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

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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 $2 \times 10^6$ cells to a constant concentration of FUra (10 $\mu$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 synthesis after FUra exposure. No differences were detected in the kinetic properties ($K_m$ for 2'-deoxyuridylate and 5,10-methylenetetrahydrofolate, 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-methylenetetrahydrofolate were decreased in HCT-8/FU7dR cells, and analysis of the chain length distribution of the polyglutamylated form of the folate cofactor in this cell line the defect in 5,10-methylenetetrahydrofolate levels is accompanied by, and possibly due to, a defect in the polyglutamylation of this cofactor. In contrast, HCT-8/FU4hR cells were similar to the parental cell line with regard to the degree of inhibition of TS by FUra and duration of inhibition after FUra removal. Labeling studies with [3H]-FUra (4 h exposure, 100 $\mu$M) showed that the incorporation of the fluoropyrimidine into RNA is significantly decreased in HCT-8/FU7dR 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 synthesis 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, levamisole, 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 in 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, definite 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 cytotoxicity. 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...
The cells/25-cnr flask (approximately 4 logs of cell kill). A progressive reduction in the time (days) at which confluence of the cultures at each to the end of the selection period. Cells were passaged (2 x 10^5 cells/ obtained resistance to FUra with either 4-h or 7-day exposure to FUra, antifolates (20). Starting from a clone of the parental cell line, we with a low selection pressure technique as previously described for (Boston, MA) and Kodak X-O-mat AR film was purchased from acrylamide and all other reagents for gel electrophoresis were purchased ^mol/h/mg) was purchased from Biopure (Boston, MA). Charcoal chased from Moravek Biochemicals (Brea, CA). Lactobacillus casei laboratories (Joña, Switzerland) and were reduced to the corresponding from Boehringer Mannheim Biochemicals (Indianapolis, IN). Poly- from Eastman Kodak. Media and sera for cell culture were obtained 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 CH_2FH_4 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 and FdUrd 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' deoxyuridine, 2'-5' deoxyuridylate, [6-^3H]FdUMP, [6-^3H]FUra, [6- ^14C]Fura, [6-^14C]FdUrd, and [methyl-^14C]thymidine were purchased from Moravek Biochemicals (Brea, CA). *Lactobacillus casei* thymidylate synthase (activity, 53.4 nmol/h/ml; specific activity, 1.4 ^14C]5-fluorouridine, [6-^14C]FdUrd, and [methyl-^14C]thymidine were purchased from Biopure (Boston, MA). Charcoal (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 Nucleic (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 colorectal 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 x 10^5 cells/ flask) and rechallenged with drug as soon as the cultures approached 100% of confluence. The initial selecting concentration was the highest that allowed regrowth of at least a few clones, starting from 2 x 10^5 cells/25-cm^2 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 monocellular 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 x 10^4 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% CO_2. 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 x10 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-h and 7-day exposures to the drugs. Each experimental point was determined in triplicate with 4 replicate controls; experiments were repeated at least twice.

**FUra Uptake.** Exponentially growing cells (10^5 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 ^g of [6-^3H]FUra (specific activity, 25 cpm/pmol) was added. The plated 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 LS801 scintillation counter. Triplicate 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 ^g MgCl_2, 1.0 mM ATP, 2.0 ^g [methyl-^14C]thymidine (82.40 Ci/mmol), and 0.2 m Tris-HCl (pH 7.6) in a final volume of 100 ^l was incubated with enzyme at 37°C for 15 min. At the end of this period, 50 ^l from each tube was spotted on DE81 filter discs (24 mm, Whatman), washed with 1 mM 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 the different procedure, which produces a higher TS yield and enzyme stability. Exponentially growing cells in 225-cm^2 tissue culture flasks were cooled on ice for 5 min, washed twice with 20 ml of ice-cold PBS, detached by trypsinization, and 5 ml of fresh medium was added. After trypsinization, and followed by addition of 15 ml of complete tissue culture medium. Keeping the cells on ice and using ice-cold reagents was essential for obtaining a higher preservation of enzyme activity. After centrifugation (5 min, 1000 rpm, 4°C) the resulting pellet was either stored at -70°C or resuspended in 1–2 ml of extraction buffer (50 mM Tris-HCl, 50 mM pH 7.4) containing 10%...
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 Vibrazor 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 for 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 on the release of tritium from [5-3H]dUMP as [3H]2H2O (30). The assay was performed in a total volume of 50 μl containing 50 μM 2'-[5-3H]deoxyuridylate (specific activity, 0.1 Ci/mmol), 250 μM 5,10-methylene tetrahydrofolate, and enzyme buffer with 50 mM Tris-HCl (pH 7.4), 5 mM formaldehyde, 50 mM NaF, 10 mM Na ascorbate, and 100 mM β-mercaptopethanol. 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 1% TCA. This allowed quantitation of TS activity remaining after FUra 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 dUMP or CH2FH4 between 1 and 150 μM or 5 and 500 μM, respectively. After correction was made for blank rates (i.e., no enzyme), the data were fit by nonlinear least squares regression to Michaelis-Menten equation by using the Enzyme Fitter program (Elsevier). IC50 values were obtained by inhibition assays in which FDUMP was added at various concentrations to the standard reaction mixture. In order to compare the IC50 values of this tight-binding inhibitor between resistant and sensitive cell lines, care was taken to use the same amount of enzyme activity for each assay. The drug concentration giving 50% inhibition of activity was obtained from plots of the percentage of remaining activity versus the logarithm of FdUMP concentration.

FdUMP-binding Assay. Cytosols were prepared as described for the catalytic assay with the exception that the extraction buffer was 200 mM Tris-HCl (pH 7.5), 70 mM β-mercaptopethanol, 100 mM NaF, and 15 mM CMP. FdUMP-binding sites were measured as described by Fernandes and Cranford (31).

In Situ TS Activity. Inhibition of thymidylate synthase activity by FUra in intact cells was measured by the in situ assay of Yalowich 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 deoxuridylate. 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.

Assay of Intracellular Folates. A modification of the radioenzymatic binding assay described by Bunni 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, detached (5-min trypsinization at 37°C), and centrifuged (5 min, 1000 × g) after addition of complete tissue culture medium. The resulting pellet was resuspended in extraction buffer that contained 50 mM Tris-HCl, 50 mM sodium ascorbate, 1 mM EDTA, and 0.25 mM succrose (pH 7.4) at a density of 1.5 × 10⁶ cells/ml. Cells were boiled for 3 min to achieve lysis and to prevent enzymatic cycling during the assay. After centrifugation (10,000 × g, 10 min, 4°C), aliquots of the supernatant were incubated with 160 μM CH2FH4 and 125 mM [3H]FdUMP in a total volume of 100 μl of the above extraction buffer. The reaction was allowed to proceed at 37°C for 30 min and stopped by cooling samples on ice. Uncomplexed radioactivity was removed by addition of a 500-μl slurry of ice-cold 3% charcoal (albumin and dextran treated) and centrifugation at 12,000 × g for 20 min. The amount of TS-bound radioactivity was then quantitated in aliquots of the supernatant after addition of 10 ml scintillation fluid. A series of standard tubes with known amounts of CH2FH4 was included in each assay. All values were corrected for binary complex (i.e., TS and [3H]FdUMP without folate cofactor) radioactivity. The plot of the fraction of bound radioactivity at each concentration of standard CH2FH4 provided a calibration curve from which molar levels of cofactor in the test samples were calculated. FH4 levels were quantitated by the same method after conversion to CH2FH4 by the addition of 6.5 mM formaldehyde. The recovery of known amounts of CH2FH4 added to cell extracts prior to boiling was 94%.

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 stacking gel; 4.5% in the running 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.
(60 min room temperature) in order to obtain protein fixation, impregnation with the fluorescent intermediate, and its precipitation in the gel (38). After the gels dried (90 min, 60°C), autoradiographs were obtained by exposure of Kodak X-AR film at -70°C for 48 h.

Incorporation of FUra into RNA. Cells were cultured 18–24 h in 150-mm dishes (approximately 10^7 cells/dish). Old medium was replaced with complete medium containing a range of concentrations (10–300 μM) of FUra labeled in position 6 with ^3H (specific activity, 2–20 μCi/μmol). At the end of the desired incubation time (4 or 24 h), cell cultures were washed thrice with PBS and either incubated for 24 h in drug-free medium or immediately harvested in 1 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol). RNA extraction (39) was performed either immediately after cell collection or after storage at −70°C for up to 10 days without any effect on RNA yield. Aliquots of the extracted RNA were placed in scintillation vials and, after addition of 5 ml of Ecolume scintillation cocktail, the incorporated radioactivity was counted in a Beckman model 5801 spectrometer. All results were normalized for RNA content as determined by measuring UV absorbance at 260 nm. Parallel 280 nm readings with determinations of 260/280 ratios excluded any protein contamination.

RESULTS

Development of Resistance

The concentrations of FUra used in the selection of resistance were 1000 μM (4-h exposure) and 15 μM (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 μM for 7-day exposure). Because 2 × 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 ± 4 versus 30 ± 2 μM, mean ± 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 ± 1.0 versus 2.0 ± 0.4 μM in the sensitive HCT-8 cells). Also, HCT-8/FU7dR cells were cross-resistant to FdUrd (ED50 for a 7-day exposure, 46.0 ± 2.5 versus 1.6 ± 0.3 nm) and, surprisingly, MTX (ED50 for 7-day exposure, 55.0 ± 7.5 versus 16.0 ± 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 ± 0.5 versus 2.0 ± 0.4 μM).

FUra Uptake

Transport of FUra into sensitive and resistant HCT-8 cells, at an extracellular concentration of 100 μM, 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 ± 0.65, 39.95 ± 0.96, and 44.81 ± 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 phosphoribosyltransferase, measured using saturating concentrations of substrates (50 μM), 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 Km values (6.5 ± 1.1, 5.3 ± 2.3, and 5.5 ± 1.1 μM in

Table 1. Activity of enzymes of pyrimidine metabolism

<table>
<thead>
<tr>
<th>Activity (nmol/mg protein/h)*</th>
<th>HCT-8/FU7dR</th>
<th>HCT-8/FU4hR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase*</td>
<td>3.3 ± 1.9</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>Thymidine kinase†</td>
<td>6.6 ± 2.7</td>
<td>5.2 ± 1.7</td>
</tr>
<tr>
<td>Uridine kinase</td>
<td>31.9 ± 9.8</td>
<td>28.6 ± 9.2</td>
</tr>
<tr>
<td>Orotate phosphoribosyltransferase</td>
<td>10.5 ± 3.7</td>
<td>10.1 ± 0.8</td>
</tr>
<tr>
<td>Thymidine phosphorylase</td>
<td>4.8 ± 0.2</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Uridine phosphorylase</td>
<td>3.8 ± 2.5</td>
<td>4.2 ± 2.6</td>
</tr>
</tbody>
</table>

* Mean ± SD of at least 2 experiments, each done in duplicate.
† 5-Fluoro-2'-deoxyuridine was used as substrate.
‡ Thymidine was used as substrate.
HCT-8, HCT-8/FU7dR, and HCT-8/FU4hR cells, respectively).

**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 after FUra exposure in intact cells, only slight differences in the degree of thymidylate synthesis inhibition between sensitive and resistant cells were observed 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 in 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).

**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>
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<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>
</tr>
<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>% free TS at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>HCT-8</td>
<td>30</td>
<td>19.0 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>25.3 ± 3.2</td>
</tr>
<tr>
<td>HCT-8/FU7dR</td>
<td>30</td>
<td>26.3 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>27.4 ± 3.2</td>
</tr>
<tr>
<td>HCT-8/FU4hR</td>
<td>30</td>
<td>26.2 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>34.7 ± 7.2</td>
</tr>
</tbody>
</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- (B) or 48-h (C) incubation in drug-free medium. M, HCT-8 cells; R, HCT-8/FU4hR cells; D, HCT-8/FU7dR cells. Columns, mean percentage of tritium released as compared to untreated controls in 3-4 experiments, each in duplicate; bars, SE.](image-url)
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, the IC50 values for in vitro inhibition of TS with FdUMP were similar for the 3 cell lines (Table 4).

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CH2FH4 (μM)</th>
<th>FH4 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-8</td>
<td>20.4 ± 0.6</td>
<td>18.6 ± 0.7</td>
</tr>
<tr>
<td>HCT-8/RU7-dR</td>
<td>21.1 ± 0.8</td>
<td>19.3 ± 0.9</td>
</tr>
<tr>
<td>HCT-8/FU4-hR</td>
<td>21.8 ± 1.0</td>
<td>19.5 ± 1.1</td>
</tr>
</tbody>
</table>

**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/FU4-hR 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...

Fig. 4. Analysis of polyglutamylated extracts from sensitive and FUra-resistant HCT-8 cells by electrophoretic separation as ternary complexes with L. casei TS and [3H]FdUMP on a polyacryamide gel in nondenaturing conditions. Lane 1, Glu 1 and Glu 4 standards; lane 2, Glu 3 and Glu 7 standards; lanes 3 and 5, parental HCT-8 cells, before and after a 3 h exposure to 100 μM LV, respectively; lanes 4 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.”

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. HCT-8 cells; □, HCT-8/FU4-hR 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.
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 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, independently of the concentration of FUra used (30–300 μM) (Fig. 5A). Incubation of cells in drug-free medium after FUra removal also resulted in an increased drug accumulation into RNA in both cell lines. However, the total amount of FUra incorporated into RNA was 3-fold lower than both the parental sensitive and the continuous infusion resistant cell line (Fig. 5B).

DISCUSSION

A large number of studies of the mechanisms of FUra resistance have been conducted in the past three decades (1). The main limitation to the clinical relevance of most of these studies has been the procedures used to select resistant cell lines. The methods used previously were aimed at obtaining highly resistant clones and usually involved a high selection pressure with increasing drug concentration. The resistant cell lines were developed in the present study to investigate the mechanisms of resistance to FUra operating under experimental conditions that closely mimic the clinical situation. The concentration of FUra used for the selection of resistance was kept constant for the six repeated treatment cycles, resulting in rapid development of low-level resistance, analogous to the clinical situation (20). This model also allows study of the possible relationship(s) between the development of resistance and the schedule of FUra administration. This issue is of crucial importance in view of the uncertainties regarding the optimal method of delivering fluoropyrimidines (1). The hypothesis that different schedules of FUra administration may lead to different mechanisms of resistance was mainly based on clinical data that pulse and continuous infusion FUra have different dose-limiting toxicities and maximum tolerated doses (16). Evidence presented in this study that supports this hypothesis derives from cytotoxicity assays that showed that cells resistant to pulse FUra still retained full sensitivity to the fluoropyrimidine given as a 7-day exposure, as well as a different pattern of cross-resistance to other drugs (FdUrd, MTX) for the two resistant cell lines. Of interest is that the cell line resistant to 7-day FUra was cross-resistant to 4-h FUra, possibly indicating that both TS inhibition and incorporation of FUra into RNA are required for cytotoxicity (data not shown). TS inhibition has long been thought to be the primary mechanism of FUra cytotoxicity (40), and either increased levels or mutations of this enzyme 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, the residual 20% 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 synthesis. 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 an altered TS with reduced affinity for FdUMP 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 was 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 potentiating effects of LV on fluorouridine 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 3 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/FU7dR 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 for FdUTP in a cell line with a decreased accumulation of FUra into DNA (51)] might explain these findings. These possibilities are under investigation. Ardal 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 of FdUMP." 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 for RNA isolation in our study results in a purer product than

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 FUra-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 polyglutamylatation 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 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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