td>HCT-8/FU7dR</td>
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<td>26.3 ± 6.1</td>
<td>143.5 ± 15.0</td>
<td>90.5 ± 7.1</td>
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<td>40.5 ± 3.5</td>
<td>70.8 ± 4.6</td>
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<tr>
<td>HCT-8/FU4hR</td>
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<td>26.2 ± 4.1</td>
<td>36.0 ± 4.0</td>
<td>44.0 ± 5.5</td>
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<td>34.7 ± 7.2</td>
<td>48.6 ± 2.3</td>
<td>37.0 ± 6.1</td>
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</table>

Fig. 2. Inhibition of in situ TS activity by FUra (4-h exposure; 10, 30, and 100 μM) in sensitive and resistant HCT-8 cells. TS activity was assayed either immediately after drug exposure (A) or after an additional 24- or 48-h (B) incubation in drug-free medium. ■, HCT-8 cells; □, HCT-8/FU4hR cells; ▼, HCT-8/FU7dR cells. Columns, mean percentage of tritium released as compared to untreated controls in 3–4 experiments, each in duplicate; bars, SE. After FUra exposure in intact cells, only slight differences in the degree of thymidylate synthesis inhibition between sensitive and resistant cells were shown at the end of a 4-h exposure to either 10 or 30 μM FUra (Fig. 2A). However, at 100 μM, complete inhibition (>95%) of thymidylate synthesis was achieved only in HCT-8 and HCT-8/FU4hR cells, while HCT-8/FU7dR cells still showed at least 10% of activity. A marked recovery of thymidylate synthesis activity occurred in HCT-8/FU7dR cells when FUra was removed and the cells were incubated in drug-free medium for 24 and 48 h (Fig. 2, B and C). In particular, at 48 h both HCT-8 and HCT-8/FU4hR cells previously exposed to 100 μM FUra still showed >90% inhibition of thymidylate synthesis activity, while under the same conditions thymidylate synthesis in HCT-8/FU7dR cells recovered to 80% of untreated controls (Fig. 2C). Exposure to 100 μM LV prior to, during, and after exposure to the fluoropyrimidine enhanced the degree of thymidylate synthesis inhibition achieved with a 4-h exposure to FUra (10–100 μM) in parental sensitive and pulse FUra-resistant cells, but this enhancement was not observed in HCT-8/FU7dR cells (Fig. 3A). While no differences were observed for the stability of thymidylate synthesis inhibition after drug removal in HCT-8 and HCT-8/FU4hR cells, the addition of LV resulted in a less profound recovery in HCT-8/FU7dR cells (Fig. 3, B and C). However, in this cell line, residual thymidylate synthesis activity after FUra removal was still significantly higher than in both sensitive and pulse FUra-resistant HCT-8 cells (26.0 ± 5.0% versus 3.0 ± 2.0% versus 3.5 ± 1.5%, at 100 μM 48 h after drug removal).
values for in vitro inhibition of TS with FdUMP were similar were determined. As shown in Table 4, only minimal differences between the 3 cell lines were detected. Furthermore, IC50 values for in vitro inhibition of TS withFdUMP were similar for the 3 cell lines (Table 4).

**TS Kinetics and Inhibition Studies**

Since a mutated enzyme would be expected to be less sensitive to inhibition by the FUra metabolite FdUMP and/or to result in a less stable ternary complex (and consequently less prolonged TS inhibition), Km values for both dUMP and CH2FH4 were determined. As shown in Table 4, only minimal differences between the 3 cell lines were detected. Furthermore, the IC50 values for in vitro inhibition of TS with FdUMP were similar for the 3 cell lines (Table 4).

**Intracellular Folate Pools**

Baseline (i.e., without previous exposure to LV) levels of FH4 were measured in the three cell lines (Table 5). FH4 levels were decreased by about one third in both resistant cell lines as compared to the parenteral line (Table 5). CH2FH4 levels were found to be decreased in HCT-8/FU7dR cells as compared to both sensitive and pulse FUra-resistant cells (Table 5). The magnitude of this reduction (approximately 60% decrease) is much greater than the intrasample variability due to the partial instability of CH2FH4 to heat treatment in the absence of excess HCHO (<20%).

Analysis of the Distribution of CH2FH4 and FH4 Polyglutamate Chain Length in Sensitive and Resistant HCT-8 Cells

A deficit in the formation of long-chain polyglutamates of CH2FH4 could result in a less stable ternary complex, leading to a less prolonged TS inhibition by FUra. This would explain both the dramatic recovery of TS activity observed in HCT-8/ FU7dR cells after FUra removal as well as the limited capability of LV to inhibit this recovery. In basal conditions, the parental cell line mainly contained heptaglutamates of CH2FH4 with a small amount of hexa- and possibly octaglutamates. In contrast, HCT-8/FU7dR cells contained only short-chain glutamates (Fig. 4). The same pattern was observed after a 4-h exposure to 100 μM LV, penta- and hexaglutamates predominated in HCT-8 cells, while no folates with >3 glutamic residuals could be detected in HCT-8/FU7dR cells (Fig. 4). In HCT-8/FU4hR cells, a significant amount of monoglutamate and diglutamates were found prior to treatment, and short-chain polyglutamates predominated after LV exposure. However, in contrast to HCT-8/FU7dR cells, this was only a quantitative difference in that this cell line also formed some penta- and hexaglutamates (data not shown).

Incorporation of FUra into RNA

In parental sensitive HCT-8 cells FUra incorporation into RNA increased linearly with increasing concentrations of the fluoropyrimidine (Fig. 5A). The amount of drug incorporated into RNA after a 4-h exposure to 300 μM was approximately 6-fold greater than that obtained with 30 μM (Fig. 5A). Incubation of cell cultures in drug-free media for 24 h after the end

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**Table 4 Kinetic parameters of thymidylate synthase from sensitive and resistant HCT-8 cells**

<table>
<thead>
<tr>
<th>Reaction conditions and analysis are described in &quot;Materials and Methods.&quot;</th>
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<tbody>
<tr>
<td>HCT-8</td>
</tr>
<tr>
<td>IC50 FdUMP (nm)</td>
</tr>
<tr>
<td>Km CH2FH4* (μM)</td>
</tr>
<tr>
<td>Km dUMP* (μM)</td>
</tr>
</tbody>
</table>

* Apparent Km values at 125 μM dUMP.

**Table 5 CH2FH4 and FH4 pool size in sensitive and FUra-resistant HCT-8 cells**

<table>
<thead>
<tr>
<th>Logarithmically growing cells were collected and resuspended at a density of 15 x 10^6 cells/ml in extraction buffer. Folate extraction and measurement were performed as described in &quot;Materials and Methods.&quot; Data represent the means ± SE of 3 experiments, each done in triplicate.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>HCT-8</td>
</tr>
<tr>
<td>HCT-8/RU7-dR</td>
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<td>HCT-8/FU4-hR</td>
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</table>

**Fig. 3. Effects of 100 μM LV on in situ TS activity inhibition by FUra (4-h exposure; 10, 30, and 100 μM) in sensitive and resistant HCT-8 cells. TS activity was assayed either immediately after FUra exposure (A) or after an additional 24- (B) or 48-h (C) incubation in drug-free medium containing 100 μM LV. [H]dUMP, HCT-8 cells; □, HCT-8/FU4hR cells; □, HCT-8/FU7dR cells. Columns, mean percentage of tritium released as compared to untreated controls of 3-4 experiments, each in duplicate; bar, SE.**

**Fig. 4. Analysis of folylpolyglutamates extracted from sensitive and FUra-resistant HCT-8 cells by electrophoretic separation as ternary complexes with L. casei TS and [3H]FdUMP on a polyacrylamide gel in nondenaturing conditions. Lane 1, Glu 1 and Glu 4 standards; lane 2, Glu 3 and Glu 7 standards; lanes 3 and 4, parental HCT-8 cells, before and after a 3 h exposure to 100 μM LV, respectively; lanes 5 and 6, HCT-8/FU7dR cells, before and after a 3 h exposure to 100 μM LV, respectively; lane 7, Glu 2 and Glu 5 standards. Polyglutamate chain length increases from the top to the bottom of the gel. Samples were prepared and gels were processed as described in "Materials and Methods."**

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In parental sensitive HCT-8 cells FUra incorporation into RNA increased linearly with increasing concentrations of the fluoropyrimidine (Fig. 5A). The amount of drug incorporated into RNA after a 4-h exposure to 300 μM was approximately 6-fold greater than that obtained with 30 μM (Fig. 5A). Incubation of cell cultures in drug-free media for 24 h after the end
of drug exposure (Fig. 5B) led to increased incorporation of FUra into RNA at both 30 μM (3-fold) and 100 μM (7-fold) concentrations. Increasing the duration of drug exposure with 30 μM FUra up to 24 h did not result in a higher incorporation of FUra into RNA (Fig. 2C). The same relationship between the concentration of FUra and its incorporation into RNA was observed in the two resistant cell lines. However, in HCT-8/ FU4hR cells, the amount of FUra incorporated into RNA after a 4-h exposure to this drug was significantly lower when compared to parental sensitive or HCT-8/FU7dR cells, respectively. The residual 20% free TS at this concentration (Table 3) was a dramatic increase in free TS was observed in HCT-8/FU4hR but not in parental cell extracts, and different slopes have been the explanation for fluoropyrimidine resistance in the majority of previous studies (1). Amplification of the TS gene resulting in higher enzyme activity is the only mechanism of resistance to fluoropyrimidine detected in human samples until now (41). In the present study, both FdUMP-binding sites and TS catalytic activity were found to be lower in the two resistant cell lines as compared to the parenteral cell line. However, this slight decrease is probably not significant with regard to the mechanism of resistance since one would expect to find an elevation, rather than a decrease, of this enzyme in resistant cells.

Although no changes in basal activity of TS and FUra-activating enzymes were detected, alterations in either the actual degree of TS inhibition achieved after FUra exposure or the stability of this inhibition over time after FUra removal could still be responsible for the FUra resistance of these two cell lines. After a 4-h exposure to FUra, TS activity was inhibited by 75–80% in the 3 cell lines, regardless of the drug concentration used (either 30 or 100 μM) (Table 3) and despite the acute induction of this enzyme observed in the 3 cell lines (approximately 5-fold increase in total intracellular TS as compared to pretreatment values, data not shown). In the parental cell line, a 4-h exposure to 100 μM FUra produces a >95% cell kill; thus, no free TS at this concentration (Table 3) was a surprising finding possibly related to TS induction following FUra treatment, suggesting that other mechanisms, in addition to TS inhibition, are involved in FUra cytotoxicity upon exposure to high doses for a short time.

Prolongation of TS inhibition after drug removal is an important determinant of fluoropyrimidine cytotoxicity (42). TS levels (43) and dUMP pools (44) have been shown to increase after FUra treatment, and either or both increases may lead to a less prolonged inhibition of thymidylate synthase. A dramatic increase in free TS was observed in HCT-8/FU7dR but not in parental sensitive and pulse FUra-resistant cells when cultures were incubated in drug-free medium after FUra exposure (Table 3). However, this assay (31), although widely used, involves a high degree of manipulation of cell extracts, and different
stabilities of TS from different cell lines could affect the results. Moreover, incorporation of TS into ternary complexes could result in a lower rate of degradation of this enzyme as compared to the enzyme not bound, leading to an overestimation of total TS as compared to free TS (43). The use of an in situ assay for TS activity overcomes these limitations and also allows a study of the kinetics of TS inhibition after FUra treatment in intact cells. This assay showed a higher sensitivity for detecting TS inhibition, making it possible to obtain a dose-response curve after a 4-h exposure to concentrations of FUra ranging from 10–100 μM. The dose-response curves for TS inhibition were similar for the 3 cell lines (Fig. 2A). However, in the HCT-8/FU7dR subline when FUra was withdrawn and cells were kept in drug-free medium, thymidylate synthesis recovered, approaching 80% (as compared to untreated controls) at 24 and 48 h (Fig. 2). These results could be due to either an altered TS with reduced affinity forFdUMP or a reduced availability of CH2FH4 polyglutamates required for stable ternary complex formation (45, 46). The former possibility was unlikely because the degree of inhibition of TS activity achieved after a 4-h exposure to FUra was similar in this cell line and in the parental cells (Fig. 2A). Furthermore, kinetic studies of TS showed that its affinity for both the substrates dUMP and CH2FH4 and the inhibitor FdUMP is approximately the same in the 3 cell lines, excluding an alteration of this enzyme as a possible reason for the less persistent TS inhibition observed in HCT-8/FU7dR cells. A further possible explanation for the recovery of TS activity observed in HCT-8/FU7dR cells is an increased synthesis of new TS protein. This possibility was excluded in that an induction of this enzyme did occur after FUra treatment, but the magnitude of this increase was similar in sensitive and FU7dR-resistant HCT-8 cells. Moreover, this phenomenon was observed at the end of FUra exposure (4 h), but 24 h (and 48 h) after drug removal total intracellular TS was similar in treated and untreated cells. The levels of CH2FH4 were found to be decreased in HCT-8/FU7dR cells, providing a possible explanation for the previous findings. The addition of 100 μM LV resulted in a higher degree of inhibition of TS activity at 10 and 30 μM FUra in both sensitive and pulse resistant cells (Fig. 3A), but the same potentiation was not observed for HCT-8/FU7dR cells (Fig. 3A). Also, although in this cell line the addition of the reduced folate resulted in a more prolonged inhibition of TS activity after FUra removal, the recovery at 24 and 48 h was still high compared to both wild-type and HCT-8/FU4hR cells. The capability of LV to only partially inhibit the recovery of TS activity might be explained by either an impaired uptake of this compound or an altered intracellular metabolism of 5-formyltetrahydrofolate. It is known that the potentiation effects of LV on fluoropyrimidine cytotoxicity are due to its conversion to CH2FH4 and FH4 after cellular uptake, and a 6-fold increase in the pool size of these reduced folates has been described in L1210 cells exposed to LV (42, 47). It has also been shown that the polyglutamylation of this cofactor results in a decreased dissociation rate of the ternary complex (48) and consequently in more prolonged TS inhibition (43). A defective uptake of LV was an unlikely explanation because a similar increase (approximately 3-fold, data not shown) in CH2FH4 and FH4 pools occurred in the three cell lines after a 3-h LV exposure. These data provide indirect evidence that neither 5-formyltetrahydrofolate transport nor intracellular conversion to other reduced folates was altered in this cell line. The analysis of the intracellular polyglutamates of CH2FH4 and FH4 clearly shows a lack of long-chain polyglutamates in the HCT-8/FU7dR cells, both in basal conditions and after a 3-h exposure to 100 μM LV. This defect is specific for this cell line in that, although HCT-8/FU4hR cells probably have a different distribution as compared to the wild type, they still show the ability to synthesize long-chain polyglutamates. The defective polyglutamylation of CH2FH4 may explain both the less prolonged inhibition of TS in this cell line and the partial lack of effectiveness of leucovorin. Since short-chain folates are retained less effectively inside the cell this might also explain the slight reduction in FH4 and CH2FH4 pools. It is of interest that Yin et al. (49) described a Hep 2 cell line naturally resistant to FUra that contained a decreased total pool size of folates and a decreased proportion of higher polyglutamate forms.

In HCT-8/FU4hR cells, TS inhibition was achieved and maintained at a fairly stable level even after FUra removal; therefore, blockade of thymidylate synthesis does not seem to play a role in the resistance that develops after high-dose short-term FUra administration. This hypothesis is consistent with the lack of cross-resistance toFdUrd and is supported by the discrepancy between the percentage of TS inhibition and percentage of cell kill achieved with a 4-h exposure to 100 μM FUra (Table 3). When compared to the parental and the HCT-8/7dR line, the amount of FUra incorporated into RNA was less in the HCT-8/FU4hR cell line, using a range of FUra concentrations and with 3 different conditions of exposure to [6-3H]FUra. Although FUTP levels were not determined, the normal values obtained for uridine kinase, as well as orotate phosphoribosyltransferase activity, make it unlikely that a decreased synthesis of this nucleotide is the reason for the defect observed in this cell line. However, either a reduction in pyrimidine monophosphate kinase or an increased phosphatase activity might lead to lower FUTP levels. The former enzyme is usually found in very high concentrations as compared to nucleoside kinases in most tumor cells (50); therefore, it is unlikely that it becomes rate limiting in FUra activation. Furthermore, the increase in FUra accumulation into RNA observed in this cell line after FUra removal would exclude an increased degradation rate or a reduced synthesis of FUTP as being the lesion responsible for this defect. Either a reduced affinity of RNA polymerase for FUTP or an increased removal of FUTP from RNA [in analogy to what has been reported forFdUTP in a cell line with a decreased accumulation of FUra into DNA (51)] might explain these findings. These possibilities are under investigation. Ardalan et al. (10) described a leukemic cell line that was resistant to FUra that had both a decreased rate of incorporation of FUra into RNA, as well as an “accelerated excretion ofFdUMP.” The incorporation of FUra into RNA was ascribed to a low rate of enzymic conversion of 5-fluorouridine 5’-monophosphate into 5-fluorouridine 5’-diphosphate, thus decreasing the amount of FUTP available for RNA synthesis. It is also evident from the data in Fig. 5 that FUra accumulation into RNA increases almost linearly with FUra concentration and with 3 different conditions of exposure to [6-3H]FUra. Although FUTP levels were not determined, the normal values obtained for uridine kinase, as well as orotate phosphoribosyltransferase activity, make it unlikely that a decreased synthesis of this nucleotide is the reason for the defect observed in this cell line. However, either a reduction in pyrimidine monophosphate kinase or an increased phosphatase activity might lead to lower FUTP levels. The former enzyme is usually found in very high concentrations as compared to nucleoside kinases in most tumor cells (50); therefore, it is unlikely that it becomes rate limiting in FUra activation. Furthermore, the increase in FUra accumulation into RNA observed in this cell line after FUra removal would exclude an increased degradation rate or a reduced synthesis of FUTP as being the lesion responsible for this defect. Either a reduced affinity of RNA polymerase for FUTP or an increased removal of FUTP from RNA [in analogy to what has been reported forFdUTP in a cell line with a decreased accumulation of FUra into DNA (51)] might explain these findings. These possibilities are under investigation. Ardalan et al. (10) described a leukemic cell line that was resistant to FUra that had both a decreased rate of incorporation of FUra into RNA, as well as an “accelerated excretion ofFdUMP.” The incorporation of FUra into RNA was ascribed to a low rate of enzymic conversion of 5-fluorouridine 5’-monophosphate into 5-fluorouridine 5’-diphosphate, thus decreasing the amount of FUTP available for RNA synthesis. It is also evident from the data in Fig. 5 that FUra accumulation into RNA increases almost linearly with the concentration of drug. Increasing the duration of exposure did not result in a similar increase. This finding is in contrast with some previous studies that report an increased accumulation of FUra into RNA over time (52, 53). The present study differs from the previous investigations in that 3 different conditions of FUra exposure (i.e., 4, 24, and 4 h followed by 24 h in drug-free medium) were compared over a range of FUra concentrations from 10–300 μM. Also, in some of the previous studies, noncytotoxic concentrations of FUra were used and the time span over which FUra accumulation into RNA was followed was often too short. Furthermore, the technique used...
for RNA isolation in our study results in a purer product than obtained by the methods used in other reports. In addition to these considerations, some of this discrepancy might be explained by the normalization of data with RNA content in these studies instead of with cell number. It is possible that when the time of exposure to FUra is prolonged TS blockade may slow down the incorporation of FUra into RNA because of an increase in the pool size of UTP due to the lack of negative feedback inhibition on pyrimidine synthetic pathway by dTTP. Alternatively, the arrest in S phase caused by inhibition of thymidylate formation might protect cells from RNA-directed cytotoxicity that has been reported to be more pronounced in the G1 phase (54).

These studies show that different schedules of FUra administration may lead to resistance via different mechanisms and also provides evidence supporting the contention that the FUra mechanism of action depends on the schedule of its administration. The clinically relevant schedules of FUra exposure used in this study resulted in two novel mechanisms of drug resistance, decreased incorporation into RNA and decreased CH2FH4 polyglutamate formation. A recent report from this laboratory described a MTX-resistant cell line which was cross-resistant to the combination FdUrd-LV and displayed a less stable TS inhibition associated with a lack of long-chain polyglutamates of CH2FH4 (24). This study is the first in which acquired resistance to FUra has been correlated with decreased levels of CH2FH4 accompanied by and probably due to a decreased polyglutamylation of this cofactor.

These results have important clinical implications. The addition of LV may not enhance the cytotoxicity of pulse FUra, at least when optimal cytotoxic concentrations of this agent are used, because this effect is mediated via thymidylate synthase inhibition. Channeling FUra into RNA using other modulating agents (MTX, PALA) may improve results when high-dose short-term administration is used. On the other hand, enhancement of FUra cytotoxicity with LV may be greater when the fluoropyrimidine is administered as a continuous infusion. Experimental support for this suggestion has recently appeared (55). Finally, these data support the concept that pulse FUra and continuous infusion FUra have different mechanisms of action. It might be possible to take advantage of the lack of cross-resistance between these schedules to treat tumors resistant to pulse FUra administration with continuous infusion FUra. Limited clinical data support this concept in that continuous infusion FUra ± LV may be effective in patients previously treated with pulse FUra (56).

REFERENCES


Novel Mechanism(s) of Resistance to 5-Fluorouracil in Human Colon Cancer (HCT-8) Sublines following Exposure to Two Different Clinically Relevant Dose Schedules

Carlo Aschele, Alberto Sobrero, Mary A. Faderan, et al.


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