Effects of folate and folylpolyglutamyl synthase modulation on chemosensitivity of breast cancer cells

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Abstract
Folylpolyglutamyl synthase (FPGS) converts intracellular folates and antifolates to polyglutamates. Polyglutamylated folates and antifolates are retained in cells longer and are better substrates than their monoglutamate counterparts for enzymes involved in one-carbon transfer. FPGS modulation affects the chemosensitivity of cancer cells to antifolates, such as methotrexate, and 5-fluorouracil (5FU) by altering polyglutamylation of antifolates and specific target intracellular folate cofactors. However, this effect may be counterbalanced by FPGS modulation-induced changes in polyglutamylation of other intracellular folate cofactors and total intracellular folate pools. We generated an in vitro model of FPGS overexpression and inhibition in breast cancer cells by stably transfecting human MDA-MB-435 breast cancer cells with the sense FPGS cDNA or FPGS-targeted small interfering RNA, respectively, and investigated the effects of FPGS modulation on chemosensitivity to 5FU and methotrexate. FPGS modulation-induced changes in polyglutamylation of both antifolates and folate cofactors and in intracellular folate pools affected chemosensitivity of breast cancer cells to pemetrexed and trimetrexate whose cytotoxic effects do or do not depend on polyglutamylation, respectively, in a predictable manner. However, the effects of FPGS modulation on the chemosensitivity of breast cancer cells to 5FU and methotrexate seem to be highly complex and depend not only on polyglutamylation of a specific target intracellular folate cofactor or methotrexate, respectively, but also on total intracellular folate pools and polyglutamylation of other intracellular folate cofactors. Whether or not FPGS modulation may be an important clinical determinant of chemosensitivity of breast cancer cells to 5FU and methotrexate-based chemotherapy needs further exploration. [Mol Cancer Ther 2007;6(11):2909–20]

Introduction
Folate mediates the transfer of one-carbon units necessary for thymidylate and purine biosynthesis and, hence, is an essential factor for DNA synthesis (1). In neoplastic cells, folate depletion and disrupted folate metabolism cause ineffective DNA synthesis, resulting in inhibition of tumor growth (2). This has been the basis for cancer chemotherapy using antifolates and 5-fluorouracil (5FU; ref. 2). Although monoglutamates are the only circulating forms of folate in blood and the only form of folate that is transported across the cell membrane, once taken up into cells, intracellular folate exists primarily as polyglutamates (1). Intra- and extracellular folate is converted to polyglutamates by folylpolyglutamyl synthase (FPGS), whereas γ-glutamyl hydrolase (GGH) removes the terminal glutamates (1). Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate-dependent enzymes (3).

Similarly, antifolates (e.g., methotrexate) are retained in cells by FPGS-induced polyglutamylation and are exported from cells after hydrolysis to monoglutamates by GGH (2, 3). As with folate, polyglutamylated antifolates are retained in cells longer, thereby increasing their cytotoxicity by extending the length of exposure (2, 3). Polyglutamylated antifolates generally have a higher affinity for and, hence, inhibit their target folate-dependent enzymes in thymidylate and purine biosynthesis to a greater extent than the monoglutamate forms (2, 3). FPGS down-regulation seems to be a mechanism of resistance to methotrexate and other antifolates (4–10). Transfection of FPGS cDNA has been shown to increase sensitivity to methotrexate and other antifolates in some mammalian cell lines (11, 12).

FPGS-induced polyglutamylation may also affect the sensitivity of tumor cells to other chemotherapeutic agents,
such as 5FU, not typically considered as antifolates. One cytotoxic mechanism of 5FU is the formation of a ternary complex involving a metabolite of 5FU [5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP)], thymidylate synthase (TS) and 5,10-methyleneetahydrofolate (5,10-methyleneTHF), thereby inhibiting TS activity with consequent suppression of DNA synthesis (13). Leucovorin (LV; 5-formylTHF), a precursor for 5,10-methyleneTHF, potentiates the cytotoxic effect of 5FU by stabilizing this inhibitory ternary complex (13). 5,10-MethyleneTHF with longer chain length polyglutamates is better retained intracellularly and is more efficient in the formation and stabilization of this inhibitory ternary complex compared with shorter chain polyglutamates (14). Decreased FPGS activity has previously been shown to confer resistance to 5FU in some human cancer cell lines (15–17). We have previously reported that FPGS overexpression significantly increases chemosensitivity of human HCT116 colon cancer cells to 5FU (18).

FPGS seems to play an important role in the sensitivity of cancer cells to antifolates and 5FU, and thus, FPGS modulation might be a potential therapeutic target for increasing sensitivity of cancer cells to these chemotherapeutic agents. However, the effect of FPGS modulation on the chemosensitivity of breast cancer cells to antifolates and 5FU is currently unknown. Furthermore, FPGS modulation affects polyglutamylation of not only antifolates and specific target intracellular folate cofactors (e.g., 5,10-methyleneTHF for 5FU), but also of all other intracellular folate cofactors. FPGS modulation also affects total intracellular folate concentration, which is an important determinant of the cytotoxic effects of antifolates and 5FU (2, 19, 20). Therefore, FPGS modulation-induced changes in intracellular folate concentrations and polyglutamylation may counterbalance the effects of FPGS modulation-induced changes in polyglutamylation of antifolates and specific 5,10-methyleneTHF. In the present study, we generated an in vitro model of FPGS modulation in breast cancer cells and determined the effects of FPGS modulation on chemosensitivity to methotrexate and 5FU. We also determined whether exogenous folate concentrations further influence the effects of FPGS modulation on the chemosensitivity to methotrexate and 5FU.

Materials and Methods

Cell Line and Culture

Human breast adenocarcinoma MDA-MB-435 cells were purchased from the American Type Culture Collection. Cells were grown either in standard RPMI 1640 (Invitrogen) containing 2.3 µmol/L folic acid or in RPMI free of folic acid supplemented with either 20 or 100 nmol/L folic acid (Sigma). Growth medium was supplemented with 10% dialyzed fetal bovine serum (FBS), which contains ~0.6 nmol/L folic acid, 2 mmol/L L-glutamine, penicillin at 100 units/mL, and streptomycin at 100 µg/mL. Cultures were maintained at 37°C in 5% CO2. The final concentrations of folic acid in medium are therefore 2.3 µmol/L, 100.6 nmol/L, and 20.6 nmol/L folic acid concentrations.

Construction and Transfection of FPGS Expression Vectors

The full-length human FPGS cDNA (21) was provided by Dr. A. Bognar (University of Toronto, Canada). The full-length human FPGS cDNA was subcloned into the EcoRI site of the eukaryotic expression vector pIRESneo (Clontech) in the sense orientation to generate the sense FPGS expression vector as described (18).

FPGS-targeted siRNA was designed according to the manufacturer’s protocol and ligated into the vector between the BamH1 and HindIII restriction sites of the pSilencer neo siRNA expression vector (Ambion). The oligonucleotides were designed encoding the desired siRNA strand: GACGGGATTCTTAGCTCT (forward) and AGAGCTAAAGAAATCCCGT (reverse), and AGAGCTAAAGAAATCCCGT (forward) and GACGGGATTCTTAGCTCT (reverse).

The pIRESneo vector containing sense cDNA and the pSilencer vector containing FPGS-targeted siRNA were stably transfected into MDA-MB-435 cells using Lipofectin (Invitrogen) according to the manufacturer’s protocol. In separate transfections, MDA-MB-435 cells were stably transfected with empty pIRESneo and pSilencer vectors as corresponding controls expressing endogenous FPGS. Transfected cells were incubated with 500 µg/mL of neomycin (Invitrogen) to select for cells that expressed the various constructs. After a population of cells was selected, individual clonal cell lines were isolated and expanded. Cells were maintained in complete medium supplemented with 500 µg/mL of neomycin. Several (>10) clones expressing the sense FPGS cDNA and FPGS-targeted siRNA and empty vectors were screened at random, and two independent clones of each construct were selected for further analysis. Data from three experiments using two independent clones of each construct were similar, and thus, the data from one experiment are presented. All analyses were done in triplicate, and at least three replicate experiments were done.

Western Blot Analysis

FPGS protein expression was determined by standard Western blot analysis as described (18) using a rabbit polyclonal antibody raised against a peptide sequence spanning amino acids 275 to 290 of human FPGS (ref. 22; Zymed) at a dilution of 1:1,000.

FPGS and GGH Enzyme Activity Assays

FPGS activity was determined by measuring the incorporation of [3H]glutamate into the polyglutamate chain of aminopterin as described (12). GGH activity was determined by incubating protein in cell lysates with methotrexate-glu4 as substrate and measuring methotrexate and its polyglutamates using high-performance liquid chromatography (HPLC) as described (23, 24).

Intracellular Folate Concentrations and Glutamate Chain Lengths

Intracellular folate concentrations for conjugase-treated and untreated samples were determined by a standard microbiological assay to determine the extent of polyglutamylation as described (18, 25).
for an additional 72 h. The concentration of 5FU ranged from 3 to 9 mol/L for methotrexate, from 0.5 to 24 mol/L for trimetrexate, from 0.5 to 24 mol/L for pemetrexed, and from 1 to 35 mol/L for trimetrexate. After 72 h, cells were fixed with trichloroacetic acid and stained with SRB protein dye. The dye was solubilized, and the A of the solution was measured at 595 nm. The results were expressed as the percentage of cell survival on the basis of the difference between A at the start and end of drug exposure according to the formula (29):

\[
\text{Survival} = \frac{[A_{\text{drug}}/A_{\text{start drug exposure}} - 1]}{[A_{\text{no drug}}/A_{\text{start drug exposure}} - 1]} \times 100%.
\]

IC50 values (i.e., the drug concentration that corresponded to a reduction in cell survival of 50% compared with survival of untreated control cells) were calculated from plots of drug concentration versus proportion of cells that survived.

**Statistical Analysis**

For continuous variables, comparisons between cells expressing the sense FPGS (Sense) and controls (Control-S) and between cells transfected with the FPGS-targeted siRNA (siRNA) and controls (Control-si) were determined using Student’s t test. \( \chi^2 \) analyses were used to examine differences in the distribution of intracellular methotrexate- and 5-methylTHF-polyglutamates between the Sense and Control-S cells and between the siRNA and Control-si cells. For the in vitro chemosensitivity analyses, plots of percentage of survival versus dose showed S-shaped curves, and therefore, the logit transformation \( \logit(p) = \ln(p/(1 - p)) \) was used. Ordinary least-squares regression was used to model the effect of log(dose) of chemotherapy and cell type on the logit-transformed proportion of cells that survived at each dose. The interaction between cell type and log (dose) was included in the model to test the hypothesis that the cell types were differentially sensitive to chemotherapy. IC50 doses and their 95% confidence intervals were calculated on the log scale from the regression results as described (30) and then back-transformed to the original scale for reporting. Relationships between medium folic acid levels and intracellular folate concentrations were examined using Pearson correlations. For all analyses, results were considered statistically significant if two-tailed \( P \) values were <0.05. Analyses were done using SAS, version 8 (SAS Institute).

**Results**

MDA-MB-435 cells expressing the sense FPGS and FPGS-targeted siRNA had significantly higher and lower steady-state levels of the FPGS protein, respectively, compared with corresponding controls expressing endogenous FPGS (\( P < 0.05; \) Figs. 1A and 2A). Cells expressing the sense FPGS had a 4.2-fold higher (\( P < 0.0001 \)) and those transfected with the FPGS-targeted siRNA had a 1.3-fold lower (\( P = 0.04 \)) FPGS activity compared with corresponding controls (Figs. 1A and 2A).

Following conjugase treatment (which allows the measurement of total intracellular folate concentration, including short- and long-chain folylpolyglutamates), intracellular folate concentration of cells expressing the sense FPGS was 1.8- to 2.1-fold higher (\( P < 0.0001 \)), whereas that of cells expressing the FPGS-targeted siRNA was 1.7- to 2.3-fold lower (\( P < 0.001 \)), than that of controls at all three folic acid concentrations (Figs. 1B and 2B). Intracellular folate concentration of samples not treated with conjugase (which allows the determination of short-chain folylpolyglutamates) was significantly higher in cells expressing the sense FPGS than that of controls only at 20 nmol/L folic acid (\( P = 0.01; \) Fig. 1B). Intracellular concentration of short-chain folylpolyglutamates was significantly lower in cells expressing the FPGS-targeted siRNA than that of controls at 100 nmol/L and 2.3 \( \mu \)mol/L (\( P = 0.023 \) and \( P = 0.0042 \), respectively), but not at 20 nmol/L folic acid (Fig. 2B).
intracellular folate concentrations correlated significantly with medium folic acid concentrations in all four FPGS clones ($r = 0.80–0.96; P < 0.01$; Figs. 1B and 2B). In contrast, intracellular concentrations of short-chain folypolyglutamates correlated with medium folic acid concentrations in cells expressing the sense FPGS ($r = 0.92; P = 0.0004$) and corresponding controls ($r = 0.62; P = 0.07$), but not in cells expressing the FPGS-targeted siRNA and corresponding controls (Figs. 1B and 2B).

Differences between mean folate concentration of conjugase-treated and untreated samples (which allows the determination of long-chain folypolyglutamates) clearly show that cells expressing the sense FPGS had significantly higher concentrations of long-chain folypolyglutamates than controls at all folic acid concentrations (at 2.3 $\mu$mol/L: Sense, 11.11 ± 0.51 versus Control-S, 5.09 ± 0.69; at 100 $\mu$mol/L: Sense, 5.68 ± 0.29 versus Control-S, 0.25 ± 0.62; at 20 $\mu$mol/L: Sense, 4.76 ± 0.40 versus Control-S, 1.54 ± 0.58; $P < 0.001$; Fig. 1B). In contrast, cells expressing the FPGS-targeted siRNA had significantly lower concentrations of long-chain folypolyglutamates than controls at 100 $\mu$mol/L and 2.3 $\mu$mol/L (at 2.3 $\mu$mol/L: siRNA, 4.28 ± 0.30 versus Control-si, 10.57 ± 1.05; at 100 $\mu$mol/L: siRNA, 2.23 ± 0.17 versus Control-si, 3.07 ± 0.10; $P < 0.002$), but not at 20 $\mu$mol/L (siRNA, 1.02 ± 0.54 versus Control-si, 3.22 ± 1.64) folic acid (Fig. 2B). Intracellular concentrations of long-chain folypolyglutamates correlated significantly with medium folic acid concentrations in all four FPGS clones ($r = 0.69–0.97; P < 0.05$; Figs. 1B and 2B).

After 24-h exposure to 1 $\mu$mol/L [3H]-methotrexate, the majority of intracellular methotrexate was found as long-chain methotrexate-polyglutamates (methotrexate-glu$_{3–6}$) in cells expressing the sense FPGS compared with controls in which the majority of intracellular methotrexate was methotrexate-glu$_1$ and methotrexate-glu$_2$ ($P < 0.01$; Table 1). In both cells expressing the sense FPGS and controls, all of the intracellular 5-methylTHF was found as long-chain polyglutamates (5-methylTHF-glu$_{4–8}$) after 24-h exposure to 20 $\mu$mol/L [3H]-5-methylTHF. FPGS overexpression was associated with a shift toward the very long-chain 5-methylTHF-polyglutamates (5-methylTHF-glu$_{7–8}$; $P < 0.01$; Table 1). In contrast, after 24-h exposure to 1 $\mu$mol/L [3H]-methotrexate, the majority of intracellular methotrexate was found as methotrexate-glu$_1$ and methotrexate-glu$_2$ in cells transfected with the FPGS-targeted siRNA compared with controls in which the majority of intracellular methotrexate was found as long-chain methotrexate-polyglutamates (methotrexate-glu$_{3–6}$; $P < 0.01$; Table 1). In both cells transfected with the FPGS-targeted siRNA and controls, all of the intracellular 5-methylTHF was found as long-chain polyglutamates (5-methylTHF-glu$_{4–8}$) after 24-h exposure to 20 $\mu$mol/L [3H]-5-methylTHF. FPGS inhibition was associated with a shift away from the very long-chain 5-methylTHF-polyglutamates (5-methylTHF-glu$_{7–8}$; $P < 0.01$; Table 1).

Cells expressing the sense FPGS had significantly lower GGH activity compared with controls at 2.3 $\mu$mol/L and 20 $\mu$mol/L ($P = 0.01$ and $P = 0.03$, respectively), but not at
100 nmol/L folic acid (Fig. 1C). In contrast, FPGS inhibition had no significant effect on GGH activity at all three folic acid concentrations (Fig. 2C).

Cells expressing the sense FPGS exhibited a significantly decreased doubling time compared with controls (43.4 ± 0.4 h versus 44.6 ± 0.2 h; \( P < 0.0001 \)), indicating a faster growth rate. Unexpectedly, cells transfected with the FPGS-targeted siRNA also had a significantly decreased doubling time compared with controls (43.4 ± 0.2 h versus 45.0 ± 0.5 h; \( P < 0.0001 \)).

As proof of principle, the effect of FPGS overexpression and inhibition on chemosensitivity of breast cancer cells to pemetrexed (positive control) and trimetrexate (negative control) was determined at 2.3 μmol/L folic acid. Pemetrexed is a novel antimetabolite that inhibits multiple enzymes involved in thymidylate and purine biosynthesis (31). Pemetrexed is polyglutamylated to the active penta-glutamate by FPGS for its cytotoxic effects (32). Trimetrexate is a nonclassic antifolate that directly inhibits dihydrofolate reductase (DHFR; ref. 32). Trimetrexate is more sensitive compared with controls at 2.3 μmol/L folic acid (Fig. 4B and E). At 20 nmol/L folic acid, chemosensitivity of cells expressing the sense FPGS to 5FU + LV, but not to 5FU, was significantly increased compared with controls (\( P < 0.05 \); Fig. 4C and F). IC₅₀ values indicate significantly reduced chemosensitivity of cells expressing the sense FPGS to 5FU + LV and 5FU at 2.3 μmol/L folic acid, but significantly enhanced chemosensitivity to 5FU at 20 nmol/L folic acid compared with controls (Table 2).

Chemosensitivity of cells transfected with the FPGS-targeted siRNA to 5FU + LV and 5FU was significantly decreased compared with controls at 2.3 μmol/L folic acid (\( P < 0.05 \); Fig. 5A and D). However, this effect was not observed at 100 nmol/L folic acid (Fig. 4B and E). At 20 nmol/L folic acid, chemosensitivity of cells expressing the sense FPGS was not significantly changed compared with controls (\( P > 0.05 \); Fig. 5C and F).

**Figure 2.** MDA-MB-435 breast cancer cells transfected with the FPGS-targeted siRNA (siRNA) had significantly lower steady-state levels of the FPGS protein (A) and lower FPGS activity (A; \( * P = 0.04 \)) compared with cells transfected with the vector alone (Control-si; endogenous FPGS). Total intracellular folate concentrations (i.e., folate levels after conjugase treatment) were significantly lower in the siRNA cells than in the Control-si cells at all three medium folic acid concentrations (B; \( * P < 0.001 \)). Intracellular concentrations of short-chain folyopolyglutamates (i.e., folate levels not treated with conjugase) were significantly lower in the siRNA cells than in the Control-si cells at 2.3 μmol/L and 100 nmol/L medium folic acid concentrations (\( * P < 0.05 \) but not at 20 nmol/L (B). Intracellular concentrations of long-chain folyopolyglutamates (i.e., differences between folate concentration of conjugase-treated and untreated samples) were significantly lower in the siRNA cells than in the Control-si cells at 2.3 μmol/L and 100 nmol/L medium folic acid concentrations (\( * P < 0.02 \) but not at 20 nmol/L (B), \( * * P < 0.0001 \) compared with non-conjugase-treated cells of the same construct grown in the same medium folic acid concentration. Intracellular concentrations of total and long-chain folyopolyglutamates correlated significantly with medium folic acid concentrations in both the siRNA (\( \dagger \dagger, r = 0.92 – 0.97, P < 0.005 \)) and Control-si cells (\( \dagger \dagger, r = 0.80 – 0.83, P < 0.01 \)). B: Intracellular concentrations of short-chain folyopolyglutamates did not correlate significantly with medium folic acid concentrations in either the siRNA or Control-si cells (B). FPGS inhibition did not significantly affect GGH activity in either the siRNA or Control-si cells (C). Columns, means; bars, SD.
IC₅₀ values indicate significantly reduced chemosensitivity of cells transfected with the FPGS-targeted siRNA to 5FU + LV and 5FU at 2.3 μmol/L folic acid and to 5FU at 20 nmol/L folic acid compared with controls (Table 2).

Chemosensitivity to methotrexate was significantly increased in both cells expressing the sense FPGS and cells transfected with the FPGS-targeted siRNA compared with corresponding controls at all three folic acid concentrations (P < 0.05; Figs. 4G–I and 5G–I). IC₅₀ values for methotrexate indicate significantly enhanced chemosensitivity of both cells expressing the sense FPGS and cells transfected with the FPGS-targeted siRNA compared with corresponding controls at all three folic acid concentrations (Table 2).

Using the IC₅₀ values obtained from corresponding controls of cells transfected with the sense FPGS or FPGS-targeted siRNA treated with 5FU or methotrexate at each folic acid concentration (Table 2), the effects of FPGS modulation on chemosensitivity to a combination treatment of 5FU and methotrexate was determined. Cells expressing the sense FPGS were more sensitive to 5FU + methotrexate compared with controls at all three folic acid concentrations (23%, 8%, and 11% lower survival at 2.3 μmol/L, 100 nmol/L, and 20 nmol/L, respectively; P < 0.0001). In contrast, cells transfected with the FPGS-targeted siRNA were less sensitive to 5FU + methotrexate compared with controls only at 100 nmol/L folic acid (15% higher survival, P < 0.0001).

### Discussion

We developed an in vitro model of FPGS overexpression and inhibition in MDA-MB-435 breast cancer cells with predictable functional consequences. Compared with controls expressing endogenous FPGS, cells expressing the sense FPGS had significantly higher FPGS expression and activity, shorter doubling time, higher concentrations of total intracellular folate, higher content of long-chain folyl- and 5-methylTHF-polyglutamates, and a shift toward long-chain methotrexate-polyglutamates. In contrast, cells transfected with the FPGS-targeted siRNA had significantly lower FPGS expression and activity, lower concentrations of total intracellular folate, lower content of long-chain folyl- and 5-methylTHF-polyglutamates, and a shift away from long-chain methotrexate-polyglutamates compared with controls expressing endogenous FPGS. Unexpectedly, however, cells transfected with the FPGS-targeted siRNA also had a shorter doubling time compared with controls. The accelerated growth rate associated with FPGS overexpression is consistent with the observed increased growth rate in cells expressing the sense FPGS compared with controls, despite the significantly higher FPGS expression and activity in the transfected cells.

### Table 1

#### (A) Effects of FPGS overexpression on 5-methylTHF- and methotrexate polyglutamate distribution

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#### Methotrexate-polyglutamate distribution (% of total methotrexate)

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#### (B) Effects of FPGS inhibition on 5-methylTHF- and methotrexate polyglutamate distribution

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intracellular folate concentrations and higher content of long-chain folylpolyglutamates, which are better substrates than short-chain folylpolyglutamates for folate-dependent enzymes involved in thymidylate and purine biosynthesis (3). This is also consistent with our prior observations made in human HCT116 colon cancer cells expressing the sense FPGS (18). However, the accelerated growth rate associated with siRNA-based FPGS inhibition is not readily explained by the observed decrease in intracellular folate concentrations and lower content of long-chain folylpolyglutamates. In fact, this observation is in direct contrast to a slower growth rate and TS suppression observed with antisense-based FPGS down-regulation in HCT116 (18) and human CCRF-CEM leukemia (33) cells. Generally, most of the observed functional consequences of FPGS overexpression and inhibition are consistent with the known biological function of FPGS and provided an appropriate in vitro model to test the effect of FPGS modulation on chemosensitivity of breast cancer cells to 5FU and methotrexate.

We hypothesized that FPGS overexpression would up-regulate GGH, whereas FPGS inhibition would down-regulate GGH to maintain intracellular homeostatic balance of folate polyglutamylation. In contrast, cells expressing the sense FPGS had significantly lower GGH activity compared with corresponding controls, whereas cells transfected with the FPGS-targeted siRNA had no significant effect on GGH activity compared with corresponding controls. We have previously reported that HCT116 cells expressing the sense FPGS had significantly higher GGH mRNA expression, whereas those expressing the antisense FPGS had significantly lower GGH mRNA expression compared with controls (18). These observations suggest a cell-specific effect of FPGS modulation on GGH unique to MDA-MB-435 cells or post-transcriptional modifications.

We first showed that the magnitude of FPGS overexpression induced in this model was sufficient to significantly enhance chemosensitivity to pemetrexed, which is one of the best substrates for FPGS and whose cytotoxic effects depend on polyglutamylation by FPGS (31). However, the degree of FPGS inhibition achieved in this model was not sufficient to significantly modulate chemosensitivity to pemetrexed. We also showed that different concentrations and polyglutamylation of intracellular folate mediated by FPGS overexpression and inhibition significantly reduced and enhanced chemosensitivity to trimetrexate, respectively, which is not polyglutamylated and, hence, does not depend on polyglutamylation for its cytotoxic effects.

**Figure 3.** A, in vitro chemosensitivity to pemetrexed. Pemetrexed is a novel antimetabolite whose cytotoxic effects depend on polyglutamylation by FPGS. MDA-MB-435 breast cancer cells transfected with the sense FPGS (Sense) were significantly more sensitive to pemetrexed compared with cells transfected with the vector alone (Control-S; endogenous FPGS; left, *, P < 0.05). However, chemosensitivity of MDA-MB-435 breast cancer cells transfected with the FPGS-targeted siRNA (siRNA) to pemetrexed was not significantly different from that cells transfected with the vector alone (Control-si; endogenous FPGS; right). B, in vitro chemosensitivity to trimetrexate. Trimetrexate is a nonclassic antifolate that does not require polyglutamylation for its cytotoxic effects; in fact, it cannot be polyglutamylated because there is no glutamate moiety. MDA-MB-435 breast cancer cells transfected with the sense FPGS (Sense) were significantly less sensitive to trimetrexate compared with cells transfected with the vector alone (Control-S; endogenous FPGS; left). In contrast, MDA-MB-435 breast cancer cells transfected with the FPGS-targeted siRNA (siRNA) was more sensitive to trimetrexate compared with cells transfected with the vector alone (Control-si; endogenous FPGS; right; *, P < 0.050). Points, means; bars, SD.
effects (32). These data collectively suggest that both polyglutamylation of antifolates and folates as well as intracellular folate concentrations modulate chemosensitivity.

FPGS overexpression was associated with decreased chemosensitivity of breast cancer cells to 5FU + LV and 5FU at 2.3 μmol/L folic acid concentration. However, this decreased cytotoxic effect of 5FU + LV and 5FU was not observed at 100 nmol/L folic acid concentration, whereas at 20 nmol/L folic acid concentration, FPGS overexpression was associated with enhanced chemosensitivity to 5FU + LV but not to 5FU. Our a priori hypothesis was that FPGS overexpression would enhance the cytotoxic effect of 5FU by increasing relative intracellular concentration of long-chain 5,10-methyleneTHF-polyglutamates, resulting in more efficient formation and stabilization of the inhibitory 5,10-methyleneTHF-TS-FdUMP ternary complex. At 2.3 μmol/L folic acid concentration, it is possible that FPGS overexpression greatly increased intracellular folate concentrations and contents of long-chain polyglutamates of other intracellular folate cofactors to a greater extent than long-chain 5,10-methyleneTHF-polyglutamates, resulting in increased thymidylate and purine biosynthesis. This might have overridden the TS-inhibiting effect of increased long-chain 5,10-methyleneTHF-polyglutamates, resulting in decreased chemosensitivity to 5FU + LV and 5FU. At 20 nmol/L folic acid concentration, the magnitude of increased contents of long-chain 5,10-methyleneTHF-polyglutamates resulting from FPGS overexpression was sufficient to overcome the effect of increased intracellular folate concentrations and contents of long-chain polyglutamates of other intracellular folate derivatives, resulting in TS inhibition and increased chemosensitivity to 5FU. However, this enhanced cytotoxic effect was observed only when a precursor for 5,10-methyleneTHF (LV) was supplied exogenously. At 100 nmol/L folic acid concentration, the competing effects of increased DNA synthesis associated with increased intracellular folate concentrations and content of long-chain foly/polyglutamates and TS inhibition associated with increased long-chain 5,10-methyleneTHF-polyglutamates seem to have canceled each other out.

Figure 4. In vitro chemosensitivity of MDA-MB-435 breast cancer cells transfected with the sense FPGS (Sense) to 5FU plus LV (A–C), 5FU alone (D–F), or methotrexate (G–I) in comparison to cells transfected with the vector alone (Control-S; endogenous FPGS). Cells were grown in medium containing 2.3 μmol/L (A, D, and G), 100 nmol/L (B, E, and H), or 20 nmol/L (C, F, and I) folic acid. Points, means; bars, SD. *, $P < 0.05$, statistically significant compared with corresponding control cells.
FPGS inhibition was associated with decreased chemosensitivity of breast cancer cells to 5FU + LV and 5FU at 2.3 μmol/L folic acid concentration. However, this decreased cytotoxic effect of 5FU + LV and 5FU was not observed at 100 nmol/L folic acid concentration, whereas at 20 nmol/L folic acid concentration, FPGS inhibition was also associated with decreased chemosensitivity to 5FU + LV but not to 5FU. Our a priori hypothesis was that FPGS inhibition would decrease the cytotoxic effect of 5FU by decreasing relative intracellular concentrations of long-chain 5,10-methyleneTHF-polyglutamates, resulting in less efficient formation and stabilization of the 5,10-methyleneTHF-TS-FdUMP ternary complex. It seems that decreased long-chain 5,10-methyleneTHF-polyglutamates might have been the predominant effect associated with FPGS inhibition, overriding the counterbalancing effect associated with decreased total intracellular folate concentrations and contents of long-chain polyglutamates of other intracellular folate derivatives and leading to decreased chemosensitivity to 5FU.

Our 5FU chemosensitivity data from the FPGS inhibition studies are generally consistent with our hypothesis, whereas those from the FPGS overexpression studies are consistent only at 20 nmol/L folic acid concentration and mostly opposite to our hypothesis. A previous study has shown that the cytotoxic effect of 5FU is directly correlated with FPGS activity in 14 human cancer cell lines (17). Another study has shown that human HCT8 colon cancer cells become rapidly resistant to 5FU over time owing to a progressive decrease in FPGS mRNA expression and activity (16). We have previously reported that FPGS overexpression significantly enhances chemosensitivity of human HCT116 colon cancer cells to 5FU, whereas antisense FPGS inhibition decreases chemosensitivity of human HCT116 colon cancer cells to 5FU (18). However, these in vitro studies were conducted only in media containing ≥2.3 μmol/L folic acid concentrations. In another study, the effect of LV, which is converted to 5,10-methyleneTHF and enters the folate pathway (13), on the chemosensitivity of human leukemia CCRF-CEM (the parent cell line with proficient polyglutamylaton) and CCRF-CEM/P (a cell line with impaired ability to form polyglutamates) to 5FU was compared (15). Both CCRF-CEM and CCRF-CEM/P cells accumulated 5,10-methyleneTHF in the presence of LV in a dose-dependent manner (15). However, at a dose of 5FU that produced only a slight decrease in cell growth, the addition of LV further inhibited the cell growth in CCRF-CEM cells, but not in CCRF-CEM/P cells (15), suggesting that the impaired polyglutamylation of 5,10-methyleneTHF was likely responsible for the lack of potentiation of the cytotoxic effect of 5FU by LV in CCRF-CEM/P cells. In contrast, three human studies have shown conflicting results concerning the role of FPGS in predicting treatment response to 5FU + LV and survival in patients with colon cancer (34–36). Our data suggest that the effect of FPGS modulation on the chemosensitivity of breast cancer cells to 5FU is highly complex and depends on intracellular folate concentrations and polyglutamylation of not only 5,10-methyleneTHF but also other folate cofactors.

FPGS overexpression resulted in increased chemosensitivity of breast cancer cells to methotrexate at all three folic acid concentrations, consistent with our a priori hypothesis that predicted that increased methotrexate polyglutamylation would enhance the cytotoxic effect of methotrexate. IC₅₀ values indicate that an increased concentration of methotrexate was required to achieve 50% inhibition among cells grown in 2.3 μmol/L compared with lower concentrations of folic acid.

Table 2. IC₅₀ values of 5FU and methotrexate in MDA-MB-435 breast cancer cells transfected with the sense FPGS and with the FPGS-targeted siRNA in comparison with corresponding control cells expressing endogenous FPGS

<table>
<thead>
<tr>
<th>Folate concentration</th>
<th>IC₅₀ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.3 μmol/L</td>
</tr>
<tr>
<td><strong>5FU + LV (μmol/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Control-S</td>
<td>6.61(5.75, 7.54)</td>
</tr>
<tr>
<td>Control-si</td>
<td>11.77(10.51, 13.35)</td>
</tr>
<tr>
<td>siRNA</td>
<td>22.85*(19.99, 26.70)</td>
</tr>
<tr>
<td><strong>5FU (μmol/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Sense</td>
<td>25.73*(22.92, 29.35)</td>
</tr>
<tr>
<td>Control-S</td>
<td>12.50(11.20, 14.12)</td>
</tr>
<tr>
<td>Control-si</td>
<td>12.61(10.78, 15.14)</td>
</tr>
<tr>
<td>siRNA</td>
<td>34.66*(27.78, 45.92)</td>
</tr>
<tr>
<td><strong>Methotrexate (nmol/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Control-S</td>
<td>28.07(25.92, 30.63)</td>
</tr>
<tr>
<td>Control-si</td>
<td>41.01(35.22, 49.45)</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with corresponding controls.

concentrations of folic acid. This suggests that increased total intracellular folate concentrations and higher contents of folylpolyglutamates associated with FPGS overexpression, which would provide an increased amount of substrates for nucleotide biosynthesis, had a competing effect on increased methotrexate polyglutamylation, which would enhance chemosensitivity to methotrexate. Furthermore, methotrexate and folate share cell entry mechanisms, and folate is a higher affinity substrate for DHFR than methotrexate (2, 3). Therefore, at high medium folate concentrations, more folate will enter cells and bind preferentially to DHFR. As a result, higher concentrations of methotrexate are required for its cytotoxic effects in high folate environments.

In contrast to our a priori hypothesis that predicted that FPGS inhibition would decrease chemosensitivity of breast cancer cells to methotrexate, FPGS inhibition enhanced the cytotoxic effect of methotrexate at 20 and 100 nmol/L folic acid concentrations. This suggests that decreased intracellular folate concentrations and contents of long-chain folylpolyglutamates induced by FPGS inhibition overwhelmed the effect of decreased methotrexate polyglutamylation and increased chemosensitivity to methotrexate. However, this effect was not observed at 2.3 μmol/L folic acid concentration, suggesting that the magnitude of intracellular folate depletion associated with FPGS inhibition at this folic acid concentration was not sufficient to modulate chemosensitivity to methotrexate.

Decreased antifolate polyglutamylation due to quantitative (4–7) or qualitative (8–10) alterations in FPGS activity has been shown to be a mechanism of resistance to methotrexate and other antifolates in human and murine leukemia cell lines. Transfection of FPGS cDNA has been shown to increase sensitivity to methotrexate in variant hamster cells that lack endogenous FPGS activity (11). FPGS gene transfer into rat and human glioma, gliosarcoma, and glioblastoma cell lines already expressing FPGS significantly enhanced cytotoxic sensitivity to methotrexate and other antifolates in vitro and in vivo (12). We have previously reported that sense FPGS overexpression and

Figure 5. In vitro chemosensitivity of MDA-MB-435 breast cancer cells transfected with the FPGS-targeted siRNA (siRNA) to 5FU plus LV (A–C), 5FU alone (D–F), or methotrexate (G–I) in comparison with cells transfected with the vector alone (Control-si; endogenous FPGS). Cells were grown in medium containing 2.3 μmol/L (A, D, and G), 100 nmol/L (B, E, and H), or 20 nmol/L (C, F, and I) folic acid. Points, means; bars, SD. *, P < 0.05, statistically significant compared with corresponding control cells.
antisense FPGS inhibition do not significantly affect chemosensitivity of human HCT116 colon cancer cells to methotrexate at the same supraphysiologic folic acid concentration (18). Our present data suggest that the effect of FPGS modulation on the chemosensitivity of breast cancer cells to methotrexate depends not only on methotrexate polyglutamylation but also on folicpolyglutamylat-

ion and intracellular folate pools. FPGS modulation affects both polyglutamylation of antifolates and folate cofactors (37). Consequently, alterations in polyglutamylation of intracellular folate cofactors as well as intracellular folate pools may diminish or even abolish the effects associated with alterations in polyglutamylation of antifolates, in particular for antifolates that are highly dependent on polyglutamylation for their cytotoxic effects (37–42).

5FU and methotrexate are combined with cyclophospha-
mide in the CMF (cyclophosphamide; methotrexate; 5-
fluorouracil) regimen for breast cancer treatment (43). Our data suggest that FPGS overexpression increases chemosensitivity of breast cancer cells to 5FU + methotrexate at all three folic acid concentrations, whereas FPGS inhibition decreases chemosensitivity to 5FU + methotrexate only at 100 nmol/L folic acid concentration. The effect of FPGS modulation on the chemosensitivity of breast cancer cells to 5FU + methotrexate likely depends on a complex interplay of intracellular folate concentrations, polyglutamylation of 5,10-methyleneTHF and other folate cofactors, and methotrexate polyglutamylation.

In conclusion, as proof of principle, we provide func-
tional evidence that FPGS modulation affects the chemosensitivity of breast cancer cells to 5FU and methotrexate. Our data suggest that the effect of FPGS modulation on the chemosensitivity of breast cancer cells to 5FU and methotrexate is highly complex and depends on intracellular folate concentrations and polyglutamylation of intracellular folate cofactors and methotrexate. Furthermore, our data suggest that the effect of FPGS modulation on the chemosensitivity to antifolates, whose cytotoxic effects depend on polyglutamylation, cannot be predicted solely based on the polyglutamylation of antifolates. Whether or not FPGS modulation may be an important clinical determinant of chemosensitivity of breast cancer cells to 5FU- and methotrexate-based chemotherapy needs further exploration.

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