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# Effects of Overexpression of γ-Glutamyl Hydrolase on Methotrexate Metabolism and Resistance[1]

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## ABSTRACT

**Intracellular metabolism of methotrexate (MTX) to MTX-polyglutamates (MTXPG) is one determinant of cytotoxicity. Steady-state accumulation of MTXPG seems to depend on the activity of two enzymes: folylpolyglutamate synthetase (FPGS), which adds glutamate residues, and γ-glutamyl hydrolase (GGH), which removes them. Overexpression of GGH would be expected to decrease intracellular MTXPG, thereby increasing efflux of MTX and decreasing cytotoxicity. Increased expression of GGH has been shown to be associated with resistance to MTX in human sarcoma cell lines and a rat hepatoma cell line. To clarify the specific role of GGH in determining MTX sensitivity, we investigated the phenotype produced by forced GGH overexpression in two cell types. Furthermore, because MTX and folic acid share metabolic pathways, we measured the effects of GGH overexpression on folic acid metabolism. The full-length cDNA for GGH, subcloned into a constitutive expression vector, was transfected into a human fibrosarcoma (HT-1080) and a human breast carcinoma (MCF-7) cell line. Compared with the clones containing an empty vector, the GGH-overexpressing cells express 15- to 30-fold more GGH mRNA, more GGH protein, and 15- to 90-fold more GGH enzyme activity. GGH overexpression altered MTX accumulation and metabolism to long-chain polyglutamates. In contrast to expectations, however, GGH overexpression did not confer resistance to short MTX exposures in either cell line. Changes in MTX metabolism were found to be balanced by alterations in accumulation and metabolism of folic acid. The ratio of MTX:folate accumulation may be a better predictor of MTX cytotoxicity than the accumulation of either alone. We conclude that, at least for these two cell lines, GGH overexpression alone is insufficient to produce clinical resistance to MTX.**

## INTRODUCTION

For four decades, MTX[3] has been an essential drug in curative regimens for patients with ALL and is presently used in the treatment of a number of other neoplastic diseases, including osteogenic sarcoma, breast cancer, head and neck cancers, and non-Hodgkin's lymphoma (1). However, achieving prolonged disease-free survival is still difficult even in chemotherapy-sensitive diseases. The efficacy of MTX, as with other antineoplastic agents, is ultimately limited by either inherent resistance or resistance acquired during the course of therapy (1).

There is increasing evidence that much of the variation in MTX sensitivity among different normal tissue types and malignancies can be accounted for by differences in how the tumor cells accumulate MTXPGs. As glutamate residues are added intracellularly to MTX by the enzyme FPGS, the additional negative charge markedly reduces efflux from the cell, thus prolonging the drug's cytotoxic effect (2). In addition, MTXPGs dissociate more slowly from the target enzyme DHFR than does MTX (3) and are potent inhibitors of thymidylate synthase, as well as enzymes necessary for purine synthesis (4). Steady-state intracellular levels of MTXPG most likely depend on the balance of the two enzymes FPGS and GGH, a lysosomal peptidase.

Differences in FPGS activity have been clearly related to differences in both MTXPG accumulation and MTX response in ALL and AML cell lines (5, 6) and in leukemic blasts from patients (7). Decreased FPGS activity is proportional to MTX resistance in subclones of the human T-ALL cell line, CEM-S (8). Reducing FPGS expression by transfection of an antisense message-containing vector significantly alters folate and MTX metabolism (9).

Recently, several lines of evidence have suggested an equally important role for GGH in regulating MTXPG accumulation. MTXPG accumulation is better predicted by the ratio of GGH:FPGS activity than by the activity of either enzyme alone (10, 11). Incubating cells with inhibitors of GGH function will augment MTXPG accumulation and MTX sensitivity in sarcomas (12) and leukemic blasts (13, 14), as well as in normal lymphocytes and fibroblasts (14). Finally, increased expression of GGH and decreased MTXPG accumulation have been demonstrated in MTX-resistant human sarcoma cell lines (15) and in a rat hepatoma cell line with antifolate resistance induced by continuous exposure to an antifolate analogue of MTX (16).

Because folates and antifolates share uptake and metabolic pathways, alterations at any step in these pathways would be expected to affect both folate and antifolate. Given that reduced folates can rescue cells from antifolate toxicity (17), alterations in intracellular folate metabolism would be expected to influence antifolate sensitivity. The recent cloning of the cDNA for human GGH (18) allows the possibility of directly testing the phenotype associated with GGH overexpression. We now report on the effects of GGH overexpression on both MTX and folate physiology as well as MTX sensitivity in two human cancer cell lines.

## MATERIALS AND METHODS

**Cell Cultures.** HT1080 (a human fibrosarcoma cell line) and MCF7 (human breast carcinoma) were obtained from American Type Culture Collection. Both cell lines were maintained at 37° in 5% $CO_2$ in folic acid-depleted RPMI 1640, supplemented with 10% FCS, and 20 nM 5-formyltetrahydrofolate (leucovorin) as the only folate source. This concentration of leucovorin is similar to physiological human plasma folate concentration and is well below the concentration of folate in standard tissue culture media, 2 μM. After transfection, constant selection pressure was maintained with 750 μg genetecin (G418)/ml.

**Construction and Transfection of the GGH Expression Vector.** The full-length cDNA for human GGH, including a region coding for a 24-amino acid leader sequence, was provided by Dr. John Galivan, New York State

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[3] The abbreviations used are: MTX, methotrexate; ALL, acute lymphoblastic leukemia; MTXPG, MTX polyglutamate; FPGS, folylpolyglutamate synthetase; DHFR, dihydrofolate reductase; GGH, γ-glutamyl hydrolase (folylpolyglutamate hydrolase); $5CH_3THF$, 5-methyltetrahydrofolate; RFC, reduced folate carrier; RT-PCR, reverse transcription-PCR; AML, acute myeloblastic leukemia.

Department of Health, Albany, New York (18). The GGH sequence was released from the pCR2 vector using the restriction enzymes *Bam*HI and *Not*I and was then ligated into the multiple cloning site of the commercially available mammalian expression vector pcDNA 3 (Invitrogen, Carlsbad, CA). This construct contains a cytomegalovirus promoter region that controls gene expression, as well as genes for ampicillin and neomycin resistance. Correct integration and orientation were established by predicted fragment sizes after multiple restriction enzyme digestions.

Five $\mu$g of GGH-containing plasmid were transfected into $10^6$ HT1080 and $10^5$ MCF7 cells. In a separate transfection, cells of each cell line were transfected with empty pcDNA 3. Transfected cells were incubated with 750 $\mu$g/ml G418 to select for presence of plasmid. After selection, colonies derived from individual clones were selected and expanded. Two clones with the GGH-containing vector from each cell line were selected at random for further analysis and comparison with the cells containing the empty vector (mock transfectants).

**Real-Time Quantitative RT-PCR.** Total cellular RNA was extracted from cells in log-phase growth using the guanidinium thiocyanate extraction method, and the concentration was determined spectrophotometrically by absorbance at 260 nm. Genomic DNA was eliminated by treating with RNase-free DNase, which was then heat-inactivated and removed by phenol-chloroform extraction. Approximately 100 ng of RNA was reverse transcribed using random primers and the cDNA Cycle kit (Invitrogen) following the manufacturer's instructions.

PCR primers and a fluorogenic probe were designed based on the known sequence of the human GGH cDNA (18), and were synthesized commercially. PCR amplification of the target sequence was performed in the Perkin-Elmer TaqMan 7700 Sequence detection system (Perkin-Elmer, Forster City, CA), using the manufacturer's TaqMan Universal PCR Master Mix and following the manufacturer's instructions. Target sequences were quantitated in real-time, during PCR, by comparison with known dilutions of plasmids containing the target sequence. PCR amplification of a rRNA sequence was performed in parallel for each sample, using a rRNA control kit (Perkin-Elmer), and was used as internal control, normalizing for the amount of total RNA entering the reverse transcription step. We have been able to show strong correlation between GGH mRNA, measured by this technique, and GGH protein measured by Western analysis, in human sarcoma and leukemia cell lines ($r$, 0.9; $P < 0.01$). The GGH primers were 5′-GCTTATTAACTGCCACAGAT-ACGTTG-3′ and 5′-GAACATTCTGCTGTGCAATGAC-3′, and the probe sequence was 5′-FAM-CGTGGCAATGCCGCTGAACTTCA-TAMRA-3′. PCR conditions were as follows: 50°C for 2 min; 95° for 10 min; and forty cycles of melting at 95° for 15 s and annealing/extending at 60° for 1 min.

**GGH Activity.** Cells were suspended in 500 $\mu$l of MTEN buffer [50 mM 2-(4-morpholino)-ethane sulfonic acid, 25 mM Tris base, 25 mM ethanolamine, 100 mM NaCl (pH 4.5)], and were lysed using three bursts from a microsonication probe for 15 s each. The suspension was then clarified by centrifugation at 12,000 $g$ for 15 min at 4°C. Total protein concentration in the supernatant was quantitated by Bradford analysis using BSA as a standard.

Hydrolase activity in cell lysates was determined as previously described (19). Briefly, 10–60 $\mu$g of protein was incubated with 50 $\mu$M substrate (MTX-$glu_4$) in MTEN buffer (pH 4.5) containing 1 mM $ZnCl_2$ and 2 mM DTT at 37°C. (Although the reaction products, MTX-$glu_{1-3}$, are substrates for GGH and can compete with MTX-$glu_4$ for binding with GGH, MTX-$glu_4$ was chosen for this assay because it has much higher affinity for the enzyme than MTX-$glu_{1-3}$, allowing greater sensitivity.) Aliquots of the reaction mixture were removed at various time points and were placed in boiling water for 5 min before centrifugation at 15,000 $\times$ $g$ for 15 min. MTXPGs in the supernatant were separated by capillary electrophoresis (19), using a Spetra-Phoresis 1000 instrument (Thermo Separation, San Jose, CA), and a 50-cm $\times$ 75-$\mu$m, inside diameter, silica capillary (Polimicro Technology, Phoenix, AZ). Separation was performed at 25°C in 20 mM sodium borate with 15 mM SDS (pH 9.5) with +20 keV. Absorbance was monitored at 300 nm. GGH specific activity was calculated as $\mu$mol of MTX-$glu_4$ hydrolyzed to MTX-$glu_{1-3}$ per min per mg of protein and was determined from time points at which less than 50% of the substrate was consumed.

**Western Analysis.** A rabbit polyclonal antibody to baculovirus-expressed GGH was generously provided by Dr. John Galivan, New York State Department of Health, Albany, New York (20). Total cellular protein was collected as above, separated by SDS-12.5% PAGE, and transferred onto nitrocellulose membranes. Prestained known proteins (Bio-Rad) were used to determine approximate molecular masses on the blot. The blots were probed with a 1:20,000 dilution of the anti-GGH rabbit antibody. A goat antirabbit IgG horseradish peroxidase conjugate (Bio-Rad) was used as a secondary antibody, and the blot was developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). A broad band of approximate molecular mass of 37 kDa was expected (20).

To test for equal loading, the blots were then stripped of antibody (by 1-h incubation in a 200-mM glycine stripping buffer with 0.1% SDS and 1% Tween 20) and reprobed with a 1:1000 dilution of mouse anti-$\beta$-Actin antibody, followed by antimouse IgG horseradish peroxidase conjugate secondary antibody.

**[$^3$H]-MTX and [$^3$H]-5$CH_3$THF Accumulation.** Cells were plated at a density of $1 \times 10^5$ cells/ml in six-well plates in 2 ml of the medium described above. Each experiment was performed in triplicate; averages are reported. After 3–4 days incubation, as the cells began to enter exponential growth, the cells were washed twice with HEPES-buffered saline solution, and the medium was replaced with 1 ml of RPMI 1640, with no serum and no folate, plus either [$^3$H]-MTX (final concentration, 1 or 10 $\mu$M) or [$^3$H]-5$CH_3$THF (final concentration, 20 nM; similar to physiological human plasma folate concentration). Thirty-five $\mu$l of $10^{-4}$ M unlabeled 5$CH_3$THF (approximately 175-fold excess) was added to three wells for each sample, to saturate nonspecific binding.

After 24 h, the cells were washed twice with iced PBS, and the medium was replaced with normal culture medium, containing 10% FCS and 20 nM leucovorin. At time 0, 6, and 24 h after washing, cells were washed twice, released by incubation with trypsin, collected, and boiled for 5 min. Intracellular content of [$^3$H]-MTX or -5$CH_3$THF was determined by scintillation counting. For 5$CH_3$THF, nonspecific binding was subtracted from each result.

Intracellular MTX and folate polyglutamates were separated on a reverse phase $C_{18}$ column as described previously (21). Radioactivity was quantitated by a Flo-One/$\beta$, on-line scintillation counter (Radiomatic Instruments & Chemical Co., Meriden, CT). Authentic MTX and folylpolyglutamates were used as internal standards.

**Intracellular Folates.** Intracellular folate content was measured by a competitive radioligand binding assay using sequential analysis and extraction techniques described previously (22), with the following modifications: $\beta$-lactoglobulin was used as a folate binder rather than hog kidney extract, and the final volume of the reaction was reduced to 0.5 ml.

**[$^3$H]Thymidine Incorporation into DNA.** Cells were plated in 6-well plates, in 2 ml of medium at a concentration of $10^5$ cells per ml. After 2 days, [$^3$H]thymidine was added for 1 h, at a final concentration of 1 $\mu$M. The cells were washed twice in iced PBS and harvested. An aliquot was counted, and the rest was suspended in 10% trichloroacetic acid and placed on ice for 30 min. After centrifugation for 15 min at 15,000 $\times$ $g$, the pellet and supernatant were separated. Radioactivity in the pellet was determined by scintillation counting. Results for each clone were divided by the total cell number and were normalized by dividing by the same ratio determined for the mock transfectant. Each experiment was performed with at least three replicates.

**Cellular Content of DHFR.** DHFR was analyzed by measuring [$^3$H]-MTX binding in whole cell extract, using techniques previously described (23). Briefly, cell pellets were suspended in 50 mM Tris buffer (adjusted to pH 8.3), containing 150 mM $\beta$-mercaptoethanol and 10 mM EDTA at 4°C, and were freeze-thawed three times. The extracts were clarified by centrifugation at 12,000 $\times$ $g$, and aliquots of these samples were exposed to [$^3$H]-MTX in the presence of excess NADPH. Unbound MTX was removed with charcoal precipitation. Because DHFR is the only intracellular protein with significant affinity for nonpolyglutamylated MTX and each DHFR molecule can bind exactly one molecule of MTX, this method allows calculation of pmol DHFR per $10^6$ cells. For each sample, specific activity was found to be linear in serial dilutions of extracts from $\sim 2 \times 10^5$ to $10^7$ cells/sample.

**Cytotoxicity.** Cells were plated in 96-well tissue culture plates, at a concentration of $10^4$ cells/ml in 100 $\mu$l of RPMI 1640, supplemented with 10% FCS, 25 nM leucovorin, and 750 $\mu$g/ml G418. All of the experiments were performed in at least triplicate. To decrease thymidine salvage from the culture medium, thymidine content was depleted by pretreatment with thymidine phosphorylase. The serum was pretreated with thymidine phosphorylase for 30 min at 37°C, (which was then inactivated by heating to 55°C for 15 min), prior

Table 1 *Expression of GGH mRNA and enzyme activity*

Expression of GGH mRNA and enzyme activity are increased in clones transfected with the GGH-containing vector compared with cells transfected with an empty vector (mock-transfectants). GGH mRNA was measured by quantitative RT-PCR. Enzyme activity was determined by measuring the ability of whole cell protein extract to hydrolyze MTX-$glu_4$.

| Cell line | Clone | GGH mRNA (relative to mock) | GGH activity (nmol substrate/min/mg protein) |
|---|---|---|---|
| HT1080 | Mock | 1 | 0.9 |
| | G10 | 23 | 87.7 |
| | G12 | 28 | 57.4 |
| MCF7 | Mock | 1 | 1.1 |
| | G5 | 17 | 16.6 |
| | G11 | 18 | 14.7 |

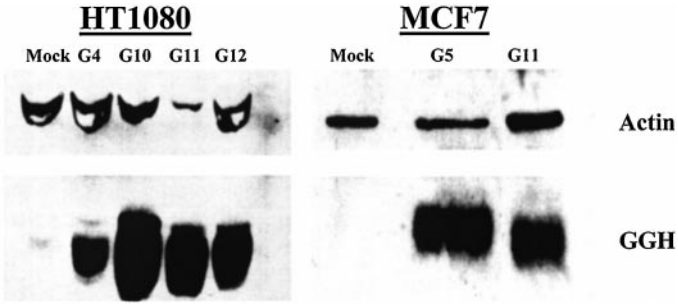


Fig. 1. GGH protein by Western analysis. Equal amounts of total cellular protein from each clone (10 $\mu$g for MCF7 and 40 $\mu$g for HT1080 clones) were separated by SDS-PAGE and probed with antihuman GGH antibody. The blots were then stripped and reprobed with antibody to $\beta$-actin. Clones transfected with GGH-containing plasmid express much more GGH protein than the mock transfectants, in which GGH is barely detectable under these conditions.

to being added to the medium. After 24-h incubation at 37° in 5% $CO_2$, MTX was added to each well, at various concentrations, in 100 $\mu$l of RPMI 1640 with no serum and no folate. After 4 h, the drug-containing media was replaced with 200 $\mu$l fresh media, containing leucovorin, serum, and G418, as above, and the cells were incubated for an additional 72 h. Cell survival was then measured by conversion of the tetrazolium salt sodium 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) to a water-soluble formazan product by mitochondrial dehydrogenases and read spectrophotometrically in a microplate reader.

**Statistics.** Two-tailed *t* tests were used to compare mean values for replicated experiments. $\chi^2$ analysis was used to examine differences in the distribution of MTX- and 5-$CH_3$THF-polyglutamates between the different GGH-overexpressing clones and the corresponding mock transfectants. A *P* of 0.05 was used as a cutoff to determine statistical significance.

## RESULTS

Compared with the mock transfectants, the clones transfected with the GGH-containing vector expressed more GGH mRNA (measured by quantitative RT-PCR) and more GGH enzyme activity in whole-cell protein extract (Table 1). More protein was detected by Western blotting with antihuman GGH antibody (Fig. 1). GGH activity in the mock-transfected HT1080 cells was similar to the activity seen in the mock-transfected MCF7 cells.

Intracellular folates were decreased in the two HT1080 clones overexpressing GGH compared with mock-transfected HT1080 cells ($P < 0.01$; Table 2). One of the two MCF7 clones overexpressing GGH had lower intracellular folates than the mock transfectant, but this did not reach statistical significance ($P = 0.06$).

Despite lower concentrations of intracellular folates in three clones, overexpression of GGH did not alter growth characteristics of HT1080 or MCF7 cells. Neither doubling time during exponential growth nor cell concentrations when cultures reached confluence were significantly affected (Table 2).

There was no evidence that the GGH-overexpressing HT1080 cells compensated for low intracellular folates by a significant increase in thymidine salvage from the media (Table 2). There was a modest increase in thymidine uptake seen in one of the two GGH-overexpressing MCF7 clones ($P < 0.05$). In both cell lines, GGH overexpression was associated with small but statistically significant alterations in DHFR expression (Table 2). DHFR was increased in MCF7 cells by 33–43%, and decreased in HT1080 cells by 46–57%.

The effects of GGH overexpression on MTX accumulation were more marked in MCF7 cells than in HT1080 (Table 3). Unmodified MCF7 cells accumulate almost 10-fold more MTX after 24-h exposure to 1 $\mu$M [$^3$H]-MTX than HT1080 cells. GGH overexpression did not significantly alter total MTX accumulation in HT1080 cells but resulted in 50–60% reduction in total MTX accumulation in MCF7 cells.

Changes in accumulation of 5-$CH_3$-[$^3$H]-THF were found to parallel those seen in MTX accumulation (Table 3). As was found with MTX, unmodified MCF7 cells accumulate ~10 times as much $5CH_3$THF as HT1080 cells. In both cell lines, GGH overexpression was associated with decreased accumulation of $5CH_3$THF, with the greater reduction noted in MCF7 cells. Because both MTX and $5CH_3$THF accumulation were decreased by GGH overexpression to the same degree, the ratio of MTX to $5CH_3$THF accumulation was not significantly changed by GGH overexpression (Table 3). After 24-h exposure to 1 $\mu$M [$^3$H]-MTX, the majority of intracellular MTX was found as long-chain MTXPG (MTX-$glu_{3-6}$) in both mock-transfected HT1080 (86%) and MCF7 cells (89%; Table 4). GGH-overexpression

Table 2 *Growth characteristics, intracellular folates, thymidine uptake, and DHFR content*

GGH overexpression did not alter doubling time during exponential growth, despite decreased intracellular folate pools. Intracellular folate, thymidine uptake, and intracellular DHFR were measured as described in the text. GGH-overexpressing clones were compared with the corresponding mock-transfected cells. Thymidine salvage was increased in only one GGH-overexpressing clone. GGH overexpression was associated with an increase in DHFR in MCF7 cells, but a decrease in HT1080.

| Cell line | Clone | Doubling time (days) | Intracellular folate (pmol/$10^6$ cells) | Thymidine uptake (%, relative to mock) | DHFR (pmol/$10^9$ cells) |
|---|---|---|---|---|---|
| HT1080 | Mock | 1.3 | 5.72 ± 1.17 | 100 ± 19 | 74 ± 10 |
| | G10 | 1.4 | 2.45 ± 0.37[a] | 78 ± 10 | 40 ± 17[a] |
| | G12 | 1.7 | 2.23 ± 0.33[a] | 101 ± 21 | 32 ± 5[a] |
| MCF7 | Mock | 1.9 | 9.87 ± 0.71 | 100 ± 19 | 293 ± 38 |
| | G5 | 2.1 | 9.61 ± 1.11 | 92 ± 19 | 419 ± 52[a] |
| | G11 | 2.2 | 7.55 ± 0.93[b] | 131 ± 19[c] | 390 ± 49[a] |

[a] $P < 0.01$.
[b] $P = 0.06$.
[c] $P < 0.05$.

Table 3 *Accumulation of MTX and 5CH$_3$THF after 24-h exposure*

Cells were exposed to tritium-labeled MTX or $5CH_3$THF for 24 h. After washing and harvesting, intracellular signal was measured by scintillation counting. GGH overexpression produced greater changes in total MTX and $5CH_3$THF accumulation in MCF7 than in HT1080. Mean values were compared with the corresponding mock-transfected clone with a two-tailed *t* test.

| Cell line | Clone | MTX accumulation (pmol/$10^6$ cells) | $5CH_3$THF accumulation (pmol/$10^6$ cells) | MTX: $5CH_3$THF ratio |
|---|---|---|---|---|
| HT1080 | Mock | 3.21 ± 1.1 | 0.66 ± 0.05 | 5.9 ± 1.0 |
| | G10 | 2.98 ± 0.4 | 0.51 ± 0.03 | 5.2 ± 0.4 |
| | G12 | 3.09 ± 0.7 | 0.55 ± 0.07 | 4.9 ± 0.4 |
| MCF7 | Mock | 26.7 ± 1.3 | 5.94 ± 0.21 | 4.5 ± 0.2 |
| | G5 | 12.9 ± 1.7[a] | 2.59 ± 0.02[b] | 5.0 ± 0.6 |
| | G11 | 10.3 ± 1.1[a] | 2.46 ± 0.13[a] | 4.2 ± 0.4 |

[a] $P < 0.001$.
[b] $P < 0.01$.

Table 4 *Long-chain polyglutamates, as a fraction of total intracellular [$^3$H]-MTX and 5-CH$_3$-[$^3$H]-THF polyglutamates*

After cells were exposed to labeled MTX or 5CH$_3$THF for 24 h, intracellular contents were separated by high-performance liquid chromatography. GGH overexpression altered accumulation of the longer-chain polyglutamates (MTX-glu$_{3-6}$ and 5CH$_3$THF-glu$_{7-8}$). Differences between GGH-overexpressing clones and the mock transfectant were analyzed by $\chi^2$ analysis.

| Cell line | Clone | MTX 1 μM MTX-glu$_{3-6}$/ total MTX (%) | MTX 10 μM MTX-glu$_{3-6}$/ total MTX (%) | 5CH$_3$THF 20 nM 5CH$_3$THF-glu$_{7-8}$/ total 5CH$_3$THF (%) |
|---|---|---|---|---|
| HT1080 | Mock | 86 | 26 | 47 |
| | G10 | 85 | 5[a] | 18[a] |
| | G12 | 71[b] | 10[c] | 30[b] |
| MCF7 | Mock | 89 | 76 | 45 |
| | G5 | 81 | 54[c] | 40 |
| | G11 | 82 | 40[a] | 33 |

[a] $P < 0.01$.
[b] $P = 0.05$.
[c] $P < 0.01$.

was associated with a shift in the intracellular MTX pool toward unmodified MTX (MTX-glu$_1$) and MTX-glu$_2$.

When cells were exposed to higher extracellular MTX concentrations, metabolism to long-chain polyglutamates was less complete, and the effects of GGH-overexpression on MTXPG distribution were more marked (Table 4). After 24-h exposure to 10 μM MTX, the fraction of intracellular MTX found as long-chain MTXPG is reduced in unmodified HT1080 cells to 26% and in MCF7 cells to 76%. In both cell lines, GGH overexpression was associated with a significant decrease in metabolism of MTX to long-chain MTXPG.

In both unmodified cell lines, all of the intracellular folate was found as long-chain polyglutamates (5-CH3THF-glu$_{4-8}$, with -glu$_{6-7}$ predominating) after 24-h exposure to 20 nM [$^3$H]-5CH$_3$THF. GGH overexpression was associated with a shift away from the very-long-chain folylpolyglutamates (-glu$_{7-8}$) among the HT1080 cells. Similar shifts was seen in MCF7 cells but were not statistically significant. In one of the GGH-overexpressing MCF-7 clones, a smaller fraction of intracellular folate was found as -glu$_8$ than in the mock transfectant ($P < 0.05$, two-tailed $t$ test; data not shown).

Despite the changes in MTX accumulation and metabolism, the GGH-overexpressing clones were not found to be resistant to short (4–6 h) exposures to MTX (Fig. 2). Compared with the mock transfectants, the GGH-overexpressing clones tended to be more sensitive to MTX. This tendency was more pronounced in MCF7, in which the IC$_{50}$ was more than 2-fold lower in the GGH-overexpressing clones (8.3 to 9.6 μM) than in the mock transfectant (22.9 μM). Longer exposures to MTX, for which sensitivity is less dependent on differences on MTXPG formation, similarly showed a lack of MTX resistance among the GGH-overexpressing cells (data not shown).

## DISCUSSION

Previous reports describe cell lines that express increased GGH activity and are resistant to antifolates. Li *et al.* described a human mesenchymal chondrosarcoma cell line (HS16) with GGH activity that was ~75% higher than HT1080 and with a corresponding relative resistance to MTX (15). Rhee *et al.* described a rat hepatoma cell line in which antifolate resistance was induced by stepwise increases in exposure to the glycinamide-ribonucleotide transformylase inhibitor DDATHF (16). This resistant subline expresses 7-fold higher GGH activity and an IC$_{50}$ for MTX that is 54 times higher than for the parental cells. It should be noted that in both reports, other changes were noted in antifolate metabolism that could be expected to affect antifolate sensitivity. In addition to increased GGH, HS16 also expresses FPGS with altered kinetics (decreased $V_{max}/K_m$) relative to HT1080 (24). In the resistant hepatoma subline, transport of MTX across the cell membrane was reduced by 80%, and transport of leucovorin by 90%, compared with the parental lines, and there was a modest (3-fold) increase in the target enzyme DHFR (14). Finally, it may be relevant that these resistant cell lines were characterized while growing in medium containing 2 μM folic acid, a concentration 50- to 100-fold higher than the physiological human plasma folate, 5CH$_3$THF. It is conceivable that, at high extracellular folate concentrations, no differences in intracellular folate (either in steady-state or after antifolate challenge) would be seen between wild-type and GGH-overexpressing cells; effects of GGH on MTX-polyglutamates would predominate and MTX resistance would result.

We now report the results of experiments that directly investigate the phenotype that results from overexpression of GGH in cells grown under conditions more similar to physiological human plasma. The cell lines HT1080 and MCF7, transfected with the full-length cDNA for human GGH in a mammalian expression vector, expressed significantly more GGH mRNA and protein than cells transfected with an empty vector. Enzyme activity in the GGH-overexpressing MCF7 and HT1080 cells was 13- to 15-fold and 64- to 97-fold higher than control cells, respectively. The GGH-overexpressing MCF-7 cells accumulate less MTX after 24-h exposure. MTX is less completely metabolized to long-chain MTXPG in the GGH-overexpressing cells in both cell lines. Nevertheless, resistance to MTX after short-term exposure was not seen in GGH-overexpressing clones of either cell line. Four-h, rather than continuous, exposure had been chosen, spe-



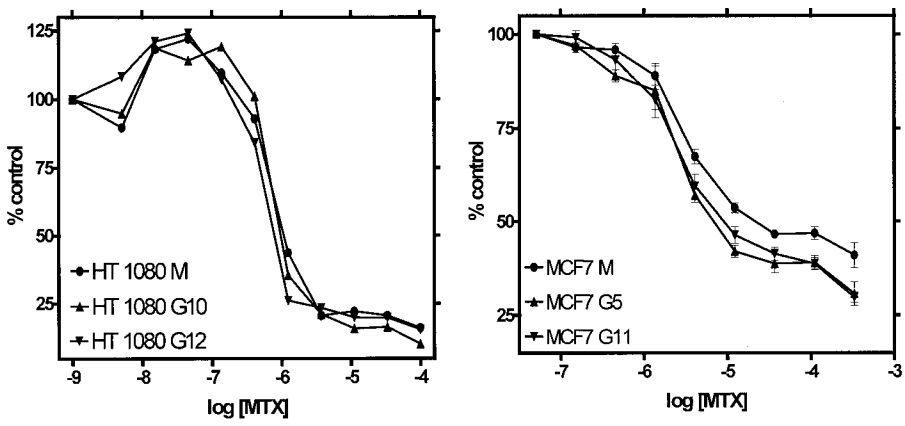


Fig. 2. GGH overexpression does not produce resistance to short exposure to MTX. Cells were exposed to MTX for 4 h followed by additional incubation in drug-free medium for at least 72 h. Cell survival was compared with wells with no drug exposure.

cifically to amplify the effects on MTX sensitivity of alterations in MTX accumulation and metabolism to MTXPG.

The proteins involved in MTX metabolism obviously evolved to process folate, not MTX. Alterations at any step in this pathway should be expected to affect intracellular folates as well as MTX. In both HT1080 and MCF7, changes in MTX accumulation caused by GGH overexpression were found to be balanced by changes in accumulation of folate. GGH overexpression did not alter the ratio of MTX to $5CH_3THF$ accumulation after 24 h exposure.

We suspect that when folate and/or antifolate metabolism is altered, either spontaneously or experimentally, this ratio (MTX:folate uptake) will predict antifolate sensitivity better than uptake of either substrate alone. As an illustration of the importance of this ratio, polymorphisms have been described in the gene for the RFC, which impair MTX transport into the cell while maintaining folate transport (25). By reducing the MTX:folate uptake ratio, cells with this RFC variant become resistant to MTX. Additionally, the ratio of MTX:folate uptake by blasts isolated from children with ALL was recently shown to correlate better with the presence or absence of relapse than uptake of either folate or antifolate alone (26). Taken together, these results underscore the importance of examining both folate and antifolate physiology when predicting how changes in cellular biochemistry will affect antifolate sensitivity.

On the basis of our data, we find it unlikely that GGH overexpression alone would be responsible for clinically relevant resistance to MTX. A tumor cell that spontaneously developed elevated GGH activity would likely be at a disadvantage relative to its sisters, in the absence of continuous antifolate selection pressure, because of decreased intracellular folates. Likely compensatory changes, *e.g.*, increased FPGS or RFC activity, would tend to increase MTX sensitivity, further decreasing the probability that the cell would survive the next exposure to MTX.

It is possible that our results are specific to the two cell types studied. There are tissue-specific differences in MTX metabolism. GGH activity varies widely across tissue types, highest in liver and kidney and significantly lower in most other tissues.

It is also possible that GGH overexpression induced compensatory changes in cellular biochemistry that could affect MTX sensitivity. Despite lower intracellular folate concentrations, no change in cell doubling time was associated with GGH overexpression, suggesting the possibility of compensatory adaptation. We did note, for example, a modest increase in thymidine salvage in the one MCF7 clone with decreased intracellular folate. Small changes in DHFR content also accompanied GGH overexpression. It seems likely, however, that the magnitude of these changes is insufficient to affect MTX sensitivity. Even in the GGH-overexpressing MCF-7 cells with decreased MTX uptake and increased DHFR content, intracellular MTX after 24-h exposure was still in molar excess of DHFR content by a factor of 26–31. Furthermore, there is evidence from other cell lines, that there is a minimum intracellular folate concentration, above which DNA synthesis and cell doubling will not correlate with folate concentration (27). It is possible that these GGH-overexpressing clones have not crossed that threshold.

Finally, it is possible that GGH overexpression is insufficient to produce MTX resistance because the majority of the cytosolic antifolate does not encounter the increased lysosomal GGH protein. The entry of MTXPG has been shown to occur by a facilitated transport mechanism that has a lower affinity for MTXPG than GGH has (28, 29). If lysosomal entry is the rate-limiting step in MTXPG hydrolysis, quantitative alterations in lysosomal GGH activity would have limited effect on cytosolic MTX concentrations, and, thus, little effect on MTX sensitivity.

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