Modulation of 5-Fluorouracil Metabolism and Cytotoxicity by Antimetabolite Pretreatment in Human Colorectal Adenocarcinoma HCT-8

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ABSTRACT

The modulation of 5-fluorouracil (FUra) metabolism by methotrexate (MTX) pretreatment in monolayer cultures of human colorectal adenocarcinoma, HCT-8, was examined and correlated to clonal growth of this cell line. There was a gradual and nearly linear total intracellular accumulation and incorporation into RNA of FUra for 30 hr in control cells. A 12-hr 10 µM MTX pretreatment before adding 100 µM FUra resulted in approximately a 3-fold increase in total FUra accumulation, 59% of which was fluorouridine triphosphate. Soluble fluorodeoxyuridine monophosphate was increased 5-fold following MTX pretreatment; however, [3H]deoxyuridine incorporation into the dime monophosphate was increased 5-fold following MTX pretreatment.

INTRODUCTION

FUra is a commonly used antimetabolite for the treatment of gastrointestinal cancer (24). Improvement in the chemotherapy of this disease may be possible by elucidating the biochemical mechanisms of FUra metabolism and cytotoxicity in continuous cultures of this cancer which then could be exploited in the design of rational drug-sequencing studies. Previous work from our laboratory demonstrated that MTX pretreatment of L1210 cells resulted in an increased intracellular accumulation of FUra and a synergistic tumor cell kill (8). This sequence-dependent drug synergism was associated with a 5-fold increase in the intracellular formation of the toxic metabolites FdUMP and FUTP. The enhanced metabolism of FUra was a consequence of the antipurine effect of MTX which resulted in a 10-fold elevation in the intracellular pool of PRPP, a cosubstrate necessary for ribosylphosphorylation of FUra. The present study extends these observations of the interaction of MTX and FUra to the cultured human colorectal adenocarcinoma, HTC-8. Effects of other investigational antimetabolites including MMPR, AZS, LAL, PALA, and PF on FUra accumulation in HCT-8 cells are also examined.

MATERIALS AND METHODS

Cells

The human colorectal adenocarcinoma HCT-8 is a well-characterized continuously growing monolayer cell line which divides every 18 hr (31). Cultures were maintained in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 10% newborn calf serum and subcultured weekly. Cells for stocks and studies were grown in 75-cm² sterile plastic culture flasks (Costar; Cambridge, Mass.) with 25 ml of medium in 5% CO2 incubators at 37°C. C cell counts were performed on a Coulter Model ZBI counter (Hialeah, Fla.).

Drug Pretreatment and Intracellular FUra Accumulation

FUra, MTX, MMPR, and AZS were purchased from Sigi Chemical Co. (St. Louis, Mo.). PF was obtained from Eli L Co. (Indianapolis, Ind.). LAL and PALA were provided by the Drug Development Branch of the Division of Cancer Treatment of the National Cancer Institute (Bethesda, Md.). [3H]FUra (Ci/mmol) was purchased from Moravek Biochemicals (City Industry, Calif.).

The intracellular accumulation of FUra was measured by the microfuge method which we have reported previously (15). Following drug pretreatment, the radiolabeled FUra was added to a concentration of 100 µM, a clinically achievable dose. Cells were harvested by rapid trypsinization (0.05% solution) and counted at the times indicated in Chart 1. Into a 0.5-ml microfuge tube were placed 0.1 ml of the labeled cell suspension. The tube was immediately centrifuged at 10,000 rpm for 15 sec to sediment the cells through a silicone-oil interface into 0.04 ml of 5% perchloric acid. The medium remaining above the silicone-oil interface contained no radioactivity; the remaining fraction contained radiolabel representing the total amount of intracellular FUra and metabolites accumulated. The oil contained no radioactivity; the remaining fraction was the FUrA-containing medium. The intracellular accumulation of FUra was linear to 6 hr (Chart 1) and is reported nmol FUra per 10⁶ cells per 6 hr in Chart 2.
colonies were rinsed with PBS, immediately stained with methanol-crystal violet (2.5%), and counted. An automated colony counter (Biotran II; New Brunswick Scientific, Edison, N. J.) was calibrated by manual counting and used to enumerate colonies. The sensitivity of the instrument was optimized to discriminate colonies ≥0.2 mm in diameter within fields of 1000 sq mm; 6 nonoverlapping fields per flask were counted with a variability less than 10% between control flasks. Clonal growth in treated conditions was recorded as mean percentage of colony count relative to untreated controls with S.D.'s ±5%.

This monolayer cloning technique enables more rapid and convenient assessment of sequential drug effects and prolonged drug exposure intervals in monolayer cell lines than by conventional soft agar methods.

**FUra Metabolism and Incorporation into RNA**

Monolayer cells given radiolabeled FUra were rinsed with cold PBS, trypsinized, counted, and then precipitated in 0.5 M perchloric acid at 4°C at variable times. The soluble extract containing free drug, nucleoside, and nucleotide derivatives was neutralized with KOH, and the salt was removed by centrifugation. The acid-insoluble precipitate was washed in 2 ml perchloric acid until the radioactivity remaining in 0.5 ml of the wash did not exceed background counts. The precipitate was then hydrolyzed in 1n KOH at 37°C for 5 hr. After reacidification, the amount of radiolabeled FUra incorporated into RNA was counted in the soluble portion of the hydrolyzed precipitate. RNA was quantitated by the orcinol reaction.

The acid-soluble cell extract was analyzed by high-pressure liquid chromatography (Altex Instruments, Berkeley, Calif.) using a Partisil SAX column (10-µm particle size; 250 × 4.6 mm) and a 2%/min linear phosphate buffer gradient (0.01 to 0.7 M NaH₂PO₄, pH 3.3) to quantitate mono-, di-, and triphosphate ribonucleotides of FUra. Absorbance was measured at 254 and 280 nm, and 1-min fractions were collected for radiochromatograms. A reverse-phase column (Partisil ODS-2) with a 0.1 M sodium acetate buffer and gradient to 4% acetonitrile was used to quantitate free intracellular FUra, 5-fluorouridine, and 5-fluorodeoxyuridine. All retention times were calibrated using either cold or radiolabeled standards. Intracellular FdUMP was measured nonenzymatically by periodate oxidation of the acid-soluble fraction of cells treated with [³H]FUra (100 µM, 6 hr) with or without 10 µM MTX pretreatment for 12 hr (Chart 2). This technique has been described in detail by Cadman et al. (8) and measures soluble-free FdUMP, which may not correspond to measurement of total complexed FdUMP as reported by Washtien and Santi (32).

### Clonal Growth of HCT-8

Cells from stock cultures were suspended by trypsinization and vigorous pipetting before 5 × 10⁶ cells were seeded into sterile flasks with medium. Cultures were drug treated (Day 2 to 3) when clusters were between 4 and 32 cells in size. We chose to delay addition of drugs until the single cells had developed clusters of 4 to 32 cells to resemble more closely tumor micrometastases in humans and rodents. It would be possible to kill up to 90% of the cells in this circumstance without any effect on the number of colonies present; thus, when an inhibitory effect on clonal growth by drug treatment is observed, it probably represents an effect far in excess of 90%. Some cells may be slowed in their growth rather than killed, and this method may not be able to easily distinguish between these 2 possibilities. However, the results are expressed as clonal growth inhibition and not cell killing. Comparison between treatment and control growth is made at Day 7 because, after this time, the control colonies become confluent, and the mean colony count begins to decline. Treated colonies continue to increase in size after 7 days, and the number of colonies ≥0.2 mm in diameter also increases.

During the treatment period, MTX and FUra concentrations and exposure intervals were kept constant. In the sequence studies, FUra was given for one of 4 successive 6-hr intervals during the 24-hr MTX exposure (Table 1). At 7 days, when untreated cultures reached the optimal mean colony density of 500 (per 1000-sq mm counting field) and median clonal diameter of ≈0.25 mm, the medium was decanted, and the

**Table 1**

<table>
<thead>
<tr>
<th>HCT-8 clonal growth</th>
<th>Mean % of control (S.D. ± 1%/9)</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>Interval (hr)</td>
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<tr>
<td>Control</td>
<td>100</td>
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<tr>
<td>MTX (0.1 µM)</td>
<td>24</td>
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<tr>
<td>FUra (10 µM)</td>
<td>6</td>
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<td>MTX (0.1 µM) + FUra (10 µM)</td>
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<td>3rd 6 20</td>
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DNA Synthesis

Intracellular thymidylate and DNA synthesis were measured by incorporation of [3H]deoxurydine (New England Nuclear, Boston, Mass.) into the acid-insoluble fraction of cultured HCT-8 cells. Cells pretreated with MTX, FURA, and sequentially combined MTX and FURA were subsequently exposed for 1 hr in culture to [3H]deoxurydine (24 Ci/mmol). The cells were washed in cold PBS, trypsinized, and counted. Known aliquots of the labeled cell suspension were precipitated with 10% trichloroacetic acid onto glass fiber filters (Whatman, Inc., Clifton, N.J.) using a MASH II apparatus (Microbiological Associates, Walkersville, Md.). The acid-insoluble precipitates were then rinsed with 20 volumes of cold PBS, and the filters were air dried and counted in vials using 7 ml of nonaqueous toluene. Incorporation of [3H]deoxurydine is linear over 1 hr. Ribonuclease digestion did not release appreciable radioactive counts from the acid precipitate of untreated cells; thus, radioactivity present in the acid-precipitable fraction was regarded as incorporated into cellular DNA and expressed as pmol/10^6 cells/hr.

PRPP Assay

Intracellular pools of PRPP in cultured HCT-8 cells were quantitated by modifications of a technique reported previously (17).

Enzyme Preparation. APRT (EC 2.4.2.7), which was used to convert [3H]adenine (30 μM; 24.5 Ci/mmol) to [3H]AMP in the presence of PRPP, was extracted from the 40 to 70% (NH₄)₂SO₄ fraction of a cell homogenate prepared from 1.5 liters of L1210 cells of a cell density of 2 to 4 x 10^5 cells/ml. This fraction was placed on a Sephadex G-150 column and eluted with 0.2 M Tris (pH 7.4)-20 mM MgSO₄ to separate the APRT from the 5'-nucleotidase activity.

Cell Extracts. Cells from which PRPP was to be determined were trypsinized, and the suspension was centrifuged at 4° for 8 min at 1200 rpm. The cell pellet was resuspended at 4° in 5 ml of a solution of Na₂HPO₄-0.9% NaCl solution, pH 7.4; 0.1 ml was removed to compute cell density, and then it was recentrifuged as before. The cell pellet was resuspended in 0.5 ml of 0.2 M Tris, pH 7.4, and placed in a boiling-water bath for 90 sec, which was the optimal time for maximum PRPP extraction. The tubes were then placed on ice and centrifuged for 4 min at 2000 rpm, and the supernatant was removed and stored at −20° until multiple determinations could be performed simultaneously.

PRPP Determination. To the reaction mixture which contains 0.02 ml of 20 mM MgCl₂ and 0.01 ml of [³H]adenine (New England Nuclear, 16 Ci/mmol) were added 0.1 ml of the PRPP cell extract and then 0.1 ml of the APRT extract. After 20 min at 19°, the reaction was stopped by adding 0.270 ml of 3 mM citric acid in methanol and placed in ice. Of this reaction mixture, 0.05 ml was spotted onto Whatman DE81 discs. The discs were then washed twice with 2 mM citric acid in 60% methanol and once with absolute methanol. This washing procedure removed greater than 99% of [³H]adenine. After air drying, the discs were placed in scintillation vials, the [³H]AMP was eluted with 1 ml 0.5 M NaCl in 1 N HCl, 8 ml of nonaqueous scintillant were then added, and the amount of [³H]AMP formed was quantitated. The amount of PRPP used in this conversion was determined by performing simultaneous controls with variable quantities of PRPP as limiting in the reaction.

Statistics

Charts 1 to 4 and Table 1 represent the means of triplicate samples from single experiments which were repeated for one to three times. Table 2 represents mean values from triplicate experiments. All calculations including S.D. were performed a Hewlett-Packard 67 programmable calculator.

RESULTS

HCT-8 cells exposed to 100 μM FURA accumulate the drug in an almost linear fashion for over 30 hr with a proportional increase of FURA incorporation into RNA (Chart 1). All subsequent experiments measuring FURA metabolites or overall accumulation utilized a 6-hr time point as a matter of technical convenience.

Chart 2 demonstrates the effect of pretreating HCT-8 cells with MTX (10 μM, 12 hr) on the metabolism of subsequently administered FURA (100 μM, 6 hr). There was almost a 3-fold increase in total intracellular FURA nucleotides. In MTX-treated cells, FURA accumulation after 6 hr resulted in 5% FUTP, 19% unconverted FURA, and 4% 5-fluorouridine, though the total quantity of all the FURA ribonucleotides was fold less in control cells, the distribution of these intracellular FURA derivatives was similar to that observed in MTX-treated cells. There was also a 5-fold increase in the amount of intracellular soluble FdUMP following MTX pretreatment.

The effects of increasing MTX pretreatment intervals on intracellular FURA accumulation and intracellular PRPP pools are shown in Chart 3. FURA accumulation was nearly maximally enhanced by 3 hr of MTX pretreatment, while PRPP lev continued to increase up to 16 times control values after 72
of MTX pretreatment. Concentrations of MTX as low as 0.1 μM required more than 12 hr of exposure to maximally enhance FUra accumulation (data not shown).

The incorporation of [3H]deoxyuridine into the acid-precipitable fraction was used to determine if the increased intracellular FdUMP in MTX-pretreated cells resulted in a further inhibition of the synthesis of thymidylate and DNA (Chart 4). The inhibition of DNA synthesis was greatest following longer MTX (10 μM) exposure time. FUra (100 μM) was a better inhibitor than 10 μM MTX; however, when MTX was given for 12 hr before adding FUra for another 6 hr, the inhibition was not significantly different from that of FUra alone.

A monolayer cloning assay was utilized to correlate FUra accumulation with tumor cell toxicity and to test the hypothesis that sequenced MTX and FUra are synergistically cytotoxic and schedule dependent. Table 1 shows that 6 hr of FUra (10 μM) did not inhibit clonal growth of HCT-8 cells while 24 hr of MTX (0.1 μM) reduced clonal growth to 60% of control. Higher concentrations of both drugs resulted in greater inhibition of clonal growth which was also synergistic when MTX preceded FUra. The drug doses represented in Table 1 were chosen because of the minimal individual inhibition on clonal growth. FUra given during the first 6 hr of the 24-hr MTX treatment period was not significantly more inhibitory than was MTX alone. However, as the 6-hr FUra dose was given later into the MTX treatment period, synergistic cytotoxicity was observed. Only after 12 hr of MTX pretreatment was the combined inhibition of MTX and FUra significantly greater than that which would be expected if the drugs were acting independently. The greatest reduction in clonal growth occurred when FUra was given during the last 6 hr of the 24-hr MTX exposure.

Other antimetabolites might be expected to alter intracellular pools of PRPP or the enzymatic ribosylphosphorylation of FUra. Table 2 compares the 3- and 18-hr pretreatment effects of 10 μM MMPP, AZS, LAL, PALA, and PF on the intracellular accumulation of FUra. Only PF inhibited FUra accumulation after both exposure intervals, while the remaining drugs enhanced FUra accumulation from 1.4- to 2.8-fold. Although complete dose-response relationships have not been determined, each of these drugs was given at toxic concentrations when measured in the monolayer cloning assay, and except for PF, their schedule dependency in combinations with FUra was found to be dose related.

**DISCUSSION**

The principle intracellular toxic metabolites of FUra are FdUMP (1, 22, 24, 26) and FUTP (10, 14, 24, 25, 27, 33) although an effect on cell membranes may result from the incorporation of FUra into nucleotide sugars which alter glycoprotein synthesis (22). FdUMP binds tightly into a ternary complex with thymidylate synthetase (EC 2.7.4.6) and 5,10-methylenetetrahydrofolate (CH₂FAH₂) leading to inhibition of DNA synthesis (24). FUTP incorporates into heterogeneous mRNA and leads to early inhibition of RNA methylation and processing, transfer RNA formation, and protein synthesis (10, 14, 24, 33). The relative cytotoxic importance of either FdUMP or FUTP varies with the particular cell type or tumor type and depends on the activity of specific enzymatic pathways (23). Decreased pools of ribose 1-phosphate and activity of pyrimidine nucleotide phosphoribosyltransferases or enzymes responsible for interconverting pyrimidine nucleotides can reverse the relative toxic effects of either FdUMP or FUTP (2, 10, 20). Under most circumstances, orotate phosphoribosyltransferase (EC 2.4.2.10) is responsible for the intracellular ribosylphosphorylation of FUra, the activity of which is limited by available pools of the cosubstrate PRPP (8, 28). As with L1210 cells, the colorectal adenocarcinoma, HCT-8, metabolizes FUra to both FdUMP and FUTP (Chart 2), which are associated with inhibition of DNA synthesis (Chart 4) and progressive incorporation into RNA (Chart 1).

Antimetabolites which inhibit salvage and *de novo* synthetic pathways can influence accumulation and toxicity of FUra in HCT-8. MTX inhibits *de novo* purine synthesis by depleting intracellular pools of reduced folate cofactors (9). Synthesis of PRPP, regulated by purine nucleotide levels (4, 13), is subsequently increased as a result of this antipurine effect by MTX. Chart 3 demonstrates that HCT-8 cells progressively accumulate PRPP in the presence of toxic levels of MTX. As was observed in L1210 cells, this increase in PRPP correlates with enhanced intracellular accumulation of FUra. Intracellular accumulation of FUra (100 μM) in untreated HCT-8 cells occurs at one-tenth the rate of untreated L1210 cells despite 10-fold greater PRPP levels (data not shown). This probably represents differences between the enzymatic kinetics for ribosylphosphorylation between these 2 cell lines. Although FUra can be converted to fluorouridine by uridine phosphorylase (EC 2.4.23) and then phosphorylated to 5-fluorouridine monophosphate by uridine-cytidine kinase (EC 2.7.1.48), this is improbable since we have shown previously that MTX pretreatment of HCT-8 cells reduces by 40% the intracellular accumulation of fluorouridine (5). A 10-fold increase in intracellular PRPP in L1210 cells maximally enhanced FUra accumulation 5-fold (7). In HCT-8 cells, a 3-fold increase in PRPP maximally enhanced FUra accumulation 3-fold. Unlike the results in L1210 cells, FUra accumulation in HCT-8 cells was nearly maximally enhanced after 3 hr of MTX pretreatment although PRPP levels continued to increase beyond that time. It is possible that, after this interval of MTX exposure in HCT-8 cells, PRPP concentrations were no longer substrate limiting for ribosylphosphorylation of FUra. Kₘ determinations of FUra ribosylphosphorylation

**Chart 4.** The effect of 10 μM MTX for variable time periods and 100 μM FUra for 6 hr on the incorporation of [3H]deoxyuridine into the acid-precipitable fraction of HCT-8 cells was examined.
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in HCT-8 cells are planned to define more precisely this observation.

We have reported previously that inhibitors of de novo purine synthesis including MMPR and AZS have been shown to increase pools of PRPP and enhance FUra accumulation and cytotoxicity in L1210 cells (30). Both MMPR and AZS inhibit the first enzyme in de novo purine synthesis, amidophosphoribosyltransferase (EC 2.4.12), resulting in decreased purine nucleotide formation and loss of feedback control on PRPP synthesis (11, 18, 31, 34).

LAL is an investigational antimetabolite which acts as an aspartate analog inhibiting de novo purine synthesis beyond the inosine 5'-monophosphate branch point, thus sparing guanine ribonucleotide levels while reducing adenine nucleotide pools (19, 21). We have also shown in L1210 cells that 10 \( \mu M \) LAL is at least as potent as MTX, MMPR, and AZS in elevating PRPP levels, resulting in enhanced FUra accumulation.\(^5\) It is possible that the increase in FUra combination in HCT-8 cells (Table 2) seen at 3 hr but not following 18 hr of LAL pretreatment is a result of early increases in PRPP pools at 3 hr followed by severe depletion of ATP pools by 18 hr which would prevent the further synthesis of PRPP necessary for FUra accumulation.

Combining MTX and FUra can produce both synergistic and antagonistic effects depending on the schedule of drug administration. MTX binds to dihydrofolate reductase (EC 1.5.1.3) preventing the regeneration of the intracellular pools of \( \text{CH}_2\text{FAH}_4 \) which are utilized for thymidylate synthesis. This reduction in \( \text{CH}_2\text{FAH}_4 \) would result in less formation of the inhibitory ternary complex containing FdUMP (24). Therefore, this sequence is theoretically antagonistic on the synthesis of thymidylate and possibly explains the results of combined MTX-FUra on \([\text{3H}]\text{deoxyuridine incorporation into acid-precipitable material (Chart 4). Likewise, FdUMP prevents depletion of CH\text{2FAH}4 pools by inhibiting thymidylate synthetase negating the antipurine effect of MTX (6, 16). Table 1 shows that synergetic toxicity in HCT-8 cells is only when FUra is combined after 12 hr of MTX pretreatment. Cloning studies, not present here, using a concentration of FUra resulting in 25% growth and a concentration of MTX resulting in 70% growth, yielded less than additive growth inhibition (25% growth) when FUra was combined during the first 6 hr interval in a 24-hr MTX treatment period. In this study as in Table 1, synergetic cytotoxicity (<15% growth) was only obtained when FUra was added after a 12-hr MTX pretreatment. Since both FdUMP and FUTP are formed after the initial ribosylphosphorylation of FUra and are enhanced by MTX pretreatment (Chart 2), it is interesting that inhibition of DNA synthesis as measured by \([\text{3H}]\text{deoxyuridine incorporation into acid-insoluble material is not enhanced by MTX pretreatment (Chart 4). These results as well as the cloning data are consistent with the hypothesis that formation of FdUMP in FUra-treated HCT-8 cells is an early metabolic occurrence antagonistic to the cytotoxic effects of MTX. The buildup of PRPP pools during 12- to 18-hr MTX pretreatment could allow for enhanced formation of FUTP, which incorporates into RNA and produces synergetic toxicity despite the antagonistic effects of increased FdUMP formation. This may partially explain the discrepancy between the nearly maximum FUra intracellular accumulation after 3 hr of MTX treatment and the maximum inhibition of clonal growth which occurred after 18 hr of MTX.

Antimetabolites which inhibit de novo pyrimidine synthesis may also act either synergistically or antagonistically with FUra but for different reasons. PALA is a transition-state analog that inhibits aspartate transcarbamylase (EC 2.1.3.2), the second enzyme of de novo pyrimidine synthesis, depleting orotate as well as uridine and cytidine nucleotide pools which are the precursors of the natural substrates competing with the toxic metabolites FdUMP and FUTP. Less significantly, PALA can produce a modest (20 to 50%) elevation of PRPP pools since PRPP is a cosubstrate for the reaction catalyzing the conversion of orotic acid to orotidine 5'-monophosphate (29). Thus, it is not surprising that PALA pretreatment augmented intracellular accumulation of FUra in HCT-8 cells (Table 2) and synergistically enhanced FUra cytotoxicity, as reported in a human mammary carcinoma cell line (3).

PF, on the other hand, reduces the accumulation of FUra into HCT-8 cells to less than 50% of control even after 3 hr of treatment (Table 2). This result also agrees with our data from L1210 cells\(^6\) and is consistent with the mechanism of action of this drug. The 5'-monophosphate derivative of PF is a competitive inhibitor of the decarboxylase activity of the same enzyme complex which ribosylphosphorylates FUra (12). In addition, the orotate pool is elevated more than 1000-fold over control values after doses of PF which reduce intracellular levels of uridine and cytidine nucleotides (7). This increase in intracellular orotate concentration is sufficient to competitively eliminate the ribosylphosphorylation of FUra by orotate phosphoribosyltransferase regardless of available PRPP pools (28).

MTX is ineffective in treating human gastrointestinal cancers, and clinical responses from FUra therapy are less than 30%. Thus, it is of considerable clinical interest to observe that sequential administration of MTX and FUra results in synergistic toxicity in the cultured human colorectal tumor, HCT-8. This information should assist in the rational design of sequential antimetabolite clinical trials in human gastrointestinal carcinoma. Evaluation of freshly procured tumor specimens from patients is currently ongoing in our laboratory to determine if these biochemical studies have any utility as a predictor of clinical tumor sensitivity.

REFERENCES


\(^a\) C. Benz and E. Cadman, unpublished results.
Modulation of FUra Metabolism in HCT-8 Cells


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