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γ -Glutamyl hydrolase modulation and folate influence chemosensitivity of cancer cells to 5-fluorouracil and methotrexate

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Background: γ -Glutamyl hydrolase (GGH) regulates intracellular folate and antifolates for optimal nucleotide biosynthesis and antifolate-induced cytotoxicity, respectively. The modulation of GGH may therefore affect chemosensitivity of cancer cells, and exogenous folate levels may further modify this effect.

Methods: We generated a novel model of GGH modulation in human HCT116 and MDA-MB-435 cancer cells and investigated the effect of GGH modulation on chemosensitivity to 5-fluorouracil (5FU) and methotrexate (MTX) at different folate concentrations *in vitro* and *in vivo*.

Results: Overexpression of GGH significantly decreased chemosensitivity of MDA-MB-435 cells to 5FU and MTX at all folate concentrations as expected. In contrast, in HCT116 cells this predicted effect was observed only at very high folate concentration, and as the folate concentration decreased this effect became null or paradoxically increased. This *in vitro* observation was confirmed *in vivo*. Inhibition of GGH significantly increased chemosensitivity of both cancer cells to 5FU at all folate concentrations. Unexpectedly, GGH inhibition significantly decreased chemosensitivity of both cancer cells to MTX at all folate concentrations. In both GGH modulation systems and cell lines, the magnitude of chemosensitivity effect incrementally increased as folate concentration increased.

Conclusion: Modulation of GGH affects chemosensitivity of cancer cells to 5FU and MTX, and exogenous folate levels can further modify the effects.

Folate has an essential role in DNA synthesis involved in thymidylate and purine biosynthesis as an important mediator of one-carbon transfer reactions (Shane, 2010). In neoplastic cells, folate depletion and disrupted folate metabolism cause ineffective

DNA synthesis, resulting in inhibition of tumour growth (Kamen, 1997). This has been the basis for cancer chemotherapy using antifolates and 5-fluorouracil (5FU; Kamen, 1997). Folate is retained intracellularly by folylpolyglutamate synthase (FPGS)-induced

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polyglutamylated, whereas γ -glutamyl hydrolase (GGH) removes the γ -polyglutamate tails attached to folate to produce the monoglutamate form and facilitate the export out of cells (Shane, 2010). Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate-dependent enzymes (Moran, 1999).

As with folate, polyglutamylated antifolates, such as methotrexate (MTX), are retained for a longer duration in cells, thereby increasing their cytotoxicity by extending the length of exposure (Kamen, 1997; Moran, 1999). Furthermore, 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 form (Kamen, 1997; Moran, 1999).

Changes in polyglutamylated of a specific intracellular folate cofactor may also affect the sensitivity of tumour cells to other chemotherapeutic agents, such as 5FU, not typically considered as antifolate. 5-Fluorouracil is a prototype of pyrimidine antagonists with several cytotoxic mechanisms, including incorporation of its metabolites as false precursor into DNA and RNA, resulting in DNA instability and interfering with RNA processing and function, respectively (Longley *et al*, 2003). Another cytotoxic mechanism of 5FU is the formation of a ternary complex involving a metabolite of 5FU (FdUMP; 5-fluoro-2-deoxyuridine-5-monophosphate), thymidylate synthase (TS) and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF), thereby inhibiting TS activity with consequent suppression of DNA synthesis (Longley *et al*, 2003). Leucovorin (LV; 5-formylTHF), a precursor for 5,10-methyleneTHF, potentiates the cytotoxic effect of 5FU by stabilising this inhibitory ternary complex, and is therefore routinely administered with 5FU in cancer chemotherapy in order to increase the intracellular pool of 5,10-methyleneTHF (Longley *et al*, 2003). 5,10-Methylenetetrahydrofolate with longer chain length polyglutamates is better retained intracellularly and is more efficient in the formation and stabilisation of this inhibitory ternary complex compared with shorter chain polyglutamates (Radparvar *et al*, 1989). Therefore, modulation of 5,10-methyleneTHF polyglutamylated may affect chemosensitivity of cancer cells to 5FU.

The effects of differences in FPGS activity and of specific FPGS modulation on drug resistance of and chemosensitivity to MTX and other antifolates (Pizzorno *et al*, 1988; McCloskey *et al*, 1991; Roy *et al*, 1997; Aghi *et al*, 1999; Zhao *et al*, 2000; Mauritz *et al*, 2002; Liani *et al*, 2003), and 5FU (Romanini *et al*, 1991; Wang *et al*, 1993; Cheradame *et al*, 1997) have been extensively studied. In general, high FPGS activity or upregulation appears to enhance chemosensitivity of cancer cells to MTX, other antifolates and 5FU, whereas low FPGS activity or downregulation seems to be a mechanism of resistance to MTX and other antifolates, as well as 5FU.

In contrast, the role of GGH modulation in influencing chemosensitivity of cancer cells to antifolates and 5FU has not yet been clearly established (Galivan *et al*, 2000; Panetta *et al*, 2002a,b; Schneider and Ryan, 2006). Increased GGH activity was identified as the mechanism of antifolate resistance in rat hepatoma cells (Rhee *et al*, 1993; Yao *et al*, 1995), human sarcoma cells (Li *et al*, 1993) and human leukaemia cells (Pizzorno *et al*, 1995). However, ectopic overexpression of GGH after transfection of MCF7 breast cancer and HT1080 fibrosarcoma cells did not confer MTX resistance (Cole *et al*, 2001). Pharmacologic GGH inhibition was observed to enhance the sensitivity of human sarcoma (Waltham *et al*, 1997) and breast cancer cells (Alabaster *et al*, 1981; Kennedy *et al*, 1983) to MTX. Inhibition of GGH induced by small-interfering RNA (siRNA) in human DLD-1 colon cancer cells enhanced sensitivity to 5FU (Sakamoto *et al*, 2008).

γ -Glutamyl hydrolase may have an important role in modulating drug resistance and chemosensitivity of cancer cells to

antifolates and 5FU by inducing changes in polyglutamylated of antifolates and a specific target intracellular folate cofactor (e.g., 5,10-methyleneTHF for 5FU), respectively. However, GGH modulation affects polyglutamylated of not only antifolates and specific target intracellular folate cofactors but also of all other intracellular folate cofactors. Modulation of GGH also affects total intracellular folate concentration, which is an important determinant of the cytotoxic effects of antifolates and 5FU (Kamen, 1997; Backus *et al*, 2000; Zhao *et al*, 2001). Therefore, the GGH modulation-induced changes in intracellular folate concentrations and folylpolyglutamylated may counterbalance the effects of the GGH modulation-induced changes in polyglutamylated of antifolates and specific 5,10-methyleneTHF. We have previously demonstrated this complex interaction in an *in vitro* model of FPGS overexpression and inhibition (Sohn *et al*, 2004b; Cho *et al*, 2007).

In the present study, we generated an *in vitro* model of GGH modulation in human HCT116 colon and MDA-MB-435 breast cancer cells, and determined the effects of GGH modulation on chemosensitivity to 5FU and MTX. We also determined whether exogenous folate concentrations further influence the effects of GGH modulation on the chemosensitivity to 5FU and MTX *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell line and culture. Human colon adenocarcinoma HCT116 and breast adenocarcinoma MDA-MB-435 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). For experiments pertaining to the generation and characterisation of an *in vitro* model of GGH modulation, both cells were grown in standard RPMI-1640 medium (Invitrogen, Gaithersburg, MD, USA) containing $2.3 \mu\text{mol l}^{-1}$ folic acid. Growth medium was supplemented with 10% dialysed fetal bovine serum (Invitrogen), $500 \mu\text{g ml}^{-1}$ Geneticin (G418; Invitrogen), 50 units ml^{-1} penicillin with $50 \mu\text{g ml}^{-1}$ streptomycin (Invitrogen) and $0.25 \mu\text{g ml}^{-1}$ fungizone amphotericin B (Invitrogen). Cultures were maintained at 37°C in 5% CO_2 . For *in vitro* chemosensitivity experiments, both cells were grown in standard RPMI-1640 medium containing $2.3 \mu\text{mol l}^{-1}$ folic acid or in RPMI-1640 free of folic acid supplemented with either 50 or 100 nmol l^{-1} 5-methyltetrahydrofolate (5-MTHF; Schircks Laboratories, Jona, Switzerland).

Folic acid is the fully oxidised monoglutamyl synthetic form of folate used in supplements and fortified foods (Shane, 2010). Folic acid is highly stable in contrast to naturally occurring folates, which are very unstable and rapidly lose their activity in foods and are easily oxidised under low pH (Shane, 2010). Folic acid is not found in nature nor is it a normal metabolite. It must be reduced and methylated to 5-MTHF before it can enter the folate cycle (Shane, 2010). Nevertheless, because of its stability folic acid is the usual form of folate used in culture media and $2.3 \mu\text{mol l}^{-1}$ folic acid, albeit a supraphysiologically high level considering that median plasma/serum concentrations of unmetabolised folic acid range from 0.50 to 1.2 nmol l^{-1} in North America in the post-folic acid fortification era depending on folic acid supplement use (Kalmbach *et al*, 2008; Bailey *et al*, 2010), is the standard amount used in most culture media. 5-Methyltetrahydrofolate is the predominant form of folate found in blood (Shane, 2010) and was therefore selected to represent nutritionally and physiologically relevant form of folate. 5-Methyltetrahydrofolate (50 nmol l^{-1}) in culture medium was selected to reflect a likely average serum folate concentration in the North American population in the post-folic acid fortification era. Large population-based surveys in North America post fortification have reported mean serum folate concentrations of 41–42 (Bailey *et al*, 2010; Pfeiffer *et al*, 2012)

and $\sim 30 \text{ nmol l}^{-1}$ higher serum folate concentrations in supplemental users (mostly from multivitamin containing 0.4 mg folic acid) compared with non-users (Pfeiffer *et al*, 2012). One study has reported a mean serum 5-MTHF concentration of 49.3 nmol l^{-1} in the North American population post fortification (Bailey *et al*, 2010). In these surveys, 75th and 95th percentile values of serum folate concentrations were ~ 60 and 105 nmol l^{-1} , respectively, largely owing to folic acid supplement use (Pfeiffer *et al*, 2012). In human clinical trials, folic acid supplementation at 0.8–1.0 mg per day increased plasma/serum folate concentrations to ~ 80 – 100 nmol l^{-1} (Bruce *et al*, 2005; Bonaa *et al*, 2006; Ebbing *et al*, 2008). Therefore, in the present study we selected 50 and 100 nmol l^{-1} 5-MTHF to represent likely average and high serum folate concentrations, respectively, in the North American population in the post-fortification era.

Construction and transfection of GGH expression vectors. The full-length human GGH cDNA (1.2 kb; GeneBank accession No. U55206; Yao *et al*, 1996) was subcloned into the *NotI* site of the eukaryotic expression vector pIRESneo (Clontech, Palo Alto, CA, USA) in the sense orientation to generate the sense GGH expression vector as described (Sohn *et al*, 2004a,b; Cho *et al*, 2007). The GGH-targeted siRNA was designed according to the manufacturer's protocol and ligated into the vector between the *BamHI* and *HindIII* restriction sites of the pSilencer neo siRNA expression vector (Ambion, Austin, TX, USA) as described (Cho *et al*, 2007). The oligonucleotides were designed encoding the desired siRNA strand: 5'-GTACTTGGAGTCTGCAGGT-3' (forward) and 5'-ACCTGCAGACTCCAAGTAC-3' (reverse), and 5'-ACCTGCAGACTCCAAGTAC-3' (forward) and 5'-GTACTTGAGTCTGCAGGT-3' (reverse). The correct integration, orientation and sequence of the sense GGH cDNA and GGH-targeted siRNA were confirmed by predicted fragment sizes after multiple restriction enzyme digestions and DNA sequencing.

The pIRESneo vector containing sense cDNA and the pSilencer vector containing GGH-targeted siRNA were stably transfected into HCT116 and MDA-MB-435 cells using Lipofectin (Invitrogen) according to the manufacturer's protocol. In separate transfections, both cell lines were stably transfected with empty pIRESneo and pSilencer vectors as corresponding controls expressing endogenous GGH. Transfected cells were incubated with $500 \mu\text{g ml}^{-1}$ 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 $500 \mu\text{g ml}^{-1}$ of neomycin. Over 10 clones expressing the sense GGH cDNA, GGH-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.

Western blot analysis. GGH and dihydrofolate reductase (DHFR) protein expression was determined by standard western analysis as described (Sohn *et al*, 2004a; Cho *et al*, 2007), using a rabbit polyclonal antibody raised against human GGH (SDI, Newark, DE, USA) at a dilution of 1:1000 and against human DHFR (Sigma, Oakville, ON, Canada) at a dilution of 1:3000. To confirm that the proteins were loaded equally, the membranes were stripped and reprobed with a mouse monoclonal antibody raised against human β -actin (Sigma) at a dilution of 1:3000. All western analyses were repeated using three different cell lysates. Densitometry of bands were determined using the public domain ImageJ (version 1.38) from the National Institute of Health available on the Internet at <http://rsbweb.nih.gov/ij>.

GGH, TS and DHFR enzyme activity assays. The activity of GGH was determined by incubating protein in cell lysate with MTX-glu₄ as substrate and measuring MTX and its polyglutamates using HPLC as described (Panetta *et al*, 2002a; Cho *et al*, 2007). The catalytic activity of TS was determined by the ³H release that occurred during the conversion of [5-³H]-dUMP to dTMP (deoxythymidine-5-monophosphate) as described (Peters *et al*, 1987). The catalytic activity of DHFR was determined using the DHFR assay kit (Sigma) as per the manufacturer's protocol. The assay is based on the ability of DHFR to catalyse the reversible NADPH-dependent reduction of DHF to THF. Enzyme assays were performed in triplicate, and three replicate experiments were performed.

Intracellular folate concentrations and glutamate chain lengths.

Intracellular folate concentrations for conjugase-treated (which allows the measurement of total intracellular folate concentration, including short- and long-chain folylpolyglutamates) and -untreated (which allows the determination of short-chain folylpolyglutamates) samples were determined by a standard microbiological assay to determine the extent of polyglutamylation as described (Sohn *et al*, 2004b; Cho *et al*, 2007). All analyses were performed in triplicate, and three replicate experiments were performed.

Doubling time calculation. Cells (8000 per well) were plated in a 96-well flat-bottom plate and grown either in RPMI-1640 medium containing $2.3 \mu\text{mol l}^{-1}$ folic acid or in RPMI-1640 free of folic acid supplemented with either 50 or 100 nmol l^{-1} 5-MTHF for 72 h. The cell population was determined using the sulphorhodamine B (SRB) optical density (OD) measurement assay (Sohn *et al*, 2004a,b; Cho *et al*, 2007). The growth rate constant k was derived using the equation $N/N_0 = e^{kt}$, where N_0 is the OD of cells at time zero and N is the OD of cells at 72 h. The same equation was used to calculate doubling time t , by setting $N/N_0 = 2$. All analyses were performed in triplicate, and three replicate experiments were performed.

In vitro chemosensitivity assay. *In vitro* chemosensitivity was determined using a modification of the SRB protein assay as described (Skehan *et al*, 1990; Sohn *et al*, 2004a,b; Cho *et al*, 2007). Briefly, 8000 cells per $100 \mu\text{l}$ RPMI-1640 medium per well were seeded in triplicate in 96-well flat-bottom plates. After 24 h, an additional $100 \mu\text{l}$ of RPMI-1640 medium containing 5FU (InvivoGen, San Diego, CA, USA) and LV (Sigma) or MTX (Sigma) were added, and cells were cultured for an additional 72 h. Medium folate concentrations were $2.3 \mu\text{mol l}^{-1}$ folic acid, or 50 or 100 nmol l^{-1} 5-MTHF. In both cell lines, the concentration of 5FU ranged from 1.5×10^{-6} to $25 \times 10^{-6} \text{ M}$, whereas the concentration of LV was held constant at $5 \times 10^{-6} \text{ M}$. The concentration range of MTX was 0.5 – 5×10^{-8} and 1.5 – $6 \times 10^{-8} \text{ M}$ in HCT116 and MDA-MB-435 cells, respectively. After 72 h, cells were fixed with trichloroacetic acid and stained with SRB protein dye. The dye was solubilised and the OD of the solution measured at 595 nm. The results were expressed as the percentage of cell survival on the basis of the difference between the OD at the start and end of drug exposure, according to the formula (Peters *et al*, 1993):

$$\text{Survival (\%)} = \frac{[(\text{OD}_{\text{drug}}/\text{OD}_{\text{start drug exposure}}) - 1]}{[(\text{OD}_{\text{no drug}}/\text{OD}_{\text{start drug exposure}}) - 1]} \times 100.$$

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 vs proportion of cells that survived. All analyses were performed in sextuplet, and at least three replicate experiments were performed for all drug treatments.

In vivo chemosensitivity. The protocol was approved by the Animal Care Committee of the University of Toronto. Twenty 6-week-old male BALB/c nu/nu mice (Charles River, Wilmington, MA, USA) received subcutaneous injections in each flank of HCT116 colon cancer cells expressing endogenous GGH or the sense GGH (1×10^6 cells per site per mouse) in 100 μ l of serum-free RPMI-1640 medium after acclimatisation to an amino acid-defined control diet containing 2 mg folic acid per kg diet for a week. When xenografts reached a volume of 80–200 mm³, mice were randomly assigned to receive the control or folic acid-supplemented (8 mg per kg diet) diet ($n = 10$ per dietary group). Within each dietary group, mice received 5FU + LV or 0.9% NaCl intraperitoneally ($n = 5$ per treatment group). 5-Fluorouracil (20 mg kg⁻¹ per day) dissolved in 100 μ l of 0.9% NaCl was administered by intraperitoneal injection for five consecutive days for a week as described (Sohn *et al*, 2004a). Leucovorin (1 mg kg⁻¹ per day) dissolved in 100 μ l of 0.9% NaCl was administered 1 h before 5FU administration by intraperitoneal injection (Sohn *et al*, 2004a). Therefore, there were four groups of mice ($n = 5$ per group): (i) treated with 0.9% NaCl and on the control diet; (ii) treated with 5FU + LV and on the control diet; (iii) treated with 0.9% NaCl and on the folic acid-supplemented diet; and (iv) treated with 5FU + LV and on the folic acid-supplemented diet.

The selected dose of 5FU and LV was determined based on the Mayo Clinic regimen (20 mg m⁻² LV (0.7 mg kg⁻¹) + 425 mg m⁻² 5FU (14 mg kg⁻¹); Buroker *et al*, 1994). Amino acid-defined diets containing different levels of folic acid constitute a standard method of providing supplemental dietary folate in rodents (Song *et al*, 2000). The control diet containing 2 mg folic acid per kg diet (0.5–1 mg of folic acid in 2000 kcal) is accepted as the basal dietary requirement for rodents (Song *et al*, 2000) and was selected to parallel the recommended daily allowance for folate in humans (0.4 mg dietary folate equivalent in humans consuming a daily average of 2000 kcal). The supplemental level of folic acid at 8 mg per kg diet approximates a total folate intake from fortified foods and daily supplement use of 1 mg folic acid and represents approximately four times the recommended daily allowance for folate in humans (i.e., 1.6 mg per day). The detailed composition of the diets has been published previously (Song *et al*, 2000). Sterilised diets and water were provided *ad libitum*. Food intake and body weights were recorded twice a week.

The tumours were measured with a digital caliper twice a week. The estimated tumour volume (V) was calculated based on the formula $W^2 \times L \times 0.5$, where W represents the largest tumour diameter in centimetres and L represents the next largest tumour diameter (Bras-Goncalves *et al*, 2000). The individual relative tumour volume (RTV) was calculated as V_x/V_1 , where V_x is the volume in cubic millimetres at a given time and V_1 is the volume at the start of treatment. Results are expressed as the mean daily per cent change in tumour volume for each group of mice.

Plasma folate concentrations. At necropsy, blood was collected for plasma folate assay. Plasma folate concentrations were determined by a standard microbiological microtitre plate assay (Sie *et al*, 2011).

Statistical analysis. For continuous variables, comparisons between cells expressing the sense GGH (Sense) and controls (Control-S), and between cells transfected with the GGH-targeted siRNA (siRNA) and controls (Control-si) were determined using the Student's *t*-test. For the *in vitro* chemosensitivity analyses, plots of percentage of survival versus dose demonstrated S-shaped curves and, therefore, the logit transformation ($\text{logit}(p) = \ln(p/(1-p))$) 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 (CIs) were calculated on the log scale from the regression results as described (Draper and Smith, 1981), and then back-transformed to the original scale for reporting.

For the *in vivo* chemosensitivity analysis, the dependent variable was tumour volume, which was log-transformed due to its skewed distribution. A generalised linear regression was used to account for the fact that each mouse had been injected with two different types of cells, and, in addition, each mouse was measured repeatedly over time. The resulting slopes estimate the change in log(volume) per day and, when back-transformed, estimate the growth rate per day. The effect of diet, treatment and/or cell types on rate of growth was included due to a significant interaction between the effect of time and the effect of one or more of the other three factors. Plasma folate concentrations were analysed by two-way ANOVA analysis, with diet and treatment as the independent variables. For all analyses, results were considered statistically significant if two-tailed *P*-values were <0.05. Analyses were performed using SPSS Statistics 17.0 (IBM SPSS, Chicago, IL, USA) and SAS version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

GGH overexpression system. HCT116 and MDA-MB-435 cells expressing the sense GGH had significantly higher steady-state levels of the GGH protein and GGH activity ($P < 0.001$) compared with corresponding controls expressing endogenous GGH (Figure 1A and C). Total intracellular folate concentration (i.e., folate levels after conjugase treatment) of HCT116 and MDA-MB-435 cells expressing the sense GGH was significantly lower compared with corresponding controls ($P < 0.01$; Figure 1B and D). Concentration of long-chain polyglutamates (i.e., differences between folate concentrations for conjugase-treated and -untreated samples) of HCT116 and MDA-MB-435 cells expressing the sense GGH was significantly lower compared with corresponding controls ($P < 0.03$; Figure 1B and D). These observations confirm the appropriate functional consequences of GGH overexpression in both HCT116 and MDA-MB-435 cells.

GGH inhibition system. HCT116 and MDA-MB-435 cells transfected with the GGH-targeted siRNA had significantly lower steady-state levels of the GGH protein and GGH activity ($P < 0.001$) compared with corresponding controls expressing endogenous GGH (Figure 2A and C). Total intracellular folate concentration of HCT116 and MDA-MB-435 cells transfected with the GGH-targeted siRNA was significantly higher compared with corresponding controls ($P < 0.001$; Figure 2B and D). Intracellular long-chain folylpolyglutamate concentration of HCT116 and MDA-MB-435 cells transfected with the GGH-targeted siRNA was significantly higher compared with corresponding controls ($P < 0.02$; Figure 2B and D). These observations confirm the appropriate functional consequences of GGH inhibition in both HCT116 and MDA-MB-435 cells.

Effects of GGH modulation on TS and DHFR. The catalytic activity of TS in HCT116 and MDA-MB-435 cells expressing the sense GGH was significantly lower compared with corresponding controls ($P < 0.01$; Table 1). The catalytic activity of TS in both cell lines transfected with the GGH-targeted siRNA was significantly higher compared with corresponding controls ($P < 0.04$; Table 1).

HCT116 and MDA-MB-435 cells expressing the sense GGH had significantly lower steady-state levels of the DHFR protein ($P < 0.05$; data not shown) and DHFR activity ($P < 0.02$; Table 1) compared with corresponding controls. In both cell lines, cells transfected with the GGH-targeted siRNA had significantly higher levels of the DHFR protein ($P < 0.02$; data not shown) and DHFR activity ($P < 0.03$; Table 1) compared with corresponding controls.

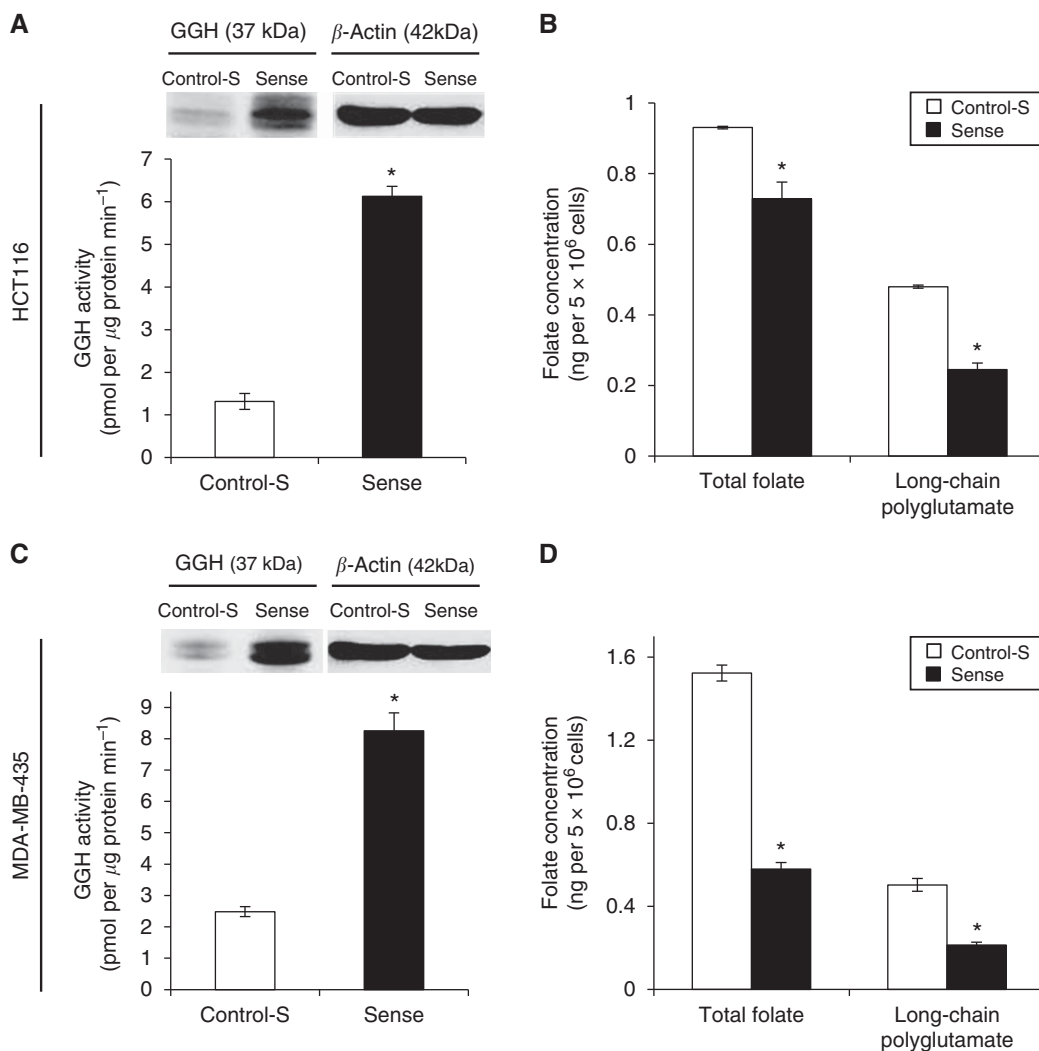


Figure 1. GGH overexpression system in HCT116 and MDA-MB-435 cells. HCT116 and MDA-MB-435 cells expressing the sense GGH (Sense) had significantly higher steady-state levels of the GGH protein and GGH activity (**A** and **C**) compared with cells transfected with the vector alone (Control-S; endogenous GGH; * $P < 0.001$). In both cell lines, total intracellular folate concentrations (i.e., folate levels after conjugase treatment) were lower in the Sense compared with corresponding controls (* $P < 0.01$; **B** and **D**). Concentrations of long-chain polyglutamates (i.e., differences between folate concentrations for conjugase-treated and -untreated samples) were significantly lower in the Sense in both cell lines compared with corresponding controls (* $P < 0.03$; **B** and **D**).

Effects of folate and GGH modulation on growth rate. In both cell lines, cells overexpressing GGH exhibited a significantly increased doubling time compared with corresponding controls at all three folate concentrations, indicating a slower growth rate ($P < 0.001$; Supplementary Table S1). This observation is consistent with the observed lower total intracellular folate and long-chain folylpolyglutamate concentrations and decreased TS activity associated with GGH overexpression.

Interestingly, the effect of GGH inhibition on growth rate was influenced by medium folate concentrations. As expected, based on the observed higher total intracellular folate and long-chain folylpolyglutamate concentrations and increased TS activity associated with GGH inhibition, HCT116 cells transfected with the GGH-targeted siRNA had a significantly decreased doubling time (i.e., a faster growth rate) compared with controls at 50 and 100 nmol l^{-1} 5-MTHF ($P < 0.001$; Supplementary Table S1). Unexpectedly, HCT116 cells transfected with the GGH-targeted siRNA had a significantly increased doubling time compared with controls at 2.3 $\mu\text{mol l}^{-1}$ folic acid ($P < 0.001$; Supplementary Table S1). Similarly, MDA-MB-435 cells in which GGH was inhibited showed a significantly decreased doubling time only at 50 nmol l^{-1}

5-MTHF but a significantly increased doubling time at 100 nmol l^{-1} 5-MTHF and 2.3 $\mu\text{mol l}^{-1}$ folic acid compared with controls ($P < 0.001$; Supplementary Table S1).

Effects of folate and GGH modulation on *in vitro* 5FU chemosensitivity. Our *a priori* hypothesis was that GGH overexpression would decrease the cytotoxic effect of 5FU by decreasing relative intracellular concentration of long-chain 5,10-methyleneTHF-polyglutamates, resulting in less efficient formation and stabilisation of the inhibitory 5,10-methyleneTHF-TS-FdUMP ternary complex. Consistent with our hypothesis, GGH overexpression significantly, albeit very modestly, decreased chemosensitivity of HCT116 cells to 5FU + LV at 2.3 $\mu\text{mol l}^{-1}$ folic acid ($P < 0.05$; Figure 3C; IC_{50} values in Supplementary Table S2). Interestingly, this effect was not observed at 100 nmol l^{-1} 5-MTHF (Figure 3B) and at 50 nmol l^{-1} 5-MTHF GGH overexpression paradoxically increased chemosensitivity of HCT116 cells to 5FU + LV ($P < 0.05$; Figure 3A; Supplementary Table S2). In contrast, consistent with our hypothesis, GGH overexpression significantly decreased chemosensitivity of MDA-MB-435 cells to 5FU + LV at 50 and 100 nmol l^{-1} 5-MTHF with a more

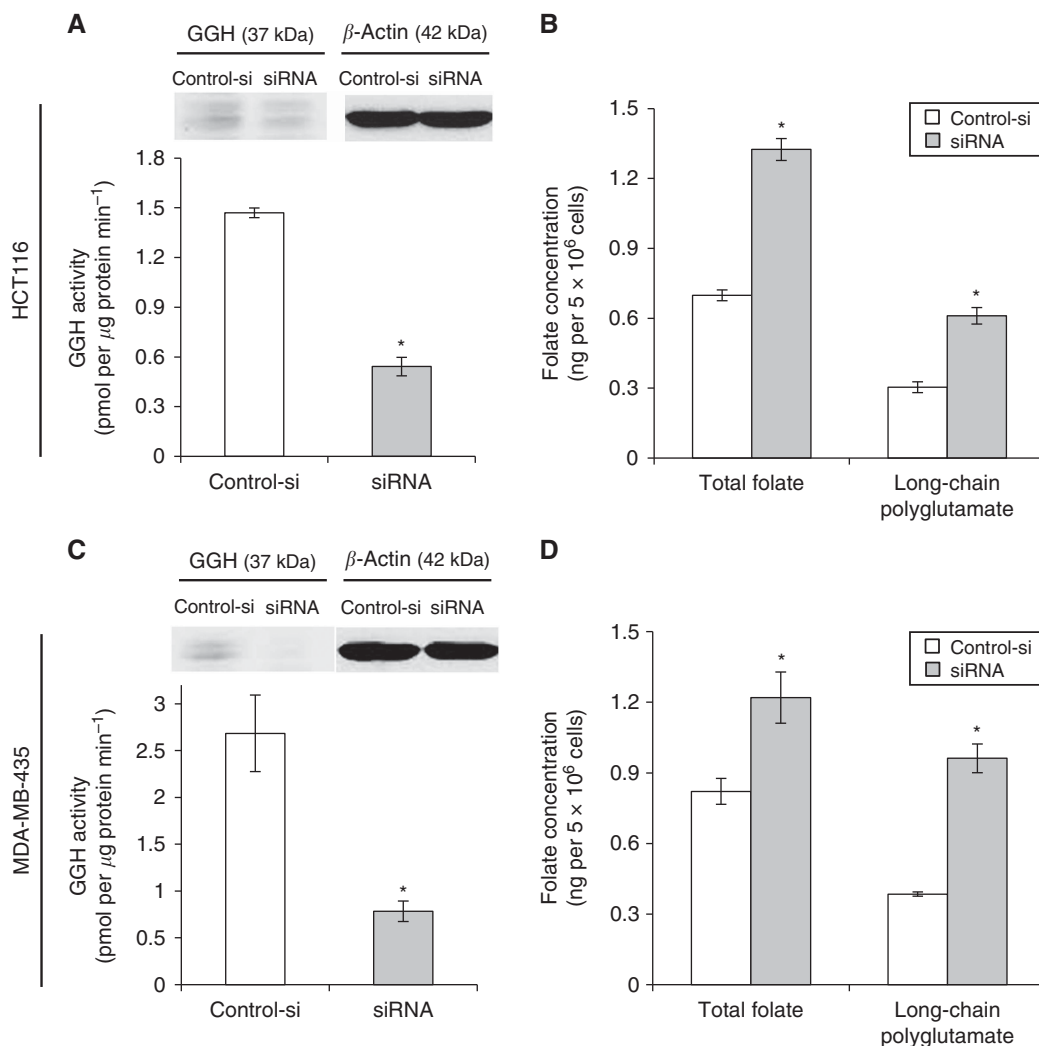


Figure 2. GGH inhibition system in HCT116 and MDA-MB-435 cells. HCT116 and MDA-MB-435 cells transfected with the GGH-targeted siRNA (siRNA) had significantly lower steady-state levels of the GGH protein and GGH activity (**A** and **C**) compared with cells transfected with the vector alone (Control-si; endogenous GGH; $*P < 0.001$). Total intracellular folate concentrations (i.e., folate levels after conjugase treatment) were significantly higher in the siRNA in both cell lines compared with corresponding controls ($*P < 0.001$; **B** and **D**). Concentrations of long-chain polyglutamates (i.e., differences between folate concentrations for conjugase-treated and -untreated samples) were significantly higher in the siRNA in both cell lines compared with corresponding controls ($*P < 0.02$; **B** and **D**).

Table 1. TS catalytic activity and DHFR activity in HCT116 and MDA-MB-435 cells transfected with the sense GGH and GGH-targeted siRNA in comparison with corresponding control cells expressing endogenous GGH

Cell line	Enzyme activity	GGH overexpression		GGH inhibition	
		Control-S	Sense	Control-si	siRNA
HCT116	TS catalytic activity (pmol h ⁻¹ per mg protein)	2067.8 ± 12.5	1246.1 ± 10.2*	694.1 ± 125.9	1005.1 ± 106.2*
	DHFR activity (a.u.)	9.3 ± 1.7	3.9 ± 0.4*	1.4 ± 0.3	4.4 ± 0.3*
MDA-MB-435	TS catalytic activity (pmol h ⁻¹ per mg protein)	845.7 ± 55.2	627.6 ± 40.7*	742.7 ± 33.4	1032.5 ± 74.4*
	DHFR activity (a.u.)	3.3 ± 0.2	1.7 ± 0.7*	0.9 ± 0.2	1.9 ± 0.4*

Abbreviations: DHFR = dihydrofolate reductase; TS = thymidylate synthase. Results are expressed as mean ± s.d., $*P < 0.05$, statistically significant compared with corresponding controls.

pronounced effect at 100 nmol l⁻¹ 5-MTHF and at 2.3 μmol l⁻¹ folic acid, which demonstrated the most prominent effect ($P < 0.05$; Figure 3D–F; Supplementary Table S3).

We hypothesised that GGH inhibition would increase the cytotoxic effect of 5FU by increasing relative intracellular concentrations of long-chain 5,10-methyleneTHF-polyglutamates,

resulting in more efficient formation and stabilisation of the 5,10-methyleneTHF–TS–FdUMP ternary complex. Consistent with our hypothesis, GGH inhibition significantly increased chemosensitivity of HCT116 cells to 5FU + LV at 50 and 100 nmol l⁻¹ 5-MTHF and 2.3 μmol l⁻¹ folic acid ($P < 0.05$; Figure 4A–C; Supplementary Table S2). Similarly, GGH inhibition

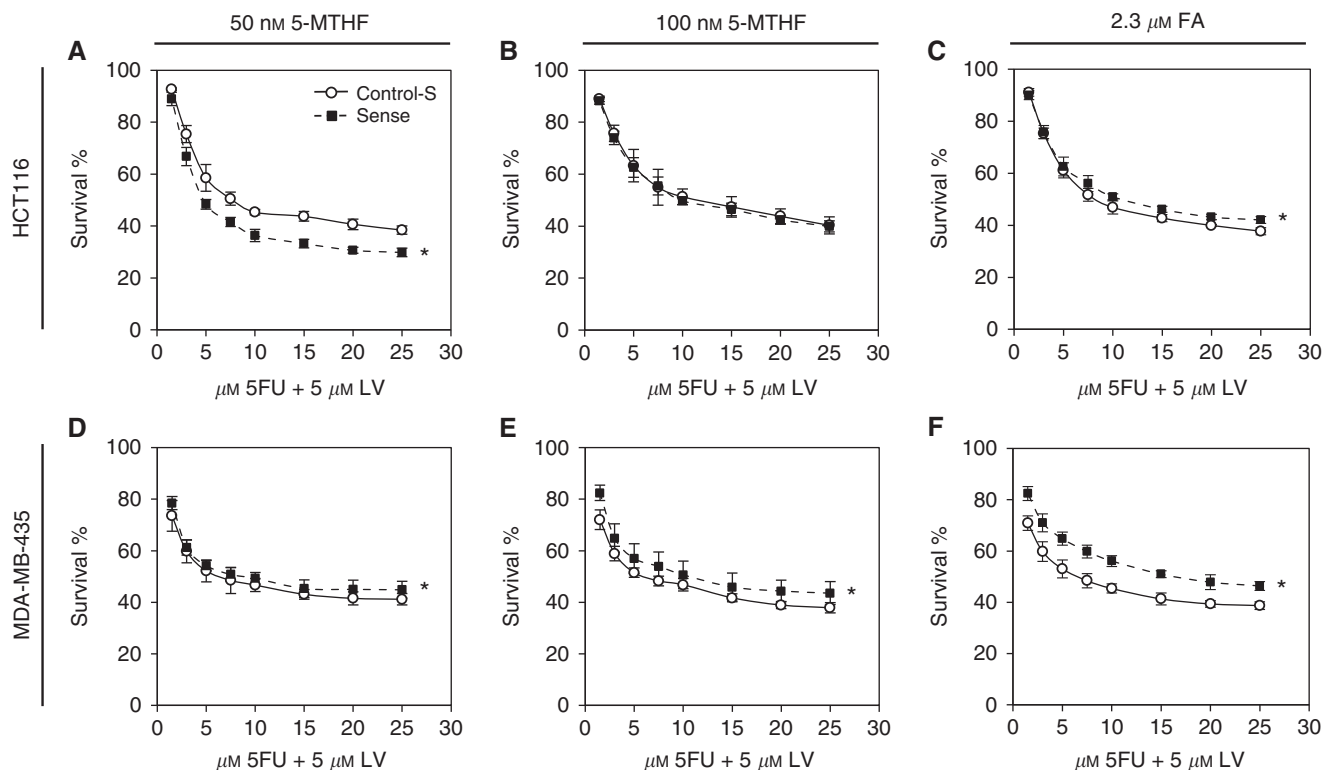


Figure 3. Effect of GGH overexpression on 5FU chemosensitivity. *In vitro* chemosensitivity of HCT116 colon and MDA-MB-435 breast cancer cells transfected with the sense GGH (Sense) to 5FU + LV in comparison with cells transfected with the vector alone (Control-S; endogenous GGH) was determined using a modification of the sulphorhodamine protein assay. Cells were grown in medium containing 50 nmol l⁻¹ 5-MTHF (A and D), 100 nmol l⁻¹ 5-MTHF (B and E) or 2.3 μmol l⁻¹ folic acid (FA; C and F). Points, means; bars, s.d.; *P < 0.05, statistically significant compared with corresponding control cells.

significantly increased chemosensitivity of MDA-MB-435 cells to 5FU + LV at 100 nmol l⁻¹ 5-MTHF and 2.3 μmol l⁻¹ folic acid ($P < 0.05$), but not at 50 nmol l⁻¹ 5-MTHF (Figure 4D–F; Supplementary Table S3). In both cell lines, the degree of the enhanced chemosensitivity to 5FU + LV was higher at 100 nmol l⁻¹ 5-MTHF than at 50 nmol l⁻¹ 5-MTHF, and was most pronounced at 2.3 μmol l⁻¹ folic acid (Figure 4A–F; Supplementary Tables S2 and S3).

Effects of folate and GGH overexpression on *in vivo* 5FU chemosensitivity. We next confirmed the *in vitro* chemosensitivity of HCT116 cells overexpressing GGH to 5FU + LV in nude mice and determined whether dietary folic acid supplementation would further influence the effect of GGH overexpression on chemosensitivity. We chose the HCT116 GGH overexpression system for the *in vivo* study for two reasons: (1) the magnitude of cell survival difference was more pronounced in this system compared with other systems and (2) the *in vitro* chemosensitivity to 5FU demonstrated an interesting modifying effect of exogenous folate. The mean plasma folate concentrations of the folic acid-supplemented mice (129.2 ± 7.7 nmol l⁻¹ in 5FU + LV-treated mice; 118.0 ± 12.7 nmol l⁻¹ in 0.9% saline-treated mice) were significantly higher than those of mice on the control diet (84.5 ± 9.3 in 5FU + LV-treated mice; 82.9 ± 4.5 in saline-treated mice) ($P < 0.001$). Treatment with 5FU + LV had no effect on plasma folate concentrations.

For each diet, the difference in the growth rates of xenografts between cell types (GGH overexpression versus control endogenous GGH) depended on treatment, and the effect of treatment depended on the cell types (P -interaction for treatment × cell types = 0.037 for 2 mg folic acid diet and < 0.0001 for 8 mg folic acid diet). When the growth rates of the xenografts in mice injected

with saline were compared, the GGH-overexpressing xenografts grew slower than the xenografts expressing endogenous GGH at both dietary folic acid levels (at 2 mg folic acid per kg diet: RTV = 0.78 (95% CI = 0.65–0.94), $P = 0.011$; at 8 mg folic acid per kg diet: RTV = 0.55 (95% CI = 0.52–0.58), $P < 0.0001$); Figure 5A and B). These data are consistent with the observed increased doubling time and thus slower growth rates associated with GGH overexpression in HCT116 cells grown at all three folate concentrations (Supplementary Table S1).

The growth of the GGH-overexpressing xenografts was inhibited more effectively by 5FU + LV than was the growth of those expressing endogenous GGH at the 2 mg folic acid level (38% inhibition (95% CI = 32–43%), $P < 0.0001$; Figure 5C). This observation is consistent with the significantly increased *in vitro* chemosensitivity of the GGH-overexpressing HCT116 cells at 50 nmol l⁻¹ 5-MTHF (Figure 3A and Supplementary Table S2). In contrast, at the 8 mg supplemental folic acid level, the growth of the xenografts expressing endogenous GGH was inhibited more effectively by 5FU + LV than was the growth of those overexpressing GGH (36% inhibition (95% CI = 21–48%), $P < 0.0001$; Figure 5D). This observation is in agreement with the significantly decreased *in vitro* chemosensitivity of the GGH-overexpressing HCT116 cells at 2.3 μmol l⁻¹ folic acid (Figure 3C and Supplementary Table S1).

Effects of folate and GGH modulation on *in vitro* MTX chemosensitivity. We predicted that the GGH overexpression-induced decreased MTX polyglutamylation would decrease the cytotoxic effect of MTX. Consistent with our hypothesis, GGH overexpression significantly decreased chemosensitivity of HCT116 cells to MTX at 2.3 μmol l⁻¹ folic acid ($P < 0.05$; Figure 6C and Supplementary Table S2). Interestingly, this effect

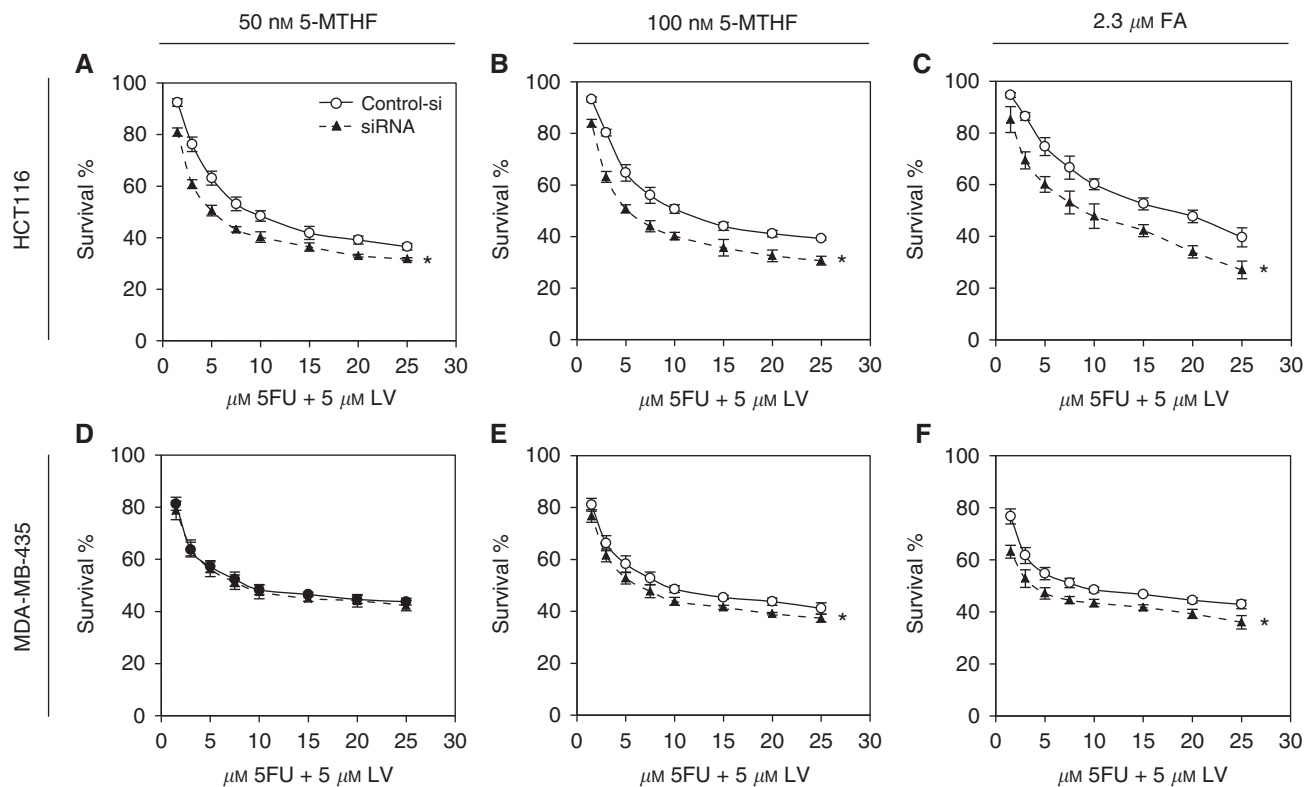


Figure 4. Effect of GGH inhibition on 5FU chemosensitivity. *In vitro* chemosensitivity of HCT116 colon and MDA-MB-435 breast cancer cells transfected with the GGH-targeted siRNA (siRNA) to 5FU + LV in comparison with cells transfected with the vector alone (Control-si; endogenous GGH) was determined using a modification of the sulphorhodamine protein assay. Cells were grown in medium containing 50 nmol l^{-1} 5-MTHF (A and D), 100 nmol l^{-1} 5-MTHF (B and E), or $2.3 \mu\text{mol l}^{-1}$ folic acid (FA; C and F). Points, means; bars, s.d.; * $P < 0.05$, statistically significant compared with corresponding control cells.

was not observed at 100 nmol l^{-1} 5-MTHF (Figure 6B), and at 50 nmol l^{-1} 5-MTHF GGH overexpression paradoxically increased chemosensitivity of HCT116 cells to MTX ($P < 0.05$; Figure 6A and Supplementary Table S2). Consistent with our hypothesis, GGH overexpression significantly decreased chemosensitivity of MDA-MB-435 cells to MTX at 50 and 100 nmol l^{-1} 5-MTHF with a more pronounced effect at 100 nmol l^{-1} 5-MTHF and at $2.3 \mu\text{mol l}^{-1}$ folic acid, which demonstrated the most prominent effect ($P < 0.05$; Figure 6D–F and; Supplementary Table S3).

We hypothesised that the GGH inhibition-induced increased MTX polyglutamylation would enhance the cytotoxic effect of MTX. Interestingly, in contrast to our hypothesis, GGH inhibition paradoxically decreased chemosensitivity of both cell lines to MTX at 50 and 100 nmol l^{-1} 5-MTHF and $2.3 \mu\text{mol l}^{-1}$ folic acid ($P < 0.05$; Figure 7A–F; Supplementary Tables S2 and S3). In both cell lines, the degree of the decreased chemosensitivity to MTX was greater at 100 nmol l^{-1} 5-MTHF than at 50 nmol l^{-1} 5-MTHF, and was most pronounced at $2.3 \mu\text{mol l}^{-1}$ folic acid (Figure 7A–F; Supplementary Tables S2 and S3).

DISCUSSION

We developed an appropriate *in vitro* model of GGH overexpression and inhibition in HCT116 colon and MDA-MB-435 breast cancer cells with predictable functional consequences (summarised in Figure 8). Compared with controls expressing endogenous GGH, cells overexpressing GGH had significantly higher GGH protein expression and activity, lower total intracellular folate concentrations and lower content of long-chain folylpolyglutamates (Figure 8A). In contrast, cells transfected with

the GGH-targeted siRNA had significantly lower GGH protein expression and activity, higher concentrations of total intracellular folate and higher content of long-chain folylpolyglutamates compared with controls expressing endogenous GGH (Figure 8B). Cells overexpressing GGH had a significantly slower growth rate compared with controls at all three folate concentrations. This is consistent with the observed decreased total intracellular folate concentrations and lower content of long-chain folylpolyglutamates, which are better substrates than short-chain folylpolyglutamates for folate-dependent enzymes involved in thymidylate and purine biosynthesis. Consistent with the growth data, cells overexpressing GGH had significantly lower TS activity and DHFR protein expression and activity. Consistent with the observed increased total intracellular folate concentrations, higher content of long-chain folylpolyglutamates, and higher TS activity and DHFR protein expression and activity, HCT116 in which GGH was inhibited exhibited a significantly faster growth rate compared with controls at 50 and 100 nmol l^{-1} 5-MTHF. Similarly, the GGH-inhibited MDA-MB-435 cells showed a faster growth rate compared with controls at 50 nmol l^{-1} 5-MTHF. Unexpectedly, HCT116 and MDA-MB-435 cells transfected with the GGH-targeted siRNA had a slower growth rate compared with controls at $2.3 \mu\text{mol l}^{-1}$ folic acid, and at 100 nmol l^{-1} 5-MTHF and $2.3 \mu\text{mol l}^{-1}$ folic acid, respectively, which is not readily explained by the observed biochemical consequences of GGH inhibition. In general, most of the observed functional consequences of GGH overexpression and inhibition are consistent with the known biological function of GGH and provided an appropriate *in vitro* model to test the effect of GGH modulation on chemosensitivity of colon and breast cancer cells to 5FU and MTX (Figure 8).

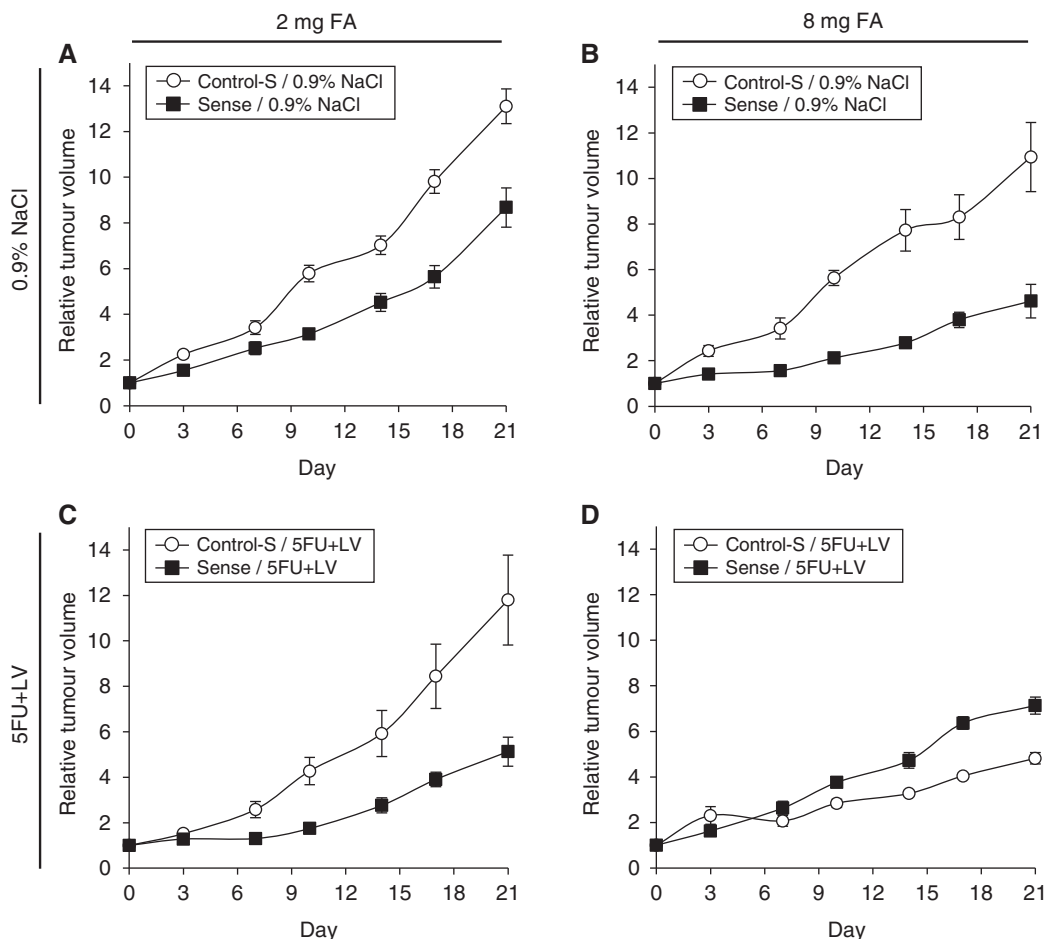


Figure 5. Effect of GGH overexpression and folic acid (FA) supplementation on 5FU chemosensitivity in nude mice. Twenty 6-week-old male BALB/c nu/nu mice received subcutaneous injections in each flank of HCT116 colon cancer cells expressing endogenous GGH (Control-S) and the sense GGH (Sense). When xenografts reached a volume of 80–200 mm³, mice were randomly assigned to receive either the control (2 mg FA per kg diet) or FA-supplemented (8 mg FA per kg diet) diet ($n = 10$ per dietary group). Within each dietary group, mice received either 5FU + LV or 0.9% NaCl intraperitoneally ($n = 5$ per treatment group). Therefore, there were four groups of mice ($n = 5$ per group): (i) treated with 0.9% NaCl and on the control diet; (ii) treated with 5FU + LV and on the control diet; (iii) treated with 0.9% NaCl and on the FA-supplemented diet; and (iv) treated with 5FU + LV and on the FA-supplemented diet. The estimated tumour volume (V) was calculated based on the formula $W^2 \times L \times 0.5$, where W represents the largest tumour diameter in centimetres and L represents the next largest tumour diameter. The individual RTV was calculated as V_x/V_1 , where V_x is the volume in cubic millimetres at a given time and V_1 is the volume at the start of treatment. Results are expressed as the mean daily per cent change in tumour volume for each group of mice. When the growth rates of the xenografts in mice injected with saline were compared, the GGH-overexpressing xenografts grew slower than the xenografts expressing endogenous GGH at both dietary FA levels: **(A)** at 2 mg FA per kg diet: RTV = 0.78 (95% CI = 0.65–0.94), $P = 0.011$; **(B)** at 8 mg FA per kg diet: RTV = 0.55 (95% CI = 0.52–0.58), $P < 0.0001$. The growth of the GGH-overexpressing xenografts was inhibited more effectively by 5FU + LV than was the growth of those expressing endogenous GGH at the 2 mg FA level (38% inhibition (95% CI = 32–43%), $P < 0.0001$; **C**). In contrast, at the 8-mg supplemental FA level, the growth of the xenografts expressing endogenous GGH was inhibited more effectively by 5FU + LV than was the growth of those overexpressing GGH (36% inhibition (95% CI = 21–48%), $P < 0.0001$; **D**).

Our *a priori* hypothesis concerning the effects of GGH modulation on 5FU chemosensitivity was based on the assumption that the GGH modulation-induced change in intracellular concentration of long-chain 5,10-methyleneTHF-polyglutamates, which in turn affects the formation and stabilisation of the inhibitory 5,10-methyleneTHF-TS-FdUMP ternary complex, might be the primary determinant of 5FU chemosensitivity. This was indeed the case for the GGH overexpression system in MDA-MB-435 cells and for the GGH inhibition system in both HCT116 and MDA-MB-435 cells (Figure 8). In these instances, folate appears to further influence the GGH modulation-induced 5FU chemosensitivity as suggested by the observation that the magnitude of change in 5FU chemosensitivity was greater in 100 nmol l⁻¹ 5-MTHF than in 50 nmol l⁻¹ 5-MTHF, which

represent a nutritionally relevant physiological range of high and normal serum/plasma folate concentrations, respectively (Figure 8). In these instances, the magnitude of change in 5FU chemosensitivity was most pronounced at 2.3 $\mu\text{mol l}^{-1}$ folic acid, which represents an extremely high supraphysiological level of an unnatural form of folate (Figure 8). For the GGH overexpression system in HCT116 cells, however, the predicted decreased 5FU chemosensitivity was observed only at 2.3 $\mu\text{mol l}^{-1}$ folic acid, whereas this effect was not observed at 100 and at 50 nmol l⁻¹ 5-MTHF, GGH overexpression was associated with paradoxically, albeit very modestly, increased 5FU chemosensitivity (Figure 8A). It appears that at 50 nmol l⁻¹ 5-MTHF, GGH overexpression decreased total intracellular folate concentrations and contents of long-chain polyglutamates of other intracellular folate cofactors to

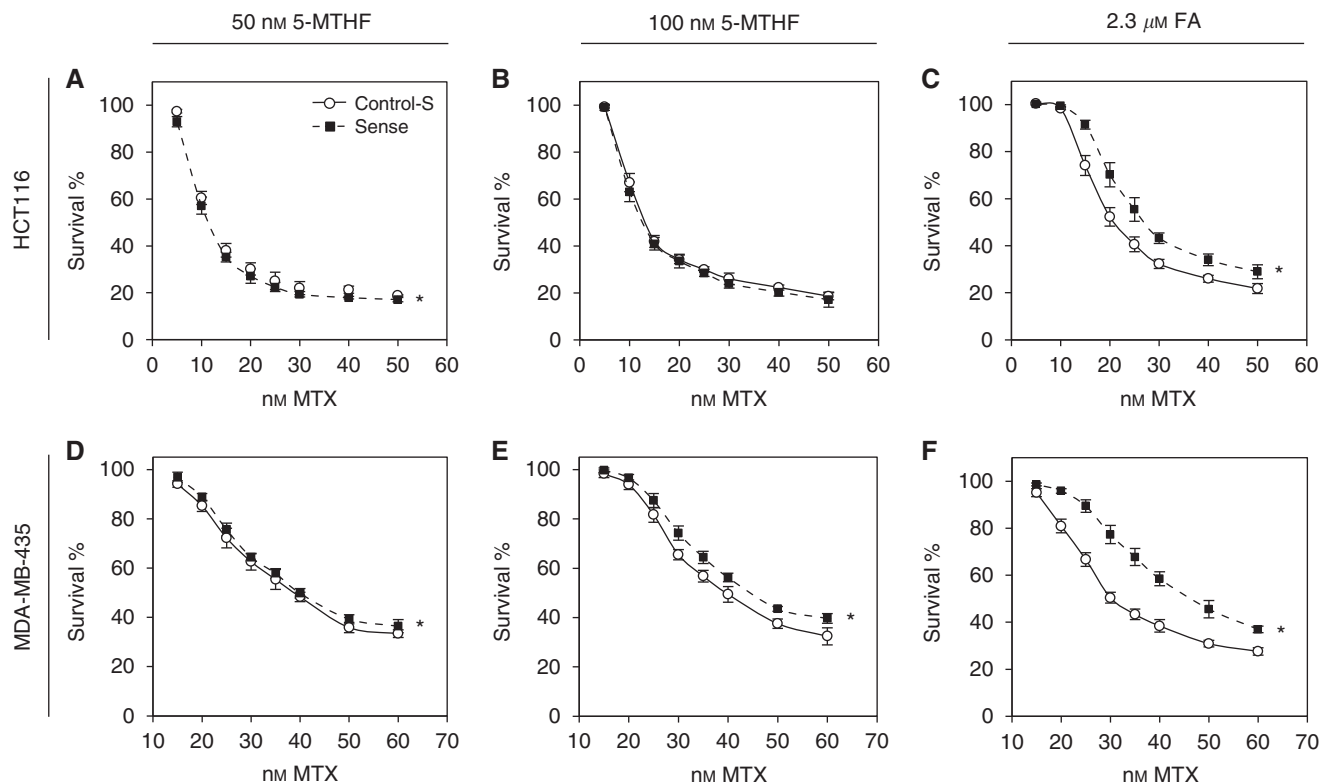


Figure 6. Effect of GGH overexpression on MTX chemosensitivity. *In vitro* chemosensitivity of HCT116 colon and MDA-MB-435 breast cancer cells transfected with the sense GGH (Sense) to MTX in comparison with cells transfected with the vector alone (Control-S; endogenous GGH) was determined using a modification of the sulphorhodamine protein assay. Cells were grown in medium containing 50 nmol l^{-1} 5-MTHF (A and D), 100 nmol l^{-1} 5-MTHF (B and E), or $2.3 \mu\text{mol l}^{-1}$ folic acid (FA; C and F). Points, means; bars, s.d.; * $P < 0.05$, statistically significant compared with corresponding control cells.

a greater extent than long-chain 5,10-methyleneTHF-polyglutamates in HCT116 cells, resulting in decreased thymidylate and purine biosynthesis. This might have overridden the effect of decreased long-chain 5,10-methyleneTHF-polyglutamates, resulting in increased 5FU cytotoxicity. At 100 nmol l^{-1} 5-MTHF, the competing effects of decreased DNA synthesis associated with decreased intracellular total folate concentrations and contents of long-chain folylpolyglutamates and of less TS inhibiting effect associated with decreased long-chain 5,10-methyleneTHF-polyglutamates seem to have cancelled each other out. We confirmed the effect of GGH overexpression on *in vitro* 5FU chemosensitivity of HCT116 cells and the modulating effect of exogenous folate levels on the GGH overexpression effect on 5FU chemosensitivity *in vivo*.

Our *a priori* hypothesis concerning the effects of GGH modulation on MTX chemosensitivity was based on the assumption that the GGH modulation-induced change in MTX polyglutamylation might be the primary determinant of MTX chemosensitivity. This was indeed the case for the GGH overexpression system in MDA-MB-435 cells (Figure 8A). In this instance, folate appears to further influence the GGH modulation-induced MTX chemosensitivity as suggested by the observation that the magnitude of change in MTX chemosensitivity was greater in 100 nmol l^{-1} 5-MTHF than in 50 nmol l^{-1} 5-MTHF, and was most pronounced at $2.3 \mu\text{mol l}^{-1}$ folic acid (Figure 8A). For the GGH overexpression system in HCT116 cells, however, the predicted decreased MTX chemosensitivity was observed only at $2.3 \mu\text{mol l}^{-1}$ folic acid, whereas this effect was not observed at 100 nmol l^{-1} and at 50 nmol l^{-1} 5-MTHF GGH expression was associated with paradoxically, albeit very modestly, increased MTX chemosensitivity (Figure 8A). This suggests that decreased

intracellular concentrations of total folate and long-chain folylpolyglutamates, which would decrease DNA synthesis, associated with GGH overexpression overwhelmed the effect of decreased MTX polyglutamylation, resulting in enhanced MTX chemosensitivity at 50 nmol l^{-1} in HCT116 cells. However, at 100 nmol l^{-1} , the magnitude of intracellular depletion of total and long-chain folylpolyglutamates associated with GGH overexpression cancelled out the counterbalancing effect of the GGH overexpression-induced decreased MTX polyglutamylation.

In contrast to our *a priori* hypothesis that the GGH inhibition-induced increased MTX polyglutamylation would enhance the cytotoxic effect of MTX, GGH inhibition significantly decreased MTX chemosensitivity at all three folate concentrations in both cell lines (Figure 8B). This suggests that increased intracellular concentrations of total folate and long-chain folylpolyglutamates, which would provide an increased amount of substrates for nucleotide biosynthesis, and increased TS and DHFR activity associated with GGH inhibition were the primary determinants of the observed decreased chemosensitivity of colon and breast cancer cells to MTX at all three folate concentrations rather than the GGH inhibition-induced increased MTX polyglutamylation, which should have enhanced MTX chemosensitivity. Nevertheless, the modulating effect of folate on the GGH inhibition-induced MTX cytotoxicity was still evident in both cell lines as the magnitude of change in MTX chemosensitivity was greater in 100 nmol l^{-1} 5-MTHF than in 50 nmol l^{-1} 5-MTHF, and was most pronounced at $2.3 \mu\text{mol l}^{-1}$ folic acid (Figure 8B).

Clinical studies investigating the role of GGH in modulating chemosensitivity to 5FU and antifolates have begun to emerge. Low GGH expression was reported to correlate with an enhanced response to 5FU-based chemotherapy in patients with metastatic

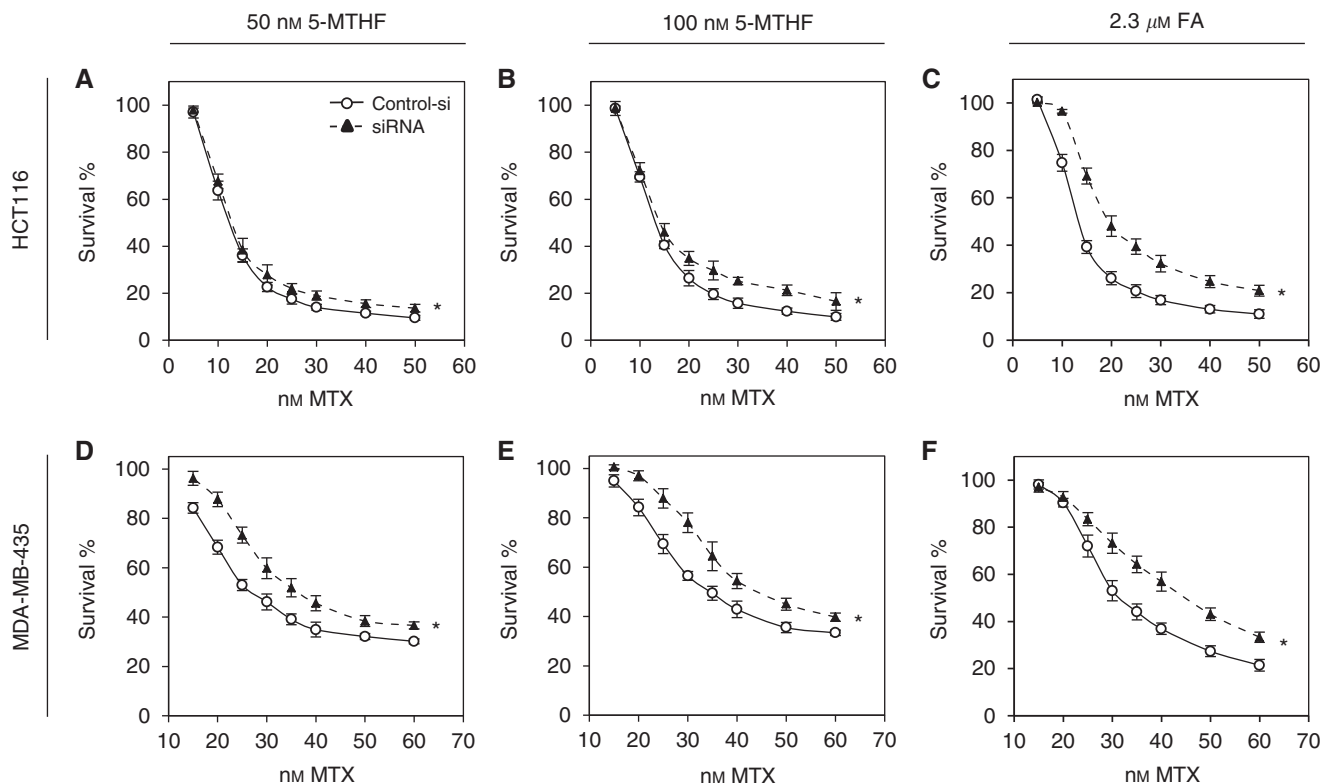


Figure 7. Effect of GGH inhibition on MTX chemosensitivity. *In vitro* chemosensitivity of HCT116 colon and MDA-MB-435 breast cancer cells transfected with the GGH-targeted siRNA (siRNA) to MTX in comparison with cells transfected with the vector alone (Control-si; endogenous GGH) was determined using a modification of the sulphorhodamine protein assay. Cells were grown in medium containing 50 nmol l⁻¹ 5-MTHF (A and D), 100 nmol l⁻¹ 5-MTHF (B and E), or 2.3 μmol l⁻¹ folic acid (FA; C and F). Points, means; bars, s.d.; *P<0.05, statistically significant compared with corresponding control cells.

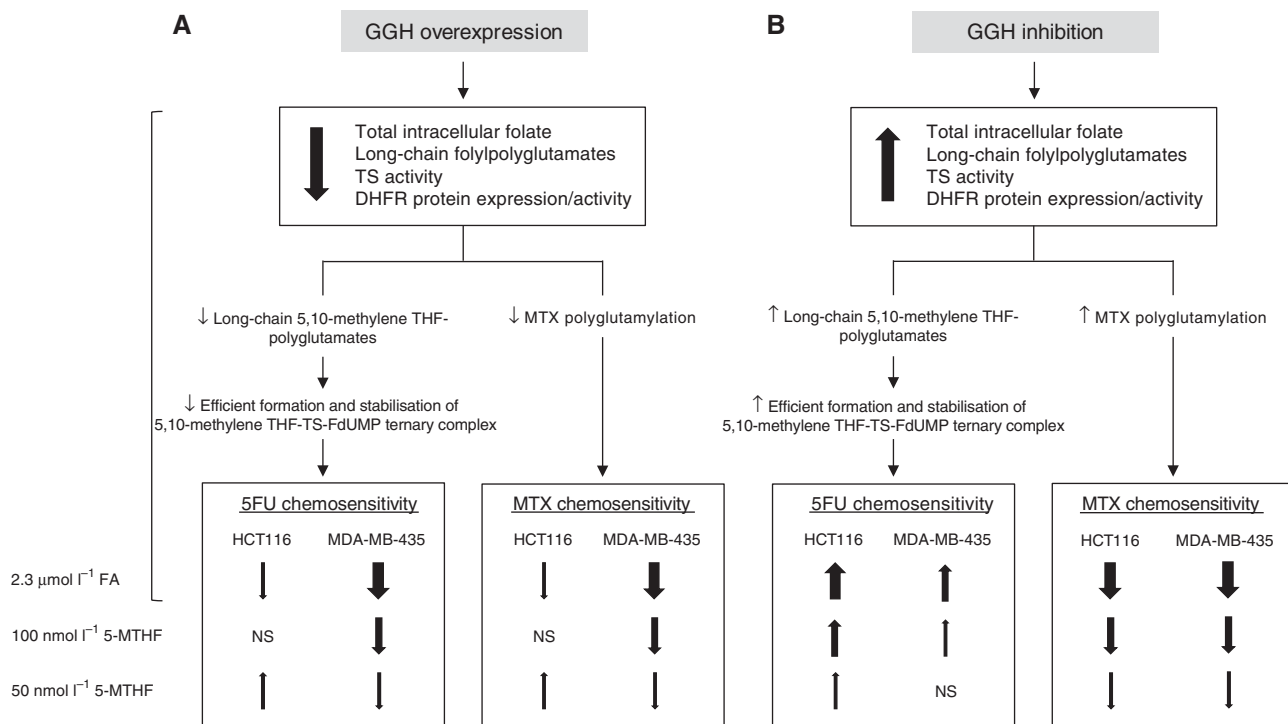


Figure 8. Summary of the effects of GGH modulation and folate. Summary of the functional consequences of GGH overexpression (A) and inhibition (B) and the effects of GGH overexpression (A) and inhibition (B) and folate on 5FU and MTX chemosensitivity in HCT116 colon and MDA-MB-435 breast cancer cells.

colorectal cancer (Nakajima *et al*, 2008). In patients with advanced pancreatic cancer treated with an oral fluoropyrimidine derivative, S-1, low GGH expression was associated with improved overall survival (Nakamura *et al*, 2011). In acute myelogenous leukemia, it was found that high GGH activity may have a role in inherent drug resistance to MTX (Rots *et al*, 1999). High GGH expression was shown to be associated with a higher risk of developing advanced toxicity to pemetrexed, a multi-targeted antifolate, in patients with advanced breast cancer (Llobart-Cussac *et al*, 2007). A recent study has reported that high GGH protein level is associated with poor prognosis and unfavourable clinical outcomes in patients with invasive breast cancer (Shubbar *et al*, 2013). Furthermore, several recently identified and characterized functionally significant genetic and epigenetic polymorphisms of GGH have been reported to predict response to and toxicity of antifolate-based treatment in patients with several cancers (Cheng *et al*, 2004; Kim *et al*, 2008; Koomdee *et al*, 2012; Smit *et al*, 2012; Silva *et al*, 2013) and inflammatory arthritis (Dervieux *et al*, 2004; van der Straaten *et al*, 2007; Hayashi *et al*, 2009; Yanagimachi *et al*, 2011; Owen *et al*, 2012; Jekic *et al*, 2013).

Intracellular folate and antifolate accumulation and metabolism, including polyglutamylolation, are affected by multiple enzymes in the highly complex folate metabolic pathway (Schneider and Ryan, 2006). Furthermore, changes in a single enzyme such as GGH induce a cascade of adaptive and compensatory changes in other enzymes in order to maintain folate and antifolate homeostasis (Shane, 2010). Thus, the attribution of changes in chemosensitivity of cancer cells to 5FU and antifolates to a single enzyme such as GGH is difficult. Most likely, GGH modulation is but one of several factors that affect cancer cells' sensitivity to 5FU and antifolates. In addition to changes in polyglutamylolation of 5,10-methyleneTHF and antifolates, GGH modulation might have also induced changes in drug uptake into and accumulation in the cells, the forward polyglutamylolation reaction by FPGS, intracellular concentration and polyglutamylolation of other folate cofactors, intracellular distribution of the various folate species between cytoplasm, lysosome and mitochondria, and/or transport mechanisms of folates and antifolates into the lysosomes where GGH resides and out of the cells by various ATP-binding cassette transporters (Schneider and Ryan, 2006). These adaptive and compensatory changes have likely had a role in modifying the effects of GGH modulation on chemosensitivity of cancer cells to 5FU and antifolates. Some of the differences between the two cell lines are likely caused by differences in this complexity. Furthermore, HCT116 and MDA-MB-435 cell lines may differ in common polymorphisms in several genes in the folate metabolic pathway and one-carbon transfer reactions such as methylenetetrahydrofolate reductase (MTHFR), TS, DHFR, methionine synthase, methionine synthase reductase, serine hydroxymethyltransferase and reduced folate carrier. This might have contributed to the observed differences between the two cell lines. In this regard, endogenous TS and DHFR activity were significantly different between the two cell lines as shown in Table 1, whereas both cell lines are heterozygous for the MTHFR C677T genotype (Sohn *et al*, 2004a).

In conclusion, as a proof-of-principle we provide evidence that GGH modulation affects chemosensitivity of colon and breast cancer cells to 5FU and MTX. Our data suggest that the effects of GGH modulation on chemosensitivity of colon and breast cancer cells to 5FU and MTX cannot be predicted solely based on the GGH modulation-induced changes in polyglutamylolation of 5,10-methyleneTHF and MTX, respectively, as we have demonstrated that exogenous folate forms and levels can further influence the effects of GGH modulation on 5FU and MTX chemosensitivity. High GGH status may predict resistance to 5FU- and MTX-based chemotherapy in breast cancer treatment, but for colon cancer this may largely depend on dietary intake and blood levels of folate.

At normal intake or blood-level folate high GGH status may enhance the response of colon cancer cells to 5FU-based chemotherapy, whereas at high intake or blood levels this enhanced chemosensitivity to 5FU may be abolished and high GGH status may even decrease 5FU chemosensitivity. On the other hand, GGH inhibition may be a potential target for increasing chemosensitivity of colon and breast cancer cells to 5FU but not to MTX. Notwithstanding the complexity of the GGH modulation-induced changes in folate and antifolate accumulation and metabolism, the potential role of GGH modulation in 5FU- and MTX-based cancer chemotherapy warrants further investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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