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MECHANISM OF LEUCOVORIN REVERSAL OF METHOTREXATE CYTOTOXICITY IN HUMAN MCF-7 BREAST CANCER CELLS

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Abstract—Previous studies have suggested that metabolic inhibition by methotrexate (MTX) is multifactorial and that cytotoxicity can be reversed by the reduced folate leucovorin. In this report we investigated the mechanism of leucovorin rescue in the MCF-7 human breast cancer cell line. Cells were exposed to various concentrations of MTX (0.5, 1.0, 3.0, and 10.0 μM) for 24 hr followed by rescue with labelled leucovorin (0.5 to 50 μM). The changes in the intracellular folate pools 24 hr following the addition of leucovorin were quantitated by high-pressure liquid chromatographic methods. The changes in the folate pools during rescue were compared with the ability of various concentrations of leucovorin to affect cellular rescue from MTX using a cloning assay. Our studies show that the total labelled intracellular folate pools increased in a log-linear fashion with respect to leucovorin exposure concentrations up to 100 μM. The degree of accumulation at a given leucovorin concentration was not significantly different in the absence or presence of MTX over the concentration range of 0.5 to 10 μM. Individual folate pool levels (tetrahydrofolate, 10-formyl tetrahydrofolate, 5-formyl tetrahydrofolate, 5-methyl tetrahydrofolate, and 5,10-methylene tetrahydrofolate) reached those present in cells not exposed to MTX at concentrations of leucovorin that were not adequate to rescue the MTX-treated cells. With exposure to concentrations of leucovorin capable of rescue, the individual folate pool levels were up to twelve times greater than those found in untreated cells, consistent with competition for catalytic activity at folate-dependent enzymes in addition to dihydrofolate reductase. The dihydrofolate pool also increased with increasing leucovorin concentration; but, unlike the reduced folates, this oxidized folate reached a maximal level that was dependent on the MTX concentration to which the cells had been exposed. This suggests that competition between MTX and leucovorin occurs at the level of dihydrofolate reductase via a competitive interaction with dihydrofolate in this intact cell system. The ability of leucovorin and its metabolites to compete with direct inhibitors of dihydrofolate reductase and other metabolically important folate-dependent enzymes appears to be associated with leucovorin rescue.

The antifolate methotrexate (MTX†) has been in clinical use for over four decades and has been applied to the treatment of a variety of neoplastic and non-neoplastic states including rheumatoid arthritis and psoriasis and as an immune-modulator in the setting of organ transplantation [1–4]. MTX is a potent inhibitor of dihydrofolate reductase (DHFR; EC 1.5.1.3) with an inhibition constant of 10–50 pM [5–7]. Many studies have shown that inhibition of dihydrofolate reductase results in a rapid accumulation of dihydrofolate with variable depletion (20–60%) of the reduced folate cosubstrates required for *de novo* purine synthesis (10-formyl-H_4PteGlu) and thymidylate synthesis (5,10-methylene-H_4PteGlu) in malignant and normal cells in both *in vitro* and *in vivo* experimental systems [8–13]. The finding that less than complete depletion of the intracellular folate pools is associated with profound inhibition of the metabolic pathways dependent on the folate cofactors as one-carbon carriers has led to the postulate that metabolic inhibition is a multifactorial event which includes folate depletion and direct inhibition of several critical enzymes by accumulated dihydrofolate (H_2PteGlu) polyglutamates and MTX polyglutamates [8–11, 14, 15]. Several investigators have described the polyglutamates of dihydrofolate and MTX as potent inhibitors of thymidylate synthase [16–18], methylene tetrahydrofolate reductase [19] and the folate-dependent enzymes of *de novo* purine synthesis [15, 20, 21]. Temporal and absolute associations between metabolic inhibition and dihydrofolate accumulation have prompted the suggestion that this metabolite may be important in the causation of metabolic inhibition [8, 10, 11, 14].

In the mid-1950s, reports by Goldin and coworkers described the use of the reduced folate leucoviron (citrovorum factor; 5-formyl-H_4PteGlu) to rescue animals treated with lethal doses of MTX. These authors noted that the therapeutic index of MTX could be enhanced by the use of high dose therapy with subsequent reduced folate rescue [22]. This strategy has since been applied to the clinical treatment of neoplasia with the assumption that high doses of drug have the potential to overcome many of the known mechanisms by which cells become antifolate resistant.

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† Abbreviations: MTX, methotrexate; H_4PteGlu, tetrahydrofolate; H_2PteGlu, dihydrofolate; and DHFR, dihydrofolate reductase.

The interaction of MTX with leucovorin has been described as competitive in both clinical and preclinical systems [23–26]. The nature of this interaction has been variously ascribed to competition for membrane transport [27–30], polyglutamation [31–33], and dihydrofolate reductase binding [34–36]. Recent evidence has demonstrated conclusively that the kinetic interaction between dihydrofolate and MTX for dihydrofolate reductase activity is competitive in nature [37]. Several investigators have recognized dihydrofolate reductase as a potentially critical locus of competitive interaction between MTX and leucovorin or the intracellular metabolites of leucovorin including reduced folates and dihydrofolate [34–36, 38]. Studies using intact cells (murine leukemia L1210 and Erlich ascites cells) have suggested that while dihydrofolate reductase is an important locus of competition with MTX, the intracellular folate metabolites from exogenously administered leucovorin were not effective competitors with either aminopterin or MTX for dihydrofolate reductase binding in cells containing high levels of the antifolate polyglutamates [10, 34].

The focus of the present study was to investigate the mechanism of leucovorin rescue with particular emphasis on the interactions between (1) MTX/dihydrofolate polyglutamates and the reduced folate substrates at folate-dependent enzymes other than dihydrofolate reductase, and (2) between MTX and dihydrofolate polyglutamates at the level of dihydrofolate reductase. These studies also provide further definition for the role of direct inhibition of folate-dependent enzymes by MTX and dihydrofolate polyglutamates.

MATERIALS AND METHODS

Materials. MTX was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). [3′,5′,7′-^{3}H]MTX (sp. act. 18 Ci/mmol) and 6-*l*-[3′,5′,7-^{3}H]leucovorin (sp. act. 40 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). The radiopurity of each compound determined by HPLC was ≥98%, and both were used without further purification. RPMI-1640, glutamine, phosphate-buffered saline, and fetal calf serum were purchased from Biofluids (Rockville, MD). Fetal calf serum was dialyzed against 0.9% NaCl for four exchanges in a 1:40 ratio with 0.9% NaCl as the dialysate. Sep-pak™ C18 cartridges and Pic Reagent A™ were purchased from Waters Associates (Milford, MA). Leucovorin (6-*d,l*-5-formyl-H_4PteGlu) was obtained from the Burroughs Wellcome Co. (Research Triangle Park, NC). β-Mercaptoethanol, methylene blue, activated charcoal, albumin (fraction V), dextran, and β-nicotinamide adenine dinucleotide phosphate, reduced form, were all purchased from the Sigma Chemical Co. (St. Louis, MO). Methanol was purchased from J. T. Baker Inc. (Phillipsburg, NJ). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ).

Cell line. An early-passage human MCF-7 breast cancer cell line was used for these experiments. The cells were grown as a continuous monolayer in 75 cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA) with RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine. All cells were grown in medium containing dialyzed calf serum for at least two passages before use in the experiments. For each of the experimental points, 1×10^6 cells were plated onto 75 cm^2 plastic tissue culture flasks, and after 96 hr of growth (60–70% confluency) the cells were used for the various experiments.

Intracellular folate pool measurements. MCF-7 cells were exposed to 0.5, 1.0, 3.0 and 10 μM concentrations of MTX for 24 hr. At the end of this period, the cells were washed two times with phosphate-buffered saline. Various concentrations of [^{3}H]leucovorin were added, along with new MTX-free medium, for an additional 24-hr period. The specific activity of the [^{3}H]-*l*-leucovorin was diluted by the addition of unlabelled (*d,l*)-leucovorin necessary to achieve the desired final concentrations. At the end of the second 24-hr period, the cells were again washed two times with ice-cold phosphate-buffered saline and then harvested in 1 mL of the saline with the aid of a rubber cell scraper.

A 100-μL aliquot was removed for subsequent protein analysis. The folates were extracted from the cell suspension according to previously published techniques [8]. Briefly, the cells were lysed and folates extracted by boiling for 90 sec in 2 mL of a 2% ascorbate, 2% β-mercaptoethanol solution, pH 6.0. The denatured protein was removed by centrifugation at 10,000 *g* for 5 min. The supernatant was then treated with 24 units (1 unit = 1 nmol of product formed/min/mg) of partially purified hog kidney polyglutamate hydrolase (sp. act. = 2 units/mg) at 37° for 30 min. The hydrolase was partially purified from fresh hog kidneys (10–13 mg protein/mL) according to published methods [39]. The solution was then subjected to a second 90-sec boil after the addition of 2 mL of the 2% ascorbate, 2% β-mercaptoethanol solution and centrifuged at 10,000 *g* for 5 min to remove the denatured proteins. The folates were then concentrated by extraction using a C18 Sep-pak cartridge, followed by evaporation under a steady stream of nitrogen. These preparations were stored for up to 72 hr in the absence of light and at −80° under nitrogen until final separation and quantitation by HPLC.

The labeled folates were separated by HPLC using a Waters model 510 pump and a Waters model 440 ultraviolet absorption detector with a fixed wavelength of 256 nm [8]. An 8 × 10 cm C-8 μBondapak column (particle size = 10 μm spherical) was developed using a flow rate of 2 mL/min under isocratic conditions. The mobile phase consisted of 80% Pic Reagent A™ [25:1 (v/v) water /Pic A], adjusted to pH 5.5 with 1 N HCl, and 20% methanol. The separated labelled folate pools were quantitated using an in-line liquid scintillation counter (model Flo-One Beta; Radiomatic Instruments, Tampa, FL). The identity of the various physiologic folates was authenticated by coelution with unlabelled standard folates injected with each assay sample. Folates were further identified by acting as substrates for specific enzymatic reactions as previously detailed [8, 9, 15]. The recovery rate for the various folates ranged from 60 to 70%, and no correction for

recovery was applied as all comparisons were made to control cells processed under identical conditions and at the same time as the treated cells. The retention times for the folates were as follows: *p*-aminobenzoate, 4–5 min; *p*-aminobenzoyl glutamate, 7.5–8 min; 10-formyl-H_4PteGlu, 10 min; H_4PteGlu, 12.5 min; 5-formyl-H_4PteGlu, 15 min; H_2PteGlu, 18.5 min; and 5-methyl-H_4PteGlu, 26 min.

5,10-*Methylene tetrahydrofolate quantitation.* The 5,10-methylene-H_4PteGlu pool was quantitated in a separate set of experiments using [^{3}H]-*l*-leucovorin [40]. For these experiments, 1×10^6 MCF-7 cells were plated onto 75 cm^2 tissue culture flasks containing RPMI medium supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine. After 72 hr (60% confluency), the cells were exposed to 0.1 and 10 μM MTX for 24 hr. At the end of the MTX incubation, the cells were washed two times with phosphate-buffered saline and the cells plated in fresh medium containing various concentrations of labelled leucovorin for an additional 24 hr. After the leucovorin incubation, the cells were again washed two times with phosphate-buffered saline and harvested. The intracellular folates were extracted as described above and separated by HPLC using a modified mobile phase consisting of 76% Pic A, adjusted to pH 4.0 with 1 N HCl, and 24% methanol. The folate pool was quantitated using an in-line scintillation counter. The retention time for 5,10-methylene-H_4PteGlu was 23 min. Authentication of the 5,10-methylene-H_4PteGlu was by coelution with standard compound and by specific metabolism of the putative folate peak to dihydrofolate in the presence of thymidylate synthase and deoxyuridylate [40].

Colony-forming assay. MCF-7 cells (800 cells/well) were replicately plated in 2 mL of RPMI supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine in 6-well, 12 cm^2/well, tissue culture plates (Costar, Cambridge, MA). These cells were found to have a cloning efficiency of 15–20%. The cells were allowed to stand for 12 hr prior to treatment with various concentrations of MTX (0, 0.5, 1.0, 3.0, and 10 μM) for 24 hr. The cells were then washed two times with phosphate-buffered saline, and new MTX-free medium containing various concentrations of leucovorin was added (0, 0.1, 0.5, 1, 5, 10, and 50 μM). The cells were then grown for 10 days until distinct colonies (>30 cells) were present. The medium was removed, and the colonies were stained [0.25% methylene blue in 50/50 methanol/water (v/v)] and counted. The percent of control for each leucovorin concentration was computed by comparing the number of colonies in MTX-treated cells to those of untreated cells. Typical control plates contained 100–150 colonies. All experiments were performed in triplicate and on at least three separate occasions.

Intracellular MTX polyglutamate measurements. MCF-7 cells were exposed to 0.5, 1, and 10 μM MTX concentrations for 24 hr. At the end of this period the cells were washed two times with phosphate-buffered saline and new MTX-free medium was added. After an additional 24-hr period, the cells were again washed two times with ice-cold phosphate-buffered saline and harvested in 1800 μL of the saline with the aid of a rubber cell scraper. The cells were transferred to a 12 × 75 mm glass tube containing 200 μL of 100% trichloroacetic acid for a final concentration of 10%. The precipitated protein was centrifuged for 15 min at 10,000 *g*. The supernatant was reserved, and the pellet was dissolved in 1 mL of 1 N NaOH for protein quantitation. The MTX polyglutamates were extracted from the supernatant according to published methods [41] and separated by HPLC using a 22-min linear gradient from 20 to 35% acetonitrile in Pic A (pH 5.5). The separated polyglutamates were quantitated by an in-line liquid scintillation counter. The identity of the various polyglutamates was authenticated by coelution with unlabelled MTX polyglutamate standards.

Cell viability. Viability of the cells after 24- and 48-hr exposures to various concentrations of MTX (0.5, 1.0, and 10 μM) was assessed by measuring the ability of the cells to exclude trypan blue dye (0.04% trypan blue in phosphate-buffered saline). For these experiments cells were grown under identical conditions as used in the folate experiments. After 24- or 48-hr exposures to MTX (0.5, 1.0, and 10 μM) the cells were washed two times with ice-cold phosphate-buffered saline, as was the case for the experiments measuring intracellular folates. The cells were then placed in trypan blue, and their viability was assessed by inspection under an inverted microscope.

Intracellular dihydrofolate reductase measurements. The measurement of dihydrofolate reductase was based on a modification of the protein binding assay described by Myers *et al.* [42]. MCF-7 cells were exposed to 10 μM MTX for 24 and 48 hr or to no MTX (control cells). The cells were harvested from 75 cm^2 tissue culture flasks by the addition of 4 cc of 0.05% trypsin/0.02% versene in Hanks' Balanced Salt Solution. The cells were pelleted by centrifugation at 700 *g* for 15 min. The pelleted cells were resuspended in 500 μL of 100 mM Tris–HCl buffer (pH 7.5), and then sonicated with a 20-sec burst from a Branson model 350 sonicator. The cell mixture was centrifuged at 10,000 *g* for 10 min. The cell supernatants were then dialyzed against 4 L of 50 mM Tris/HCl buffer (pH 8.5) for a 24-hr period to remove MTX bound to DHFR. Control cells treated with 10 μM MTX for only 1 hr were done in parallel with experimental samples to demonstrate the efficiency of free enzyme recovery. We found >95% recovery of free enzyme compared to control cells not exposed to MTX and not subjected to dialysis. Following dialysis, 0.12 mg of cytosolic protein was added to a reaction tube containing Tris–HCl buffer (pH 7.5), 100 mM; [^{3}H]MTX, 0.15 μCi in a total volume of 200 μL and incubated at 37° for 3 hr. The extended incubation with [^{3}H]MTX allowed for complete exchange to take place with any remaining unlabelled drug bound to DHFR. For the binding assay, β-nicotinamide adenine dinucleotide phosphate, reduced form 50 mM, was added to the reaction mixture, and the tubes were vortexed and allowed to equilibrate for 10 min at room temperature. After ternary complex formation, the unbound drug was removed by the

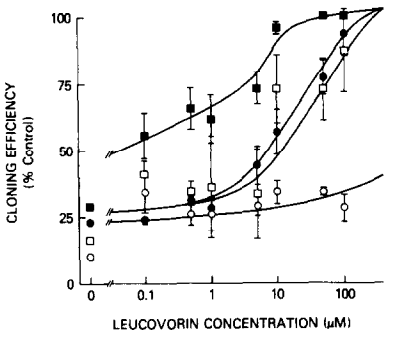

Fig. 1. Effect of various concentrations of leucovorin on colony formation of MCF-7 cells, following a 24-hr exposure to MTX. The breast cells were grown in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and 2 mM glutamine at an initial plating density of 500–1000 cells/plate. After 12 hr, the cells were treated for a 24-hr period with various MTX concentrations: (■) 0.5 μM; (□) 1 μM; (●) 3 μM; and (○) 10 μM. At the end of the 24-hr MTX exposure, the cells were washed two times with phosphate-buffered saline and placed in drug-free medium containing various concentrations of leucovorin. Colonies were allowed to grow for a 10-day period prior to staining and counting. Cloning efficiency was determined by comparing the number of colonies formed in MTX-treated cells to those of a control without MTX, but with exposure to the corresponding leucovorin concentration. Typical control plates contained 80–150 colonies/plate. Each experimental point is the mean ± SE of three to six separate experiments performed in triplicate.

addition of 50 μL of charcoal slurry (activated charcoal, 10 g; bovine serum albumin, fraction V, 2.5 g; and 0.1 g of high molecular weight dextran in 100 mL H_2O), followed by vortexing and immediate centrifugation at 10,000 *g* for 20 min. A 150-μL aliquot of the supernatant was counted in a Packard scintillation counter after the addition of 10 mL of liquid scintillation mixture.

Protein measurement. A 100-μL aliquot of cell suspension was sonicated with five 3-sec bursts using a Branson model 350 sonicator equipped with a microtip. The cell debris was pelleted by centrifugation at 10,000 *g* for 10 min, and the protein in the supernatant was quantitated using the method of Bradford [43].

Calculations. Disintegrations per minute (dpm) obtained by counting an aliquot of the extracted labelled folates were converted to total intracellular folate content in pmol/mg protein by dividing total dpm/flask of cells by total protein/flask of cells and then dividing by the specific activity of the labelled leucovorin: (dpm/flask)/(mg protein/flask)/(dpm/pmol folate) = pmol folate/mg protein.

RESULTS

Colony-forming assay. To determine the concentration of leucovorin required to rescue MCF-7 cells exposed to four concentrations of MTX (0.5, 1, 3, and 10 μM) for 24 hr, we performed a cloning assay. The plot of cloning efficiency versus leucovorin concentration is shown in Fig. 1. We found that leucovorin alone was toxic at concentrations in excess of 50 μM such that this concentration reduced colony formation by 20%. The control to which the MTX-treated cells were compared consisted of cells treated in an identical fashion as the MTX cells including exposure to the appropriate concentrations of leucovorin. Treatment with 0.5 μM MTX required 5–10 μM leucovorin to achieve rescue of 90% of the cells, while 3 μM MTX-treated cells required 50–100 μM for 90% rescue. Cells treated with 10 μM MTX were not rescued substantially despite using leucovorin concentrations up to 100 μM.

Cell viability. Since we were measuring intracellular folate pools in cells after 24 and 48 hr of MTX exposure, we wanted to document the viability of the population of cells after these brief exposures. In contrast to the effects of MTX on the ability of the cells to clone after the MTX exposure (Fig. 1), we found that the cells maintained greater than 90% viability after 24- and 48-hr exposures to concentrations of MTX up to 10 μM.

Intracellular MTX polyglutamates. Since it has been suggested that polyglutamation of MTX may play an important role in the metabolic inhibition of folate-dependent enzymes, we looked at the profile of MTX polyglutamates in MCF-7 breast cells treated with various concentrations of MTX (Fig. 2). We measured the polyglutamate levels 24 hr after rescue and 48 hr from the start of MTX treatment. The total amount of MTX in the cells treated with 0.5, 1, and 10 μM MTX was 11.5, 21.8 and 41.9 pmol/mg respectively. The higher polyglutamates including MTX-Glu_3–MTX-Glu_5 represented between 88 and 94% of the total MTX with measurements of 10.3, 19.2 and 39.5 pmol/mg for 0.5, 1, and 10 μM MTX respectively. We also measured the total amount of the higher MTX polyglutamates 24 hr after exposure to various concentrations of leucovorin (48 hr after the start of MTX exposure). We found that the addition of 1, 10 and 50 μM leucovorin resulted in a decrease in MTX polyglutamates to approximately 50% of levels obtained without the addition of leucovorin (Table 1). To compare the amount of MTX in the cells with the dihydrofolate reductase binding capacity, we measured the amount of dihydrofolate reductase in the MCF-7 cells (Fig. 2). We found the amount of dihydrofolate reductase to be 3.4 ± 1.5 pmol/mg for these cells. We also measured the amount of DHFR 24 and 48 hr after exposure to 10 μM MTX and found no change in the total levels. Thus, the amount of higher polyglutamates substantially exceeded the binding capacity (up to 12-fold), depending on the concentration of MTX to which the cells had been exposed.

Intracellular folate pools. In an effort to understand the mechanism of leucovorin rescue and the competitive nature of this process, we investigated alterations in the intracellular folate pools during rescue. The intracellular folate pools in cells labelled with leucovorin (0.1 μM) prior to any treatment with MTX (unperturbed) were composed of 10-formyl-H_4PteGlu (12.7 ± 2.7 pmol/mg), H_4PteGlu (8.1 ± 2.8 mol/mg), 5-methyl-H_4PteGlu (12.0 ± 2.5 pmol/mg), 5-formyl-H_4PteGlu (1.1 ± 0.9 pmol/mg), and 5,10-methylene-H_4PteGlu (1.7 ± 0.07 pmol/

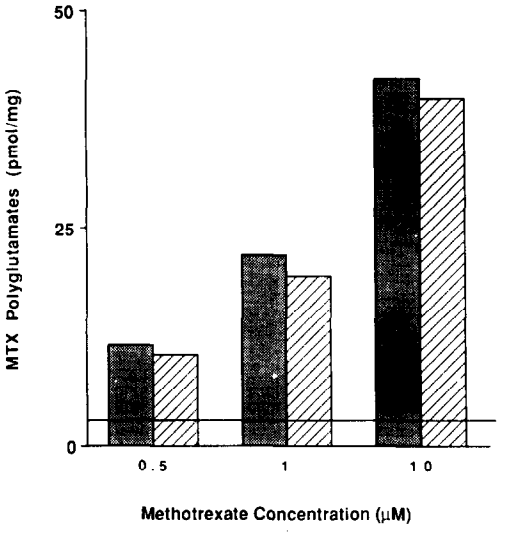

Fig. 2. MTX polyglutamate levels in the MCF-7 breast cells 24 hr after rescue and 48 hr from the start of MTX treatment. MCF-7 breast cells were grown in RPMI 1640 medium containing 10% dialyzed fetal calf serum and 2 mM glutamine. The cells were initially plated at a density of 1×10^6 cells/plate and allowed to grow until they reached 50–60% confluency (3–4 days). The cells were then exposed to 0.5, 1.0 and 10 μM concentrations of MTX for 24 hr, washed two items in phosphate-buffered saline, and placed in drug-free medium for an additional 24 hr. At the end of the washout period, the cells were harvested and the MTX polyglutamates quantitated by HPLC. The total polyglutamates (pmol/mg) for 0.5, 1, and 10 μM MTX exposures are represented by the solid bars; the higher polyglutamates from Glu_3 to Glu_5 are represented by the hatched bars. The line drawn at the bottom of the graph represents the total amount of dihydrofolate reductase in the breast cells as determined by the radiolabelled MTX binding assay. The experimental results are the means of two separate experiments performed in duplicate. Standard deviation of the experiments was <10% for 1 and 10 μM MTX treated cells and 25% for those treated with 0.5 μM MTX.

mg). Dihydrofolate was not detectable in these untreated cells. The total intracellular folate pool size was 37.2 ± 9.5 pmol/mg.

We next studied the folate pools during rescue of the cells with various concentrations of [^{3}H]leucovorin from either a 24-hr treatment with 0.5, 1, 3, and 10 μM MTX or without prior treatment with MTX. We found that the total labelled intracellular folate pools (reduced plus oxidized) increased in a log-linear fashion as a function of increases in leucovorin concentration (Fig. 3). This relation was apparent for resue from all concentrations of MTX, as well as for cells not exposed to MTX. Previous studies [8] have shown that a 24-hr exposure of the MCF-7 breast cells to labelled leucovorin results in an equilibration of the label within the cells, suggesting that quantitation of the labelled folates is an accurate measure of the endogenous folate pool as well as that contributed by the exogenously added folate. We found that the level of total folates in cells exposed to concentrations of leucovorin required to rescue from MTX greatly exceeded those found in cells growing in an unperturbed state. Figure 4 illustrates the changes in each of the measured reduced folate pools during rescue with increasing concentrations of leucovorin for each of the MTX concentrations studied (panels B–E). Also illustrated is the change in folate pools in cells that were not exposed to MTX (panel A). As in unperturbed cells, the majority of the intracellular folates consisted of 10-formyl-H_4PteGlu, H_4PteGlu, and 5-methyl-H_4PteGlu. The 5-formyl-H_4PteGlu pool was small and remained so despite increasing leucovorin concentrations suggesting that this folate was metabolized rapidly upon entry into the cell and that the cell population studied was metabolically active. The major folate pools, however, increased with increasing leucovorin concentrations, most significantly, the 10-formyl-H_4PteGlu and H_4PteGlu pools. These folates reached levels found in unperturbed cells (15 pmol/

Table 1. MTX polyglutamate formation in human breast carcinoma cells (MCF-7)

MTX pre-exposure (μM)	Leucovorin exposure (μM)	Total MTX (pmol/mg)	Higher MTX polyglutamates* (pmol/mg)
1	0	23.0 ± 5.2†	22.4 ± 6.0
	1	15.2 ± 6.3	13.1 ± 5.5
	10	15.6 ± 4.8	13.3 ± 4.2
	50	10.4 ± 2.0	8.2 ± 1.4
10	0	49.1 ± 9.3	43.1 ± 5.2
	1	28.0 ± 5.3	20.1 ± 3.0
	10	30.0 ± 7.9	22.2 ± 6.3
	50	26.3 ± 2.0	20.1 ± 2.8

MCF-7 breast carcinoma cells were grown to 60–70% confluency in RPMI-1640 medium with 10% dialyzed fetal calf serum and 2 mM glutamine. Cells were exposed to 1 or 10 μM MTX for 24 hr followed by washing two times in phosphate-buffered saline. The cells were placed into fresh MTX-free medium, and various concentrations of leucovorin were added for an additional 24 hr. At the end of the folate exposure, the cells were again washed two times in phosphate-buffered saline and harvested. The MTX polyglutamates were separated by HPLC and quantitated by an in-line scintillation counter.

* Higher polyglutamates = MTX-Glu_3–MTX-Glu_5.

† Each value is the mean ± SE of three to four independent experiments.

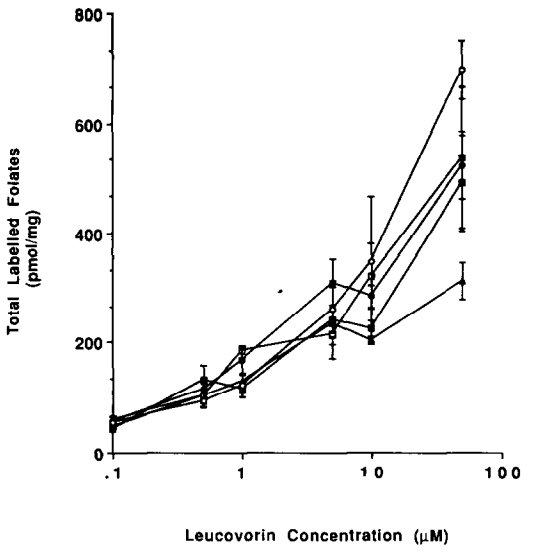

Fig. 3. Total labelled folate pool levels during leucovorin rescue in MCF-7 cells treated with or without MTX for 24 hr. The MCF-7 breast cells were plated at an initial density of 1×10^6 cells/plate in RPMI 1640 medium containing 10% dialyzed fetal calf serum and 2 mM glutamine. After 4 days of growth (60–70% confluency), the cells were exposed to several concentrations of MTX: (△) 0 μM; (■) 0.5 μM; (□) 1 μM; (●) 3 μM; and (○) 10 μM. After 24 hr in drug, the cells were washed two times in phosphate-buffered saline and placed in drug-free medium containing various concentrations of radiolabelled leucovorin. After an additional 24 hr, the cells were harvested and the labelled folate pools separated and quantitated by HPLC. Each experimental point is the mean ± SE of four to six independent experiments.

mg) at concentrations of leucovorin (0.5 to 1.0 μM) that did not result in rescue from any of the studied MTX exposures. In general, the concentrations of leucovorin required for MTX rescue (90%) resulted in intracellular reduced folate concentrations that substantially exceeded those in unperturbed cells (up to 12-fold).

In a separate set of experiments, the amount of 5,10-methylene-H_4PteGlu was measured in the breast cancer cells that were exposed to 1 and 10 μM MTX or no MTX for 24 hr, followed by leucovorin at concentrations ranging from 1.0 to 50 μM (Fig. 5). The 5,10-methylene-H_4PteGlu accumulated in proportion to the leucovorin rescue concentration, reaching levels in control cells at concentrations of leucovorin less than that required for rescue from MTX cytotoxicity. The absolute 5,10-methylene-H_4PteGlu level for any leucovorin exposure appeared to be highest in the cells treated with MTX versus untreated cells.

We evaluated the changes in the dihydrofolate pool levels during leucovorin rescue, as illustrated in Fig. 6. We found that, as with the other folates, dihydrofolate pools increased with increasing leucovorin concentrations; however, unlike the reduced folate pools, dihydrofolate appeared to reach a maximal (plateau) level that was directly dependent on the MTX exposure concentration. In cells that were not preexposed to MTX, detectable dihydrofolate levels were not apparent regardless of the leucovorin concentration (data not shown). The maximal dihydrofolate levels reached by cells treated with 0.5, 1.0, and 3.0 μM MTX were 50, 100, and 130 pmol/mg respectively. In cells treated with 10 μM MTX, dihydrofolate levels continued to increase with concentrations of leucovorin up to 50 μM.

DISCUSSION

These studies illustrate that the interaction between MTX and leucovorin in the human breast cell line (MCF-7) appears to be competitive in nature. This finding is in concert with other well-characterized systems [23–26]. Preliminary experiments demonstrate that the higher MTX polyglutamates (Glu_3–Glu_5) represent almost all of the MTX in the cells during the 24 hr of leucovorin rescue following the removal of the extracellular MTX. We also found that the higher polyglutamates exceed the amount of dihydrofolate reductase by up to 12-fold, depending on the MTX exposure. This finding supports the ability of leucovorin to overcome metabolic inhibition despite the presence of intracellular MTX polyglutamate concentrations in excess of the dihydrofolate reductase binding capacity.

In addition to inhibition of dihydrofolate reductase, the polyglutamates of MTX and dihydrofolate have been described as direct inhibitors of a number of folate-dependent enzymes including thymidylate synthase (K_i MTX Glu_5 = 0.047 μM; $H_2PteGlu_5$ = 3.9 μM), aminoimidazolecarboxamide ribonucleotide (K_i MTX Glu_5 = 5.89 μM; $H_2PteGlu_5$ = 2.89 μM), and glycinamide ribotide transformylases (K_i MTX Glu_5 = 22.0 μM; $H_2PteGlu_5$ = 8.7 μM) and methylene tetrahydrofolate reductase (K_i MTX Glu_5 = 1.0 μM; $H_2PteGlu_5$ = 0.04 μM) [15–21]. Cells not exposed to MTX had intracellular reduced folate concentrations that approximated those in MTX-treated cells for any given leucovorin exposure. Presumably, since the transport of the leucovorin is identical in control and treated cells (MTX removed from the medium), the accumulation of the reduced intracellular folates would not be expected to vary. However, the level of accumulated intracellular reduced folates associates with rescue did vary with the MTX exposure. For example, rescue from 0.5 and 3.0 μM MTX occurred at leucovorin concentrations of 5 and 50 μM. The total labelled reduced folates associated with these rescue concentrations were 242 ± 72 and 520 ± 61 pmol/mg respectively. Further, the absolute intracellular amount of reduced folates associated with the process of rescue (90%) was in excess of that required by the cells for growth in their unperturbed state. These findings suggest that simple repletion of the intracellular folate pools to levels found in untreated cells is insufficient to affect cellular rescue. Rather, leucovorin rescue appears to require levels of the reduced folates in excess of those in untreated cells. It is conceivable that these high levels of reduced folates are required to act as competing substrates for the folate-dependent reactions which may be

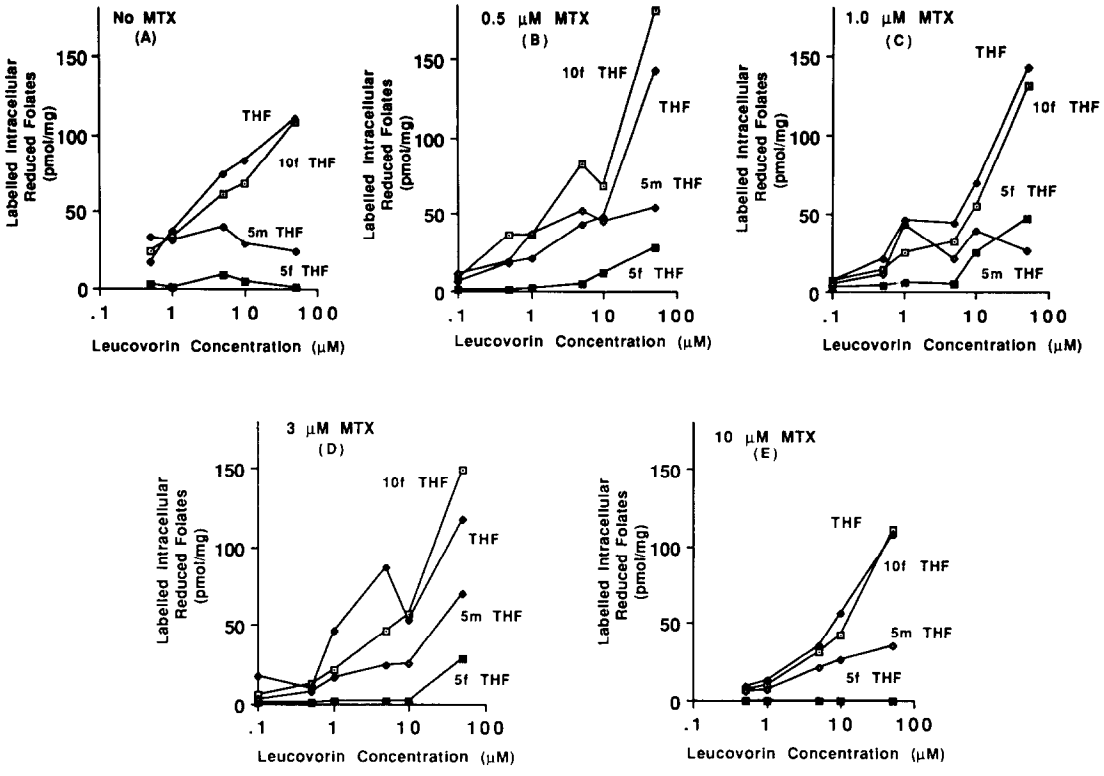

Fig. 4. Changes in the reduced folate pools during leucovorin rescue from various concentrations of MTX. MCF-7 breast cancer cells were grown to a confluency of 50–60% in RPMI 1640 medium with 10% dialyzed fetal calf serum and 2 mM glutamine. Cells were treated for 24 hr with MTX followed by washing in phosphate-buffered saline, and then an additional 24-hr exposure in various concentrations of radiolabelled leucovorin. Panel A represents the effect of increasing leucovorin on the reduced folate pools of MCF-7 cells in the absence of MTX pretreatment. Panels B–E represent the folate pool profiles 24 hr after rescue with various concentrations of leucovorin and 48 hr after the start of the MTX treatment: 0.5, 1.0, 3.0 and 10 μM. The folate pools were separated and quantitated by HPLC immediately after the 24-hr rescue period with leucovorin. Abbreviations: THF, tetrahydrofolate; 10f THF, 10-formyl tetrahydrofolate; 5f THF, 5-formyl tetrahydrofolate; and 5m THF, 5-methyl tetrahydrofolate. Each experimental point is the mean of six to eight independent experiments. The standard error values for the various reduced folate levels ranged from 5 to 20%.

under the direct inhibitory influence of MTX polyglutamates and/or dihydrofolate polyglutamates. It should also be noted that leucovorin exposure resulted in a decrease in intracellular MTX polyglutamates in a dose-dependent fashion. This effect would also impact cellular rescue with leucovorin.

Intracellular dihydrofolate levels were also found to increase in concert with increases in the exposure concentrations of leucovorin. However, unlike the reduced folates which increased continuously with increasing extracellular leucovorin, the dihydrofolate levels appeared to approach a maximal level beyond which further increases did not occur despite further increments in leucovorin rescue concentrations. The plateau level of dihydrofolate appeared to be directly related to the MTX exposure concentration. This result is in contrast with the reduced folate pools whose intracellular level for any given leucovorin concentration was relatively independent of the MTX concentration with the exception of 5,10-methylene-H_4PteGlu treated with 10 μM MTX followed by 50 μM leucovorin. This suggests that cellular rescue is associated with an intracellular concentration of dihydrofolate adequate to compete with MTX and MTX polyglutamates for dihydrofolate reductase activity. This postulate would predict that, due to competition at the level of dihydrofolate reductase, dihydrofolate levels associated with cellular rescue should be directly related to the MTX concentration to which the cells were exposed. While these studies support a role for competition between MTX and dihydrofolate at the locus of dihydrofolate reductase, they do not rule out the potential role of the accumulated reduced folates to contribute to the ultimate reactivation of this enzyme.

MTX has been shown to be a pure competitive inhibitor of dihydrofolate reductase with respect to dihydrofolate [37]. Dihydrofolate has been shown to be capable of competing with MTX for dihydrofolate reductase activity in cell-free systems using isolated enzyme preparations [34–36]. Based on mathematical models of the folate-dependent metabolic pathways, several investigators have postulated that the generation of dihydrofolate pools

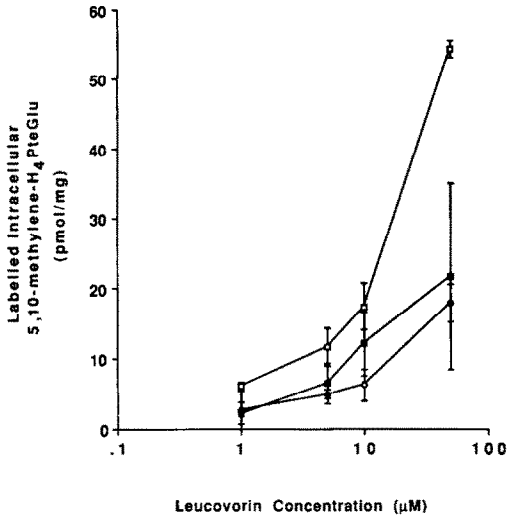

Fig. 5. Determination of 5,10-methylene-H_4PteGlu pools in MCF-7 breast carcinoma cells during leucovorin rescue. One million MCF-7 cells were plated onto 75 cm^2 tissue culture flasks in RPMI-1640 medium containing 10% dialyzed fetal calf serum and 2 mM glutamine. After 96 hr of growth, the cells were treated with either no MTX (○), 1 μM MTX (■), or 10 μM MTX (□). After 24 hr, the cells were washed and placed into drug-free medium supplemented with 1–50 μM [^{3}H]leucovorin. Following the 24-hr rescue period, the cells were washed and harvested; 5,10-methylene-H_4PteGlu was fractionated by HPLC and quantitated by liquid scintillation counting. Each point is the mean ± SE of three to four independent experiments.

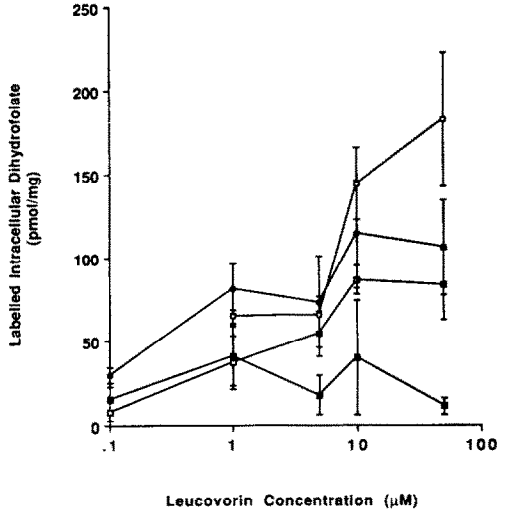

Fig. 6. Changes in the dihydrofolate pools during leucovorin rescue after a 24-hr exposure to MTX. The MCF-7 breast cells were plated into RPMI 1640 medium with 10% dialyzed fetal calf serum and 2 mM glutamine and grown to 60–70% confluency. The cells were treated with MTX [(■) 0.5 μM; (□) 1 μM; (●) 3 μM; and (○) 10 μM] for a 24-hr period, washed in phosphate-buffered saline, and then exposed to radiolabelled leucovorin for an additional 24 hr. The dihydrofolate pools were extracted, separated, and quantitated by HPLC following the 24-hr exposure to leucovorin. Each experimental point is the mean ± SE of six to eight separate experiments.

adequate to compete with MTX for dihydrofolate reductase activity is a prerequisite for leucovorin rescue [35, 36]. The studies in the present report serve as an additional evidence for the importance of such a competition in intact human neoplastic cells and suggest that competition may occur (1) between reduced folates and MTX/dihydrofolate polyglutamates at folate-dependent enzymes other than dihydrofolate reductase, and (2) between MTX and dihydrofolate polyglutamates at the level of dihydrofolate reductase.

The apparent paradoxical role of dihydrofolate as an effector of both metabolic inhibition and leucovorin rescue may be explained by its interaction with dihydrofolate reductase as a substrate versus the interaction with other folate-dependent enzymes where it appears to be an inhibitor. As the amount of dihydrofolate increases with increasing leucovorin concentrations, presumably, the dihydrofolate-mediated inhibitions persist as the inhibitor increases in proportion with the competing reduced folate substrates. At rescue concentrations of leucovorin, the dihydrofolate concentrations plateau, while reduced folates continue to increase, thereby effectively competing with dihydrofolate for activity of the folate-dependent enzymes. The dihydrofolate plateau allowing effective competition is presumably related to the concentration of dihydrofolate needed to compete with MTX for dihydrofolate reductase activity.

It appears that cellular rescue depends upon the generation of both reduced folates and dihydrofolate in adequate quantities to compete with the various metabolic inhibitors generated in cells treated with MTX. The fact that normal and malignant cells vary in their abilities to generate MTX polyglutamates may explain the selective nature of leucovorin rescue [41, 44]. While the MTX polyglutamates are similar to MTX in inhibitory activity against dihydrofolate reductase, they are substantially more potent than MTX as inhibitors of other folate-dependent enzymes. Cells that are able to generate MTX polyglutamates would be expected to require greater amounts of reduced folates to compete with these metabolites for the successful reconstitution of folate-dependent metabolism. In addition to furthering an understanding of the process of leucovorin rescue in malignant cells, these studies are consistent with the concept that direct inhibition of folate-dependent enzymes plays a role in the mechanism of action of MTX.

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