Evidence for Direct Inhibition of de Novo Purine Synthesis in Human MCF-7 Breast Cells as a Principal Mode of Metabolic Inhibition by Methotrexate*

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We have investigated the role of dihydrofolate (H₂PteGlu) accumulation in the inhibition of de novo purine synthesis by methotrexate (MTX) in human MCF-7 breast cancer cells. Previous studies have shown that cytotoxic concentrations of MTX that inhibit dihydrofolate reductase produce only minimal depletion of the reduced folate cofactor, 10-formyltetrahydrofolate, required for purine synthesis. At the same time, de novo purine synthesis is totally inhibited. In these studies, we show that 10 μM MTX causes inhibition of purine synthesis at the step of phosphoribosylaminomimidazolecarboxamide (AICAR) transformylase, as reflected in a 2–3-fold expansion of the intracellular AICAR pool. The inhibition of purine synthesis coincides with the rapid intracellular accumulation of H₂PteGlu, a known inhibitor of AICAR transformylase. When the generation of H₂PteGlu is blocked by pretreatment with 50 μM 5-fluorodeoxyuridine (FdUrd), an inhibitor of thymidylate synthase, MTX no longer causes inhibition of purine synthesis. Intermediate levels of H₂PteGlu produced in the presence of lower (0.1–10 μM) concentrations of FdUrd led to proportional inhibition of purine biosynthesis, and the exogenous addition of H₂PteGlu to breast cells in culture re-established the block in the purine synthesis in the presence of FdUrd and MTX. The early phases of inhibition of purine biosynthesis could be ascribed only to H₂PteGlu accumulation. MTX polyglutamates, also known to inhibit AICAR transformylase, were present in breast cells only after 6 h of incubation with the parent compounds and were not formed in cells preincubated with FdUrd. The lipid-soluble antifolate trimetrexate, which does not form polyglutamates, produced modest 10-formyltetrahydrofolate depletion, but caused marked H₂PteGlu accumulation and a parallel inhibition of purine biosynthesis. This evidence leads to the conclusion that MTX and the lipid-soluble analog trimetrexate cause inhibition of purine biosynthesis through the accumulation of H₂PteGlu behind the blocked dihydrofolate reductase reaction.

It produces cytotoxic effects through the inhibition of the de novo synthesis of purines, thymidylate, and certain amino acids (1). Many investigations have demonstrated that inhibition of purine synthesis is an important component in MTX-induced cytotoxicity. Whereas either purine (hypoxanthine) or thymidine can partially rescue murine sarcoma 180 cells, both are required to effect complete rescue from the toxic effects of MTX (2, 3), a conclusion corroborated with work in vitro and in vivo systems using normal and malignant murine and human cell lines (4–10). Finally, more recent studies have demonstrated the ability of inosine alone to obviate completely the toxicity of mice treated with high-dose MTX (10 μg/h for 72 h), whereas thymidine alone was ineffective (11). Each of these studies suggests an important role for the inhibition of de novo purine synthesis in the cytotoxic action of MTX.

The previous concept of MTX action has been that the drug inhibits the metabolic pathways by depleting reduced folates as a consequence of its primary action, the inhibition of dihydrofolate reductase (EC 1.5.1.3, 5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase). In the face of dihydrofolate reductase inhibition, the intracellular pools of reduced folate are converted to H₂PteGlu by the thymidylate synthase reaction and cannot be reduced to the H₂PteGlu state required for cofactor activity. This hypothesis provides a mechanism whereby reduced folates would ultimately become trapped as H₂PteGlu and metabolic reactions requiring reduced folate coenzymes would cease. This indirect mechanism of metabolic inhibition fails to account for the competitive nature of Leucovorin rescue (1, 4, 12, 13) and is inconsistent with evidence that certain reduced folate coenzyme pools are relatively preserved during exposure to MTX (14–16). In particular, we found that 5-methyl-H₂PteGlu and H₂PteGlu were the only reduced folates depleted by greater than 50% in the presence of MTX; 10-formyl-H₂PteGlu, the required folate coenzyme for de novo purine synthesis, was preserved at 80% of control levels for up to 21 h during exposure of human MCF-7 breast cells to 1 μM MTX, a concentration that markedly curtailed de novo purine synthetic activity. In addition, we found that the rapid accumulation of H₂PteGlu in the MCF-7 breast cells following MTX exposure correlated temporally with the inhibition of de novo purine synthetic activity and that the polyglutamates of this oxidized folate as well as MTX polyglutamates potently inhibit AICAR transformylase (17). These studies suggest that inhibition of the de novo purine pathway may occur through direct enzyme inhibition by the polyglutamates of H₂PteGlu and/or MTX rather than by depletion of 10-formyl-H₂PteGlu. This study provides further evidence for a direct inhibition of the folate-requiring purine synthetic enzymes in that it demonstrates a close temporal and quantitative relationship between the ac-

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Methotrexate (MTX); 4-amino-10-methylpteroylglutamic acid) has gained widespread use for the treatment of cancer.

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* The abbreviations used are: MTX, methotrexate; H₂PteGlu, dihydrofolate; H₃PteGlu, tetrahydrofolate; AICAR, 5-aminolevulinate-4-carboxamide-1-β-D-ribofuranosyl-5'-monophosphate; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; FdUrd, 5-fluorodeoxyuridine.
cumulation of H,PteGlu and the inhibition of de novo purine synthesis under a variety of conditions. Moreover, this report lends additional support for the concept of direct inhibition of AICAR transformylase by dihydrofolate polyglutamates as the principal mechanism of de novo purine inhibition by MTX.

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**EXPERIMENTAL PROCEDURES**

**Materials**

Methotrexate, methotrexate polyglutamates (Glu, Glu), and tri-metatrexate acetate/glucuronate were supplied by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). [3H]Methotrexate (specific activity, 18 Ci/mmol) and [3',5',7,9-3H]folate (specific activity, 40 Ci/mmol) were purchased from Mound Radiochemicals Inc. (Brea, CA). [2-14C]Glycine (specific activity, 42.3 mC/mmole) was obtained from Du Pont-New England Nuclear. Sep-Pak C18 cartridges and Pic reagent A were purchased from Waters Associates (Milford, MA). 5-Fluoro-2-deoxyuridine (FUDr), thymidine, adenosine, guanine, guanosine, and 5-aminoadenozole-4-carboxamide-1-p-d-ribofuranylosyl-5-monophosphate (AICAR) were purchased from Sigma. Tetrabutylammonium phosphate was purchased from Eastman Kodak. AICAR transformylase was purified 300-fold from human breast cancer cell line (specific activity, 0.3 pmol/min/mg) (17). All other chemicals were of the highest purity available and were purchased from either Fisher or Sigma.

**Methods**

**Cell Culture**—A late-passage, mycoplasma-free human MCF-7 breast cancer cell line was used for all experiments requiring intact cells. The cells were grown in T-75 tissue culture flasks (Falcon Labware, Oxnard, CA) in 15 ml of RPMI 1640 (Biofluids, Rockville, MD) medium with 2 mmol/L glutamine and 10% heat-inactivated (56°C for 30 min), dialyzed fetal bovine serum. Fetal bovine serum was dialyzed against 0.9% NaCl in a 1:40 ratio for 5-24 h exchanges. The cells were grown for 72 h in media from the previous day, and harvested in 1 ml amounts of time, followed by three washes with ice-cold PBS. The cells were then harvested in 1 ml of PBS with a rubber policeman. 100-μl aliquots of the cellular suspension were used to measure total protein, and the intracellular folates were extracted from the remainder of the suspension. The extracted folates were treated with partially purified hog kidney conjugase, concentrated using a Sep-Pak C18 cartridge, and loaded onto an HPLC column for separation and quantitation of the H-labeled intracellular folate pools.

**Intracellular MTX Polyglutamates—MTX polyglutamates were measured by plating MCF-7 cells onto T-75 tissue culture plates, and after 72 h of growth, the cells were exposed to various drugs and [3H]MTX (specific activity, 21 Ci/mmol). The cells were then washed three times with PBS, followed by harvesting in 5 ml of ice-cold PBS with a rubber policeman. The cells were sedimented at 200 × g for 10 min, and the supernatant was decanted. One ml of 10% trichloroacetic acid was added to the cell pellet, and the precipitated protein was used as a measure of total cell protein. The MTX polyglutamates found in the supernatant were concentrated using a Sep-Pak C18 cartridge and loaded onto an HPLC column for separation and quantitation according to published methods (19).

**Intracellular Folate Pool Measurements**—IntraceUular folate pools were measured by plating MCF-7 cells onto T-75 tissue culture plates, and after 72 h of growth, the cells were exposed to various combinations of drugs and [3H]MTX (specific activity, 21 Ci/mmol). The cells were then washed three times with PBS, followed by harvesting in 5 ml of ice-cold PBS with a rubber policeman. The cells were sedimented at 200 × g for 10 min, and the supernatant was decanted. One ml of 10% trichloroacetic acid was added to the cell pellet, and the precipitated protein was used as a measure of total cell protein. The MTX polyglutamates found in the supernatant were concentrated using a Sep-Pak C18 cartridge and loaded onto an HPLC column for separation and quantitation according to published methods (19).

**Intracellular AICAR Transformylase**—The catalytic activity of AICAR transformylase was measured using a spectroscopic assay (20). A 1-ml reaction cuvette contained 0.02 unit of 300-fold purified human AICAR transformylase (1 unit = 1 μmol of H,PteGlu formed per min at 37°C), the folate cosubstrate, 1-10-formyl-H,PteGlu, and various concentrations of inhibitor in 25 mM KCl, 50 mM 2-mercaptoethanol, and 50 mM Tris/HCl, pH 7.4. After a 10-min equilibration period at 37°C, the reaction was initiated with the addition of 50 nmoI of AICAR. The reaction velocity was measured as the change in optical density at 298 nm using an extinction coefficient for the reaction of 19,700 cm⁻¹ molar. The intracellular AICAR transformylase activity was measured by [14C]Glycine Labeling—Intracellular nucleotide pools were measured by plating MCF-7 cells as outlined above with the addition of [14C]Glycine (specific final activity, 5.17 mCi/mmol). After 72 h of growth, the cells were treated with 10 μM MTX or trimetrexate for various timed intervals and washed three times with ice-cold PBS, and the nucleotides were extracted with 1 ml of 10% perchloric acid. The nucleotides were then neutralized with 1 N KOH and separated by an HPLC system based on that described by Brown and Parks (21). We used a Waters HPLC model 6000-A solvent delivery system, Model 440 absorbance detector, and a Model 720 system controller and data module. We used a Waters No. 8 convex gradient with a flow rate of 1 ml/min. The gradient time was 50 min from 100% low-concentration eluent to 100% high-concentration eluent. After completion of the gradient, the column was eluted for an additional 40 min with 100% high-concentration eluent, and peaks were identified by the use of radioactivity standards. To quantitate the incorporation of [14C]glycine into AICAR, we collected the effluent in 1.0-min (1 ml) fractions and determined radioactivity in each fraction by liquid scintillation spectrometry. The retention time for AICAR was found to be 40 min.

**Radiolabeled AICAR Transformylase**—For these experiments, MCF-7 cells, 3 days after replating, were treated with 10 μM MTX or trimetrexate for various times prior to harvesting in 1 ml of ice-cold PBS. The cells were disrupted by sonication with three 2-s bursts from a Branson sonifier 350 equipped with a microtip. Follow-ing 10-min centrifugation at 2000 × g, a 100-μl aliquot was reserved for protein analysis, and the supernatant was then decanted and used for nucleotide analysis. The extracted nucleotides were then concentrated using a Sep-Pak C18 cartridge as follows. The Sep-Pak cartridge was washed with 10 ml of methanol, followed by 10 ml of double-distilled water, and then 2 ml of 5 mM tetrabutylammonium phosphate, pH 5.5, followed by an addition of water to 3 ml, and then loaded onto the cartridge and washed with 5 ml of water. Three ml of methanol was then used to elute the nucleotides from the Sep-Pak cartridge and concentrated using a small column, followed by drying under a stream of nitrogen. The dried nucleotides were resuspended in 50 μl of water and separated by HPLC as described above.

**Purine Synthetic Activity**—De novo synthetic activity was measured according to published methods (18). MCF-7 breast cells (2 × 10⁶ cells) were plated onto T-75 tissue culture flasks (Falcon Labware, Oxnard, CA) in 15 ml of RPMI 1640 (Biofluids, Rockville, MD) medium with 2 mM glutamine and 10% heat-inactivated (56°C for 30 min), dialyzed fetal bovine serum. Fetal bovine serum was dialyzed against 0.9% NaCl in a 1:40 ratio for 5-24 h exchanges. The cells were grown for 72 h after plating (50-70% confluency) prior to use in any experiments. Furthermore, all cells were grown in dialyzed fetal bovine serum for at least two passages prior to their use in experiments.

**De novo Purine Synthetic Activity**—De novo purine activity was measured according to published methods (18). MCF-7 breast cells (2 × 10⁶ cells) were plated onto T-75 tissue culture flasks (Falcon Labware, Oxnard, CA) in 15 ml of RPMI 1640 (Biofluids, Rockville, MD) medium with 2 mM glutamine and 10% heat-inactivated (56°C for 30 min), dialyzed fetal bovine serum. Fetal bovine serum was dialyzed against 0.9% NaCl in a 1:40 ratio for 5-24 h exchanges. The cells were grown for 72 h after plating (50-70% confluency) prior to use in any experiments. Furthermore, all cells were grown in dialyzed fetal bovine serum for at least two passages prior to their use in experiments.

Radioisotope dilutional effects were minimized by relatively long incubation times (1 h) and a high mass of external glycine (final concentration in media, 0.13 mM). To ensure that the observed changes in de novo purine activity were not the result of changes in the intracellular specific activity of [14C]glycine, intracellular glycine pools were measured using an automatic amino acid analyzer (Beckman Instruments) in control cells and cells exposed to MTX (1 μM) and thymidine (10 μM) for 2, 5, and 24 h. The specific radioactivity in MTX-treated cells was found to be indistinguishable from control cells under these experimental conditions. The glycine content in

Glu, refers to the total number of glutamyl residues in the compound, e.g. MTX-Glu = 4-amino-10-methylpteroylglutamyl-y-glutamic acid.
cells were processed simultaneously. As illustrated in Fig. 1 the assay system described under the "Methods," we found which of the two enzymes was inhibited by the antifolate.

Intracellular levels of AICAR were measured by two different techniques after exposure of the MCF-7 cells to 10 μM MTX or trimetrexate for various timed intervals: 1) direct measurement of the AICAR pool using UV detection of the nucleotide separated by HPLC, and 2) [14C]glycine pool labeling, followed by nucleotide separation on HPLC and quantitation by scintillation counting of the peak identified as AICAR. For all experiments, control (unexposed) cells and experimental cells were processed simultaneously. As illustrated in Fig. 1 (A and B), both techniques demonstrate a 2.3-fold increase in the intracellular level of AICAR when the MCF-7 cells were exposed to either MTX or trimetrexate. Using the direct UV measurement of intracellular AICAR, the mean amount of this nucleotide found in the control MCF-7 cells was 6.1 ± 1.2 pmol/mg of cytosolic protein (n = 5). These studies suggest that inhibition of the de novo purine pathway occurs at the level of AICAR transformylase.

Whereas MTX is capable of forming polyglutamates that are potent inhibitors of AICAR transformylase, trimetrexate cannot form polyglutamates; we next determined whether trimetrexate may also be an inhibitor of this enzyme. Using the assay system described under the "Methods," we found that trimetrexate had no effect on the velocity of the reaction catalyzed by purified human AICAR transformylase at concentrations of drug up to 10^{-4} M (data not shown). Thus, the inhibition of AICAR transformylase could not be ascribed to direct effects of the antifolates or their polyglutamates on the enzyme.

Additional evidence against direct inhibition of AICAR transformylase by MTX polyglutamates came from the following experiments. 1) For the rate of formation of MTX polyglutamates in MCF-7 cells, we exposed MCF-7 cells to 1 μM MTX for various times, monitored the activity of the de novo purine pathway using [14C]glycine incorporation into the products of the pathway (adenine/guanine) (Fig. 4), and correlated these findings with the rate of formation of the higher polyglutamates of MTX (Glu3). As depicted in Fig. 4, the activity of the purine pathway is only 27% of untreated control cells after 2 h of exposure and 10% after 5 h. Examination of the rate of higher MTX polyglutamate formation revealed no higher MTX polyglutamates after 2 h of exposure and only 30 ± 5.7% of the intracellular MTX in the form of higher polyglutamates after 6 h. After 24 h of drug exposure, 68 ± 4.5% of the intracellular MTX is in the higher polyglutamate form. 2) To determine the effect of trimetrexate on de novo purine synthetic activity, since trimetrexate does not undergo polyglutamation and does not inhibit AICAR transformylase directly, we chose to study its effects on purine pathway activity and related these effects to changes in the intracellular folate cofactor pools. Fig. 2 (inset) indicates that purine synthesis is rapidly inhibited by exposure to trimetrexate. Control cells had a base-line rate of de novo purine synthesis of adenine and guanine nucleotides of 3.7 ± 0.6 nmol/h/mg (n = 6). Similar to our previous studies of the folate cofactor pools after MTX exposure, purine pathway inhibition correlates temporally with rapid accumulation of H4PteGlu, whereas there is relative preservation of the 10-formyl-H4PteGlu pool, the required cosubstrate for the folate-requiring enzymes of the de novo purine pathway (Fig. 2). During trimetrexate exposure, other changes in the intracellular folate pools closely mimicked the changes produced by MTX: 5-methyl-H4PteGlu was rapidly depleted, and 10-formyl-H4PteGlu accumulated. The latter compound is presum...
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Fig. 2. Effect of 1 μM trimetrexate on the intracellular folate pools in MCF-7 breast cells. Human breast cells were labeled with [3H]folic acid (final specific activity, 0.9 Ci/mmol) for 72 h, followed by exposure to 1 μM trimetrexate for 2 and 5 h. The folate pools in control cells are depicted on the ordinate at time 0. 'F', 5-methyl-H4PteGlu; 'M', H2PteGlu; 'O', 10-formyl-H4PteGlu; 'C', 10-formyl-H2PteGlu. Also detected in control cells, but not in trimetrexate-treated cells, were H2PteGlu (7.8 ± 2.0%) and 5-formyl-H2PteGlu (5.4 ± 1.5%). All cells contained folate (14.3 ± 4.2%) (not shown on graph). 5,10-Methylene-H4PteGlu could not be accurately quantitated using these experimental methods as it coeluted with 5-methyl-H4PteGlu, but is known to represent a small fraction (<3%) of the total folate pools in this cell line (see Footnote 3). The total folate pool in the control cells was 15.6 ± 2.4 pmol/mg, and there was less than a 13% fall in the total pool in the cells treated with drug for 5 h. The inset depicts the de novo purine activity in MCF-7 cells treated with trimetrexate for the stated time intervals using the incorporation of [14C]glycine into purine nucleotides as a measure of activity. The error bars represent the standard error of three independent experiments.

In addition, both control and experimental cells had constant levels of folate (14.3 ± 2%). Control cells also contained H2PteGlu (7.8 ± 2%) and 5-formyl-H2PteGlu (5.4 ± 1.5%), but these folates were not detectable in cells exposed to trimetrexate. These early effects of trimetrexate on purine synthesis and intracellular folates closely parallel those produced by MTX (14).

Because of the temporal coincidence of intracellular dihydrofolate accumulation and purine pathway inhibition and the lack of correlation of purine inhibition with MTX polyglutamate formation or cosubstrate depletion, we investigated the possibility that the intracellular accumulation of H2PteGlu might be responsible for direct inhibition of the purine pathway. Since thymidylate synthase is the only known biochemical reaction that generates H2PteGlu, we chose to modulate the intracellular level of dihydrofolate by treating MCF-7 cells with variable concentrations of the halogenated pyrimidine FdUrd prior to constant exposures to 1 μM MTX. 5-Fluoro-2-deoxyuridine monophosphate, a metabolite of FdUrd, is a potent and specific inhibitor of thymidylate synthase; and, as such, the amount of 5,10-methylene-H2PteGlu (the folate cosubstrate for thymidylate synthase) oxidized to H2PteGlu is inversely correlated with the concentration of FdUrd used for pretreatment. To ensure that effects on the de novo purine pathway were not the result of pyrimidine deprivation or FdUrd-related toxicity, all cells treated with FdUrd were simultaneously incubated with 10 μM thymidine, which effectively reversed the toxic effects of thymidylate synthase inhibition. Variable inhibition of thymidylate synthase activity as a function of FdUrd concentration can be demonstrated by the measurement of intracellular folate pools after drug treatment. Fig. 3 illustrates the actual measurement of the intracellular folates 5-methyl-H4PteGlu (a prevalent reduced folate that is rapidly depleted upon antifolate exposure) and H2PteGlu with various permutations of FdUrd/dThd pretreatment and MTX exposure. Control cells had high levels of 5-methyl-H4PteGlu and undetectable levels of H2PteGlu (first bar), and these findings are undisturbed by a 24-h treatment with 50 μM FdUrd, 10 μM dThd (second bar). Exposure to 1 μM MTX in the absence of a 4-h pretreatment with FdUrd/dThd leads to a rapid decrease in the 5-methyl-H4PteGlu pool with a parallel accumulation of H2PteGlu (third bar). Four-h pretreatment with 10 μM dThd and 1, 10, and 50 μM FdUrd results in a progressive preservation of the reduced folate pools such that at the highest FdUrd concentration (50 μM), the pools are unaffected by MTX (fourth to sixth bars).

Fig. 4 depicts the activity of the de novo purine synthetic pathway after various durations of incubation with 1 μM MTX following an initial 4-h preincubation with 10 μM thymidine and various concentrations of FdUrd. For each time point, a parallel experiment was performed exposing the cells to the identical concentration of thymidine and FdUrd, and this was used as the control for each MTX time point. When cells were pretreated with 50 μM FdUrd, a condition that prevented accumulation of H2PteGlu (Fig. 3, sixth bar), de novo purine pathway activity was unaffected by subsequent MTX. Conversely, in the absence of pretreatment with the fluorinated pyrimidine, MTX caused a marked accumulation of H2PteGlu and an equally dramatic inhibition of purine pathway activity. Pretreatment with intermediate concentrations of FdUrd (0.1, 1, and 10 μM) allowed intermediate effects by MTX on H2PteGlu accumulation and purine pathway activity.

An analysis of the 10-formyl-H4PteGlu pool for each of the
points, cells were pretreated with 10 \mu M dThd and FdUrd but no MTX, and the controls were processed for 4 h prior to timed exposures to 1 \mu M MTX. The correlation between the preservation of the 10-formyl-H,PteGlu pool and exposure to FdUrd was found to be 20.4% ± 3.1 pmol/mg. The inset represents a linearization of the data plotting the log percent control of purine activity versus intracellular H,PteGlu. The error bars represent the standard error of three to six independent determinations.

**Fig. 5.** Correlation between intracellular dihydrofolate and de novo synthetic activity. For each point, the purine activity was modulated by a 4-h pretreatment with 10 \mu M dThd and various concentrations of FdUrd (0.1-50 \mu M), followed by timed exposures to 1 \mu M MTX. In each case, the amount of H,PteGlu (as a percent of the total folate pool) accumulation in the cells was quantitated and plotted as a function of de novo purine synthetic activity. The mean total intracellular folate concentration was found to be 20.4 ± 3.1 pmol/mg. The inset represents a linearization of the data plotting the log percent control of purine activity versus intracellular H,PteGlu. The error bars represent the standard error of three to six independent determinations. The standard error for the H,PteGlu determinations was less than 10%.

FdUrd exposures (0.1, 1, and 10 \mu M) followed by 24 h of MTX showed a mean pool preservation of 78 ± 7%. There was no correlation between the preservation of the 10-formyl-H,PteGlu pool and exposure to FdUrd. Exposure to 1 \mu M MTX in the absence of FdUrd resulted in an 80 ± 13% 10-formyl-H,PteGlu hydrofolate pool.

For each of the experimental points shown in Fig. 4, the amount of intracellular H,PteGlu accumulation under each of the conditions was measured and the correlation of de novo purine pathway activity as measured by [14C]glycine incorporation. The correlation is shown in Fig. 5. Fig. 5 (inset) is a replot of the data using the log of the percent of purine activity as a function of H,PteGlu concentration, a plot that yields a log linear relationship with a correlation coefficient of 0.96.

The relation between H,PteGlu and de novo purine activity shown in Fig. 4 was derived from experiments in which cells were exposed to 1 \mu M MTX for various times up to 21 h and various FdUrd concentrations. Whereas brief exposures to MTX (2-3 h) result in negligible polyglutamate formation, it would be expected that prolonged exposures would result in a significant generation of MTX polyglutamates. These polyglutamates may have additional inhibitory effects on purine synthesis beyond those expected for H,PteGlu alone; however, Fig. 4 reveals no conclusive evidence for such an effect. However, there was a constant tendency for increased inhibition of purine synthesis activity at a given intracellular H,PteGlu level for 24-h MTX exposures versus the 2- and 5-h exposure points (Fig. 4). To investigate further this question, we measured the formation of MTX polyglutamates in cells exposed to 1 \mu M MTX for 21 h following 4-h treatments with various permutations of thymidine and/or FdUrd. These results are illustrated in Table I and reveal that negligible quantities of MTX polyglutamates are formed under the pretreatment conditions used for these studies. Thymidine (10 \mu M) alone was sufficient to diminish the conversion of MTX into its higher polyglutamate forms by 40% compared to control experiments, and the addition of FdUrd, even at the lowest concentrations used in these studies (0.1 \mu M), resulted in a 94% reduction in MTX polyglutamate formation.

As additional support for the concept of H,PteGlu acting as a direct inhibitor of purine synthesis, we performed the following experiment. MCF-7 cells were preincubated for 4 h with 10 \mu M thymidine and 50 \mu M FdUrd, followed by 1 \mu M MTX. As previously shown (Fig. 4), this combination results in no accumulation of H,PteGlu and maintenance of de novo purine synthetic activity at control levels. Subsequent incubation of cells under these conditions with increasing amounts of exogenously added H,PteGlu at 1, 3, 10, 20, and 100 \mu M results in inhibition of de novo purine synthesis in a time- and dose-dependent fashion, as shown in Fig. 6.

**DISCUSSION**

These studies indicate that de novo purine synthesis in MCF-7 breast cells is rapidly inhibited by cell exposure to either methotrexate or the lipid-soluble antifolate trimetrexate and that inhibition occurs at the level of the folate-requiring enzyme AICAR transformylase. Moreover, inhibition of this enzyme cannot be accounted for by reduced folate.

**Table I**

| Pre-exposure (h) | Total MTX | Glu, Glu | Glu, Glu | Glu, Glu, Glu |
|------------------|-----------|----------|----------|----------------|
|                  | mmol/g    |          |          |                |
| None             | 21.5      | 1.8      | 6.0      | 6.4            | 5.3            | 2.0            | 13.7            |
| dThd (10 \muM)   | 18.0      | 4.2      | 5.7      | 4.8            | 2.6            | 0.7            | 8.1             |
| FdUrd (1 \muM)   | 6.3       | 3.5      | 1.5      | 1.2            | 0.3            | 0.2            | 1.5             |
| dThd (10 \muM)   | 5.0       | 3.2      | 0.9      | 0.7            | 0.2            | 0.0            | 0.9             |
| MTX (0.1 \muM)   | 4.9       | 3.8      | 0.8      | 0.3            | 0.0            | 0.0            | 0.3             |
| dThd (10 \muM)   | 4.2       | 3.2      | 0.8      | 0.2            | 0.0            | 0.0            | 0.2             |
| dThd (10 \muM)   | 4.7       | 4.2      | 0.5      | 0.0            | 0.0            | 0.0            | 0.0             |
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MTX pools and H$_2$PteGlu polyglutamates potently inhibit AICAR transformylase in cell-free systems (17). Inhibition constants are strongly dependent on the glutamylated state of the folate cosubstrate 10-formyl-H$_4$PteGlu; but even in the presence of 10-formyl-H$_2$PteGlu, inhibitory intracellular levels of MTX polyglutamates and H$_2$PteGlu polyglutamates are readily attained in the presence of modest concentrations of MTX (1-2 μM) (14, 16, 19, 29, 30). Other investigators have also demonstrated that MTX and H$_2$PteGlu polyglutamates are potent inhibitors of other folate-requiring enzymes, including thymidylate synthase (31-35) and methylene tetrahydrofolate reductase (36, 37). Several additional lines of evidence implicate H$_2$PteGlu polyglutamates as the entity responsible for inhibition of the purine pathway. Trimetrexate, which has no direct inhibitory effects on AICAR transformylase and is incapable of forming polyglutamates, nonetheless caused an increase in H$_2$PteGlu and inhibition of purine synthesis. Furthermore, purine synthesis inhibition occurs within 2 h of exposure to MTX, at a time when MTX polyglutamates are not detectable in MCF-7 cells (19, 29). Intracellular folate pool measurements in cells treated with either antifolate reveal a rapid accumulation of H$_2$PteGlu that coincides with inhibition of purine synthesis. These findings all strongly suggest that the accumulation of H$_2$PteGlu polyglutamates may be the factor responsible for inhibition of de novo purine synthesis during antifolate exposure.

To test this hypothesis, we designed a series of experiments that would allow modulation of intracellular H$_2$PteGlu levels by FdUrd, an inhibitor of thymidylate synthase. Thus, a correlation could be made between the intracellular accumulation of H$_2$PteGlu and inhibition of de novo purine synthesis. FdUrd (50 μM) prior to MTX completely inhibited thymidylate synthase activity and thereby prevented any H$_2$PteGlu accumulation. Under these conditions, purine synthesis was unaffected by MTX. Lower concentrations of FdUrd pretreatment allowed greater accumulations of H$_2$PteGlu and proportional decreases in purine synthesis. As alluded to under "Results," cells exposed to MTX for 24 h appear to consistently demonstrate a greater inhibition of purine synthesis at a given H$_2$PteGlu concentration when compared to cells exposed for 2 or 5 h (Fig. 5). As there is an insignificant formation of MTX polyglutamates under the experimental conditions, it is conceivable that an additional purine inhibitor is being generated during the prolonged exposures. As we have shown in cells exposed to either trimetrexate or MTX (14, 18), a new folate, formyl-H$_2$PteGlu, can be identified. Preliminary observations suggest that this folate may be an inhibitor of human glycaminide ribonucleotide transformylase (28). The addition of exogenous H$_2$PteGlu to cells blocked by pretreatment with 50 μM FdUrd and 1 μM MTX (a condition that had no effect on purine synthetic rate) led to inhibition of purine synthesis. The amounts required for this inhibition could not be directly compared to inhibition constants derived from cell-free experiments as the glutamylated state and the extent of uptake of the added H$_2$PteGlu were not defined.

Since inhibition of de novo pyrimidine synthesis is also an important component of the cytotoxicity of antifolates, the observations need to be followed by a parallel set of experiments designed to define the relative contributions of folate depletion versus direct enzyme inhibition of thymidylate synthase, the critical de novo pyrimidine synthetic enzyme. This enzyme requires 5,10-methylene-H$_4$PteGlu, and measurement of this pool during antifolate exposure would be essential.

The concept that H$_2$PteGlu may act as a controller of metabolic pathways is not novel. Matthews and Baugh (36)...

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**Fig. 6.** Effect of exogenous dihydrofolate on de novo purine synthesis in MCF-7 cells pretreated with 50 μM FdUrd, 10 μM dThd, and 1 μM MTX. MCF-7 cells were preincubated with 50 μM FdUrd and 10 μM dThd for 4 h, followed by 1 h of a 1 μM MTX exposure. These cells were then treated with various doses of H$_2$PteGlu for 3 or 5 h, and the de novo purine activity measured by labeled glycine incorporation into adenosine and guanine nucleotides as described previously. Purine activity in the experimental cells was compared to control cells exposed to an identical regimen of FdUrd/dThd/MTX but no exogenous H$_2$PteGlu. Purine activity in the control cells was measured simultaneously with the experimental cells. The error bars represent the standard error of at least four separate determinations.

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C. J. Allegra and J. Baram, unpublished observation.
and Matthews and Haywood (37) found that the polyglutamates of H₂PteGlu were potent inhibitors of methylene tetrahydrofolate reductase, one of several cytotoxic enzymes utilizing 5,10-methylene-H₂PteGlu. These investigators postulated that intracellular H₂PteGlu pools would expand in response to increased thymidylate synthesis activity and, by inhibiting methylenetetrahydrofolate reductase, act as a physiologic mechanism for preserving 5,10-methylene-H₂PteGlu for additional thymidylate synthesis.

Whereas the reported experiments strongly suggest a role for H₂PteGlu as the mediator of the inhibitory effects of antifolates on purine synthesis, they do not exclude the potential role of MTX polyglutamates in these processes. Clearly, the early inhibition of purine synthesis is best explained by the accumulation of H₂PteGlu, but the experiments that examined late effects on this pathway employed conditions that inhibited MTX polyglutamate formation. Pretreatment of MCF-7 cells with FdUrd/thymidine or FdUrd alone markedly curtailed the polyglutamation of MTX. This finding is in contrast to that reported by McGuire et al. (38), who found that pretreatment of a human leukemia cell line with concentrations of FdUrd up to 50 μM had no effect on the ability of these cells to polyglutamate subsequently administered MTX. Our results would support the attenuation of MTX polyglutamation by FdUrd pretreatment as a potential explanation for the antagonism reported by others for the sequential use of FdUrd, followed by MTX. Based on clinical observations, one possible explanation for the differences observed in the two cell lines may be a relative insensitivity of the leukemic cells compared to the breast cells to the inhibitory effects of FdUrd. The MTX polyglutamates are capable of inhibition of dihydrofolate reductase, and, by virtue of their normal intracellular half-life (39), are critical in maintaining the intracellular H₂PteGlu pools. Sensitivity to MTX has been correlated with cellular capacity for polyglutamation by a number of investigators (19, 40–43). In addition to sustaining inhibition of dihydrofolate reductase, MTX polyglutamates are likely contributors to the direct inhibition of enzymes other than dihydrofolate reductase given their potency of interaction with metabolically important folate-requiring enzymes such as AICAR transformylase (17) and thymidylate synthase (31–33). Inhibition of these distal enzyme sites by MTX and H₂PteGlu polyglutamates and the greater formation of both in malignant cells may explain the selectivity of antifolate action and Leucovorin rescue. Furthermore, the suggestion that the antifolates are exerting their effects through direct inhibition of enzymes distal to dihydrofolate reductase would support the development of inhibitors directed at critical folate-requiring enzymes other than dihydrofolate reductase.

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