Over expression of the mitochondrial folate and glycine-serine pathway: a new
determinant of methotrexate selectivity in tumors.

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Abstract

Previous studies have documented the roles of transport via the reduced folate carrier, retention via polyglutamylation and increased levels of the target enzyme, dihydrofolate reductase in sensitivity to methotrexate. Recent studies have shown that the mitochondrial enzymes in the cellular metabolism of serine, folate and glycine are overexpressed in a subset of human cancers and that their expression is required for tumor maintenance. In this Perspective, we propose that expression of mitochondrial enzymes in the metabolism of serine and glycine, in addition to those involved in folate metabolism, are determinants of the response to methotrexate. Further we show that Myc activation in tumors is associated with up regulation of these enzymes. We propose that patients whose tumors that show this phenotype will be sensitive to folate antagonists targeting thymidylate or purine biosynthesis.
**Introduction**

Previous studies have documented the roles of transport via the reduced folate carrier (RFC), retention via polyglutamylation and increased levels of the target enzyme, dihydrofolate reductase in sensitivity to the folate antagonists methotrexate (MTX) pralatrexate and pemetrexate (1). Although these factors explain in part why tumor cells are sensitive or resistant to antifolates, they do not fully account for selectivity. Two recent publications have now provided insight for why some tumor cells, as compared to normal replicating cells, are sensitive to MTX: cancer cells that undergo metabolic reprogramming and are characterized by rapid proliferation and up regulate glycine consumption and metabolism are potently inhibited by MTX while normal cells with similar proliferative rates are not as sensitive.

The contribution by Jain et al (2) showed that metabolic reprogramming that occurs in cancer may lead to higher expression of the mitochondrial glycine biosynthetic pathway and up regulation of mitochondrial folate enzymes that include serine hydroxymethyl transferase (SHMT2), methylenetetrahydrofolate dehydrogenase (MTHFD2) and tetrahydrofolate synthetase (MTHFD1L) (see Fig1). The up regulation of these mitochondrial enzymes correlated with increased proliferation, while the folate cytosolic enzymes were not up regulated. Unlike the trifunctional cytosolic enzyme, MTHFD1, that contains methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase activity, the mitochondrial enzyme MTHFD2 is a bifunctional enzyme that contains methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activity. Formyltetrahydrofolate synthetase activity is encoded by a separate enzyme, MTHFD1L.
These enzymes contribute to the synthesis and utilization of glycine, methylene THF, and N-10 formyl THF for de novo purine and thymidylate biosynthesis (Fig. 1).

Supporting the importance of the above pathway is the high expression of mitochondrial 1-carbon pathway components in embryos. A developmental period marked by high cell proliferation rates, embryogenesis requires significant amounts of protein, lipid and nucleic acid synthesis, of similar nature to neoplastic cells. MTHFD1L was found to be up regulated in mouse embryos, with the mitochondria providing greater than 75% of the 1-carbon units present in the cytoplasm used for purine synthesis and other biosynthetic pathways (3). MTHDF1L and MTHFD2 are targets of microRNA miR-9, which may act as a tumor suppressor in regard to these and other genes (4). High expression of miR-9 suppresses levels of MTHFD1L and MTHFD2 and miR-9 has been found to be down regulated in breast cancer cell lines (4). Increasing miR-9 levels or knocking down MTHFD2 both have anti-proliferative and pro-apoptotic effects in tumor cell lines, which suggests that inhibitors of MTHFD2 may demonstrate similar effects.

Zhang et al (5) isolated tumor initiating cells from primary non-small cell lung cancers (NSCLC) and found that up regulation of the enzyme glycine decarboxylase and other glycine-serine enzymes was associated with increased rates of proliferation and poorer outcome in patients with Non-Small Cell Lung Cancer (NSCLC). Remarkably, cDNA of the glycine decarboxylase gene transformed 3T3 cells. Large increases in the levels of thymidylate synthase (TS) (6) and dihydrofolate reductase (DHFR) (unpublished) by transfection of immortalized cells can also cause transformation, and these findings are in accord with the idea that up regulation of nucleotide synthesis is associated with
metabolic reprogramming and increased tumor cell proliferation. The details of how this increase in enzymes involved in nucleotide biosynthesis is associated with malignant transformation are not clear. In the study by Zhang et al, the mitochondrial SHMT2 and MTHFD2 and MTHFD1L enzyme levels were not assessed, and emphasis was focused on the role of glycine decarboxylase to generate methylenetetrahydrofolate, the one-carbon donor for thymidylate biosynthesis. As the glycine decarboxylase enzyme complex is also located in mitochondria of mammalian cells (7), this further demonstrates the role of the mitochondria in proliferation by supplying tumor cells with precursors for pyrimidine biosynthesis. In this regard, an earlier report showed that transformation imposes a stress on cancer cells in that the demand for nucleotide formation is difficult for the cell to meet (8). As suggested by both Jai et al and Zhang et al, inhibitors of glycine decarboxylase and or serine hydroxymethyl transferase may be novel targets for tumors in which proliferation is driven by over expression of these enzymes.

**Sensitivity to methotrexate in vitro and in vivo**

Here, we provide evidence to show that known potent and clinically approved inhibitors of thymidylate and purine biosynthesis, that include DHFR inhibitors MTX and pralatrexate and the thymidylate synthase inhibitors 5-fluorouracil and pemetrexed, show selectivity to rapidly proliferating tumors in patients with overexpression of genes coding for folate metabolism enzymes. To test this hypothesis, we analyzed in vitro data reported by the Genomics of Drug Sensitivity in Cancer at the Sanger Institute (9) to determine if upregulation of the mitochondrial folate enzymes correlated with sensitivity to MTX, a potent inhibitor of dihydrofolate reductase and when converted intracellularly to polyglutamates (10), an inhibitor of two enzymes of purine synthesis,
phosphoribosylglycinamide formyltransferase (GARFT) and phosphoribosylaminoimidazolecarboxamide formyltransferase (AICART). A strong correlation between increased expression of seven genes and lower IC50 for MTX was observed (Fig. 2a), including the genes coding for the folate mitochondrial enzymes $SHMT2$ ($p$-value=$3.8\times10^{-4}$) and $GLDC$ ($p$-value=$5.4\times10^{-3}$), as well as $SLC19A1$ (coding for the RFC, $p$-value=$4.1\times10^{-3}$), $DHFR$ ($p$-value=$3.6\times10^{-5}$-$5.4\times10^{-4}$), $TYMS$ (TS, $p$-value=$6.9\times10^{-8}$), $GART$ (GARFT, $p$-value=$3.6\times10^{-3}$-$1.7\times10^{-2}$) and $PHGDH$ ($p$-value=$1.5\times10^{-2}$). These data support the hypothesis that MTX and potentially other inhibitors of thymidylate and purine biosynthesis could be used to target cancers with high rates of proliferation.

To provide further evidence we analyzed a study reporting the response of acute lymphoblastic leukemia (ALL) patients to MTX treatment (11). This study reports the leukemia cell counts before and after treatment together with the leukemia gene expression profiles before treatment. We used the fold change reduction in leukemia cell count as a quantification of sensitivity to MTX treatment. A strong correlation was observed between the sensitivity to MTX treatment and increased expression of the genes coding for the folate mitochondrial enzymes $SHMT2$ ($p$-value=$1.6\times10^{-2}$) and $MTHFD2$ ($p$-value=$3.8\times10^{-2}$) as well as the gene $SLC19A1$ ($p$-value=$1.1\times10^{-2}$) coding for the RFC (Fig. 2b). Surprisingly, the expression of $DHFR$ did not correlate with the response to MTX, underscoring the need to include the folate mitochondrial enzymes in the investigation of the mechanisms of response to MTX.

**What malignancies may be expected to be sensitive to methotrexate?**
Another key aspect is to have an estimate of the patient population size that could be targeted using this approach. To this end, we used as a proxy a set of genes coding for folate/serine/glycine metabolism enzymes (folate metabolism: \textit{SLC19A1, DHFR, TYMS, GART, ATIC, FPGS}; serine synthesis: \textit{PHGDH, PSAT1, PSPH}; cytosolic serine/glycine/folate metabolism: \textit{SHMT1, MTHFD1}; and mitochondrial serine/glycine/folate metabolism: \textit{SHMT2, MTHFD2, MTHFD2L, AMT, DLD, GLDC}).

We hypothesize that cancers overexpressing a subset of these genes are more dependent on folate metabolism and, therefore, could benefit from treatment with MTX. However, we note that this signature is not a predictor of sensitivity to MTX, but rather a rough quantification of the potential benefit from stratified therapy with MTX. For example, Fig. 2d shows the expression of the folate metabolism genes in a cohort of breast cancer patients. The breast cancer patients can be divided in three groups. On the left we have the subset of cancers with a significant up-regulation (Fig. 2d, Up) of the folate metabolism gene signature, characterized by the concomitant expression of most genes in the gene signature. The color-coded P-value on the right emphasizes in red those genes that tend to be co-expressed. This group of patients is likely to benefit from MTX therapy because their cancers appear to be dependent on folate metabolism. In contrast, the subset of cancers on the right manifest a significant down-regulation of the folate metabolism gene signature (Fig. 2d, Down) and the corresponding patients are not expected to benefit from MTX therapy. Finally, there is an intermediate group with a mixed phenotype (Fig 2d, Intermediate) whose response cannot be anticipated unless we know which specific enzyme mediates the response to MTX in this specific cancer subtype.
Extending this type of analysis to other cancer types, we observe that about 25% of cancers manifest a significant up-regulation of the folate metabolism gene signature and are likely to benefit from treatment with MTX (Table 1). There are some variations depending on the cancer subtype, with colorectal cancers and lymphomas reaching slightly more than 30% to the lowest value of about 10% in prostate cancers.

The concomitant expression of genes encoding for enzymes in folate metabolism indicates a common regulatory mechanism. It is known that several serine, folate and glycine metabolism genes are targets of c-Myc (\textit{PHGDH, PSPH, SLC19A1, DHFR, TYMS, GART, SHMT1, MTHFD1, MTHFD2, FGPS and GCSH} \cite{12, 13}), and that their expression is required for cell proliferation. Once again, we used gene signatures as a proxy to quantify the activation of c-Myc targets \cite{12} and cell proliferation \cite{14}. Burkitt’s lymphoma is a typical example of a Myc driven cancer \cite{15}. Indeed, from our analysis of a published dataset \cite{16}, 88% and 68% of lymphoma classified as Burkitt’s lymphoma (BL) and atypical BL, respectively, manifest a significant upregulation of the c-Myc gene signature (Fig. 2c). Furthermore, as previously noticed \cite{16}, there is a group of diffuse large B-cell lymphoma (DLBCL) that is also Myc driven. Indeed, 44% of the DLBCL manifest a significant up-regulation of the c-Myc signature. Focusing on the folate metabolism genes, the P-value column in Fig. 2c highlights in red those genes that are frequently overexpressed (2-fold or higher) in the group of lymphoma with a significant Myc signature up-regulation. In addition to \textit{DHFR} (\textit{p-value}=1.5\times10^{-7}-2.4\times10^{-3} depending on the microarray probe), we note the serine synthesis genes \textit{PHGDH} (\textit{p-value}=9.7\times10^{-13}), \textit{PSAT1} (\textit{p-value}=6.1\times10^{-4}), \textit{PSPH} (\textit{p-value}=1.5\times10^{-4}) and the mitochondrial gene \textit{SHMT2} (\textit{p-value}=1.4\times10^{-2}). The correlation between the folate/serine/glycine...
metabolism, c-Myc and proliferation gene signatures is actually a universal feature of human cancers (17). We also note a significant correlation between the up-regulation of the c-Myc and proliferation signatures and the response to MTX in both the in vitro and in vivo studies (Fig. 2a and b, respectively). More precisely, in the sensitive group there is a significant number of samples manifesting an up-regulation of the c-Myc (in vitro, \( p\)-value=5.1\( \times 10^{-4} \); in vivo, \( p\)-value=2.6\( \times 10^{-4} \)) and proliferation (in vitro, \( p\)-value=6.4\( \times 10^{-6} \); in vivo, \( p\)-value=1.5\( \times 10^{-2} \)) signature.

**Conclusions**

It will be of importance to learn if known potent and clinically approved inhibitors of thymidylate and purine biosynthesis, that include DHFR inhibitors MTX and pralatrexate and the thymidylate synthase inhibitors 5-fluorouracil and pemetrexed, show selectivity to rapidly proliferating tumors in patients with overexpression of genes coding for mitochondrial folate metabolism enzymes. Based on the concomitant over expression of folate metabolism genes, we estimate that around 25% of patients with cancer manifest this phenotype, and determination of this phenotype will allow patients to be selected for treatment with inhibitors of thymidylate and purine biosynthesis.
References


Figure/Table Legends

Figure 1. The role of the mitochondria in the generation of purines and thymidylate for DNA synthesis. Reactions 1, 3, 4 and 5 occur in both the mitochondria and the cytoplasm, with reaction 2 limited only to the mitochondria. Reaction 1 and 2 are catalyzed by SHMT2 and the glycine cleavage system, respectively. In the cytoplasm, reactions 3, 4 and 5 are carried out by the trifunctional enzyme (MTHFD1); in the mitochondria, two enzymes are required (MTHFD2 catalyzes reactions 3 and 4 and MTHFD1L catalyzes reaction 5). The enzyme 10-formyl tetrahydrofolate dehydrogenase (not shown), has been reported to be absent in most cancer cells (10). (1) serinehydroxymethytransferase; (2) glycine oxidase complex; (3) 5-10 methylenetetrahydrofolate dehydrogenase; (4) methenyltetrahydrofolate cyclohydrolase; (5) formyltetrahydrofolate synthetase.

Figure 2. Heat map showing the expression of genes coding for enzymes in folate metabolism (blue: underexpressed, red: overexpressed). a) A collection of 515 tumor derived cell lines sorted in decreasing order of their sensitivity for MTX, based on data from Ref. (9). The samples were divided into two groups: Sensitive (1/3 of samples with lowest IC50) and Other (remaining samples). The right column shows a color-coded quantification of the P-value for enrichment of samples with high expression (2-fold or above) in the Sensitive group, where red indicates significantly enriched, the brighter the more significant, and blue not significantly enriched. b) A collection of 161 ALL sorted in decreasing order of their sensitivity to MTX, based on data from Ref. (11). The samples were divided into two groups: Sensitive (1/3 of samples with highest fold decrease in the leukemia cell count) and Other (remaining samples). The right column shows a color-coded quantification of the P-value as in a). c) A collection of 221 lymphomas grouped by subtype and sorted in decreasing order of c-Myc gene signature, based on data from Ref. (16). The right column shows a color-coded quantification of the P-value for enrichment of samples with high expression (2-fold or above) among samples with a significant up-regulation of the c-Myc signature. d) Samples from 508 breast cancers, based on data from Ref. (18). The samples were divided in three groups with significant up-regulation (Up), non-significant (Intermediate) and significant down-regulation (Down) of the folate metabolism gene signature. The right column shows a color-coded quantification of the P-value for enrichment of samples with high expression in the Up group.

Table 1. Prevalence of samples with concomitant expression of folate metabolism genes across different cancer types. The PubMed reference (PMID) and Gene Expression Omnibus (GEO) of the gene expression profiles source are reported as well.
### Table 1

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Figure 1

Mitochondria

Serine \rightarrow \text{(1)} \rightarrow \text{Glycine}

THF \rightarrow \text{(2)} \rightarrow \text{CH}_2\text{-THF}

ATP \rightarrow \text{(3)} \rightarrow \text{CH}^+\text{-THF}

ADP \rightarrow \text{(5)} \rightarrow \text{CHO-THF}

Cytoplasm

Purine Biosynthesis

THF \rightarrow \text{Thymidylate Biosynthesis}

CH}_2\text{-THF} \rightarrow \text{Purine Biosynthesis}
Figure 2

a) Tumor derived cell lines

b) ALL

c) Lymphomas

d) Breast cancers
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