The Influence of Methotrexate Pretreatment on 5-Fluorouracil Metabolism in L1210 Cells*

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Pretreatment of L1210 cells with methotrexate in concentrations which produced free intracellular methotrexate and near maximal inhibition of dihydrofolate reductase resulted in an enhancement of intracellular 5-fluorouracil (FUra) accumulation. This enhancement of FUra accumulation was maximum (5-fold increase) after a 6-h exposure to 100 μM methotrexate. The nucleotide derivatives of FUra, including 5-fluoro-2'-deoxyuridylic acid, and 5-fluorouridine-5'-triphosphate were also increased nearly 5-fold following methotrexate treatment. In cells pretreated with methotrexate, there was an increase in intracellular 5-phosphoribosyl-1-pyrophosphate pools which ranged from 2 to 8 times control values following concentrations of methotrexate between 0.1 μM and 10 μM. Both the increase in 5-phosphoribosyl-1-pyrophosphate and FUra accumulation could be prevented by the addition of Leucovorin (N5-formyltetrahydrofolate) at concentrations which rescued cells from the inhibitory effects of methotrexate. Pretreatment with 6-methylmercaptopurine riboside, which inhibits amidophosphoribosyltransferase, the first committed step in de novo purine synthesis, also resulted in a similar elevation in 5-phosphoribosyl-1-pyrophosphate pools and enhancement of FUra accumulation. If the 5-phosphoribosyl-1-pyrophosphate pools were reduced following methotrexate pretreatment by the addition to the cultures of hypoxanthine, which utilizes 5-phosphoribosyl-1-pyrophosphate for the conversion to IMP, the intracellular accumulation of FUra was not enhanced. Also, if the inhibitor of 5-phosphoribosyl-1-pyrophosphate synthetase, 7-deazaadenosine, was given to cultures with methotrexate, there was no increase in 5-phosphoribosyl-1-pyrophosphate pools, nor enhancement of FUra accumulation. In addition, when 5-fluoro-2'-deoxyuridine was added with the methotrexate to cell cultures, there was no increase in 5-phosphoribosyl-1-pyrophosphate pools, nor enhancement of intracellular FUra accumulation.

These results indicate that the ability of methotrexate to enhance FUra accumulation was probably the consequence of the antipurine effect of methotrexate which resulted in a reduction of the complex feedback inhibition on 5-phosphoribosyl-1-pyrophosphate synthetase and utilization. The resultant increased 5-phosphoribosyl-1-pyrophosphate pools were then capable of being utilized for the conversion of FUra to 5-fluorouridylate, the possible rate-limiting step in FUra intracellular metabolism and the major determinant of the rate of intracellular FUra accumulation. When methotrexate preceded FUra, there was synergistic cell killing as determined by soft agar cloning. The exact mechanism of this sequential synergistic antitumor activity may be the result of the enhanced incorporation of FUra into RNA, since the increased 5-fluoro-2'-deoxyuridylate which is formed is unlikely to increase substantially the inhibition of dTMP synthesis induced by methotrexate pretreatment.

Methotrexate and 5-fluorouracil are frequently used in combination for the treatment of cancer, especially in women with breast cancer (1). Antagonism between these two drugs, regardless of drug sequence, on their respective ability to inhibit dTMP synthesis (2, 3) have resulted in concern for the continued concurrent use of these drugs (4). Synergistic antitumor activity on experimental tumors in rodents, however, has been observed when methotrexate preceded FUra (5-7). These conflicting reports indicated that methotrexate and FUra interactions could also be occurring at metabolic sites other than those which influence the formation of dTMP.

The presumed major antitumor derivative of FUra, 5-fluoro-2'-deoxyuridylate, requires the reduced folate, N5,10-methylenetetrahydrofolate, for covalent binding to thymidylate synthetase (EC 2.1.1.45). The result is inhibition of dTMP synthesis (8-10). Because the inhibition of dihydrofolate reductase (EC 1.5.1.3) by methotrexate pretreatment prevents the regeneration of N5,10-methylenetetrahydrofolate, FdUMP is unable to form the ternary complex necessary for prolonged inhibition of dTMP synthesis. Ullman et al. (3) has proposed this mechanism as evidence against the use of this sequence for the treatment of malignancy. Methotrexate, which is a folate analogue, can promote binding of FdUMP to thymidylate synthetase, but the maximal effect is only 21% as efficient as N5,10-methylenetetrahydrofolate (11). This methotrexate-formation of ternary complex has not yet been identified in vitro, and is unlikely to be the total explanation for the in vivo synergism observed in animals when methotrexate is given before FUra.

Bowen et al. (2) have shown that, when 5-fluoro-2'-deoxyuridine precedes methotrexate, theoretical antagonism also occurs. The inhibition of thymidylate synthetase by FdUMP leads to dUMP accumulation (12), and also prevents the oxidation of N5,10-methylenetetrahydrofolate to the inactive dihydrofolate derivative which normally occurs during the methylation of dUMP to dTMP catalyzed by thymidylate synthetase. Therefore, the subsequent administration of methotrexate and inhibition of dihydrofolate reductase would have little effect on the already inhibited dTMP synthesis.

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1 The abbreviations used are: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2'-deoxyuridine; FdUMP, 5-fluoro-2'-deoxyuridylate; FUMP, 5-fluorouridylate; dUrd, 2'-deoxyuridine.
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The effect of the direct inhibition of thymidylate synthetase by FdUMP diminished, there would, however, be 1 increased dUMP pools and 2 available N10-methylentetrahydrofolate pools which could be used for dTMP synthesis in spite of the methotrexate-induced inhibition of dihydrofolate reductase.

Thereafter, the plateaus to synergistic antitumor effects observed when methotrexate is administered prior to FdUMP in the peritoneal cavity are attributable by the known biochemical interactions that occur with thymidylate synthetase and must be due to other interactions not previously described. We have found that methotrexate at doses which maximally inhibit dihydrofolate reductase and, subsequently, dTMP formation lead to a 5-fold increase in the intracellular accumulation of FUrA, primarily as the nucleotide derivatives, and that this sequence results in an augmented incorporation of FUrA derivatives into RNA. This enhanced intracellular metabolism of FUrA that follows methotrexate treatment is a consequence of the intracellular increase of 5-phosphoribosyl-1-pyrophosphate pools and the result of purine synthesis inhibition that occurs with these doses of methotrexate. Portions of this work have been reported in preliminary form elsewhere (13-18).

MATERIALS AND METHODS

Drugs—[6-3H]FUrA (2 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL, and used for intracellular accumulation and ribonucleotide studies. The higher specific activity [6-3H]FUrA (25 Ci/mmol), [2-14C]FdUMP (18 mCi/mmol), [2-3H]FdUMP (20 mCi/ mmol), and [6-3H]FdUr (18 Ci/mmol) were obtained from Moravek Biochemicals, City of Industry, CA. [6-3H]dThd (21.9 Ci/mmol) which was used to evaluate thymidylate synthase function following methotrexate treatment, [2-3H]dadenine (24.5 Ci/mmol) which was used for the 5-phosphoribosyl-1-pyrophosphate assay, and [1-14C]glycine (20 mCi/mm) which was used to evaluate purine synthesis, were produced by New England Nuclear. The methotrexate and N-phosphorylacetamido-L-aspartate were provided by the National Cancer Institute, Bethesda, MD. Pyrazofurin was a gift of Eli Lilly Co., Indianapolis, IN. All other nonradioabeled compounds were purchased from Sigma.

Cells—L1210 murine leukemia cells, with a doubling time of 10 to 12 h, were maintained as stationary suspension cultures in Fischer's medium plus 10% horse serum, transferred twice weekly, and kept at 37°C in a 5% CO2 atmosphere. Tests performed monthly were negative for mycoplasma contamination. All experiments were performed with cells which had been inoculated at 1 to 3 × 10^6 cells/ml and had been in the logarithmic phase of growth for 10 to 20 h, which corresponded to 3 to 5 × 10^6 cells/ml. Exponential growth of cells was performed with a model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

Cloning—The biological antitumor effect of methotrexate and FUrA was determined by cloning L1210 cells in soft agar by the technique we previously reported (19, 20). Following the indicated single drug exposure to logarithmically growing cell cultures, the second drug was added for the specified time. The drug-containing medium was then removed after centrifuging at 1000 × g for 5 min at 37°C. The cell pellet was resuspended in drug-free medium and then recentrifuged as before. This washing procedure was repeated twice to remove any extracellular drug before cloning.

Fifty cells were pipetted into 10-ml culture tubes which contained 2 ml of liquified agar (37°C) and 3 ml of drug-free Fischer's medium plus 15% horse serum. The tubes were capped and placed upright and incubated at 37°C in a 5% CO2 atmosphere. The amount of agar in the culture medium was 0.088 g/100 ml; the consistency of this mixture does not allow cell settling but does permit cell growth. Cells that remain viable after the drug exposure, as defined by having the continued capability to divide and produce progeny, will form individual cell colonies after 10 days of incubation. All clones were counted with an inverted microscope. Each clone contained between 20 and 250 cells, with the majority containing between 20 and 100 cells. Clones were not observed in the cloning medium after the 10-day incubation indicating all viable cells had developed into clones. The per cent viability is the ratio of clones formed from drug-treated cultures to clones formed from untreated cultures, multiplied by 100. The cloning efficiency of L1210 cells in this system was 15%. All experiments were done in triplicate on three separate occasions. Mean values are shown; the maximum range was ±5%.

Total Intracellular FUrA Accumulation—Logarithmically growing cells (2 to 5 × 10^6 cells/ml) were exposed to methotrexate in concentrations from 0.1 to 100 μM for 3 h. After the appropriate methotrexate exposure time, 50 ml of the cell suspension was centrifuged at 1000 × g for 5 min at 37°C. The drug-containing medium was removed and the cell pellet gently resuspended in 2 ml of the initial drug-containing supernatant to which was added 0.1 mI of an N5-(methoxyethyl)adenosine-10-β-D-ribofururonoside-barbiturate (pH 7.0, 10 μM). Over the course of an hour, 0.1 ml aliquots of this cell suspension were gently placed in a 0.4-ml microcentrifuge tube containing 0.04 ml of 0.5 M HClO4 at the tip, which was overlayed with 0.1 ml of a silicone mineral oil mixture of 84:16 proportion (the silicone fluid used was Hi-phenyl 125, DC560, from Wm. F. Nye, Inc., Bedford, MA); the mineral oil used was light mineral oil from Invivex, Chagrin Falls, OH. The tube was then immediately centrifuged at 13,000 rpm for 15 s. The silicone/mineral oil interface allowed the cells to pass into the HClO4-containing tip but kept the drug-containing medium at the top of the tube. The time necessary for complete separation of cells was determined by microscopic examination of the medium and was 15 s. The tubes were then quickly frozen in an ethyleneglycol/dry ice bath, cut at the liquid interface into three fractions: the medium, the oil, and the cell pellet. Radioactivity in each fraction was quantitated; radioactivity was only present in the cell pellet and medium fractions. All experiments were done in duplicate three times. The maximum range of the mean values shown was ±8%. A separate experiment was designed to demonstrate the fractional cell metabolism of FUrA in a drug-containing cell culture medium from entering the tip of the microtube. [3H]FUrA (1 μCi/100 μg) was added to the medium containing cells. Radioactivity was only detected in the medium-containing section of the microtube and not in the cell pellet following centrifugation. Therefore, the radioactivity in the cell pellet fraction is an accurate representation of the total intracellular FUrA which includes the base as well as nucleotide derivatives.

Identical cloning procedures as outlined above as well as trypan blue exclusion determinations were performed on a control group of cells which had been centrifuged from 50 ml of cell suspension following the FUrA-accumulation studies. There was no reduction in cloning efficiency, and greater than 99% of the cells excluded the dye, indicating the high concentration of the cells for the duration of these experiments did not adversely affect cell viability.

Nucleotide Pools—Fifty milliliters of cells under identical conditions as outlined above were exposed first to 1 μM methotrexate for 3 h and then 3 μM FUrA (2 Ci/mmol) for an additional hour. The cells were then centrifuged at 100 g for 5 min, the supernatant was discarded, and the cells were resuspended in phosphate-buffered 0.9% NaCl. To remove any extracellular drug, the cell suspension was washed twice as before. The final cell pellet was precipitated in 1 ml of 0.5 N HClO4 and centrifuged at 1000 × g. The supernatant which contained the nucleotides was neutralized with 4 N KOH, the salt was removed by centrifugation, and the final supernatant was stored at −20°C. All ribonucleotide assays were evaluated consecutively by high pressure liquid chromatography (Altex model 110 A pump microprocessor-controlled gradient system, Altex Scientific, Inc., Berkeley, CA) with a linear gradient (0.01 to 1.0 M) of NaHPO4 (pH 3.31) at a flow rate of 0.9 ml/min on a Whatman Partisil SAX (particle size, 16 μm) column 25 cm × 4.6 mm. Absorbance was recorded at 254 and 280 nm and 0.5-ml fractions were collected of the entire column flow. FUrA, FdRd, FdUMP, FUMP,UMP, UDP, UDP-Glc, and UTP were used as unlabeled markers.

Acid-soluble fractions were also prepared for FdUMP analysis from cells under identical conditions and treatment, except that FUrA of higher specific activity was used (25 Ci/mmol). Perchlorate oxidation of this fraction (12 μM NaHPO4 at 37°C for 30 min, followed by 0.4 M CH3NO2 plus 0.01 N NaOH for 15 min) cleaved the base and phosphates from the ribonucleotides but not the deoxyribonucleotides. On the SAX column, the FUMP, and FdUMP were inseparable and eluted together after periodate treatment. Separation of these two nucleotides, however, was achieved on a polystyrene column (BA-X4, James Benson, Reno, NV) eluted at 50°C with CH3COONa (pH 7.0, 0.5 M) at a rate of 1 ml/min. The only radioactivity remaining after periodate treatment was in the FUMP and FdUMP void volume. The radioactivity appeared in the FUMP region. When the periodate-treated sample was recromatographed on the SAX column, radioactivity was only present in the void volume and the monophosphate region, indicating the oxidation process had
efficiently eliminated the FUDP and FUTP derivatives below detectability. [2-14C]FUDP (20 mCi/mmol) at 70,000 cpm was reduced to background counts (40 cpm) by periodate oxidation, indicating that reactivity was greater than 99.9%. Similarly, whereas [2-14C]FUTP (18 mCi/mmol) at 70,000 cpm was unaffected by this oxidation process.

**FURA Incorporation into RNA**—The acid-precipitable fraction of the cells prepared for ribonuclease analysis was washed repeatedly with cold 5% trichloracetic acid, until no radioactivity was detected in any of this supernatant (six washes). The precipitate was then separated into the RNA, DNA, and protein fractions by the methods of Trakellis and Axelrod (21) and the radioactivity in the RNA and DNA fractions was determined and related to micrograms of D-ribose and deoxyribose by the orcinol (22) and diphenylamine reactions (23), respectively.

**dUrd Incorporation**—Cells were prepared as before for the FURA-accumulation studies and, at designated times after adding [6-3H]-dUrd (21.9 Ci/mmol) to a concentration of 1 µM, 0.025 ml of the cell suspension was placed in harvesting plates (Limburo Scientific, Hamden, CT) and automatically sucked onto a Reeve angle 904AH glass filter strip (Whatman) by use of a MASH cell harvester (Microbiological Associated, Walkersville, MD). The acid-insoluble fraction was then precipitated on the filter strips with 10 ml of 10% trichloracetic acid, washed twice with 10% trichloracetic acid, and then with 5% trichloracetic acid to remove any nonprecipitated label. After drying at room temperature, the cell-precipitated areas were placed in Omni counting vials, (Whatman) and radioactivity was quantitated with a Packard Tricarb liquid scintillation spectrometer (Packard Instrument Co.).

**RESULTS**

**FURA Intracellular Accumulation**—The intracellular FURA accumulation was enhanced following methotrexate exposure and was dependent on the pretreatment concentration of methotrexate (Fig. 1). The maximum enhancement of FURA accumulation was after a 6-h exposure for all methotrexate concentrations. The enhancement of FURA accumulation following methotrexate exposure occurred with concentrations of FURA as high as 1 mM. Leucovorin (N"formyltetrahydrofolate) at a concentration which reversed the methotrexate-induced inhibition of dihydrofolate reductase (24). The same enzymatic method was used to determine the specific activity of dihydrofolate reductase. Protein content was established by the method of Lowry et al. (25).

**Purine Synthesis**—The incorporation of [1-14C]glucose (20 mCi/mmol) into purine bases of nucleic acid was used as a measure of de novo purine synthesis. Cells under identical conditions as outlined in the previous sections were divided into 100-m1 aliquots and either exposed to 10 µM methotrexate, 10 µM FUDr, or both drugs together for 3 h, or they were used as controls. After the 2-h drug exposure, [1-14C]glucose was added to a concentration of 10 µCi/ml. The cells were centrifuged at 10,000 × g for 5 min, and the supernatant was discarded and the cell pellet was precipitated in 1.5 ml of 1 N HClO4 until no radioactivity was detected in 1 ml of 0.5 N HClO4. The samples were then kept at 100°C for 1 h to depurinate the nucleic acid. The supernatant, following centrifugation at 1000 × g, was neutralized with 4 N KOH. The soluble extract was then analyzed on the chromatography system previously described but using an ODS-2 column (Whatman) eluting with 0.1 M sodium acetate, pH 4.6, and an acetonitrile gradient from 0 to 7.5% over 30 min. Radioactivity was quantitated. Glucose appeared in the void volume and did not interfere with the analysis.

**5-Phosphoribosyl-1-pyrophosphate Assay**—Intracellular 5-phosphoribosyl-1-pyrophosphate of L1210 cells after variable drug and exposure conditions is approximated by modifications of a previously reported technique (26). Adenine phosphoribosyltransferase (EC 2.4.2.7) was extracted from the 40 to 70% (NH4)2SO4 fraction of a cell homogenate prepared from 0.6 liter of an L1210 culture at a density of 6 × 106 cells/ml. Following centrifugation at 10,000 × g for 20 min, the supernatant was placed on a G-150 Sephadex column and eluted with 0.2 M Tris, pH 7.4, 20 mM MgSO4, to separate the adenine phosphoribosyltransferase from the 5'-nucleotidase activity. Twenty milliliters of each cell culture from which 5-phosphoribosyl-1-pyrophosphate was to be determined was centrifuged at 4°C for 5 min at 12,000 × g. The cell pellet was resuspended in 5 ml of a HClO4, 0.9% NaCl (pH 7.4); 0.1 ml was removed to compute cell density, and then the pellet was centrifuged 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 s to extract the 5-phosphoribosyl-1-pyrophosphate. Extraction was performed at 15-s intervals to 5 min; the optimal time for maximum 5-phosphoribosyl-1-pyrophosphate quantitation was 90 s. The tubes were then placed on ice and centrifuged for 4 min at 2,000 rpm. the supernatant was removed and stored at −20°C until multiple determinations could be performed simultaneously within 3 weeks. The quantity of the 5-phosphoribosyl-1-pyrophosphate in these extracts exceeded 90% of the original values following storage at −20°C for 3 weeks; by 6 weeks the quantitation had decreased to 75% of these initial values.

To the reaction mixture, which contained 0.1 ml of 0.2 M Tris, pH 7.4, 0.02 of 20 mM MgSO4, and 0.005 ml of [2-3H]adenosine (24.5 Ci/mol) to achieve 30 µM, was added 0.1 ml of the 5-phosphoribosyl-1-pyrophosphate cell extract and then 0.1 ml of the adenine phosphoribosyltransferase extract. The adenine phosphoribosyltransferase converts [2-3H]adenosine to [3H]AMP in the presence of the 5-phosphoribosyl-1-pyrophosphate. After 20 min at 37°C, the reaction was stopped by adding 0.265 ml of 3 M citric acid in methanol and the 3-ml tube containing this assay mixture was placed in ice. One DE81 discs (Whatman) was spotted 0.05 ml of this reaction mixture. The discs were then washed twice with 2 ml of 2 M citric acid in 60% methanol and then with absolute methanol. This washing procedure removed greater than 99% of [3H]adenosine. The discs were dried at room temperature and then placed in scintillation vials, the [3H]AMP was eluted with 1 ml of 0.5 M NaCl in 1 N HCl, and then 8 ml of scintillant was added (15 g of 2,5-diphenyloxazole, 0.3 g of 1.4-bis[2-(5-phenyloxazolyl)]benzene, 1 liter of Triton X-100, and 2 liters of toluene) and counted. The amount of [3H]AMP formed was quantitated. The amount of 5-phosphoribosyl-1-pyrophosphate used in the conversion of adenine to AMP was determined by performing simultaneous controls with known quantities of 5-phosphoribosyl-1-pyrophosphate.
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FIG. 1. Accumulation of FUra into L1210 cells following varying concentrations of methotrexate (MTX). Suspension cultures at 2 x 10^6 cells/ml were exposed to the indicated concentrations of MTX for 3 h. [3H]FUra (2 Ci/mmol) was added to 3 μM and the intracellular accumulation was determined as outlined under “Materials and Methods.”

0.076 μmol of tetrahydrofolate/min/mg of protein, 13.5 times greater than that of the wild or methotrexate sensitive (S) line, which had a specific activity of 0.0056 μmol of tetrahydrofolate/min/mg of protein. The RR line did not transport methotrexate. In addition, the specific activity of dihydrofolate reductase was 0.315 pmol of tetrahydrofolate/min/mg of protein. Augmentation of FUra accumulation in the R cells occurred only following methotrexate concentrations which resulted in free intracellular methotrexate. Free intracellular methotrexate was never present in the RR cell line even following 100 μM methotrexate; nor did methotrexate enhance the rate of FUra accumulation in this cell line (Fig. 5). Therefore, free intracellular methotrexate must be present for enhanced FUra accumulation to occur.

Cloning—Synergistic killing of L1210 cells resulted when methotrexate preceded FUra, but not if the sequence was reversed (Table I). If cells were exposed to 100 μM Leucovorin 2 h after being exposed to 1 μM methotrexate but 1 h before being treated with 1 μM FUra, the viability was equivalent to nondrug-treated cells. 6-Methymercaptopurine riboside also resulted in a synergistic killing of cells when given before FUra. The possible explanation of this observation is examined in subsequent experiments.

Nucleotide Pools—The ribonucleotides and FdUMP were increased 5-fold following a 3-h exposure to 1 μM methotrexate (Table II). No radioactivity was detected following periodate oxidation in the regions which would correspond to FdUDP and FdUTP.

Incorporation of FUra into RNA—Because of the increased quantities of FUTP in response to methotrexate, it was not unexpected to find that the FUra content of RNA from methotrexate-treated cells was 5-fold greater than that present in control cells (Table III). Radioactivity was not detected in the DNA or protein cell fractions. It is unlikely

FIG. 2. The effect of Leucovorin on methotrexate-enhanced intracellular FUra accumulation in L1210 suspension cultures. Leucovorin (100 μM, □) for 1 h did not alter [3H]FUra accumulation (5 μM, 2 Ci/mmol) from control cultures (○). Methotrexate (■) (0.1 μM) for 3 h resulted in enhanced FUra accumulation; however, when Leucovorin (100 μM) was added to the methotrexate-treated cells for 1 more h, the FUra accumulation was not enhanced (■). The effect of higher concentrations of methotrexate on intracellular FUra accumulation was only partially reversed with this concentration of Leucovorin.

FIG. 3. The relationship of free intracellular methotrexate (unbound to dihydrofolate reductase) and the per cent inhibition of [3H]dUrd incorporation into the acid-precipitable cell fraction. The free intracellular methotrexate concentration ([MTX]) and the per cent inhibition of the incorporation of [3H]dUrd into the acid-precipitable fraction of L1210 cells were determined in L1210 cells after a 3-h exposure to doses of methotrexate ranging from 0.1 to 100 μM.
that a 5-fold increase in RNA synthesis is occurring in response to methotrexate treatment. To evaluate this possibility \([1-\text{H}^4]\)glycine (20 mCi/mmol, 10 \(\mu\)M) incorporation into purine bases of nucleic acid was determined, and found to be reduced by 86% following identical methotrexate treatment. The salvaging of performed purines could account for some dilution of the glycine incorporation into RNA. Because purines are incorporated into both DNA and RNA, the reduction in glycine incorporation into nucleic acid in these experiments is only an indication that RNA synthesis, as well as DNA synthesis, is reduced. The precise magnitude of this reduction of RNA synthesis cannot be accurately ascertained from this data.

5-Phosphoribosyl-1-pyrophosphate Levels and \(\text{FURA}\) Metabolism—An increase in 5-phosphoribosyl-1-pyrophosphate levels did occur in cells exposed to methotrexate and was related to the dose and time of methotrexate exposure (Table IV). The increase in 5-phosphoribosyl-1-pyrophosphate levels was also correlated with the increased rate of intracellular \(\text{FURA}\) accumulation (Fig. 6). These results suggested that the enhancing effect of methotrexate on \(\text{FURA}\) accumulation was related to an increased conversion of \(\text{FURA}\) to FUMP by the transfer of the 5-phosphate ribose moiety from 5-phosphoribosyl-1-pyrophosphate. Several experiments were performed to evaluate this possibility. Hypoxanthine requires 5-phosphoribosyl-1-pyrophosphate for conversion to the nucleotide derivative, IMP. Therefore, hypoxanthine would be expected to decrease intracellular 5-phosphoribosyl-1-pyrophosphate pools and reduce the methotrexate effect on both 5-phosphoribosyl-1-pyrophosphate levels and \(\text{FURA}\) accumulation. Table IV shows that 10 \(\mu\)M hypoxanthine for 1 h following 3 h of 1 \(\mu\)M methotrexate reduced the previously elevated 5-phosphoribosyl-1-pyrophosphate levels that were present after a 3-h exposure of 1 \(\mu\)M methotrexate. This concentration of

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\text{FIG. 4. The relationship of free intracellular methotrexate (unbound to dihydrofolate reductase) and intracellular \(\text{FURA}\) accumulation into \text{L1210} cells. The concentration of free intracellular methotrexate (\([\text{MTX}]_{\text{b}}\)) which resulted from a 3-h exposure to concentrations of methotrexate ranging from 0.1 to 100 \(\mu\)M was determined and correlated with the rate of intracellular \([3\text{H}]\text{FURA}\) (2 Ci/mmol, 3 \(\mu\)M) accumulation that resulted from this methotrexate pretreatment.}
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\text{FIG. 5. \(\text{FURA}\) accumulation into two methotrexate resistant mutants of \text{L1210}. \text{L1210 R} had normal methotrexate transport but the specific activity of dihydrofolate reductase was 0.076 \(\mu\)mol of tetrahydrofolate/min/mg of protein. \text{L1210 RR} did not transport methotrexate and also had a specific activity of this enzyme which was increased to 0.315 \(\mu\)mol of tetrahydrofolate/min/mg of protein. The sensitive line (\text{L1210 S}) had a specific activity of 0.0066 \(\mu\)mol of tetrahydrofolate/min/mg of protein. Methotrexate from 0.1 to 100 \(\mu\)M was added for 3 h before adding \([3\text{H}]\text{FURA}\) (2 Ci/mmol, 3 \(\mu\)M). The only enhancement seen in \text{L1210 R} was when free intracellular, nondihydrofolate reductase-bound methotrexate (\([\text{MTX}]_{\text{b}}\)) was present. In the \text{L1210 RR} there was no augmentation of \(\text{FURA}\) accumulation and no \([\text{MTX}]_{\text{b}}\) following methotrexate treatment. Inhibition of \([3\text{H}]\text{Urd}\) into the acid-precipitable fraction of these resistant cell lines was only observed when \([\text{MTX}]_{\text{b}}\) was present.}
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hypoxyanithine given after 3 h of 1 μM methotrexate also reduced the intracellular FURA accumulation to rates observed in control cells (Table V). These interactions among 5-phosphoribosyl-1-pyrophosphate, FURA, and hypoxyanithine provides an explanation for the recent observation that hypoxyanithine and other purine bases which utilize 5-phosphoribosyl-1-pyrophosphate for their metabolic conversions to nucleotides can prevent or reduce FURA cytotoxicity (29, 30).

Pyrazofurin, which, as the monophosphate, is a tight-binding inhibitor of orotidylate decarboxylase (K = 5 nM, EC 4.1.1.23) (31, 32), did not affect 5-phosphoribosyl-1-pyrophosphate values (Table IV), but this drug did prevent FURA accumulation in both the methotrexate-treated and control cells (Table V). The orotate accumulation that occurs following pyrazofurin treatment could be competing with FURA for the available 5-phosphoribosyl-1-pyrophosphate and, therefore, conversion to their respective monophosphorylated nucleotide derivatives. This is unlikely to be the entire cause of the marked reduction in FURA accumulation which followed pyrazofurin treatment, however, because the 5-phosphoribosyl-1-pyrophosphate levels which would be expected to be depleted in the presence of excess orotate were unaffected. These persistent 5-phosphoribosyl-1-pyrophosphate levels could be the result of the antipurine effect of pyrazofurin (30). N-(phosphonacetyl)-l-aspartate, which inhibits aspartate transcarbamylase (EC 2.1.3.2) results in reduced orotate levels (34); therefore, it was not unexpected that this drug resulted in some augmentation of FURA accumulation (Table V). Uridine at 100 μM also resulted in a modest enhancing effect of FURA accumulation of a similar magnitude as that which followed N-(phosphonacetyl)-l-aspartate treatment. This, too, is not unexpected, since this concentration of uridine inhibits de novo pyrimidine synthesis and also reduces orotate formation (35). Flourouridine accumulation studies were performed to determine if this nucleoside might be responsible for the enhanced FURA accumulation which followed methotrexate. This nucleoside would first need to be formed by uridine phosphorylase (EC 2.4.1.3) acting on FURA (36) and then subsequently be phosphorylated by uridine-cytidine kinase (EC 2.7.1.48) (37) to form FUMP. Following methotrexate's influence on 5-Fluorouracil metabolism.

**Table II**

| Nucleotide | Control | Methotrexate |
|------------|---------|--------------|
|            | pmol/10⁶ cells |            |
| FURA       | 0.5     | 0.45         |
| FUMP       | 0.25    | 1.75         |
| FUdUMP     | 0.45    | 2.50         |
| FUTP       | 1.20    | 5.50         |
| FdUMP      | 0.027   | 0.134        |

**Table III**

| Time | 0 μM methotrexate | 1 μM methotrexate |
|------|-------------------|-------------------|
| h    | cpm/μg RNA        |                   |
| 0.5  | 200               | 225               |
| 1    | 400               | 800               |
| 2    | 800               | 2200              |
| 4    | 1500              | 4000              |

**Table IV**

| Drug 1 | Drug 2 | Concentration | Exposure | PRPP |
|--------|--------|---------------|----------|------|
|        |        | 1 μM          | h        | ng/10⁶ cells |
|        | 0      | 7 ± 1.2       |          |      |
| MTX    | 0.1    | 3             | 14.1     |      |
| MTX    | 1      | 3             | 32.0     |      |
| MTX    | 1      | 3             | 52.2     |      |
| MTX    | 1      | 1             | 10.0     |      |
| MTX    | 1      | 2             | 20.0     |      |
| MTX    | 1      | 3             | 32.0     |      |
| MTX    | 1      | 5             | 59.0     |      |
| MTX    | 1      | 24            | 10.3     |      |
| Hyp    | 1      | 1             | 3.8      |      |
| MTX    | Hyp    | 10            | 1        | 1    |
| MTX    | PF     | 5             | 3        | 12.2 |
| MTX    | PF     | 5             | 3        | 40.0 |
| Tbc    | 10     | 1             | <0.7     |      |
| Tbc    | 10     | 10            | 2        | 2.0  |
| MMPR   | 1      | 4             | 110.2    |      |
| FdUrd  | 100    | 3             | 10       |      |
| FdUrd  | MTX    | 100           | 3        | 10   |

**Table V**

| Drug 1 | Drug 2 | Concentration | Exposure | FURA accumulation |
|--------|--------|---------------|----------|-------------------|
|        |        | 1 μM          | h        | pmol/10⁶ cells    |
|        | 0      | 0.025         |          |      |
| MTX    | 1      | 0.083         |          |      |
| MTX    | 10     | 0.108         |          |      |
| Hyp    | 10     | 0.013         |          |      |
| MTX    | Hyp    | 10            | 1        | 1    |
| PF     | PALA   | 10            | 1        | 1    |
| PF     | MTX    | 5             | 3        | UD   |
| PF     | Tbc    | 5             | 3        | UD   |
| PF     | MMPR   | 10            | 1        | 3    |
| PF     | FdUrd  | 100           | 3        | 1    |
| PF     | MTX    | 100           | 3        | 3    |

**Materials and Methods**

The FURA incorporation into RNA of logarithmically growing L1210 cells following methotrexate treatment. Following 3 h exposure to 1 μM methotrexate, ['H]FURA (2 Ci/mmol) was added to 3 μM and the RNA was separated at 0.5, 1, 2, and 4 h. The experimental conditions were as outlined under "Materials and Methods." These are the mean values of three experiments with the greatest range being ±15%.
Methotrexate's Influence on 5-Fluorouracil Metabolism

5-Phosphoribosyl-1-pyrophosphate—The purine synthetic rate, as measured by the incorporation of $[1^{-14}C]$glycine into the adenine and guanine bases of nucleic acids, was reduced by 85% following a 2-h exposure to 10 μM methotrexate. The likely explanation of the mechanisms by which methotrexate leads to an elevation of 5-phosphoribosyl-1-pyrophosphate is the result of the inhibition of de novo purine synthesis. The following experiments were performed to test this hypothesis.

7-Deazaadenosine (tubercidin) is a nucleoside analogue which does not require 5-phosphoribosyl-1-pyrophosphate for metabolic conversion but does inhibit 5-phosphoribosyl-1-pyrophosphate synthetase (EC 2.7.6.1) (38). The 5-phosphoribosyl-1-pyrophosphate synthetase activity was determined in L1210 cells as described under "Materials and Methods." The rate of [3H]FUra accumulation was linear for the duration of these studies; mean values are shown. The range was ±8%.

| Drug          | Concentration (μM) | Control in adenine and guanine pmol [14C]glycine into Ade and Gua per 10^6 cells in 1 h |
|---------------|--------------------|-----------------------------------------------------------------------------------------------|
| MTX           | 10                 | 15                                                                                           |
| FdUrd         | 100                | 100                                                                                          |
| FdUrd and MTX | 100 and 10         | 130                                                                                          |

Table VI

Effect of a 3-h exposure of methotrexate and fluorodeoxyuridine on the incorporation of $[1^{-14}C]$glycine into purine bases

The effect of methotrexate (MTX) and FdUrd on the incorporation of $[1^{-14}C]$glycine into the adenine and guanine bases of nucleic acid of L1210 cells. Both drugs were incubated for 3 h either separately or together. The values represent the mean of three experiments; the range was ±25%. Control was 0.20 ± 0.05 (S.D.) pmol of [14C]glycine into Ade and Gua per 10^6 cells in 1 h.

Fig. 7. The proposed metabolic interaction between methotrexate and FUra metabolism. Enzymatic steps: 1, aspartate transcarbamoylase; 2, orotate phosphoribosyltransferase; 3, orotidylate decarboxylase; 4, thymidylate synthetase; 5, dihydrofolate reductase; 6, amidophosphoribosyltransferase; 7, glycaminidase ribonucleotide transformylase; 8, aminooimidazole carboxamidribonucleotide transformylase; PRPP, 5-phosphoribosyl-1-pyrophosphate; THF, tetrahydrofolate; DHF, dihydrofolate; PR, 5-phosphoribosyl ribose, PALA, N-(phosphonoacetyl)-L-aspartate; PF, pyrazofurin; MTX, methotrexate; MPPR, 6-methylmercaptopurine riboside.
Methotrexate's Influence on 5-Fluorouracil Metabolism

5-fluorouracil (FUra) is phosphorylated by adenosine kinase (EC 2.7.1.20), and in the nucleotide forms inhibits amidophosphoribosyltransferase (EC 2.4.2.14) (39). The consequence of this reaction is inhibition of de novo purine synthesis and increased levels of 5-phosphoribosyl-1-pyrophosphate (Table IV). 6-Methylmercaptopurine riboside like methotrexate, also resulted in enhanced intracellular FUra accumulation (Table V) and probably accounts for the cytotoxic effect when this drug preceded FUra (Table I).

Finally, the pretreatment of cells with 5-fluoro-2-deoxyuridine (FdUrd), which is phosphorylated by thymidine kinase (EC 2.7.1.21) to FdUMP (40), the derivative which inhibits thymidylate synthetase, prevented the effects of methotrexate on purine synthesis, 5-phosphoribosyl-1-pyrophosphate pools, and intracellular FUra accumulation (Tables IV to VI).

**DISCUSSION**

These findings demonstrate that the metabolism of FUra by L1210 cells can be altered with methotrexate pretreatment. The increased amounts of the phosphorylated derivatives of FUra which are formed indicate that either the entry of FUra into cells has increased or the activity of a metabolic process has been enhanced. Since the quantity of the base, FUra, within methotrexate-treated cells was unchanged from control cells, it is unlikely that the effect being observed is related to the transport of FUra into the cells.

FUra conversion to nucleotides can occur via three metabolic pathways. FUra can react with ribose-1-phosphate in the presence of uridine phosphorylase to form FUrU (30) which is then phosphorylated to FUMP by uridine-cytidine kinase (37). The intracellular accumulation of [14C]FUra into L1210 cells was reduced in response to methotrexate pretreatment and previously reported by us (18). Therefore, these sequential metabolic conversions of FUra are not responsible for the enhanced metabolism of FUra to nucleotides observed in methotrexate-treated cells. FUra can also be converted to FdUrd by the deoxyribose phosphorylases (36). This nucleoside is then phosphorylated to FdUMP by thymidine kinase (40). However, since FdUDP was not observed and because ribonucleotide reductase cannot convert FdUDP to FUDP (41), which was increased in the methotrexate-treated cells, this pathway is inconsistent with our findings. The only metabolic pathway which is consistent with our data is the conversion of FUra directly to FUMP by orotate phosphoribosyltransferase (42). This reaction requires 5-phosphoribosyl-1-pyrophosphate as a co-substrate for the source of the 5-phosphate ribose moiety. The FUMP can then be converted to the other nucleotide forms, including FUTP and FdUMP, the cytotoxic metabolites (Fig. 7).

The proposed mechanism by which methotrexate influences this metabolic conversion of FUra is the increased 5-phosphoribosyl-1-pyrophosphate levels which occur in methotrexate-treated cells. Reyes (42) has shown that the rate of FUMP formation from FUra by orotate phosphoribosyltransferase is increased with increasing concentrations of 5-phosphoribosyl-1-pyrophosphate. Reyes also demonstrated that this conversion of FUra to FUMP was nearly completely inhibited by orotate, which also requires the same enzyme and co-substrate to form the nucleotide, OMP. We have examined the $K_m$ values of FUra and orotate conversion to the 5'-phosphate nucleotide derivative by orotate phosphoribosyltransferase in these L1210 cells and found them to be 520 μM and 12 μM, respectively. (The enzymatic changes following methotrexate treatment will be published in detail elsewhere.) The large difference between these $K_m$ values indicate that small changes in orotate levels within the cell could greatly affect the initial metabolic step of FUra. This may explain the marked enhancement of intracellular FUra accumulation within cells exposed to N-(phosphonomethyl)-L-aspartate which can reduce intracellular orotate levels (34). The inhibitory effects of pyrazofurin, an inhibitor of OMP decarboxylase, on FUra intracellular accumulation is probably the result of the increased orotate levels in pyrazofurin-treated cells (31). Although OMP cannot be decarboxylated to UMP in the presence of this drug, this nucleotide is converted to the nucleoside, orotidine, which is increased (31). Therefore, even though the levels of 5-phosphoribosyl-1-pyrophosphate are not depleted in response to pyrazofurin, there is a constant utilization of this co-substrate by the preferred substrate orotate.

Our studies indicate that the modulating effect of methotrexate on FUra metabolism is the result of the inhibition of de novo purine synthesis. Methotrexate's influence on purine synthesis is not a new concept (43, 44), although the cell killing from methotrexate has generally been considered to be secondary to the effect of this drug on the formation of dTMP (44). An increase in the concentration of the purine nucleotides can reduce 5-phosphoribosyl-1-pyrophosphate generation (45); therefore, a reduction in these modulating nucleotides might lead to an increased synthesis of 5-phosphoribosyl-1-pyrophosphate. Although we did not quantitate the concentrations of purine nucleotides in our studies, we did demonstrate that the synthesis of adenine and guanine bases which were incorporated into nucleic acid was reduced in the methotrexate-treated cells. The other possible mechanism by which 5-phosphoribosyl-1-pyrophosphate levels are increased in response to inhibition of the purine synthetic rate is from a decreased utilization of 5-phosphoribosyl-1-pyrophosphate which is normally used for purine synthesis. Cells which were deficient in hypoxanthine-guanine phosphoribosyltransferase and were unable to salvage hypoxanthine or guanine had increased levels of 5-phosphoribosyl-1-pyrophosphate even though the synthesis of 5-phosphoribosyl-1-pyrophosphate was normal (46). The elevation of 5-phosphoribosyl-1-pyrophosphate in these cells was thought to be the result of 5-phosphoribosyl-1-pyrophosphate underutilization. The activity of 5-phosphoribosyl-1-pyrophosphate synthetase, however, was increased 3-fold in human fibroblasts exposed to the antifolate, aminopterin (47). In this study, as in ours, the increase in 5-phosphoribosyl-1-pyrophosphate levels was prevented if hypoxanthine, which utilizes 5-phosphoribosyl-1-pyrophosphate for conversion to IMP, was also present in the cell cultures exposed to the antifolate. The FUra metabolism of cells exposed to 6-methylmercaptopurine riboside, which inhibits the first committed enzymatic step in de novo purine synthesis, was identical with that which followed methotrexate treatment. This observation is consistent with what would be expected if inhibition of de novo purine synthesis is the major determinant of 5-phosphoribosyl-1-pyrophosphate levels.

The enhanced FUra metabolism and the reduction in purine synthesis in cells exposed to methotrexate can be prevented if FdUrd is given with methotrexate to the cell cultures. This suggests that if the utilization of the tetrahydrofolate pools for dTMP synthesis can be prevented by a direct inhibitor of thymidylate synthetase, FdUMP, purine synthesis can continue for the duration of these studies in the presence of
methotrexate. In normal cells, N^5,10-methylenetetrahydrofolate is oxidized to a metabolically inactive dihydrofolate derivative only during the methylation of dUMP to dTMP, a reaction catalyzed by thymidylate synthetase. Dihydrofolate is then reduced by dihydrofolate reductase to a tetrahydrofolate derivative which can now undergo conversion to one of several metabolically active tetrahydrofolate compounds. The tetrahydrofolate compounds used for de novo purine synthesis are not oxidized and therefore can be converted to metabolically active derivatives independent of dihydrofolate reductase.

The consequence of the inhibition of dihydrofolate reductase by methotrexate on folate metabolism would be a gradual depletion or alteration of the tetrahydrofolate pool sizes from the continued utilization of N^5,10-methylenetetrahydrofolate for dTMP synthesis without an equivalent regeneration of a tetrahydrofolate. Eventually, the purine synthetic rate would decrease because of this alteration in the tetrahydrofolate pools. However, the alteration of the tetrahydrofolate pools could be prevented by the direct inhibition of the process which converts N^5,10-methylenetetrahydrofolate to the inactive dihydrofolate form. The known inhibitory effects of the major nucleotide form of FdUMP, FdUMP, on thymidylate synthetase and the ability of this drug to prevent the inhibitory effects of methotrexate on purine synthesis in our studies is consistent with this hypothesis.

Leucovorin (N^5-formyltetrahydrofolate) can reduce the effectiveness of the methotrexate-induced inhibition of dihydrofolate reductase (44, 48) presumably by replenishing the tetrahydrofolate pools. A recent report by Moran et al. (49) has shown that concentrations of Leucovorin (0.1 μM) which are unable to rescue L1210 cells from the lethal effects of 10 μM methotrexate are capable of preventing this cytotoxicity if 3 μM FdUrd and 5.5 μM dThd were also present in the cell cultures. Presumably, the FdUrd reduced the utilization of N^5,10-methylenetetrahydrofolate and, therefore, less exogenous tetrahydrofolate was needed to maintain the tetrahydrofolate pools at a level required to support purine synthesis. The dThd provided an exogenous source for dTMP, the synthesis of which was inhibited by the FdUrd metabolite, FdUMP. These studies illustrate the dynamic relationship among the folate pools, purine synthesis, and dTMP synthesis, and are concepts consistent with our proposed complex interactions between methotrexate and FURA metabolism.

The exact mechanism responsible for the synergistic cell killing observed in cell cultures treated with methotrexate followed by FURA can not be answered from our studies. Legitimate reasons why there might be less ternary complex formation among FdUMP, N^5,10-methylenetetrahydrofolate and dTMP synthetase have already been discussed. However, the increased incorporation of FURA into RNA of cells pretreated with methotrexate could be contributing to this enhanced cell killing, perhaps by altering further the functional RNA defects that occur with FURA (50–53).

Further investigations need to be performed to determine if the biochemical modulations that were observed in L1210 cells also occur in human neoplastic and normal cells. In addition, evaluation of tissue selectivity to this drug sequence is important if a major therapeutic benefit is to be expected without increasing toxicity to normal tissues.

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