Effects of folylpolyglutamate synthetase modulation on chemosensitivity of colon cancer cells to 5-fluorouracil and methotrexate


Background: Poly(γ-glutamate synthetase (FPGS) converts intracellular folates and antifolates (for example, methotrexate (MTX)) 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. Polyglutamylation of intracellular 5,10-methylenetetrahydrofolate may also enhance the cytotoxicity of 5-fluorouracil (5-FU) by allowing more efficient formation and stabilisation of the inhibitory ternary complex involving thymidylate synthase and a 5-FU metabolite.

Aim: We investigated the effects of FPGS modulation on the chemosensitivity of colon cancer cells to 5-FU and MTX.

Methods: Human HCT116 colon cancer cells were stably transfected with the sense or antisense FPGS cDNA or blank (control). FPGS protein expression and enzyme activity, growth rate, intracellular folate content and composition, and in vitro chemosensitivity to 5-FU and MTX were determined.

Results: Compared with cells expressing endogenous FPGS, those overexpressing FPGS had significantly faster growth rates and higher concentrations of total folate and long chain folate polyglutamates while antisense FPGS inhibition produced opposite results. FPGS overexpression significantly enhanced, whereas FPGS inhibition decreased, chemosensitivity to 5-FU. No significant difference in chemosensitivity to MTX was observed.

Conclusions: These data provide functional evidence that FPGS overexpression and inhibition modulate chemosensitivity of colon cancer cells to 5-FU by altering intracellular folate polyglutamylation, providing proof of principle. Thus FPGS status may be an important predictor of chemosensitivity of colon cancer cells to 5-FU-based chemotherapy, and FPGS gene transfer may increase the sensitivity of colon cancer cells to 5-FU-based chemotherapy.

Folate mediates the transfer of one carbon units necessary for the de novo biosynthesis of purines and thymidylate, and hence is an essential factor for DNA synthesis. While 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. Intracellular folate is converted to polyglutamates by folylpolyglutamate synthetase (FPGS), while γ-glutamyl hydrolase (GGH) removes the terminal glutamates. Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate dependent enzymes.

Similarly, antifolates (for example, methotrexate (MTX)) are retained in tumour and normal cells by FPGS induced polyglutamylation, and are exported from cells after hydrolysis to monoglutamates by GGH. As with folate, polyglutamylated antifolates are retained in cells longer, thereby increasing their cytotoxicity by extending the length of exposure. Polyglutamylated antifolates generally have a higher affinity for and hence inhibit their target folate dependent enzymes in the thymidylate and purine biosynthetic pathways (for example, thymidylate synthase (TS)) to a greater extent than the monoglutamate forms. Decreased antifolate polyglutamylation due to quantitative or qualitative alterations in FPGS activity has been shown to be a mechanism of resistance to MTX and other antifolates in human and murine leukaemia cell lines. Transfection of FPGS cDNA has been shown to increase sensitivity to MTX in variant hamster cells that lack endogenous FPGS activity.

FPGS gene transfer into rat and human glioma, gliosarcoma, and glioblastoma cell lines already expressing FPGS significantly enhanced sensitivity to MTX and other antifolates. FPGS induced polyglutamylation may also affect the sensitivity of tumour cells to other chemotherapeutic agents not typically considered as antifolates such as 5-fluorouracil (5-FU). One cytotoxic mechanism of 5-FU is the formation of a ternary complex involving 5-fluoro-2-deoxyuridine-5-monophosphate (FdUMP; a metabolite of 5-FU), TS, and 5,10-methylenetetrahydrofolate (5,10-methylenethf), thereby inhibiting TS activity with consequent depletion of intracellular thymidylate and ultimately suppression of DNA synthesis. Leucovorin (LV), a precursor for 5,10-methylenethf, potentiates the cytotoxic effect of 5-FU by stabilising the inhibitory 5,10-methylenethf-TS-FdUMP ternary complex. 5,10-Methylenethf with longer chain length polyglutamates is better retained intracellularly and is more efficient in the formation and stabilisation of the inhibitory 5,10-methylenethf-TS-FdUMP ternary complex compared with shorter chain polyglutamates. Decreased FPGS activity has previously been demonstrated to confer resistance to 5-FU in some human cancer cell lines.

Abbreviations: AS, antisense; FdUMP, 5-fluoro-2-deoxyuridine-5-monophosphate; FPGS, folylpolyglutamate synthetase; 5-FU, 5-fluorouracil; GGH, γ-glutamyl hydrolase; LV, leucovorin; MTX, methotrexate; OD, optical density; S, sense; THF, tetrahydrofolate; TS, thymidylate synthase; VA, vector alone
FPGS appears to play an important role in the sensitivity of cancer cells to antifolates and 5-FU and thus FPGS modulation might be a potential therapeutic target for increasing sensitivity of cancer cells to these chemotherapeutic agents. However, definitive functional evidence supporting the effect of FPGS modulation on the chemosensitivity of colon cancer cells to MTX and 5-FU is currently lacking. Therefore, we generated an in vitro model of FPGS modulation in colon cancer cells and determined the effect of FPGS overexpression and inhibition on the chemosensitivity of colon cancer cells to MTX and 5-FU.

MATERIALS AND METHODS

Cell line and culture

Human colon adenocarcinoma HCT116 cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA). HCT116 cells exhibit microsatellite instability due to an inactivating MLH1 mutation, contain a k-ras protooncogene mutation, and lack p53 and APC mutations. Cells were grown in RPMI-1640 medium (Invitrogen, Gaithersburg, Maryland, USA) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, penicillin 100 U/ml, and streptomycin 100 mg/ml. Cultures were maintained at 37°C in 5% CO₂.

Construction and transfection of the sense and antisense FPGS expression vector

The full length human FPGS cDNA was provided by Dr A Bognar (University of Toronto, Toronto, Canada). The full length human FPGS cDNA was subcloned into the EcoRI site of the eukaryotic expression vector pIREneo (Clontech, Palo Alto, California, USA) containing a CMV promoter and a neomycin resistance gene expression cassette in the sense and antisense orientation to generate the sense and antisense FPGS expression vectors, respectively. Correct integration, orientation, and sequence of the sense and antisense FPGS cDNAs were confirmed by predicted fragment sizes after multiple restriction enzyme digestions and DNA sequencing. The pIREneo vector containing the sense or antisense FPGS cDNA was stably transfected into HCT116 cells using Lipofectin (Invitrogen) according to the manufacturer’s protocol. In a separate transfection, HCT116 cells were stably transfected with empty pIREneo vector (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 neomycin 500 μg/ml. Several (>10) clones expressing the sense and antisense FPGS cDNA and empty vector were screened at random, and two independent clones of each construct were selected for further analyses. Data from two experiments using two independent clones of each construct were similar and thus the data from one experiment are presented.

Western blot analysis

FPGS, TS, p53, and p21 protein expression was determined by standard western analysis, as described previously.28 using a rabbit polyclonal antibody raised against a peptide sequence spanning amino acids 275–290 of human FPGS27 (Zymed, San Francisco, California, USA) at a dilution of 1:200, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000, a sheep polyclonal antibody against human TS (Rockland Immunochemicals, Gilbertsville, Pennsylvania, USA) at a dilution of 1:2000. All western analyses were repeated using three different cell lysates.

FPGS and TS activity assay

FPGS activity was determined by measuring incorporation of [1H]glutamate into the polyglutamate chain of aminopterin, as described previously.20 The catalytic activity of TS was determined by H release that occurred during conversion of [5-3H]-deoxyuridine-5-monophosphate to deoxythymidine-5-monophosphate, as described previously.20 Enzyme assays were performed in triplicate and repeated using three different cell lysates.

Intracellular folate concentrations and determination of glutamate chain lengths

Intracellular folate concentrations were determined by a standard microbiological microtitre plate assay using Lactobacillus casei29 for both conjugase treated and untreated samples to determine the extent of polyglutamylation as L casei grow in proportion to the amount of mono-, di-, tri- and, to some extent, tetra-glutamylated folate in the samples, and conjugase treatment results in cleavage of the polyglutamylated chain to yield these short chain length forms of folate. This method has been used in previous studies to determine overall pool of long chain versus short chain polyglutamates.30 All analyses were performed in triplicate and repeated using three different cell lysates.

Real time quantitative reverse transcription-PCR

Total cellular RNA was extracted using the RNeasy MidiKit (Qiagen, Mississauga, Ontario, Canada). cDNA was generated from total RNA using random primers and the SuperScript III RNase H-Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) primers for GGH were constructed based on the human GGH cDNA sequence31 and were synthesised by ACGT (Toronto, Ontario, Canada) as follows: forward, 5'-GCA ACA GAT ACT GTT GAC GTG G3'; reverse, 5'-ATG GAA ATT GGC AGT CAG AGG-3'. Real time PCR was performed using the LC FastStart DNA Master SYBR Green 1 Kit in the LightCycler rapid thermal cyther system (Roche Diagnostics, Laval, Quebec, Canada) and the melting curve was determined as described.32 All PCR reactions were performed in triplicate, and experiments were repeated three times.

Doubling time calculation

Cells (8000 per well) were plated in 96 well plates and grown in RPMI-1640 medium with 10% fetal bovine serum for 72 hours. The cell population was determined using the sulforhodamine B (SRB) optical density (OD) measurement assay. 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 hours. The same equation was used to calculate the 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 previously.20 21 33 Briefly, 8000 cells per 100 μl RPMI-1640 medium per well were seeded in triplicate in 96 well flat bottom plates (Costar, Cambridge, Massachusetts, USA). After 24 hours, an additional 100 μl of RPMI-1640 medium containing MTX (Schircks, Jona, Switzerland) or 5-FU (InvivoGen, San Diego, California, USA) in combination with LV (Sigma Aldrich Canada, Oakville, Ontario, Canada) were added, and cells were cultured for an additional 72 hours. The
concentration of 5-FU was varied, with concentrations ranging from $1.5 \times 10^{-6}$ M to $25 \times 10^{-6}$ M, whereas the concentration of LV was held constant at $5 \times 10^{-6}$ M. LV was added to simulate the standard 5-FU based chemotherapy used in the treatment of colorectal cancer. The concentration of MTX was varied, with concentrations ranging from $3.5 \times 10^{-9}$ M to $5 \times 10^{-8}$ M. After 72 hours, 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 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:

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

IC$_{25}$, IC$_{50}$, and IC$_{75}$ values (that is, the drug concentration that corresponded to a reduction in cell survival by 75%, 50%, and 25%, respectively, compared with survival of untreated control cells) were calculated from plots of drug concentration versus proportion of cells that survived. All analyses were performed in triplicate, and three replicate experiments were performed.

**Statistical analysis**

Comparisons among cells expressing sense FPGS, antisense FPGS, and endogenous FPGS were determined using one way analysis of variance. Tukey’s honestly significance difference test was used for pairwise comparisons. 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 (sense FPGS, endogenous FPGS, and antisense FPGS) 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. IC$_{25}$, IC$_{50}$, and IC$_{75}$ doses and their 95% confidence intervals were calculated on the log scale from the regression results, as described previously, and then back transformed to the original scale for reporting. For all analyses, results were considered statistically significant if two tailed p values were <0.05. Analyses were performed using SAS, version 8 (SAS Institute, Cary, North Carolina, USA).

**RESULTS**

HCT116 cells expressing the sense and antisense FPGS had significantly higher and lower steady state levels of the FPGS protein, respectively, compared with those expressing endogenous FPGS (p<0.001) (fig 1A). HCT116 cells expressing the sense FPGS had an 8.2-fold higher, whereas those expressing the antisense FPGS had a 2.3-fold lower, FPGS activity compared with those expressing endogenous FPGS (p<0.001) (fig 1B). HCT116 cells expressing the sense and antisense FPGS grew faster and slower, respectively, than those expressing endogenous FPGS, as reflected by a significantly decreased and increased doubling time, respectively (32.0 (0.6) hours (sense) v 34.9 (0.4) hours (endogenous) v 36.4 (0.7) hours (antisense); p<0.001).

Following conjugase treatment (which allows measurement of total folate content, including short and long chain polyglutamates), intracellular folate concentration of HCT116 cells expressing the sense FPGS was 30% higher, whereas that of those expressing the antisense FPGS was 21% lower, than that of cells expressing endogenous FPGS (p<0.0001) (fig 2). Intracellular folate concentration of samples not treated with conjugase (which allows determination of short chain polyglutamates) was not significantly different between cells expressing the sense FPGS and those expressing endogenous FPGS and between cells expressing the antisense FPGS and cells expressing endogenous FPGS (fig 2). However, there were significantly more short chain polyglutamates in cells expressing the sense FPGS compared
appropriately affected the glutamate chain lengths of intracellular folate pool.

Cells expressing the sense FPGS had significantly higher, whereas those expressing the antisense FPGS had significantly lower, GGH mRNA expression, compared with those expressing endogenous FPGS (p < 0.05) (fig 3).

In vitro chemosensitivity to 5-FU plus LV was significantly different among HCT116 cells transfected with the three different FPGS constructs (p < 0.0001) (fig 4A). In vitro chemosensitivity of HCT116 cells expressing the sense FPGS to 5-FU plus LV was significantly enhanced compared with those expressing endogenous or the antisense FPGS (p < 0.0001) (fig 4A). In contrast, in vitro chemosensitivity of HCT116 cells expressing the antisense FPGS was significantly decreased compared with those expressing endogenous or the sense FPGS (p = 0.0122 and p < 0.0001, respectively) (fig 4A). IC_{25}, IC_{50}, and IC_{75} values for 5-FU indicate significantly enhanced chemosensitivity of HCT116 cells expressing the sense FPGS and reduced chemosensitivity of those expressing the antisense FPGS compared with cells expressing endogenous FPGS (table 1).

Overall, there was no significant difference in in vitro chemosensitivity to MTX among HCT116 cells transfected with the three different FPGS constructs (p = 0.548) (fig 4B). IC_{25}, IC_{50}, and IC_{75} values for MTX, however, suggest that antisense FPGS inhibition enhances, whereas sense FPGS overexpression decreases, chemosensitivity of HCT116 cells to MTX compared with cells expressing endogenous FPGS (table 1).

We wished to ascertain that the observed effects on chemosensitivity of HCT116 cells to 5-FU and MTX were secondary to alterations in FPGS and not an unintended effect of FPGS modulation on intrinsic TS protein expression and activity. TS is a critical target for 5-FU and MTX, and its expression level is an important predictor of chemosensitivity of cancer cells to 5-FU and MTX.\textsuperscript{10} TS protein expression and catalytic activity were similar among HCT116 cells expressing the sense, antisense, and endogenous FPGS that were not treated with 5-FU or MTX (data not shown). The effect of FPGS modulation on two molecular determinants of chemosensitivity, p53 and p21,\textsuperscript{36–38} was also investigated. FPGS overexpression downregulated, whereas FPGS inhibition upregulated, p53 and p21 protein expression compared with endogenous FPGS (fig 5).
DISCUSSION

We developed an in vitro model of FPGS overexpression and inhibition in HCT116 colon cancer cells with predictable functional consequences. Compared with HCT116 cells expressing endogenous FPGS, those expressing the sense FPGS had significantly higher FPGS expression and activity, higher GGH expression, faster growth rates, higher concentrations of total intracellular folate, and higher content of long chain polyglutamates. In contrast, HCT116 cells expressing the antisense FPGS had significantly lower FPGS expression and activity, lower GGH expression, slower growth rates, lower concentrations of total intracellular folate, and lower content of long chain polyglutamates compared with those expressing endogenous FPGS. These observed metabolic and 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 overexpression and inhibition on chemosensitivity of colon cancer cells to 5-FU and MTX.

Using this system, we have shown that FPGS overexpression enhances, whereas FPGS inhibition decreases, chemosensitivity of colon cancer cells to 5-FU plus LV. The most likely explanation for this observation is that FPGS overexpression enhanced the cytotoxic effect of 5-FU by increasing relative intracellular concentrations of longer chain length 5,10-methyleneTHF polyglutamates, resulting in more efficient formation and stabilisation of the inhibitory 5,10-methyleneTHF-TS-FdUMP ternary complex. In contrast, FPGS inhibition decreased the cytotoxic effect of 5-FU by decreasing relative intracellular concentrations of longer chain length 5,10-methyleneTHF polyglutamates, resulting in less efficient formation and stabilisation of the 5,10-methyleneTHF-TS-FdUMP ternary complex. However, we did not provide direct evidence that FPGS modulation lead to different glutamate chain lengths of 5,10-methyleneTHF because of technical difficulties in measuring intracellular 5,10-methyleneTHF. Furthermore, we did not determine and compare the rate of formation and dissociation and the concentration of the 5,10-methyleneTHF-TS-FdUMP ternary complex among HCT116 cells transfected with the three different FPGS constructs. However, indirect evidence from a prior study supports the proposed mechanism by which FPGS modulation might have affected chemosensitivity of colon cancer cells to 5-FU. In that study, the effect of LV, which is converted to 5,10-methyleneTHF and enters the folate pathway, on chemosensitivity of human leukaemia CCRF-CEM (the parent cell line with proficient polyglutamylation) and CCRF-CEM/P (a cell line with impaired ability to form polyglutamates) to 5-FU was compared. Both CCRF-CEM and CCRF-CEM/P cells accumulated 5,10-methyleneTHF in the presence of LV in a dose dependent manner. However, at a dose of 5-FU that produced only a slight decrease in cell growth, addition of LV further inhibited the cell growth in CCRF-CEM cells, but not in CCRF-CEM/P cells, suggesting that the impaired polyglutamylation of 5,10-methyleneTHF was likely responsible for the lack of potentiation of the cytotoxic effect of 5-FU by LV in CCRF-CEM/P cells.

The observed effect of FPGS modulation on chemosensitivity to 5-FU is consistent with the existing in vitro evidence that suggests that the cytotoxic effect of 5-FU is directly correlated with FPGS activity in 14 human cancer cell lines. Another in vitro study has shown that human colon cancer HCT8 cells become rapidly resistant to 5-FU over time owing to a progressive decrease in FPGS mRNA expression and activity. In contrast, three small human studies have shown conflicting results concerning the role of FPGS activity and mRNA expression in predicting treatment response to 5-FU+LV and survival in patients with colon cancer.

Table 1 IC₂₅, IC₅₀, and IC₇₅ values of 5-fluorouracil (5-FU) and methotrexate (MTX) in HCT116 colon cancer cells expressing sense (FPGS-S), endogenous (vector alone (VA)), and antisense (FPGS-AS) folylpolyglutamate synthetase (FPGS)

<table>
<thead>
<tr>
<th></th>
<th>25% survival</th>
<th>50% survival</th>
<th>75% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₂₅ 95% CI</td>
<td>IC₅₀ 95% CI</td>
<td>IC₇₅ 95% CI</td>
</tr>
<tr>
<td>5-FU  (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPGS-S</td>
<td>3.90 (3.30, 4.63)</td>
<td>1.64 (1.26, 2.14)</td>
<td>0.35 (0.26, 0.47)</td>
</tr>
<tr>
<td>VA</td>
<td>17.03 (15.39, 18.83)</td>
<td>3.03 (2.71, 3.37)</td>
<td>0.54 (0.42, 0.68)</td>
</tr>
<tr>
<td>FPGS-AS</td>
<td>59.83 (48.83, 73.30)</td>
<td>4.57 (4.15, 5.02)</td>
<td>0.69 (0.47, 1.01)</td>
</tr>
<tr>
<td>MTX  (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPGS-S</td>
<td>13.93 (10.99, 17.67)</td>
<td>9.54 (8.18, 11.13)</td>
<td>6.54 (6.05, 7.07)</td>
</tr>
<tr>
<td>VA</td>
<td>13.18 (12.38, 14.04)</td>
<td>8.82 (8.49, 9.16)</td>
<td>5.89 (5.79, 6.00)</td>
</tr>
<tr>
<td>FPGS-AS</td>
<td>10.95 (10.32, 11.61)</td>
<td>7.53 (7.29, 7.79)</td>
<td>5.18 (5.10, 5.27)</td>
</tr>
</tbody>
</table>

IC₂₅, IC₅₀, and IC₇₅ values (that is, drug concentration that corresponded to a reduction in cell survival by 75%, 50%, and 25%, respectively) were calculated from plots of cell survival versus drug concentration.

Figure 5 Effect of folylpolyglutamate synthetase (FPGS) modulation on p53 and p21. p53 is integrally involved in cell cycle control, DNA repair, and apoptosis while p21 is a member of the cyclin dependent kinase inhibitor family, which inhibits G₁ and S phase progression. p53 promotes cell cycle arrest in late G₁ through upregulation of p21 in response to cell injury, thereby allowing time for repair, whereas in the event that DNA damage is more severe and non-reparable, p53 performs its alternative role of moving the cell into apoptosis. FPGS overexpression (FPGS-S) downregulated, whereas FPGS inhibition (FPGS-AS) upregulated, p53 and p21 protein expression compared with endogenous FPGS (vector alone (VA)) in HCT116 cells (densitometry of bands: p53:β-actin ratio 1.9 (FPGS-S) v 3.1 (VA) v 4.1 (FPGS-AS); p21:β-actin ratio 1.7 (FPGS-S) v 2.5 (VA) v 3.2 (FPGS-AS)).
Large clinical trials are therefore necessary to investigate the potential clinical utility of FPGS status as a predictor of treatment response and a prognostic indicator in colon cancer patients receiving 5-FU based chemotherapy.

FPGS modulation had no significant overall effect on the chemosensitivity of colon cancer cells to MTX, in contrast with our hypothesis that FPGS induced changes in the glutamate chain lengths of MTX would affect chemosensitivity of colon cancer cells to MTX by altering intracellular MTX retention and affinity of MTX for its target folate dependent enzymes in the thymidylate and purine biosynthetic pathways. However, the overall pattern of chemosensitivity to MTX and the IC₅₀, IC₃₀ and IC₁₅₀ values of MTX suggest that FPGS inhibition may enhance, whereas FPGS overexpression may decrease, chemosensitivity of colon cancer cells to MTX. Our data contrast with prior observations, which suggested that FPGS downregulation leads to resistance, whereas FPGS overexpression enhances chemosensitivity, to MTX and novel antifolates in other cancer cell lines. It is possible that our results are specific to the cell type studied because there are tissue specific differences in MTX metabolism and in FPGS expression and activity. Also, colon cancer cells are generally not sensitive to MTX. Another possible explanation is that the effect of the FPGS induced changes in the glutamate chain lengths of MTX might have been nullified by similar changes in the glutamate chain lengths of intracellular folate induced by FPGS modulation.

We have found that FPGS overexpression downregulates, whereas FPGS inhibition upregulates, p53 and p21 expression in untreated HCT116 cells. Whether or not FPGS modulation induced p53 and p21 changes played a role in altering chemosensitivity of HCT116 cells to 5-FU was not determined in the present study. Given the very small magnitude of changes in p53 and p21 protein expression, however, it is highly unlikely that they contributed significantly to altered chemosensitivity. Nevertheless, the direction of changes of p53 and p21 is consistent with the observed alterations in intracellular folate concentrations resulting from FPGS modulation (that is, the effect of intracellular folate level on DNA damage and consequent p53 activation and p21 upregulation).

At present, mechanisms by which FPGS expression and activity are decreased or qualitatively altered in cancer cells resistant to MTX and 5-FU are not clearly elucidated. To date, only one putative mutation in FPGS has been identified. Therefore, it is of great interest to elucidate genetic, epigenetic, and other potential mechanisms of FPGS regulation in tumours.

In conclusion, our study provides evidence that FPGS overexpression enhances, whereas FPGS inhibition decreases, chemosensitivity of colon cancer cells to 5-FU. Our data suggest that FPGS status may be an important determinant, and hence a useful clinical predictor, of the chemosensitivity of colon cancer cells to 5-FU based chemotherapy. Furthermore, our data provide, for the first time, evidence suggesting that FPGS gene transfer may be a potential target for increasing sensitivity of colon cancer cells to 5-FU based chemotherapy. In contrast, FPGS modulation appears to have no significant effect on the chemosensitivity of colon cancer cells to MTX. However, our data based on a single colon cancer cell line need to be confirmed in other colon cancer cell lines.

ACKNOWLEDGEMENTS
This project has been supported by an operating grant (grant #14126) and New Investigator award from the Canadian Institutes of Health Research (to YIK).


Amin AB. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. Chem Res 2001;88:590-7.


Amin AB. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. Chem Res 2001;88:590-7.
