Mechanism of Thymidylate Synthase Inhibition by Methotrexate in Human Neoplastic Cell Lines and Normal Human Myeloid Progenitor Cells*

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We have studied the roles of 5,10-methylentetrahydrofolate (5,10-methylene-H4PteGlu) depletion and dihydrofolate (H2PteGlu) accumulation in the inhibition of de novo thymidylate synthesis by methotrexate (MTX) in human MCF-7 breast cancer cells. Using both a high pressure liquid chromatography system and a modification of the 5-fluoro-2'-deoxyuridine-5'-monophosphate radioenzymatic binding assay, we determined that the 5,10-methylene-H4PteGlu pool is 50–60% depleted in human MCF-7 breast cancer cells following exposure to 1 μM MTX for up to 21 h. Similar alterations in the 5,10-methylene-H4PteGlu pools were obtained when human promyelocytic HL-60 leukemia cells and normal human myeloid precursor cells were incubated with 1 μM MTX. The H2PteGlu pools within the MCF-7 cells increased significantly after 15 min of 1 μM MTX exposure, reaching maximal levels by 60 min. Thymidylate synthesis, as measured by labeled deoxyuridine incorporation into DNA, decreased to less than 20% of control activity within 30 min of 1 μM MTX exposure. The inhibition of thymidylate synthesis coincided temporally with the rapid intracellular accumulation of H2PteGlu, a known inhibitor of thymidylate synthase. Furthermore, inhibition of this pathway was associated in a log-linear fashion with the intracellular level of dihydrofolate. These studies provide further evidence that depletion of the thymidylate synthase substrate 5,10-methylene-H4PteGlu is inadequate to account completely for diminished thymidylate synthesis resulting from MTX treatment. Our findings suggest that acute inhibition of de novo thymidylate synthesis is a multifactorial process consisting of partial substrate depletion and direct enzymatic inhibition by H2PteGlu polyglutamates.

Methotrexate (MTX); 4-amino-N10-methylpteroylglutamic acid is a potent inhibitor of the enzyme dihydrofolate reductase (EC 1.5.1.3; 5,8,7,8-tetrahydrofolate:NADP+ oxidoreductase) and produces its cytotoxic effects through the inhibition of the de novo synthesis of purines, pyrimidines, and certain amino acids (1). The relative importance of inhibition of de novo purine versus thymidylate biosynthesis as the lethal process in the cytotoxic mechanism of MTX has not been entirely resolved. It is clear that both purine and thymidine sources are required in the murine S180 cell line to produce complete rescue from the cytotoxic effects of MTX. This finding has been confirmed with work in both in vitro and in vivo systems employing normal and malignant murine and human cell lines (2–10).

It has been classically proposed that the MTX-mediated inhibition of dihydrofolate reductase results in the depletion of intracellular pools of reduced folates due to ongoing oxidation via thymidylate synthase (11–16). The folate-dependent enzymes of de novo purine and thymidylate biosynthesis require 10-formyl-H4PteGlu and 5,10-methylene-H4PteGlu, respectively. Reduced folate depletion would result in the inhibition of these critical metabolic pathways. A number of recent studies have suggested, however, that certain reduced folate substrate pools are relatively preserved during exposure to MTX. An earlier report by Sur et al. (17) found an initial rise in 5,10-methylene-H4PteGlu levels followed by a plateau at 60–80% of control levels following exposure of Krebs ascites cells to MTX in vivo. Previous work in our own laboratory has demonstrated that 10-formyl-H4PteGlu, the required folate cofactor for de novo purine synthesis, was preserved at 80% of pretreatment values following a 1 μM MTX exposure of human MCF-7 breast cancer cells for up to 21 h. We also found that the rapid accumulation of dihydrofolate polyglutamates following MTX exposure was associated with the inhibition of de novo purine biosynthesis (18, 19). Further more, both MTX polyglutamates and dihydrofolate polyglutamates are potent inhibitors of both 5-aminomimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate transformylase (20) and thymidylate synthase (21, 22). In support of these findings, Matherly et al. (23), using murine L1210 cells in culture, observed increased dihydrofolate levels without depletion of 10-formyl-H4PteGlu following MTX exposure. They found that both de novo purine and thymidine biosynthesis were inhibited and suggested that depletion of 10-formyl-H4PteGlu could not account entirely for the inhibition of purine synthesis. They concluded that accumulation of dihydrofolate polyglutamates and MTX polyglutamates was playing a critical role in the inhibition of purine synthesis. More recently, Bunni et al. (24) demonstrated that depletion of 5,10-methylene-H4PteGlu could not explain the diminished growth or inhibition of thymidylate synthesis in MTX-treated L1210 cells. They showed that the MTX-mediated inhibition of growth and thymidylate synthesis inhibition was based on a modest accumulation of dihydrofolate and concluded that
the ratio of dihydofolate to total reduced folates most closely correlated with both growth and thymidylate synthesis inhibition. Recent studies using computer modeling of the folate-dependent pathways in human MCF-7 breast cancer cells also support the concept that inhibition of thymidylate synthesis cannot be explained entirely by folate depletion but may occur as a result of direct inhibition of thymidylate synthase by MTX polyglutamates and dihydrofolate polyglutamates (25).

The present study was undertaken to investigate further the effects of intracellular folate depletion and dihydrofolate formation following MTX exposure on de novo thymidylate synthesis. Since 5,10-methylene-H$_4$PteGlu intracellular pools are small, we have used two independent assay systems for quantitating this reduced folate. The first assay is a modification of our original method for quantitating intracellular folates using HPLC (18, 19), and the second method is a modification of the ternary complex assay developed previously by Priest and co-workers (17, 24, 26). We have applied these techniques to the study of intracellular 5,10-methylene-H$_4$PteGlu pools during cell growth and following MTX exposure. The results of this study suggest that inhibition of thymidylate biosynthesis following MTX exposure is multifactorial, consisting of a partial depletion of 5,10-methylene-H$_4$PteGlu and direct inhibition by the accumulated dihydrofolate polyglutamates.

**EXPERIMENTAL PROCEDURES**

**Materials**

Methotrexate was obtained from the Drug Synthesis and Chemistry Branch, NCI. Folate standards including dl-tetrahydrofolic acid (H$_4$PteGlu) and dihydrofolic acid (H$_2$PteGlu) were purchased from Sigma. dl-5,10-Methylentetrahydrofolic acid (5,10-methylene-H$_4$PteGlu) was prepared as described previously (19). [3',5',7,9-$^3$H]Folate (specific activity, 40 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [6-$^3$H]Deoxyuridine (specific activity, 23 Ci/mmol) was obtained from ICN (Irvine, CA). Adenosine and guanosine were purchased from Sigma. Pit Reagent A, C$_6$H$_6$Bondapak HPLC columns, and C$_{18}$ Sep-Pak cartridges were purchased from Waters Associates (Milford, MA). All other chemicals were of the highest purity available and were purchased from Sigma. Thymidylate synthase (5.2 units/mg) was purified from Lactobacillus casei cells according to methods described previously (27) and was a generous gift of Dr. John Galivan (Div. of Laboratories and Research, New York State Dept. of Health, Albany, NY).

**Cell Culture—Human promyelocytic leukemia HL-60 cells and human MCF-7 breast cancer cells were maintained in T-75-cm$^2$ tissue culture flasks (Falcon Labware, Oxnard, CA) in RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with 10% dialyzed fetal bovine serum (GIBCO) and 2 mM glutamine (Biofluids, Inc.). Sterile flasks were incubated in a humified 5% CO$_2$ incubator at 37 °C. All cells were grown for at least two passages in 10% dialyzed fetal bovine serum before use in any experiments.

**Purification of Human Myeloid Precursor Cells—Heparinized bone marrow samples were collected by aspiration from the posterior iliac crest of normal volunteers after informed consent was obtained. The mononuclear cell population was separated using a Ficoll-Hypaque gradient centrifugation, and the fraction enriched in myeloid precursors was obtained by an affinity-rosetting technique as described previously (28).

**Methods**

**Measurement of Intracellular Folate Pools** Intracellular folate pools were measured according to methods published previously (18). Briefly, human promyelocytic leukemia HL-60 cells and human breast cancer MCF-7 cells, at an initial density of 1 x 10$^6$ cells/flask, were plated onto T-75-cm$^2$ tissue culture flasks with 2.2 ml of the sterility culture medium (Biofluids, Inc.). Sterile cultures were incubated in a humidified 5% CO$_2$ incubator at 37 °C. All cells were grown for at least two passages in 10% dialyzed fetal bovine serum before use in any experiments.

**Control experiments were performed to determine the extent of recovery of 5,10-methylene-H$_4$PteGlu. Known concentrations of 5,10-methylene-H$_4$PteGlu were added to cytosolic extracts of MCF cells, which were then subjected to the same lysis and assay process as described above. Recovery for 5,10-methylene-H$_4$PteGlu by this HPLC assay was 36 ± 8%.

**Determination of 5,10-Methylene-H$_4$PteGlu Pools by Radioenzymatic Assay—The second method used to measure 5,10-methylene-H$_4$PteGlu pools in cell-free extracts was a modification of the radioenzymatic assay developed by Priest and co-workers (24, 26). This assay is based upon the binding of 5,10-methylene-H$_4$PteGlu by thymidylate synthase and [1H]$^4$FdUMP to form a stable ternary complex. After treating MCF-7 human breast cancer cells with various concentrations of MTX for 2, 5, and 24 h, cells were harvested by sedimentation in 1.25 ml of cold extraction buffer. The extracts containing exogenous 5,10-methylene-H$_4$PteGlu were then reboiled and subjected to the same assay process as the drug-treated cell extracts. All samples were incubated at 37 °C for 30 min, denatured by the addition of 1% sodium dodecyl sulfate, and boiled for 5 min. C-8 Bond Elute minicolumns (Analytichem, Harbor City, CA) were used to separate bound from free radiolabeled ligands. The columns were first washed with 2 ml of methanol followed by 2 ml of water and then loaded with samples. The columns were washed three times with a total of 8 ml of phosphate-buffered saline to elute unbound ligands, and the ternary complex eluted with 2 ml of a mixture containing 60% acetonitrile and 0.1% trifluoroacetic acid, pH 2.5. The total amount of binding [1H]$^4$FdUMP was determined by adding 10 ml of scintillant (3a70B, Research Products Co., Mount Prospect, IL) to each sample and counting tritium radioactivity in a Packard Tri-Carb liquid scintillation counter.

Control experiments were performed to determine the extent of recovery of 5,10-methylene-H$_4$PteGlu by the radioenzymatic assay. A known concentration of 5,10-methylene-H$_4$PteGlu was added to cytosolic extracts of MCF cells that had been initially lysed in the extraction buffer. The extracts containing exogenous 5,10-methylene-H$_4$PteGlu were then reboiled and subjected to the same assay process as the drug-treated cell extracts. Recovery of 5,10-methylene-H$_4$PteGlu by this assay system was 78 ± 4%.

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**FIG. 1. Authentication experiments for 5,10-methylene-H₄PteGlu (peak 1).** The intracellular folate pools of MCF-7 cells were labeled for 96 h during growth with 2.25 μM [³H]folic acid. Following exposure, the labeled folates were extracted as described under "Methods." HPLC separation at pH 4 and radiolabeled peak collections were performed on cells after the extracted radiolabeled folates were subjected to the following treatments: panel A, control cells with no treatment; panel B, cells following a 6-h incubation at 21 °C with purified *L. casei* thymidylate synthase in 100 mM 2-mercaptoethanol and 50 mM KH₂PO₄ (pH 7.2); panel C, cells following a 6-h incubation at 21 °C under identical conditions as in panel B with the addition of 10⁻⁵ M dUMP. Each of the three experiments contained the same amount of unlabeled standard, 5,10-methylene-H₄PteGlu, which was added to the extracted radiolabeled folates prior to the various treatments. Peak 1, 5-formyl-H₄PteGlu; peak 2, H₄PteGlu; peak 3, H₃PteGlu; peak 4, 5,10-methylene-H₄PteGlu.

**TABLE I**

Stability of 5,10-methylene-H₄PteGlu pools following 1 μM MTX exposure

<table>
<thead>
<tr>
<th>Treatment [Formaldehyde]</th>
<th>5,10-Methylene-H₄PteGlu μmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.83 ± 0.24*</td>
</tr>
<tr>
<td>1 μM MTX</td>
<td>4.20 ± 0.70</td>
</tr>
<tr>
<td>+</td>
<td>1.27 ± 0.04</td>
</tr>
<tr>
<td>-</td>
<td>1.21 ± 0.01</td>
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* S.E. of two independent determinations in duplicate.

mmol). The cells were then denatured with 2 ml of 10% trichloracetic acid, washed four times with phosphate-buffered saline, and finally the precipitated macromolecules were solubilized with 1 ml of 1 M NaOH. A 0.5-ml aliquot of the solubilized preparation was assayed for radioactive counts by dissolving in 10 ml of scintillant (Ready-Solv) and counting in a liquid scintillation counter. The remainder of the preparation was assayed for protein content according to the method of Bradford (29).

**RESULTS**

**Extraction and Quantitation of Folates by HPLC**—We have demonstrated previously that HPLC can separate the intracellular folate pools. However, it had not been possible to separate the 5,10-methylene-H₄PteGlu fraction adequately. Changing the pH of the mobile phase to 4 allowed isolation of the 5,10-methylene-H₄PteGlu peak that eluted at 23 min, as shown in Fig. 1. Proof for the identity of this folate was based on two criteria: 1) coelution with standard 5,10-methylene-H₄PteGlu at pH 4, and 2) biochemical properties consistent with this specific folate. Authentication of the radiolabeled peak as 5,10-methylene-H₄PteGlu was performed in a set of experiments shown in panels A-C of Fig. 1. Panel A represents the control 5,10-methylene-H₄PteGlu level extracted from MCF-7 cells. Panel B shows no change in this folate peak when the cell extract is incubated with purified *L. casei* thymidylate synthase. With the addition of both dUMP and purified *L. casei* thymidylate synthase to MCF-7 cell extracts, 5,10-methylene-H₄PteGlu is metabolized and, as seen in panel C, there is complete disappearance of the original peak. Furthermore, a prominent peak eluting at 16 min is now present, representing significant formation of dihydrofolate. A similar set of experiments was performed using the MCF-7 cells after exposure to 1 μM MTX, and again, the radiolabeled peak at 23 min disappeared with addition of both dUMP and thymidylate synthase to the cell extract (results not shown).

We next examined the stability of the extracted 5,10-methylene-H₄PteGlu fraction to determine if significant interconversion to tetrahydrofolate (H₄PteGlu) was occurring during the extraction procedure. As shown in Table I, when control MCF-7 cells were extracted in the presence of 1 μl/ml 37% (w/v) formaldehyde, significantly increased levels of 5,10-methylene-H₄PteGlu resulted as compared with MCF-7 cells extracted in the absence of formaldehyde, indicating conversion of tetrahydrofolate to 5,10-methylene-H₄PteGlu. However, when MCF-7 cells were treated with 1 μM MTX for 2 h, which results in depletion of tetrahydrofolate pools, and...
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FIG. 2. Intracellular 5,10-methylene-H₄PteGlu pools in human cells following exposure to 1 μM MTX. The intracellular 5,10-methylene-H₄PteGlu pools of normal human myeloid progenitor, human promyelocytic leukemia HL 60, and MCF-7 human breast cancer cells were labeled for 96 h during growth with 2.25 μM [³H]folic acid. The cells were exposed to 1 μM MTX for various time periods up to 21 h prior to folate extraction. After cell harvesting, the 5,10-methylene-H₄PteGlu fraction was extracted and quantitated by measuring the radiolabeled folate peak separated by HPLC. Each point represents the mean of three to six experiments in duplicate, and error bars represent the standard error.

Effects of MTX on Intracellular Folate Pools—We initially examined the influence of 1 μM MTX on the 5,10-methylene-H₄PteGlu pool within the MCF-7 human breast cancer cell line. MCF-7 cells were preincubated for 96 h with 2.25 μM [³H]folic acid and then exposed to 1 μM MTX for various time intervals up to 21 h. Fig. 2 shows the relative 5,10-methylene-H₄PteGlu levels prior to and following MTX exposure. The absolute value of intracellular 5,10-methylene-H₄PteGlu for control non-drug-treated MCF cells was 1.7 pmol/mg. A rapid decrease in this folate pool occurred after brief exposures and essentially remained constant at 40% control levels after 5 h of MTX. We also determined the effect of 1 μM MTX on this specific folate pool in normal human myeloid precursor and human promyelocytic leukemia cells. Fig. 2 shows that the resulting changes in intracellular 5,10-methylene-H₄PteGlu for control non-drug-treated MCF cells was 1.7 pmol/mg. A rapid decrease in this folate pool occurred after brief exposures and essentially remained constant at 40% control levels after 5 h of MTX. We also determined the effect of 1 μM MTX on this specific folate pool in normal human myeloid precursor cells and the human promyelocytic HL-60 leukemia cells, the 5,10-methylene-H₄PteGlu levels decreased to 55–60% control levels following 21 h of MTX exposure.

Similar experiments on the MCF 7 human breast cancer cell line were performed using a modification of the radioenzymatic assay described by Priest and co-workers. (24, 26, 30). As shown in Fig. 3, the 5,10-methylene-H₄PteGlu level, which was 3.4 pmol/mg in control untreated cells, decreased significantly within 30 min of a 1 μM MTX exposure and remained constant at 30% control levels after 2 h of MTX exposure.
**FIG. 4.** Time course inhibition of de novo thymidylate synthesis following MTX exposure of MCF-7 cells. MCF-7 cells were grown in RPMI 1640 medium with 10% dialyzed fetal bovine serum, 2.25 μM folic acid, and 10 μM adenosine. The cells were exposed to varying concentrations of MTX for various periods of time. De novo thymidylate synthetic activity was measured by [3H]deoxyuridine incorporation into DNA as described under "Methods." Thymidylate synthase activity in the experimental cells was compared with control cells, and the controls were processed at the same time with the experimental cells. The error bars represent the standard error of three to six independent experiments.

(panel B). Similar results were obtained with 10⁻⁷ M MTX (panel C). However, in the presence of 10 μM MTX, the resulting 5,10-methylene-H₄PteGlu pools remained higher at both 2 and 5 h as compared with those levels obtained with 10⁻⁷ and 10⁻⁸ M MTX and remained at 50% control levels after 24 h (panel A).

**De Novo Pyrimidine Metabolism during MTX Exposure—** We next attempted to correlate the changes in folate pools resulting from exposure to MTX with alterations in thymidylate biosynthesis. As shown in Fig. 4, thymidylate synthetic activity, as measured by labeled deoxyuridine incorporation into DNA, decreased to less than 20% of control levels within 30 min to 1 h of MTX exposure at concentrations of 10⁻⁷-10⁻⁵ M. At lower concentrations of MTX we found that [3H]deoxyuridine incorporation fell to <40% of control values within 8-10 h of MTX exposure. The mean thymidylate synthase activity in control cells was found to be 800 nmol/mg/min.

The amount of intracellular H₂PteGlu accumulating after timed exposures of 0.25, 1, 2, and 5 h to various MTX concentrations (10⁻⁵-10⁻³ M) was measured and correlated with thymidylate synthetic activity. This relationship is presented in Fig. 5 and shows that thymidylate synthase activity decreases as the amount of intracellular H₂PteGlu increases. In addition, the inset in Fig. 5 displays a replott of the data using the log of the percent of thymidylate synthase activity as a function of H₂PteGlu concentration, a plot that yields a linear relationship (r = 0.96).

In addition to the relationship described above, we noted a close temporal association between intracellular dihydrofolate and de novo thymidylate synthetic activity. As shown in Fig. 6, the rapid increase in intracellular dihydrofolate to 35% of the total folate pool was associated with a significant decrease in thymidylate synthase activity to less than 10% control values within 30 min of 10 μM MTX exposure. Moreover, this figure indicates that when thymidylate synthase activity is completely inhibited, the generation of dihydrofolate becomes self-limiting, as evidenced by the plateau levels to 40-45% of the total folate pool. Similar results were obtained when human MCF-7 breast cancer cells were exposed to 0.1 and 1 μM MTX (data not shown). For each of the experimental points shown in Fig. 5, the cellular content of 5,10-methylene-H₄PteGlu was measured and correlated with thymidylate synthetic activity. The correlation coefficient was only 0.37 (data not shown). However, since H₂PteGlu and 5,10-methylene-H₄PteGlu are both critical factors in determining the activity of thymidylate synthase, we calculated the ratio of H₂PteGlu to 5,10-methylene-H₄PteGlu and compared this value with thymidylate synthase activity. The correlation is shown in Fig. 7, which is a replott of the data using the log of the percent of thymidylate synthase activity as a function of the ratio of H₂PteGlu to 5,10-methylene-H₄PteGlu, a plot that yields a linear relationship (r = 0.98). When data points from Fig. 5 representing 1 h or less MTX exposure were included in a similar analysis, there remained a significant correlation (r = 0.94) between thymidylate synthase inhibition and the ratio of H₂PteGlu to 5,10-methylene-H₄PteGlu (results not shown).

**DISCUSSION**

In order to gain insight into the mechanisms by which antifolate agents exert their cytotoxic action, the development of sensitive assays to measure the intracellular folate pools is essential. We have described previously a reliable and relatively simple HPLC technique for the quantitation of the intracellular folate pools. However, we were initially unable...
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FIG. 5. Correlation between intracellular dihydrofolate and de novo thymidylate synthetic activity. The thymidylate synthetic (TS) activity for each experimental point was determined by timed exposures (0.25, 1, 2, and 3 h) and various concentrations of MTX ($10^{-8}$-$10^{-5}$ M). In each case, the amount of H$_2$PteGlu (as a percent of the total folate pool) accumulation in the cells was determined and plotted as a function of de novo thymidylate synthetic activity. The mean total intracellular folate concentration was 20.4 ± 3.1 pmol/mg. The inset represents a linearization of the data plotting the log percent control of thymidylate synthase activity versus intracellular H$_2$PteGlu. The error bars represent the standard error of three to six independent experiments.

Our findings indicate that inhibition of de novo thymidylate
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**FIG. 6.** Temporal correlation between intracellular dihydrofolate and de novo thymidylate synthetic activity. The accumulation of $H_2PteGlu$ (○) as a percent of the total intracellular folate pool and the thymidylate synthetic activity (■) for each experimental point were determined by timed exposures (0.25, 1, 2, and 5 h) to 10 μM MTX. The error bars represent the standard error of three to six determinations.

**FIG. 7.** Correlation between thymidylate synthase activity and the ratio of 5,10-methylene-$H_2PteGlu$ to $H_2PteGlu$. The thymidylate synthase (TS) activity for each point was determined by timed exposures (0.25, 1, 2, and 5 h) to MTX ($10^{-9}$ to $10^{-5}$ M) as outlined in Fig. 5. In each case, the amount of both $H_2PteGlu$ (as a percent of the total folate pool) and 5,10-methylene-$H_2PteGlu$ (as a percent of control) accumulation in the cells was quantitated. The ratio of $H_2PteGlu$ to 5,10-methylene-$H_2PteGlu$ was calculated and then plotted as a function of thymidylate synthase activity. The inset represents a linearization of the data plotting the log percent control thymidylate synthase activity versus the ratio of $H_2PteGlu$ to 5,10-methylene-$H_2PteGlu$. 

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synthesis occurs rapidly and at a time when the intracellular levels of MTX polyglutamates are relatively small in MCF-7 breast cancer cells. Earlier studies by Jolivet et al. (32) revealed that synthesis of the higher MTX polyglutamates (Glu6) in MCF-7 cells was initially detectable only after 6 h of MTX exposure. Our measurement of H$_2$PteGlu pools, however, revealed a rapid accumulation (14.7 μM) within the MCF-7 cells after 1 h of MTX exposure, which was temporally associated with inhibition of thymidylate synthase activity. It had been shown previously that dihydrofolate polyglutamates are competitive inhibitors of thymidylate synthase (33, 34). Hämälä et al. (22) have determined recently that the Km for the thymidylate synthase enzyme isolated from MCF-7 cells in the presence of 5,10-methylene-H$_2$PteGlu is 3.9 μM. Thus, the intracellular concentration of dihydrofolate polyglutamates generated after 1-h MTX exposure is 3.8-fold higher than its Km for thymidylate synthase. These results suggest then that the early build-up of dihydrofolate polyglutamates would result in significant inhibition of thymidylate synthase. Analysis of the relationship between the ratio of dihydrofolate to 5,10-methylene-H$_2$PteGlu and thymidylate synthase inhibition as shown in Fig. 7 revealed a significant temporal association. As was the case for the experiments included in this figure (up to 5 h of MTX exposure), there remained a significant correlation (r = 0.94) between thymidylate synthase inhibition and the ratio of dihydrofolate to 5,10-methylene-H$_2$PteGlu when data points for 1 h or less MTX exposure, where little polyglutamylamates occurred, were included. The importance of H$_2$PteGlu on thymidylate synthase inhibition is supported further by the folate cycle kinetic model reported recently by Morrison and Allegre (25). Their computer model emphasizes the importance of H$_2$PteGlu as an inhibitor of thymidylate synthase in order to account for the relative preservation of 5,10-methylene-H$_2$PteGlu pools seen after MTX exposure. Exclusion of this inhibition would thus result in a substantial depletion of the 5,10-methylene-H$_2$PteGlu pools.

Matthews and colleagues (35, 36) have shown that H$_2$PteGlu polyglutamates are potent inhibitors of methylenetetrahydrofolate reductase, a cytosolic enzyme that utilizes 5,10-methylene-H$_2$PteGlu. They hypothesized that H$_2$PteGlu pools would expand with increased de novo pyrimidine synthesis and subsequently feedback inhibit methylenetetrahydrofolate reductase. This process would preserve 5,10-methylene-H$_2$PteGlu levels and maintain thymidylate synthase activity. Moreover, since H$_2$PteGlu polyglutamates inhibit thymidylate synthase, its accumulation in the presence of MTX is self-limiting and would prevent further depletion of 5,10-methylene-H$_2$PteGlu. This important relationship may explain the initially paradoxical results obtained when MCF-7 cells were incubated with varying concentrations of MTX. As shown in Fig. 2, there is greater preservation of 5,10-methylene-H$_2$PteGlu levels resulting from 10 μM MTX at both 2- and 5-h exposure, respectively, as compared with the values obtained with 0.1 and 1 μM MTX. At the higher MTX concentration, it may be postulated that there is a more rapid generation of intracellular H$_2$PteGlu polyglutamates and MTX polyglutamates. As a result, inhibition of both thymidylate synthase and methylenetetrahydrofolate reductase would be hastened, leading to an enhanced preservation of 5,10-methylene-H$_2$PteGlu.

In summary, our studies suggest that the process by which MTX inhibits de novo thymidylate biosynthesis is complex. It is clear that depletion of the reduced folate substrate 5,10-methylene-H$_2$PteGlu does occur but is inadequate to account completely for inhibition of thymidylate formation. The rapid accumulation of H$_2$PteGlu appears to be more closely associated with inhibition of thymidylate synthesis. However, since the levels of 5,10-methylene-H$_2$PteGlu and H$_2$PteGlu are metabolically linked to one another, it appears that a model that completely describes the cytotoxic effects of MTX on de novo thymidylate biosynthesis must consider these two folate pools simultaneously. As shown in Fig. 7, inhibition of thymidylate synthesis following early exposures to MTX (≤5 h) is dependent upon both the accumulation of H$_2$PteGlu along with the reduced levels of 5,10-methylene-H$_2$PteGlu. With longer MTX exposures (>5 h), the inhibitory role of MTX polyglutamates must be considered. MTX polyglutamates inhibit dihydrofolate reductase, and as a result of their long intracellular half-life, can maintain the intracellular levels of H$_2$PteGlu. In addition, previous studies have shown that MTX polyglutamates themselves are potent noncompetitive inhibitors of thymidylate synthase (21). Recent studies have shown that 12 h after MTX exposure, MCF-7 breast cancer cells and normal myeloid precursor cells begin to metabolize dihydrofolate to 10-formyldihydrofolate (18, 28). Enzyme kinetic studies revealed that 10-formyldihydrofolate polyglutamate, like the parent dihydrofolate polyglutamate, is a potent competitive inhibitor of thymidylate synthase (22). Thus, although the exact contribution of 10-formyldihydrofolate to the cytotoxicity of MTX remains to be determined, this novel folate has the potential to enhance inhibition of thymidylate synthase with prolonged MTX exposures.

Direct inhibition of thymidylate synthase by H$_2$PteGlu polyglutamates and MTX polyglutamates may provide further insight into the competitive process of leucovorin rescue (19, 21). These findings suggest that the rescue process by leucovorin cannot be attributed to simple repletion of reduced folates. Instead, it appears that leucovorin can be metabolized within the cell to 5,10-methylene-H$_2$PteGlu polyglutamate, which may then compete with MTX polyglutamates for thymidylate synthase or other folate-dependent enzyme activity. Finally, our findings that the antifolates exert their cytotoxic effects through direct inhibition of folate-dependent enzymes other than dihydrofolate reductase provide rationale for the ongoing design and development of new antifols directed at these critical folate-requiring enzymes. N$^5$-Propargyl-5,6-dideazafolic acid (CB-3717), a tight-binding inhibitor of thymidylate synthase (37, 38), and 5,10-dideazatetrahydrofolate, an inhibitor of glycinamide ribonucleotide transformylase (39, 40), are two such antifols presently undergoing active preclinical study.

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